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Table of Content

African Journal of Microbiology Research Vol. 5(18), pp. 2550-2554, 16 September, 2011
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Review

New chimeric anti-tubercular dendrimers with self-delivering property

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Tuberculosis is the second lethal infectious disease caused by *Mycobacterium* species. This pathogens could cause severe disease like tuberculosis and leprosy in human. Today there are a number of anti tuberculosis agents utilized in treatment of this disease but multiple drug resistance is one of the major problems that end to failure in treatment. Dendrimers are synthetic, high branched polymers with a number of functional groups that could bind to different macromolecules like drugs, oligosaccharides that makes it appropriate for target drug delivery. Today, some type of dendrimers like Jeffamines are developed possessing self antimicrobial activity. Hypothesis: we propose that combination of ethambutol with 3rd generation of Jeffamine core based dendrimer (P3) and mannose could both create a complex compound with high potency against *mycobacterium* species that is targeted to macrophages via interaction between lectin receptors on immune cells and mannose molecules on outer branch of P3.

Key words: Tuberculosis, dendrimers, Jeffamines, ***Mycobacterium***, ethambutol

INTRODUCTION

Tuberculosis (TB) is the second most lethal infectious disease (Sosnik et al., 2010). TB is an infectious disease that could be fatal caused by mycobacteria, generally ***Mycobacterium tuberculosis*** in humans (Vinay et al., 2007). The most contaminated part of body is attributed to lungs even though other parts are also infected to some less extent. Unfortunately the most contaminations remain undiagnosed because the infection is in latent phase and only 10% of them become active that could take the life of patients in 50% of cases if untreated. The usual ways of spreading is through coughing, sneezing or split (Konstantinos, 2010). The most common symptoms comprising fever, weight loss, and chronic cough associated with blood tinged sputum and night sweats. The rate of infection in the world is increasing that is typically in developed countries (WHO, 2009).

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Abbreviations: TB, Tuberculosis; **PAMAM,** polyamidoamine; **CD,** cyclic oligossacardies; **CFUs,** colony-forming unit

Concepts

We propose that ethambutol (an antimycobacterium agent) could be replaced with one or several of the functional groups on the branches of P3, which also possess antimycobacterium effect parallel to self-target delivery property meanwhile a mannose is bound to one or several other branches (Figure 1) that could interact with its receptors on macrophages resulting in development of a highly potent compound that is targeted to immune cells properly.

Mycobacterium tuberculosis

Mycobacterium is a member of Actinobacteria class. This group of pathogens accounts as hazardous bacteria and could potentially cause severe diseases like tuberculosis and leprosy in mammals (Ryan and Ray, 2004). As introducing in body, they could present no significant signs. For this reason, today many people

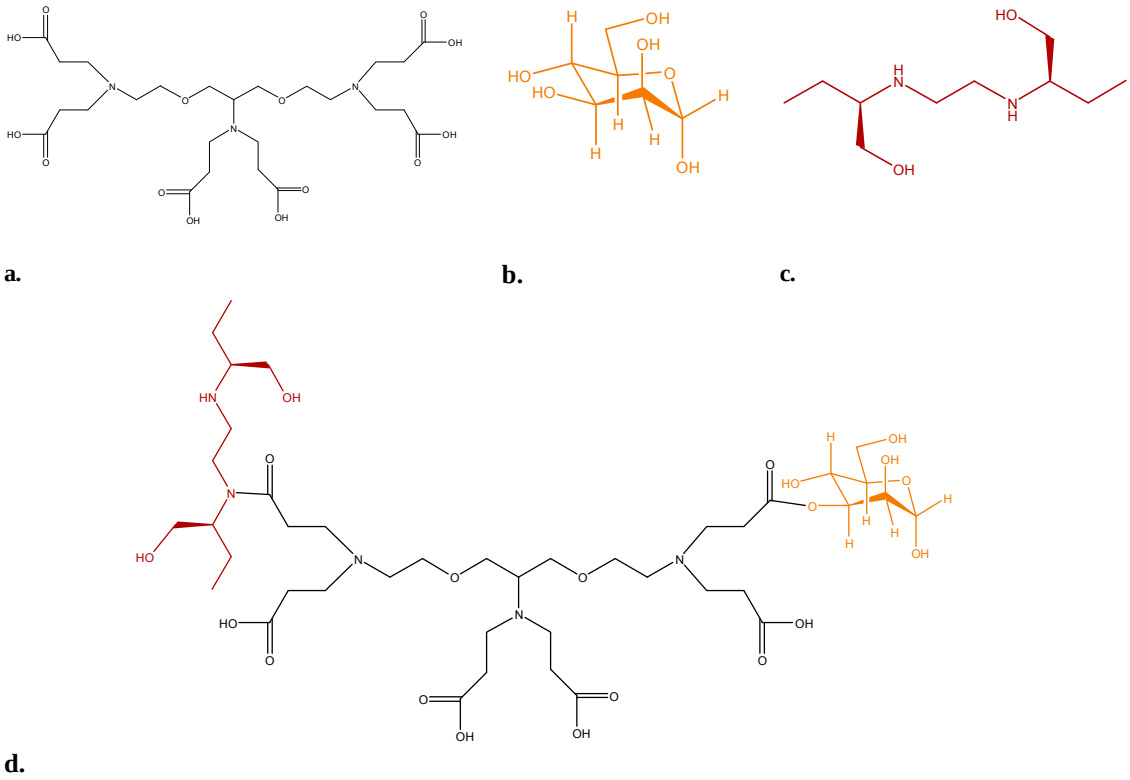

Figure 1a. The structure of 3rd generation of Jeffamine core based dendritic (P3), b. The structure of mannose, c. The structure of ethambutol, d. The general structure of 3rd generation of Jeffamine core based dendritic (P3), which also possess antimycobacterial effect parallel to self target delivery property, in combination with ethambutol and mannose as novel antimycobacterial agent.

around the world are infected with ***M. tuberculosis*** while they know nothing about their disease. ***M. tuberculosis*** possesses a cell wall dissimilar to Gram negative and positive that is resistant to many antibiotics resulting in difficulty in treatment plane. In addition to cell wall that gives the pathogen unique properties against antibiotic drugs, other factors also are effective in virulence of this pathogen including its surface and secreted proteins (Woolridge, 2009). Invasion and replication into alveolar macrophages is the primary and main stages of disease that happens before this pathogen is distributed (Shirzad et al., 2008).

Drugs used against *Mycobacterium tuberculosis*

There are several problems in treatment of TB that essentially get backs to the unique structure of the TB that could cause drug resistance (Acharya et al., 1967; Migliore et al., 1966; Acharya and Goldman, 1966). Rifampicin and isoniazid are among the most frequently used drugs in the treatment of tuberculosis that should be administered for about 6 to 24 months. If a patient is diagnosed in latent infection, only one antibiotic is used but in active form, multiple drugs are exploited in order to lessen the rate of antibiotic resistance (CDC, 2003). Ethambutol is another drug used as a bacteriostatic antimycobacterial drug prescribed in treatment of tuberculosis that is more explained in the next parts. One of the impediments in treatment of tuberculosis efficiently is the long time – period of treatment and high price of pills that could contribute to failure and development of multi drug resistant strains (Sosnik et al., 2010). Thus, strategies like design and development of novel formulations along with novel antimicrobial compounds that could reduce the length of treatment and reduce the resistance are inevitable.

Dendrimers

Dendrimers are synthetic, highly branched macromolecules with nanometer dimensions and are characterized by structural perfection (Anil et al., 2002). Advent of dendrimers as drug delivery systems in nanoscale has attracted much interest for studying controlled drug delivery systems (Umesh et al., 2006). They are monodisperse and usually highly symmetric, spherical compounds. The main features of dendrimers is related to functional groups on the molecular surface, though, some dendrimers possess internal functionality (Antoni et al., 2009; McElhanon and McGrath, 2000; Liang and

Fréchet, 2005). Dendrimers are investigated as carrier for drug delivery system (Anil et al., 2002). Among such diverse dendrimers, polyamidoamine (PAMAM) are much more studied. Researchers throughout the world investigated such dendrimers in different aspect. In one study (Malik et al., 2000) the relation between structure and bio-compatibility of PAMAM has been studied. They reported that cationic dendrimers are not suitable candidate as drug carrier because of their hemolytic and cytotoxic properties at even relatively low concentrations as well as having short half life and are cleared quickly from the body. Contrary, anionic PAMAM dendrimers possess longer half life while every less generation number, the more half life. In one study, it was defined that with increase in molecular weight and size of dendrimers, the extravasation across microvascular endothelium is also enhanced. Because of the toxicity of cationic PAMAM it is essential to replace the amino groups on surface area with neutral or anionic groups to eliminate toxicity and prevent its accumulation in liver (Jevprasesphant et al., 2003). In another study (Bhadra et al., 2003), polyethylene glycol was added to dendrimer structure to improve drug delivery of anticancer agnates. By this way, it was found that PEGylated dendrimers dominated by some more advantages in comparison to previous generations. The first superiority is related to finding more drug loading capacity via creating more functional groups on the outer surface that lead to much more drug interaction. Second advantage is attributed to diminishing hemolytic toxicity of dendrimers. It has been demon-strated that dendrimers can pass through GI membranes and are also valuable while deliver in the GI is dependent to pH and enzyme. Dendrimer also could improve the pharmacokinetics profile of drugs. The positive role of dendrimers in transdermal flux and enhancement of corneal retention time is also proved. However, regardless of all these benefits, the toxicological features of dendrimers should not be ignored and must be evaluated comprehensively (Aulenta et al., 2003; Bosman et al., 1999; Esfand and Tomalia, 2001; Patri et al., 2002; Cloninger, 2002; Gillies and Frechet, 2005). In recent times, water-soluble dendrimers have been exploited for the targeting of rifampicin, chloroquine and lamivudine to macrophages for treat-ment of tuberculosis (Briones et al., 2008). Generally, small molecules are uptaken by different mechanism comprising phagocytosis, fluid phase pinocytosis or by receptor-mediated endocytosis. Since phagocytosis mechanism take places for molecules with at least 500 nm in size, cellular uptake of dendrimers are not dependent to phagocytosis as their size of 20-30 nm (Rupper and Cardelli, 2001). In addition, receptor-mediated endocysis is ruled out when there is no ligand on the outer surface of dendrimer. Existence of -OH groups in outer surface of dendrimers disturb any ionic interaction with cell membrane (Chirila et al., 2002).

The only supposed mechanism for cellular uptake of dendrimers is fluid phase endocytosis through non-specific interactions. This mechanism was verified when no dendrimer went through cells as fluid phase endocytosis inhibitor was added to the media (Kolhe et al., 2004). Eventually, after entering into cell, the polymer-drug conjugate would be cleaved by hydrolytic enzymes in the lysosomal compartment, producing high concentration of drug in cell (Llyod, 2000).

Lectin and oligosaccharides

Dendrimers mostly PAMAMs are considered as suitable candidates for delivery of anti-TB agents because of their unique structure. In order to target the drug delivery to macrophages Kumar et al. (2006) developed a novel generation of rifampin loaded dendrimers that were modified with mannose molecules on surface. Mannose molecules could bind to their receptors on macropaghes that result in improved their uptake (Kumar et al., 2006). High haemolysis levels derived from amine-termina dendrimers could hinder their clinical application while mannosylation could dramatically decrease the hemolytic toxicity (Sosnik et al. 2010). In another study, combination of dendrimers with cyclic oligosaccharides (CD) was investigated. CD is composed of 12 D-(+)-glucopyranose units linked by α-(1–4) bonds. The CD has an outer hydrophilic and an inner hydrophobic part that permits hydrophobic molecules be placed in inner part resulting in elevated solubility (Loftsson and Duchene, 2007; Brewster and Loftsson, 2007). In one study, addition of CD to the dendrimers increased the solubility of the poorly-water soluble nitroimidazole P-824 as a new anti-TB drug (Lenaerts et al., 2005). Even though enhanced solubility was not observed with rifampin but such complexes could improve the thermal stability of the drug as well as enhanced passing of the drug through the wall of the bacilli (Lindenberg et al., 2004). Norfloxacin in solution is not effective against ***M. bovis***, while its combination with a dextran polymer bound to mannose could lessen the number of colony-forming unit (CFUs) in the lungs of mice infected with ***M. bovis*** (Roseeuw et al., 2003).

Antimicrobial activity of water soluble dendritic macromolecules

Several types of water soluble dendrimers are designed and developed based of poly(propyleneoxide) amines (Jeffamines) (P1). P1-core and branched units were developed from both methylacrylate and ethylenediamine (P2-P9, and generations 0-3 with -NH2, -COOH functionalities) (Metin, 2009). The unique architecture of dendrimers along with multiple functional groups gives impetus researchers to use such structures for designing as both antimicrobial agents (Chen and Cooper, 2000).

The above specific dendrimer is a dendritic poly-chelatogens starting from P1 as an initial core com-posed of amide connectivity without internal hydrolytic cleavage (Tomalia et al., 1986; Beezer et al., 2003; Newkome et al., 1992). The antimicrobial activity of Jeffamines is proved against some Gram-negative and Gram-positive such as ***Mycobacterium smegmatis*** (Wikler et al., 1993; Collins et al., 1989).

Ethambutol

Ethambutol is one of drugs utilized in treatment of TB bacilli. Its mechanism of action is through blocking the cell wall via inhibiting enzyme arabinosyl transferase that results in arabinogalactan synthesis disruption. There-fore, the permeability of cell wall would be increased (Yendapally, 2008). It is usually prescribes in combination with other anti-tubercular drugs, like isoniazid, and rifampicin.

DISCUSSION

Since TB is one of the life-threatening diseases and is spreading out in developing countries (Sosnik et al., 2010; Vinay et al., 2007) it is necessary to combine old drugs with new ones to produce high potent compounds to overcome multiple drug resistance that accounts for as one of the most important impediments in treatment of tuberculosis. Even though the role of dendrimers as drug delivery system have been reported by many researchers (Anil et al., 2002; Umesh et al., 2006; Antoni et al., 2009; Malik et al., 2000), but there are some type of dendrimers (Jeffamine core) with anti-tubercular properties (Metin, 2009) that could potentially be utilized in treatment of tuberculosis in a highly efficient manner. Therefore, it is concluded that combination of dendrimers and an anti-tubercular agent like ethambutol (Figure 1) could serve synergistic effect on tuberculosis species; even though targeting the dendrimer-ethambutol conjugate to desired immune cells like macrophages could also provide better effectiveness with improved therapeutic profile. To achieve this aim, exploiting mannose molecules as oligo-saccharide ligands on outer surface of dendrimers could target this complex to lectin receptors on macrophages. We think that using this strategy to practice, could facilitate tuberclosis treatment and prevent multiple drug resistance.

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Review

Current understanding of *de novo* synthesis of bacterial lipid carrier (undecaprenyl phosphate): More enzymes to be discovered

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An isoprenoid named undecaprenyl phosphate (Und-p) is the only known lipid carrier in bacteria. It is involved in the translocation of hydrophilic intermediates of cell wall components across the hydrophobic phospholipid bilayer of the cell membrane for subsequent polymerization in cell wall synthesis. Und-p is made available by both *de novo* synthesis and recycling. Evidences are emerging that in addition to dephosphorylation of undecaprenyl pyrophosphate (Und-pp), the phosphorylation of undecaprenol (Und-OH) into Und-p exists as an alternative pathway in Gram-positive bacteria but not in Gram-negative bacteria. This review gives an overview of the current knowledge in the synthesis of Und-p. It also hypothesizes the presence of yet to be identified Und-pp phosphatases at the inner cytoplasmic membrane that function in addition to a known phosphatase, named undecaprenyl pyrophosphate phosphatase (Upp-P) in *de novo* synthesis of Und-p. As the processes involved in cell wall synthesis remains the most promising antimicrobial therapeutic means, a more thorough understanding of the synthesis of the bacterial lipid carrier will not only improve the knowledge of cell wall synthesis but may also lead to the identification of potential drug targets and vaccine candidates.

Key words: Lipid carrier, undecaprenyl phosphate, undecaprenol, undecaprenol kinase, cell wall synthesis.

INTRODUCTION

Polyisoprenoids are polymers of five carbon isoprene units used as lipid carriers across the three domains of life (Jones et al., 2009). At the centre of the synthesis of bacterial cell wall is an isoprenoid lipid carrier named undecaprenyl phosphate (Und-p). It is involved in the translocation of glycan biosynthetic intermediates of carbohydrate polymers across the hydrophobic phospholipid bilayer of bacteria cell membrane to the externally located site of polymerization during cell growth, and division (Lennarz and Scher, 1972; Bouhss et al., 2008). The intermediates of peptidoglycan lipopolysaccharide, teichoic acid and enterobacterial common antigen are all translocated by the Und-p, reflecting its importance to the life of the cell. As such, the availability of Und-p has been shown to be a limiting factor and a site of control in the biosynthesis of cell wall components (Higashi et al., 1970; Anderson et al., 1972; Tatar et al., 2007; Valvano, 2008). Und-p has long been known to be derived from dephosphorylation of its precursor, undecaprenyl pyrophosphate (Und-pp) by the action of Und-pp phosphatases. These phosphatases have drawn much attention lately because of the essential role they play in the availability of the lipid carrier. The first Und-pp phosphatase gene to be characterized was identified in ***Escherichia coli*** and named ***uppP*** (formerly ***bacA***) (Cain et al., 1993; El Ghachi et al., 2004). Biochemical characterization showed its importance as it accounts for about 75% of Und-pp phosphatases activity in ***E. coli*** while bioinformatics reveals that ***uppP*** is largely distributed as single copy in most bacteria

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Abbreviations: DMAPP, Dimethylallyl pyrophosphate; **FPP,** farnesyl pyrophosphate; **G3P,** glyeraldehyde -3-phosphate; **GPP,** geranyl pyrophosphate; **IPP,** isopentenyl pyrophosphate; **MEP,** 2-C-methyl-erythritol-4-phosphate; **Und-pp,** undecaprenyl pyrophosphate; **Und-p,** undecaprenyl phosphate.

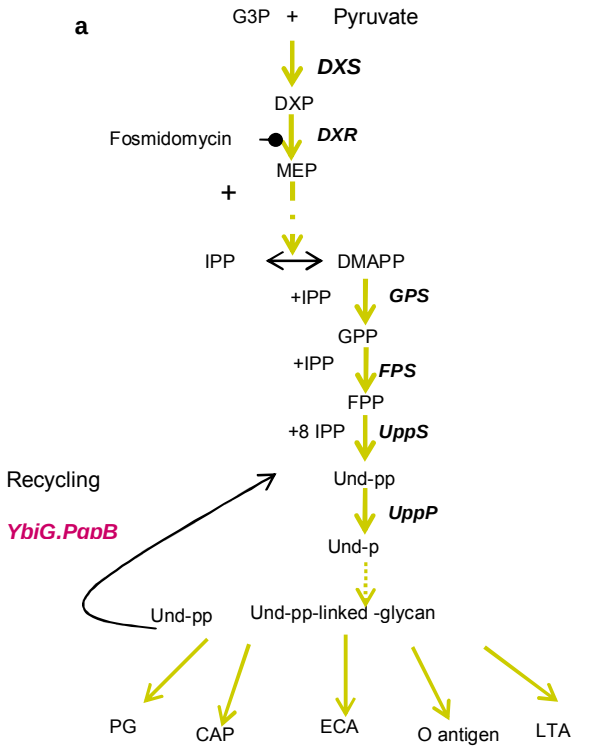

Figure 1. Synthesis of Und-p as a lipid carrier in ***E. coli***. ***E. coli*** uses the mevalonate pathway in generating IPP, Isopentenyl pyrophosphate and DMAPP, Dimethylallyl pyrophosphate. The key enzymes involved in the pathway are in bold italics. G3P: Glyeraldehyde -3-phosphate, GPP: Geranyl pyrophosphate, FPP: Farnesyl pyrophosphate, MEP: 2-C-methyl-erythritol-4-phosphate, Und-pp: Undecaprenyl pyrophosphate, Und-p: Undecaprenyl phosphate, GPS: Geranyl pyrophosphate synthase, FPS: Farnesyl pyrophosphate synthase, DXS: deoxy-xylulose-5-phosphate synthase, DXR: deoxy-xylulose-5-phosphate reductoisomerase, UppS: Und-pp synthase, UppP: Und-pp phosphatases. PG: Peptidoglycan, CAP: Capsule, ECA: Enterobacteria common antigen, LTA: Lipoteichoic acid translocated by Und-p. Fosmidomycin is indicated to inhibit DXR. Figure adapted with modification from (Tatar et al., 2007).

(El Ghachi et al., 2004). Unsurprisingly, no copy of this gene was detected in ***Mycoplasma*** which lacks cell wall. Despite the essentiality of the function of this gene, many studies have shown that its deletion from the genome is not lethal in both Gram-positive and Gram-negative bacteria (Chalker et al., 2000; El Ghachi et al., 2004; Rose et al., 2004; Bernard et al., 2005).

To explain the non-essentiality of UppP, some members of type 2 phosphatidic acid phosphatase family (PAP2) (Stukey and Carman, 1997) were later demonstrated to be involved in dephosphorylating Und-pp in ***E. coli***, ***Bacillus subtilis*** and ***Cupriavidus metallidurans*** (El Ghachi et al., 2005; Tatar et al., 2007; Touze et al., 2008; Hynninen et al., 2009). Since the availability of Und-p is essential for the survival of bacteria, a thorough understanding of all the enzymes involved in both its ***de novo*** synthesis and recycling will give a more complete overview of the bacterial cell wall biosynthesis in addition to the possible discovery of new drug targets. Moreover, elucidating the potential of additional enzymes known to possess Und-pp phosphatase activities may be beneficial in proffering solution to the challenging cases of bacterial drug resistance or unavailability of efficient vaccines.

This review emphasizes the contribution of undecaprenol kinase to the ***de novo*** synthesis of the essential Und-p in Gram-positive bacteria. It also hypothesizes the presence of unknown Und-pp phosphatases at the inner cytoplasmic membrane that functions together with UppP in ***de novo*** synthesis of Und-p in ***E. coli***. Although Und-p is involved in various processes of cell envelope biosynthesis, peptidoglycan synthesis is used as a typical example of the ***de novo*** synthesis and recycling of Und-p in this review. Due to the implication of UppP in virulence and antibiotic resistance in some bacteria, a focus is also made on its potential as a drug target as well as in vaccine development against pathogenic bacteria including ***Mycobacterium tuberculosis.***

Biosynthesis of undecaprenyl phosphate

All isoprenoids including Und-p originate from two five-carbon building blocks which are isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Swiezewska and Danikiewicz, 2005; Valvano, 2008). These two molecules are products of two distinct biosynthetic pathways: the 2-C-methyl-erythritol 4-phosphate pathway (MEP) which forms IPP and DMAPP from glyceraldehyde-3-phosphate and pyruvate (Rohmer et al., 1993; Kuzuyama, 2002; Eisenreich et al., 2004) and the mevalonate pathway (MVA) which involves the formation of IPP and DMAPP from acetyl coA with mevalonate as an intermediate (Schroepfer, 1981; Hunter, 2007).

A major intermediate in the synthesis of polyisoprenoids is farnesyl pyrophosphate (FPP). It is made of fifteen carbon atoms resulting from the head to tail condensation of IPP and DMAPP by geranyl pyrophosphate phosphatase, followed by a further addition of IPP by farnesyl pyrophosphate synthase (Ogura and Koyama, 1998; Liang et al., 2002). The first committed step in the synthesis of bacterial Und-p is catalyzed by undecaprenyl pyrophosphate synthase (UppS), an essential prenytransferase that catalyses the addition of eight C5 IPP units in cis configuration unto all ***trans*** FPP to produce Und-pp (Shimizu et al., 1998; Liang et al., 2002). In order to generate the functional Und-p, Und-pp is dephosphorylated by Und-pp phosphatases (Figure 1).

The process of identifying the gene(s) encoding the essential Und-pp phosphatase activity started with the finding that the overexpression of two genes namely ***bcrC*** and ***bacA*** (now referred to as ***uppP***) in ***E. coli*** resulted in bacitracin resistance (Cain et al., 1993). Cells overexpressing ***uppP*** were able to resist up to 200 µg ml^{-1} bacitracin that completely lysed control cells ((El Ghachi et al., 2004). Bacitracin is a mixture of related polycyclic peptides (Johnson et al., 1945) that binds tightly to the pyrophosphate motif of Und-pp in the presence of divalent cations thereby preventing its access to phosphatases. In the presence of bacitracin, the supply of Und-p is affected, consequently leading to lysis of the cell due to impaired cell wall formation (Kanof, 1970; Stone and Strominger, 1971; Storm, 1974).

280-fold increase in Und-pp phosphatase activity was observed in membrane extracts of ***E. coli*** overexpressing ***uppP*** compared with control cells, supporting its role as an Und-pp phosphatases (Cain et al., 1993; El Ghachi et al., 2004). It was thus explained that overexpression of UppP enhanced its competition with bacitracin for the pyrophosphate motif of Und-pp and accounts for the observed bacitracin resistance (El Ghachi et al., 2004). Amino acid sequence analysis shows that UppP possess a large cytoplasmic loop that is conserved in all bacteria (El Ghachi et al., 2005). Due to the cytoplasmic location of UppS (the enzyme that synthesizes Und-pp), and the conserved cytosolic loop of UppP it has been suggested to be involved in ***de novo*** synthesis rather than in the recycling of Und-p (El Ghachi et al., 2004; Tatar et al., 2007).

E. coli Δ***uppP*** null mutant did not show any significant growth or morphological defect, neither was its sensitivity to bacitracin affected (El Ghachi et al., 2004). However, the Und-pp phosphatase activity in the mutant was reduced by 75%. These observations showed its importance, but non-essentiality as well as non exclusiveness in generating Und-p. The non-essentiality of ***uppP*** homologues was also observed in Gram-positive bacteria namely ***Staphylococcus aureus***,

Streptococcus pneumoniae and ***B. subtilis*** and acid fast bacteria; ***M. tuberculosis***, ***M. bovis*** BCG, and ***M. smegmatis*** (Cain et al., 1993; Chalker et al., 2000; Rose et al., 2004; Bernard et al., 2005). Computational analysis shows that paralogues of ***uppP*** do not exist in any of these bacteria (El Ghachi et al., 2005). It was thus later demonstrated that some integral membrane enzymes from type 2 phosphatidic acid phosphatase family (PAP2) possess Und-pp phosphatase activities sufficient to sustain the growth of bacterial cells and are responsible for the viability of ***uppP*** mutants (El Ghachi et al., 2005). In ***E. coli***, these include LpxT and YbjG, which had been previously uncharacterized, and PgpB, a phosphatidyl glycerol phosphate phosphatases (Tatar et al., 2007; Touze et al., 2008). PbrB in ***Cupriavidus metallidurans***, and BcrC in ***B. subtilis*** have also been reported to possess Und-pp phosphatase activities (Bernard et al., 2005; Hynninen et al., 2009).

Recycling of undecaprenyl phosphate

De novo biosynthesis of Und-p is known to occur in small quantities and cells have been shown to rely on the recycling of previously used carrier (Higashi et al., 1967; van Heijenoort 2001). Some ***E. coli*** proteins have been implicated in Und-p recycling and are described later.

One of the enzymes reported to be involved in Und-p recycling is an ***E. coli*** enzyme named LpxT. It uses Und-pp as a phosphate donor in the formation of lipid A 1-diphosphate species releasing Und-p as a product (Stukey and Carman, 1997; Touze et al., 2008). Using a thermo-sensitive variant of MsbA, a lipid A flippase that is impaired at temperatures above 37°C (Doerrler et al., 2001) and temperature shift assay, it was reported that LpxT is functional only when MsbA is active (Touze et al., 2008). This confirms that the involvement of LpxT in the dephosphorylation of Und-pp actually takes place at the periplasm, an action that corresponds to the recycling rather than ***de novo*** synthesis of Und-p (Touze et al., 2008).

Another enzyme YbjG, was also shown to be involved in Und-p recycling in ***E. coli***. A sandwich fusion was constructed in which PhoA, an alkaline phosphatase that folds correctly only at the reducing condition of the periplasm was inserted between Arg139 and Val140 close to the location of catalytic His145 of YbjG. With this construct, the alkaline phosphatases activity of PhoA was observed. In addition, topological model prediction of YbjG orientates its active site in the periplasmic compartment. This thus implicates YbjG in recycling rather than in de novo synthesis of Und-p (Tatar et al., 2007).

With similar experiments, the catalytic site of PgpB, an ***E. coli*** phosphatidyl glycerophosphate phosphatase which could also dephosphorylate Und-pp was found to be located in the periplasm (Touze et al., 2008). ***pgpB*** truncated at the 3' end to various lengths was fused with β-lactamase gene. Fusions with the amino acids located in the periplasm resulted in ampicillin resistance and the corresponding amino acid residues were shown to be located at the catalytic site of PgpB (Touze et al., 2008).

Also, PbrB, biochemically characterized to dephosphorylate Und-pp, was also reported to depend on PbrA, a P-1B type ATPase for its effect on lead resistance in ***C. metallidurans*** (Hynninen et al., 2009). PbrA acts as an efflux pump extruding divalent metal ions including lead ions from the cytosol (Borremans et al., 2001; Hynninen et al., 2009). The dependence of PbrB on PbrA suggests that

it relies on PbrA to extrude lead ions from the cytosol before it transfers phosphate group from Und-pp to the extruded lead ions thereby precipitating the ions in the phosphate form, a known mechanism of lead precipitation in bacteria (Levinson et al., 1996; Levinson and Mahler, 1998; Mire et al., 2004; Hynninen et al., 2009). It can therefore be implied that the active site of PbrB is located in the periplasm thus contributing to recycling of Und-p in this bacterium (Hynninen et al., 2009).

The various enzymes outlined above functions in recycling of Und-p in ***E. coli***. However, no enzyme has been reported in its recycling in Gram-positive bacteria. Notably, BcrC of ***B. subtillis*** uses its Und-pp phosphatase activity to confer resistance to bacitracin (Bernard et al., 2005). The introduction of a copy of ***bcrC*** into a strain carrying double deletion of ***bceA***, (an efflux pump) and ***bcrC*** (∆***bceA***∆***bcrC***) resulted in 8 fold increase in IC_{50} to bacitracin (0.9 to 7.4 µM) while the introduction of a copy of ***bcrC*** into ***bcrC*** single mutant (∆***bcrC***) (with intact ***bceA***) resulted into a 13 fold increase (31 to 420 µM) (Bernard et al., 2005). It therefore appears that the function of BcrC in conferring bacitracin resistance depends somewhat on the presence of BceA in this bacterium and thus may play role in Und-p recycling. Further experiments are needed verify this.

More enzymes to be discovered

In ***E. coli***, the simultaneous inactivation of three genes, ***uppP***, ***ybjG*** and ***pgpB*** was reportedly lethal (El Ghachi et al., 2005). It was thus suggested that the lethality of the triple mutation is an indication that UppP, PgpB and YbjG contributed to the total Und-pp phosphatase activity in E. coli (Touze et al., 2008). In further investigations, an ***E. coli*** mutant with conditional expression of ***uppP*** was constructed. In the mutant, ***uppP*** was supplied to double deletion mutant with genotype, ***ΔuppPΔybjG*** by plasmid ***p***MAK705***uppP*** whose expression is impaired at 42°C. A third mutation was introduced by deleting the chromosomal copy of ***pgpB*** resulting in a triple mutant with genotype ***ΔuppPΔybjGΔpgpBpMAK705uppP.*** As such, the triple mutant was viable at 30°C, but not at 42°C (El Ghachi et al., 2005). This further suggested that the three genes ***uppP***, ***ybjG*** and ***pgpB*** contribute to the total Und-pp phosphatases activities in ***E. coli.***

E. coli mutants with genotypes ***ΔuppPΔybjGΔpgpBpMAK705uppP,*** ***ΔuppPΔybjG*** and ***ΔuppPΔpgpB*** were reported to be viable without change in growth rate (El Ghachi et al., 2005; Touze et al., 2008). This suggests that a single chromosomal copy of ***uppP***, pgpB or ybjG respectively in the mutants was enough for survival and sustenance of growth in E. coli (El Ghachi et al., 2005).

However, as described previously, the active sites of YbjG and PgpB face the periplasm and thus are implicated in recycling. The growth and survival of the ***E. coli*** cells in the absence of UppP (presumably functioning in ***de novo*** synthesis) and only a single chromosomal copy of either YbjG or PgpB implicated only in recycling requires an explanation. It may be that it is not only UppP that functions as Und-pp phosphatase in ***de novo*** synthesis of Und-p. This is because the cell needs the production of Und-p ***de novo*** before recycling takes place. Figure 2 depicts the scenario.

In addition, the cell wall of ***E. coli ΔuppP*** mutant appeared intact as implied from the unchanged growth rate and sensitivity to antibiotics observed (El Ghachi et al., 2005). The cell walls of ***S. aureus*** and ***S. pneumoniae uppP*** mutants also appeared to remain intact as judged by flow cytometry, microscopy, unchanged growth rate and sensitivity to osmotic stress (Chalker et al., 2000). However, while the sensitivity of ***E. coli ΔuppP*** mutant to bacitracin and other antibiotics remained unchanged, there were 16 and 1,000 fold increase in sensitivities of ***S. aureus*** and ***S. pneumoniae uppP*** mutants respectively to bacitracin alone but not to other antibiotics including those targeting the cell wall or cell membrane such as vancomycin and polymyxin B (Leutgeb, 1969; Chalker et al., 2000).

For the hypersensitivities to bacitracin observed with ***S. aureus*** and ***S. pneumoniae ΔuppP*** mutants, the authors explained that Und-p is produced solely by dephosphorylation of Und-pp. Therefore, treatment with bacitracin sequestered the available Und-pp, accounting for the hypersensitivity to bacitracin (Chalkern et al., 2000). If Und-p is produced solely by Und-pp phosphatase, it is expected that the deletion of ***uppP*** would have been lethal due to the absence of Und-p produced ***de novo***, since ***uppP*** is present as a single copy in these bacteria. And if other phosphatases perform the function of UppP, thus rescuing the cell from its deletion and ensuring the observed intact cell wall, there should be no change in sensitivity to any antibiotic (bacitracin inclusive) in these bacteria as observed in ***E. coli ΔuppP*** mutants. An explanation for the hypersensitivity to only bacitracin among other antibiotics by Gram-positive ***S. aureus*** and ***S. pneumoniae ΔuppP*** mutants will fill a knowledge gap that may be existing in bacterial cell wall biosynthesis.

It is reasonable to hypothesize that there should be a bypass pathway that does not require dephosphorylation of Und-pp that functions to provide the essential lipid carrier (Und-p), in Gram-positive bacteria. The pathway may account for the intact cell walls of ***S. aureus*** and ***S. pneumoniae ΔuppP*** mutants and the observed hypersensitivity to only bacitracin among others. It can also be hypothesized that if the active site of UppP faces the cytoplasm as widely speculated, other unknown

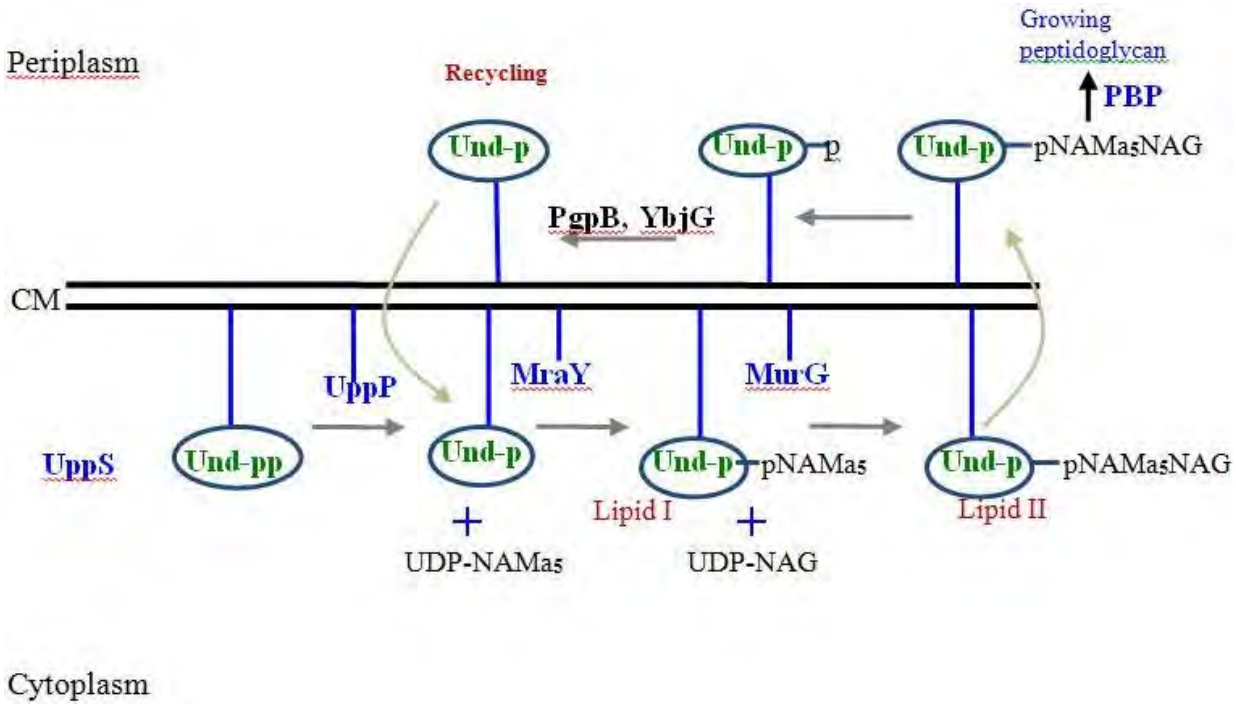

Figure 2. This diagram illustrates ***de novo*** synthesis and recycling of Und-p typified by peptidoglycan synthesis.

phosphatases with active sites facing the cytoplasm functions in addition to UppP in ***de novo*** synthesis of Und-p.

Contribution of undecaprenol kinase to the pool of the lipid carrier

The hypersensitivity of the ***S. pneumoniae*** and ***S. aureus uppP*** mutants to only bacitracin (Chalker et al., 2000) despite intact cell walls can be explained if the Und-p needed for survival is generated by other enzymes apart from UppP in these bacteria**.** However, in the presence of bacitracin, these other enzymes could not compete effectively with bacitracin for Und-pp to generate Und-p and thus could not confer resistance. This other enzyme is likely involved in the conversion of undecaprenol (Und-OH) to Und-p by a kinase.

The presence of abundant Und-OH had long been detected in the membranes of some Gram-positive bacteria such as in ***S. aureus*** and ***S. pyogenes*** (Strominger et al., 1970), ***Enterococcus faecalis***, ***Lactobacillus plantarum*** (Thorne and Kodicek, 1966; Gough et al., 1970; Thorne, 1973) and ***Listeria monocytogenes*** (Vilim et al., 1973). Recently, the amount of Und-OH in the cell wall of ***S. aureus*** was estimated to be 70 $nmolg^{-1}$ of cell dry weight. It was also reported to be the predominant form of undecaprenoids in this bacterium. In contrast, Und-OH was not detectable in the membrane extracts of ***E. coli*** (Barreteau et al., 2009).

The conversion of Und-OH to Und-p has been reported in membrane extracts of Gram-positive bacteria: ***S. aureus*** and ***L. plantarum*** (Higashi et al., 1970; Kalin and Allen, 1979). In addition, rather than phosphorylating diacylglycerol, homologue of ***E. coli*** diacylglycerol kinase, DgkA of ***B. subtillis*** functions as undecaprenol kinase while its sequence and conserved functional residues have a high degree of similarities with DgkA of ***S. aureus***, ***S. mutans*** and ***S. pneumoniae*** (Jerga et al., 2007). A higher efficiency of phosphate group transfer to Und-OH compared to diacylglcerol was also observed with DgkA homologue of ***S. mutans*** (Lis and Kuramitsu, 2003; Shibata et al., 2009). These suggest that DgkA of Gram-positive bacteria converts Und-OH to Und-p. Phylogenetic tree presented in Figure 3 shows a clear distinction of DgkA homologues in representative Gram-positive and Gram-negative bacteria and agrees with sequence alignment reported by Jerga et al. (2007). It may therefore be possible that DgkA homologues in Gram-positive bacteria are the Und-OH kinases contributing to ***de novo*** synthesis of Und-p.

Supporting the involvement of undecaprenol kinase in the provision of Und-p is the finding that ***S. mutans dgkA*** mutant was found to exhibit increased sensitivity to bacitracin, reduced virulence and stress resistance than the parental strain (Shibata et al., 2009). The Und-p provided by DgkA might have ensured the translocation of cell wall intermediates thereby compensating the loss of UppP in the ***S. aureus*** and ***S. pneumoniae*** ***Δuppp*** mutants. However the phosphatases activity needed for the conferment of bacitracin resistance might have been lost following the deletion of ***uppP*** (Kanof, 1970; Stone and Strominger, 1971).

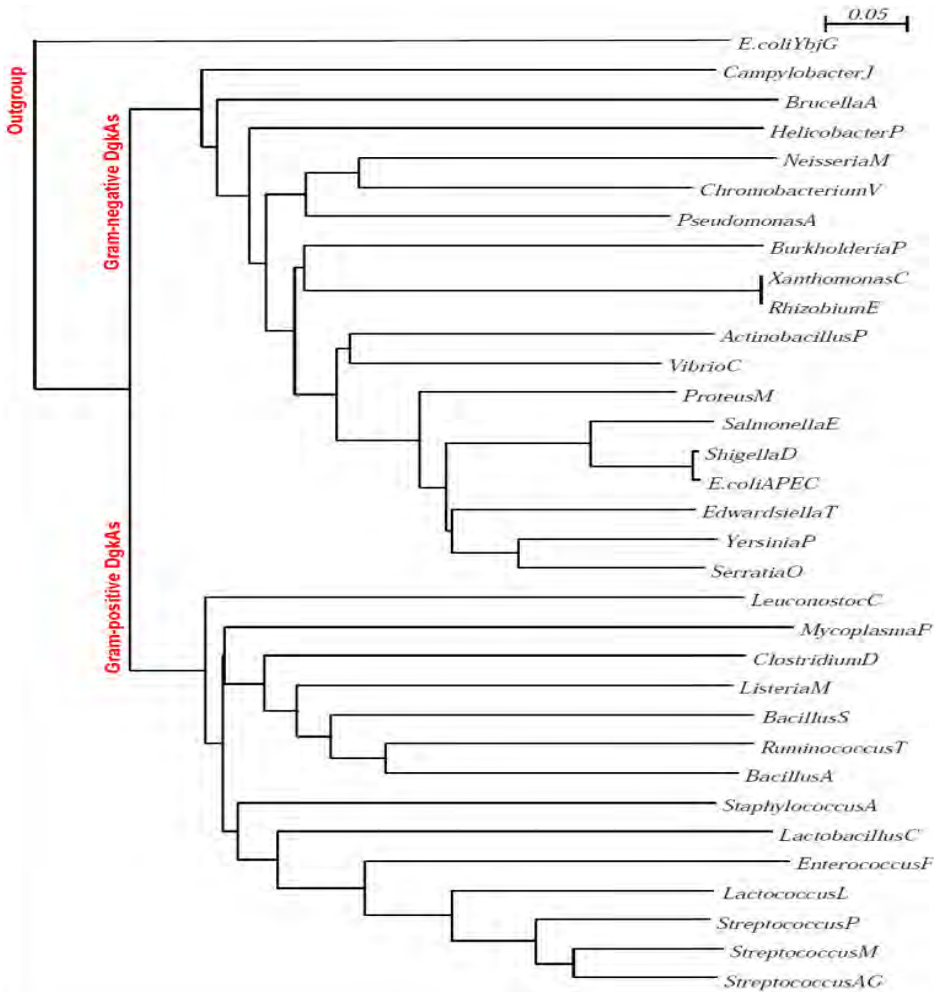

Figure 3. Phylogenetic tree (created using ClustalX 2.0.12 with default parameters) of DgkAs in Gram-positive and Gram-negative bacteria. ***E.coli*** YbjG (Und-pp phosphatases) is used as an outgroup. ***LactococcusL*** (***Lactococcus lactis***), ***EnterococcusF*** (***Enterococcus faecium), ClostridiumD (Clostridium difficile), StaphylococcusA (Staphylococcus aureus), BacillusA (Bacillus anthracis),StreptococcusAG (S agalactiae), ListeriaM (Listeria monocytogenes), StreptococcusP (S pneumonia), StreptococcusM (S. mutans),BacillusS (Bacillus subtillis), RuminococcusT (Ruminicoccus torques), LactobacillusC (Lactococcus casei), LeuconostocC (L. citreum), MycoplasmaP (Mycoplasma penetrans), SerratiaO (Serratia odorifera), E.coliAPEC (E.coli APEC), BrucellaA (Brucella abortus),YersiniaP (Yersinia pestis), SalmonellaE (Salmonella enterica), ShigellaD (Shigella dysenteriae), CampylobacterJ (Campylobacter jejuni), BurkholderiaP (Burkholderia pseudomallei), RhizobiumE (Rhizobium etli), HelicobacterP (Helicobacter pylori), VibrioC (Vibrio cholera), ProteusM (Proteus mirabilis),SynechococcusSP (Synechococcus sp.), EdwardsiellaT (Edwardsiella tarda), ActinobacillusP (Actinobacillus pleuropneumoniae), PseudomonasA (Pseudomonas aeruginosa), ChromobacteriumV (Chromobacterium violaceum), XanthomonasC (Xanthomonas campestris), NeisseriaM (Neisseria meningitides).*** The tree reveals a clear distinction between the DgkAs of the two groups of bacteria . This supports that, a different role for DgkA in Gram-positive bacteria may not limited to ***S. mutans*** and ***B. subtilis*** in which it has been characterized

Figure 4 gives a pictorial view of possible involvement of an Und-OH (DgkA) when ***uppP*** is deleted in ***S. aureus*** and ***S. pneumoniae***.

The origin of Und-OH in Gram-positive bacteria is yet unclear. The only known pathway in the generation of bacteria undecaprenoids is through the essential UppS enzyme whose product is Und-pp as shown in Figure 1. This suggests that Und-OH might have been generated from Und-pp or Und-p. Und-OH, originally absent, was detected in ***E. coli*** membrane extracts only after treatment

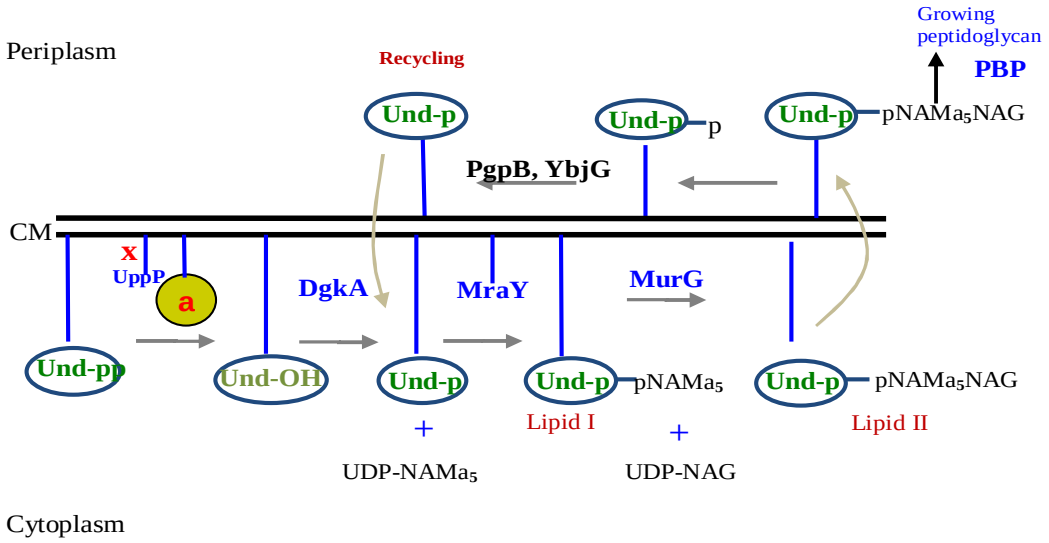

Figure 4. Scheme for a bypass pathway when ***uppP*** is deleted (depicted by red 'X') in ***S. aureus*** and ***S. pneumoniae***. Contribution of undecaprenol kinase (DgkA) to ***de novo*** pool of Und-p in Gram-positive bacteria typified by peptidoglycan synthesis. a?: an unknown phosphatase functioning in the synthesis of Und-OH which will then be phosphorylated into functional Und-p by DgkA. CM: inner cell membrane, Und-OH: Undecaprenol , PgpB ,YbjG: recycling enzymes, UppS: Und-pp synthase ,UppP: Und-pp phosphatase in ***de novo*** synthesis, Und-p: undecaprenyl phosphate, Und-pp: undecaprenyl pyrophosphate, NAG: N-acetyl glucosamine, $NAMa_5$:N-acetyl muramic acid pentapeptide. MraY: phospho-N-acetylmuramoyl-pentapeptide transferase, MurG: Glycosyl transferase. Lipid I: Und-p-p- $NAMa_5$, Lipid II: Und-p-p- $NAMa_5$NAG.

with colicin M which degrades both lipid I (Und-p-$pNAMa_5$) and lipid II (Und-p-$pNAMa_5$NAG) (El Ghachi et al., 2006; Barreteau et al., 2009). This supports the notion that Und-OH is formed from either Und-pp or Und-p and that, the enzymes that catalyses this reaction may be present in Gram-positive bacteria but absent in Gram-negative bacteria. The discovery of enzymes playing this role designated at ***a*** in Figure 4 will thus be highly informative. Characterization of DgkA homologues of ***S. aureus*** and ***S. pneumoniae*** as possible Und-OH kinases and coinactivation of ***dgkA*** and ***uppP*** in Gram-positive bacteria will help in clarifying the contribution of DgkA to cell wall synthesis in these bacteria.

Unknown undecaprenyl pyrophosphate phosphatase in *de novo* synthesis of undecaprenyl phosphate in bacteria

The essentiality of undecaprenyl pyrophosphate synthase (UppS) which is committed to the synthesis of Und-pp (Keenan and Allen, 1974; Shimizu et al., 1998; Apfel et al., 1999), coupled with absence of Und-OH in ***E. coli*** (Barreteau et al., 2009) brings to mind that the pathway of dephosphorylating Und-pp into Und-p is important in this bacterium. Essentially, the survival of ***E. coli*** cells with single copy of either YbjG or PgpB (recycling) in the absence of UppP (***de novo*** synthesis) suggests the presence of yet to be identified phosphatases functioning ***de novo*** together with PgpB or YbjG to ensure survival.

Computational analysis shows that, while UppS is present in ***Treponema pallidum*** and ***Rickettsia sp*** as in other cell wall containing bacteria, UppP which performs the immediate downstream action is absent. Since UppP is absent, the dephosphorylation of Und-pp into functional Und-p may be performed by other enzymes in ***T. pallidum*** and ***Rickettsia sp*** if the active sites of YbjG or PgpB present in these bacteria are in the periplasmic space as demonstrated in ***E. coli***.

Additionally, the subinhibitory concentration of fosmidomycin (Figure 1) partially impaired the activity of deoxy-xylulose-5-phosphate reductase (an enzyme functioning upstream of Und-pp synthesis), thereby reducing the amount of Und-pp formed de novo. ***E. coli mutant*** with simultaneous deletion of ***uppP*** and ***pgpB*** exhibited 54% survival as compared to 73% survival observed with wild type (Tatar et al., 2007). Owing to the absence of ***uppP*** in the mutant, there should be another enzyme that functions in ***de novo*** dephosphorylation of the residual Und-pp left after fosmidomycin treatment. This other phosphatase coupled with the recycling by enzymes like YbjG and LpxT may account for such a relatively high survival of the Δ***uppP***Δ***pgpB*** mutant compared to wild type.

However, the lethality of ***E. coli*** triple mutant (***ΔuppPΔybjGΔpgpB***) indicates that the contribution of the proposed alternative Und-pp phosphatases (still present in the triple mutant) may not be enough to sustain cell growth without efficient recycling. This needed recycling might

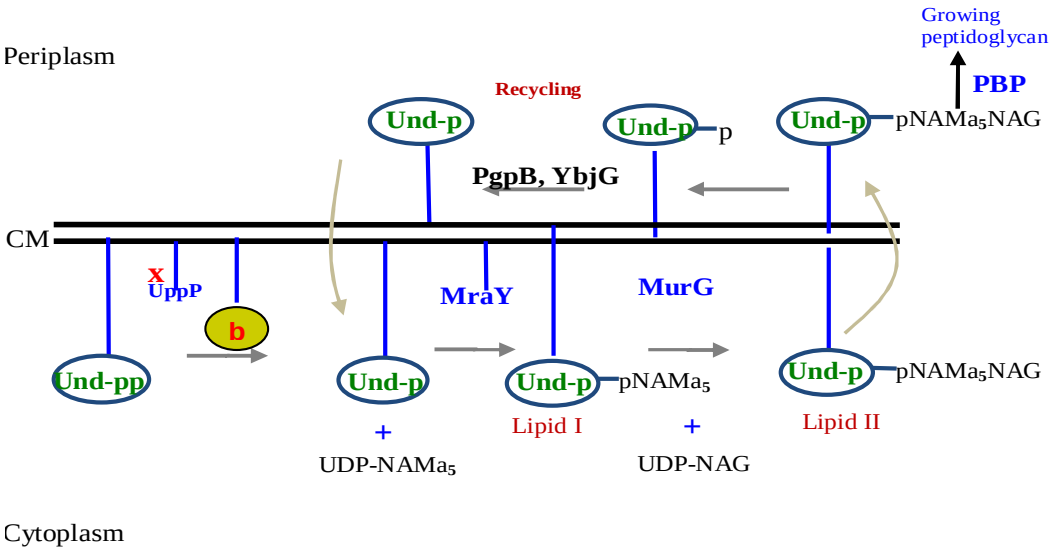

Figure 5. Probable contribution of other Und-pp phosphatases to the ***de novo*** pool of Und-p in ***E. coli*** when uppP is deleted (depicted by red'X') typified by peptidoglycan synthesis. **b**: Unknown Und-pp phosphatases in the generation of Und-p. CM: inner cell membrane, Und-OH: Undecaprenol , PgpB ,YbjG: recycling enzymes, UppS: Und-pp synthase, UppP: Und-pp phosphatase in ***de novo*** synthesis, Und-p: undecaprenyl phosphate, Und-pp: undecaprenyl pyrophosphate, NAG: N-acetyl glucosamine, $NAMa_5$:N-acetyl muramic acid pentapeptide. MraY: phospho-N-acetylmuramoyl-pentapeptide transferase, MurG: Glycosyl transferase. Lipid I: Und-p-p- $NAMa_5$, Lipid II: Und-p-p- $NAMa_5$,NAG.

have been provided by the PgpB or YbjG in the viable ***E. coli ΔuppPΔybjG, ΔuppPΔpgpB*** mutants respectively**.** It is therefore proposed that, in the absence of UppP, the unknown Und-pp phosphatases dephosphorylate the Und-pp produced by UppS ***de novo*** to provide the needed Und-p. The yet to be identified Und-pp phosphatases may account for the intact cell wall of ***E. coli ΔuppP*** mutants.

Figure 5 depicts the probable involvement of the unknown phosphatases in ***de novo*** synthesis of Und-p in the absence of UppP in ***E.coli***.

Possible involvement of UppP in vaccine or drug target screening

In addition to its clear role in cell wall synthesis, homologues of UppP in different bacteria have been implicated in other functions such as virulence, antibiotic resistance and biofilm formation (Chalker et al., 2000; Rose et al., 2004; Touze et al., 2008; Hynninen et al., 2009; Vandal et al., 2009). As such its involvement in the development of vaccine and in drug screening should be considered.

Role in virulence

While there was no change in cell wall integrity, the ***S. aureus*** and ***S. pneumoniae uppP*** mutants were shown to be attenuated in mouse model of infection (Chalker et al., 2000). ***S. pneumoniae uppP*** mutant displayed 4.6 log10 attenuation in comparison to wild type, a value approaching the maximum attenuation of 6 logs earlier detected in ***S. pneumoniae*** respiratory tract infection model (Chalker et al., 2000). Similar observation was reported in H37Rv, the pathogenic strain of ***M. tuberculosis*** (Rose et al., 2004). ***M. tuberculosis*** (H37Rv) Rv2136c mutant exhibited reduced virulence in immunocompromised $Rag1^{-/-}$ mice. The median survival of mice infected with H37Rv wild type was 26 days while that of the mice infected with the mutant was as long as 70 days . Similarly, $IFN\gamma^{-/-}$ mice were less susceptible to the mutant strain (median survival of 80 days) than to the wild type (median survival of 30 days) (Rose et al., 2004).

In a related work, it was shown that H37Rv, with a single transposon disruptive insertion in Rv2136c did not exhibit any difference in morphology as depicted with scanning and transmission electron microscope (Vandal et al., 2009). However, the mutant was shown to be 11 fold and 19 fold more susceptible to heat and hydrogen peroxide respectively than the wild type. Severe attenuation was also observed ***in vivo*** as demonstrated by complete clearance of the bacteria after 56 days post infection (Vandal et al., 2009). Although ***Mycobacterium bovis*** (BCG) is an attenuated strain, faster clearance of the mutant than the wild type in the mouse model of infection suggests the contribution of the gene to survival in host cells (Rose et al., 2004).

As also stated by (Chalker et al., 2000), these findings thus suggest that, although other enzymes compensates

for the loss of ***uppP*** homologues in these bacteria in ensuring intact cell walls, they could not compensate for its probable involvement in virulence.

Role in antibiotic resistance

M. ***tuberculosis*** mutant with transposon insertion in Rv2136c shows 32 fold, 8 fold and 2 fold increased susceptibility erythromycin, rifampin and streptomycin respectively (Vandal et al., 2009). The association of UppP homologues and PAP2 enzymes with bacitracin resistance has also been reported largely in bacteria such as ***E. coli***, ***Bacillus sp***, ***Streptococcus sp***., and ***S. aureus*** among others (Chalker et al., 2000; El Ghachi et al., 2004; Rose et al., 2004; El Ghachi et al., 2005). Although no increased susceptibility to other antibiotics investigated was observed with ***uppP*** null mutants of ***S. aureus*** and ***S. pneumoniae*** (Chalker et al., 2000), further studies are needed to elucidate the involvement of UppP in antibiotic resistance in other bacteria.

Coupled with its role in virulence and antibiotic resistance, the absence of UppP in eukaryotic cells makes it an attractive potential antimicrobial drug target. Drugs targeting UppP may perform synergistic functions.

A major mechanism of ***M. tuberculosis*** resistance to antibiotics is the complex cell wall (Zhang and Yew 2009). For example, fosmidomycin successfully inhibited the essential mycobacterial deoxy-xylulose-5-phosphate reductase (Figure 1) ***in vitro*** but was reportedly inactive against whole ***M. tuberculosis*** cells. The resistance observed was attributed to lack of antibiotic uptake (Dhiman et al., 2005; Henriksson et al., 2007; Brown and Parish, 2008). In essence, focusing on proteins involved in cell wall synthesis as drug target in ***M. tuberculosis*** is still an important area demanding attention. In support of possible synergistic role, it was earlier reported that sub-inhibitory concentration of bacitracin supposedly inhibiting the function of Rv2136c resulted in about tenfold increase in susceptibility of ***M. tuberculosis*** to clarithromycin, an antibiotic that had been classified as ineffective against ***M. tuberculosis*** (Bosne-David et al., 2000). All possible drug targets (Eoh et al., 2009) including Rv2136c should therefore be evaluated in this bacteria. Such drugs can synergize the currently used drugs by reducing the number of drugs required to treat the disease or shorten the required length of time of drug usage.

Similarly, increasing resistance of ***S. pneumoniae*** to commonly used antibiotics including macrolides and penicillins has been reported (Chalker et al., 2000; Ortqvist et al., 2005). Drugs that can target UppP homologue of ***S. pneumoniae*** thus weakening the cells as observed by reduced virulence of ***S. pneumoniae*** ∆***uppP*** mutant may provide a synergistic effect thus improving the efficacy of compounds currently used to combat the bacteria.

CONCLUSION AND OPEN RESEARCH ISSUES

In addition to the lack of outer cell membrane, the hypersensitivity of ***S. aureus*** and ***S. pneumoniae*** ∆***uppP*** mutants to only bacitracin among other antibiotics may be attributed to the lack of Und-pp phosphatase activity required to compete with bacitracin for resistance. The intact cell wall observed despite deletion of ***uppP*** homologue was also explained to be most probably due to the presence of Und-OH kinase that generates the essential Und-p alternatively from Und-OH. Investigation into the enzymes involved in the generation of Und-OH from either Und-p or Und-pp in Gram-positive bacteria will be informative as the conversion of Und-OH to the essential Und-p appears to play a significant role in the biosynthesis of cell wall components.

De novo synthesis of Und-p was also suggested to involve other yet to be identified enzymes with active sites located in the cytoplasm. Discovery of this proposed enzyme will give an improved picture of the enzymes playing role in cell wall biosynthesis as well as adaptation of the bacteria to varying environment such as exposure to antibiotics. Additionally, there is need for experimental evidence to ascertain the cytoplasmic location of the conserved loop of UppP in bacteria and thus, its involvement in ***de novo*** synthesis of Und-p.

The potential use of UppP homologues as well as DgkA homologues of Gram-positive bacteria as drug targets in synergy with other currently used but ineffective antibiotics in the treatment of resistant pathogenic bacteria also warrants further investigations. Importantly, the study of bacterial isoprenoids metabolism has only been done in few representative bacteria, studies in more Gram-positive and Gram-negative bacteria are needed for better understanding of cell wall synthesis.

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Review

Advances in microbial heterologous production of flavonoids

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Flavonoids are important plant specific secondary metabolites that are synthesized from the general phenylpropanoid pathway and have many pharmaceutical and nutraceutical functions. Separation of pure flavonoids from plants material is very difficult and chemical syntheses of flavonoids require extreme reaction conditions and toxic chemicals. However, in the past decades, engineered microbes are becoming increasingly important as recombinant production platforms. Cultivation of the recombinant strains in rich medium offers another choice for the production of flavonoids. In the present review, we cover the main achievements that the multi-gene pathway of phenylpropanoid is introduced as microorganisms to heterologously produce flavonoids, analyze the key factors affecting heterologous production of flavonoids in microbes, and discuss the new research prospect.

Key words: Flavonoids, metabolic engineering, natural products, synthetic biology.

INTRODUCTION

Flavonoids, containing a 15-carbon phenylpropanoid core (Figure 1), are plant secondary metabolites that occur in a large variety of plants, fruits and vegetables (Turnbull et al., 2004). Flavonoids have significant properties and applications, such as antioxidant activity that confers beneficial effects on coronary heart disease, cancer, and allergies (Shaik et al., 2006; Steinmetz and Potter, 1996). Flavonoid-derived compounds have drawn much attention from scientists and researchers, who are keen on their native nutraceutical properties or who use them as starting formulations for market pharmaceuticals (Fowler and Koffas, 2009).

Till date, the low yield of flavonoids yet remains a bottleneck for its large scale applications. When these compounds are separated from plant materials, the availability of plant flavonoids is first limited by seasonal and regional variations. Moreover, it continues to be a major challenge for separating pure flavonoid compounds from plants material because of the low concentrations of certain flavonoids and numerous similar natural products in plants (Du et al., 2010).

In addition, the chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals (Park et al., 2009). To date, mass production of flavonoids from plant cell or tissue cultures have been reported for a few species, while the economic feasibility has not been established due to engineering challenges in large-scale cultivation and difficulties to maintain the cell viability during long period (Kobayashi et al., 1993; Zhong et al., 1991).

Combinatorial biosynthesis is a new tool for production of rare and expensive natural products (Chang and Keasling, 2006; Hutchinson, 1994), and can be used in both simple and complex transformations without the tedious blocking and deblocking steps that are common in enantio and regioselective organic synthesis (Wang et al., 2010). These methods in flavonoids biosynthetic pathway are attractive targets for metabolic engineering processes to enhance the production of flavonoids.

This article reviews the main works done on the microbial synthetic flavonoids in the decades, including the optimization of synthetic route, carrier, and culture conditions, the selection of strains, and synthetic biology

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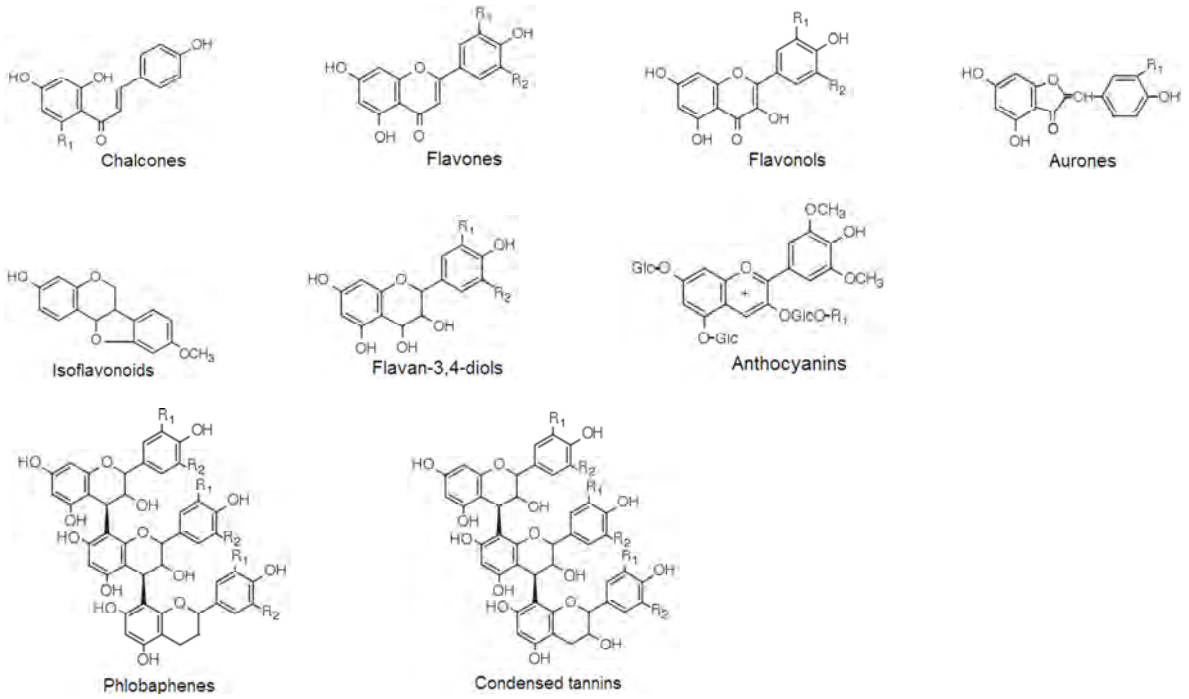

Figure 1. The biosynthesis of nine major classes of flavonoid derivatives starting with general phenylpropanoid metabolism. R_1= H or OH; R_2= H or OH.

used for production of flavonoids is also discussed.

Synthetic pathway and diversity of flavonoids

In the plant phenylpropanoid pathway (Figure 2), phenylalanine ammonia lyase (PAL) is the first enzyme in the general phenylpropanoid pathway, which catalyzes phenylalanine to yield cinnamic acid; Cinnamic acid is hydroxylated by the action of cinnamate-4-hydroxylase (C4H) to 4-coumaric acid. Then 4-coumaric acid is activated to 4-coumaroyl-CoA by 4-coumarate:coenzyme A ligase (4CL); Chalcone synthase(CHS) is a plant-specific polyketide synthase that uses a starter CoA-ester, which catalyzes the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaroyl-CoA to yield naringenin chalcone, the precursor of a large number of flavonoids. In the last stages of the biosynthesis of flavonoids, naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) (Weisshaar and Jenkins, 1998).

Compared with the plant pathway, the heterologous expression of phenylpropanoid biosynthetic pathway (Figure 3) has many characteristics as follows: the PAL from ***Rhodotorula rubra*** can use both phenylalanine and tyrosine as substrates to synthesize flavonoids, which increases the selectivity of substrates (Kyndt et al., 2002; Rosler et al., 1997; Scott et al., 1992). Simultaneously, the combinatorial biosynthesis of flavonoids bypasses the C4H step. Because the C4H is a membrane-bound cytochrome P-450 hydroxylase, its activation requires molecular oxygen and a reducing equivalent from NADPH delivered via cytochrome P-450 reductase (Hotze et al., 1995; Pompon et al., 1996). Some microorganisms lack the cytochrome P-450 hydroxylase.

Additionally, some researchers reported the ScCCL that encodes a cinnamate/coumarate: CoA ligase from the actinomycete ***Streptomyces coelicolor A3(2)***, which can directly attach CoA to both cinnamic acid and 4-coumaric acid (Hwang et al., 2003). By increasing the selectivity of substrates and shortening the reaction steps, the heterologous large-scale production of targeted flavonoids has been improved. These strategies also provide reference for the heterologous synthesis of other natural products in microorganisms.

Based on the synthesis of the precursor of flavonoids in the above flavonoids synthetic route, the structural diversities of flavonoids could originate from various modification reactions. Typical flavonoids modification reactions are mediated by O-methyltransferases (OMT), glycosyltransferases (GT), and cytochrome P450 etc (Ibrahim et al., 1998; Kim et al., 2006b; Schuler and Werck-Reichhart, 2003; Winkel-Shirley, 2001). Kim et al., 2006a) have reported the characterization and expression of SaOMT-2 from ***Streptomyces avermitilis*** MA-4680; SaOMT-2 transfers the methyl onto the 7- hydroxyl group of isoflavones, daidzein and genistein, and the flavones, kaempferol, apigenin, and quercetin, as well as the flavanone naringenin, which make various compounds only by means of using one gene.

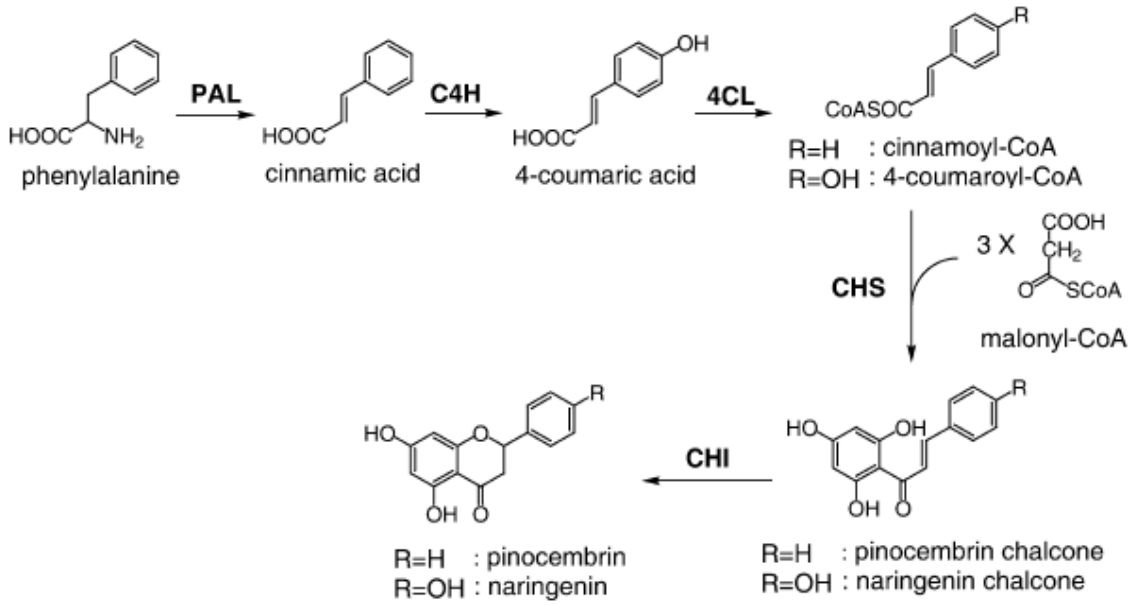

Figure 2. The schematic in the complete synthetic pathway of flavonoids in plant. Enzyme names are abbreviated as follows: Phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumaroyl : CoA-ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI).

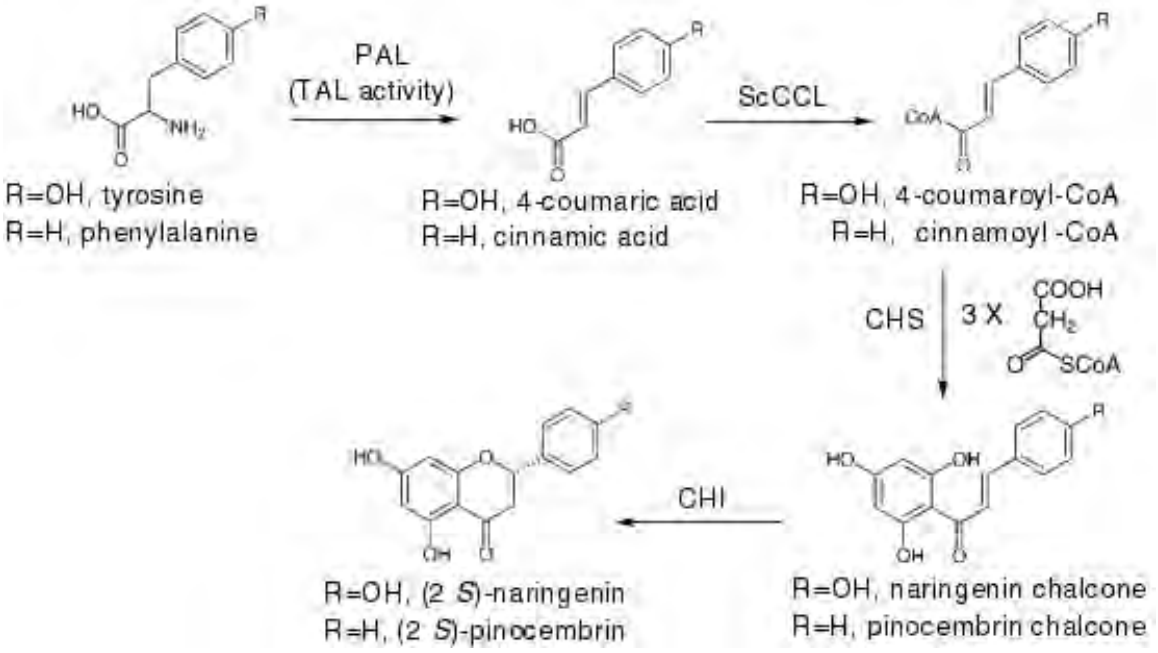

Figure 3. The heterologous biosynthetic flavonoids pathway in microorganism. PAL can also use tyrosine as a substrate; ScCCL attaches CoA to both cinnamic acid and 4-coumaric acid at the same efficiency.

In a previous study, genes representing enzymes of flavonoids pathway were individuallly cloned into yeast expression cassettes, then these cassettes were randomly combined on yeast artificial chromosomes. So these new combinatorial chromosomes create a variety of flavonoids producing pathways in a single transformation of yeast, which also presents a novel strategy for synthesis of diverse flavonoids ***in vivo*** (Michael et al., 2009). Only recently, construction of a multiplasmid approach for producing unnatural plant polyketides in ***Escherichia coli*** is very useful; incubation of the recombinant ***E. coli*** with exogenously supplied carboxylic

acids led to production of 36 unnatural fl vonoids and stilbenes (Katsuyama et al., 2007b).

Strains

Escherichia coli

E. coli is widely used as model systems and considered as the primary prokaryotic host for the expression of heterologous genes due to its extensive genetic characterization (Krings and Berger, 1998). Moreover, most of its biological processes are well understood and there are extensive genetic tools readily available for its gene manipulation (Rodriguez et al., 2003). It is also firstly chosen as host and to heterologously produce the flavonoids by designing and constructing the artificial phenylpropanoid biosynthetic pathways.

Now about 0.75 mg/L of pinocembrin and 0.45 mg/L of naringenin could be produced with ***E. coli*** (Hwang et al., 2003). However, the yields of fl vonoids were too low for large-scale production. This possibly resulted from the inefficient carbon fl x from glucose, the amino acid precursors toward the phenylpropanoid biosynthetic pathway and the low amount of malonyl-CoA in ***E. coli*** cell.

Saccharomyces cerevisiae

Jiang et al. (2005) chose ***S. cerevisiae*** as the eukaryotic heterologous host to successfully produce the flavonoids after Ro and Douglas began to reconstitute the early steps of the phenylpropanoid pathway in ***S. cerevisiae*** (Ro and Douglas, 2004). In the ***S. cerevisiae*** AH22 strain that coexpressed PAL, 4CL, and CHS, approximately 7 mg/L of naringenin and 0.8 mg/L of pinocembrin could be produced. The yield in ***S. cerevisiae*** was higher than in ***E. coli*** which the phenylpropanoid pathway was firstly chosen to express.

The key factor is that ***S. cerevisiae*** has some advantages over ***E. coli*** for expressing certain eukaryotic heterologous proteins. Yeast system is not only capable of performing posttranslational modifications of the eukaryotic proteins but also has many similar intracellular compartments to plant cells. In addition, yeast has been shown to be an excellent host for CYP activity ***in vivo*** (Bayoumi et al., 2008; Humphreys et al., 1999; Jiang and Morgan, 2004; Pompon et al., 1996; Szczebara et al., 2003).

Other strains

Streptomyces venezuelae has a rapid growth, relative ease of genetic manipulation, abundant supply of substrates (Jung et al., 2006; Park et al., 2008; Yoon et al., 2002) and produces a wide range of important secondary metabolites (Pfeifer and Khosla, 2001), so it is also used as a robust hoterologous host for plant flavonoids production (Table 1).

Phellinus igniarius is a medicinal mushroom containing many bioactive compounds, and is viewed as a attractive alternative for the efficient production of secondary metabolites (Zhong, 2005). Zhu et al. (2010) have constructed an expression vector containing ***Vitreoscilla*** hemoglobin gene, which supplies more oxygen for the aerobic organisms growth, for the fi st successful and signific nt heterologous production of fl vonoids in ***P. igniarius*** (Table 1).

Molecular biology technology used in the heterologous production of flavonoids

Knockout of related genes

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It is reported that, using the λ Red Recombinase/FLP system to knock out the udg gene encoding for UDP-glucose dehydrogenase, the endogenous UDP-glucose consumption pathway could be eliminated (Leonard et al., 2008). By the above molecular biological technology, UDP-glucose intracellular concentration was extremely improved, which resulted in the increment of the production of flavanones and anthocyanins to 700 and 113 mg/L, respectively.

Combination of promoter and target genes

The promoter often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used in the the synthesis of flavonoids such as T7 promoter (Kaneko et al., 2003), ermE* promoter (Park et al., 2009), GAL1 promoter (Yan et al., 2005) (Figure 4). It needs to choose the suitable promoter according to the specificity of host. In addition, every gene from the phenylpropanoid pathway is cloned under the control of the promoter, especially in ***E. coli*** and ***S. venezuelae***. By employing a ribosomebinding sequence (rbs) in front of each gene, the yields of pinocembrin and naringenin could be enhanced strongly.

Over expression of malonyl-CoA

The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids (Davis et al., 2000). Through the coordinated overexpression of acetyl-CoA carboxylase genes from ***Photorhabdus luminescens***, Leonard et al. (2007) have augmented the intracellular malonyl-CoA pool and increased the production of pinocembrin, naringenin, and eriodictyol in 36 h up to 429, 119 and 52 mg/L, respectively. Moreover, the introduction of ***R. trifolii*** MatB and MatC genes allowed for the

Table 1. Heterologous production of flavonoids in various kinds of microbial.

End-product	Precursor molecule	Gene	Host organism	Level of production (mg/L)	Reference
Pinocembrin Naringenin	Phenylalanine Tyrosine	PAL, ScCCL, CHS, RBS	***E. coli***	0.75 0.45	(Hwang et al., 2003)
Naringenin	Tyrosine	TAL, 4CL, CHS	***E. coli***	20.8	(Watts et al., 2004)
Pinocembrin Naringenin	Phenylalanine Tyrosine	PAL, ScCCL, CHS, CHI, ACC	***E. coli***	58 57	(Miyahisa et al., 2005)
Pinocembrin Naringenin	Phenylalanine Tyrosine	PAL, 4CL, CHS	***S. cerevisiae***	0.8 7	(Jiang et al., 2005)
Apigenin Luteolin Genkwanin	Phenylpropanoid acids	4CL, CHI, CHS, FSI, OMT	***E. coli***	0.415 0.01 0.208	(Leonard et al., 2006b)
Pinocembrin Naringenin Eriodictyol	Cinnamic acid ***p***-Coumaric acid Caffeic acid	C4H, 4CL, CHI, CHS	***S. cerevisiae***	16.3 28.3 6.5	(Yan et al., 2005)
Naringenin Dihydrokaem-pferol Kaempferol	***p***-Coumaric acid	4CL, CHS, CHI, FHT, FLS	***E. coli***	2.4 2.4 0.3	(Leonard et al., 2006a)
Chrysin Galangin Apigenin Kaempferol	Phenylalanine Tyrosine 	pET-PT7-4GS(05,3) ACC, FNSI, F3H, FLS	***E. coli***	9.4 1.1 13.0 15.1	(Miyahisa et al., 2006)
Genistein	Tyrosine	PAL, ScCCL, CHS, CHI, IFS	***E. coli*** and ***S. cerevisiae*** cells	6	(Katsuyama et al., 2007a)
Flavanone Dihydroflavonol	***p***-coumaric acid	4CL, CHS, CHI, FHT	***S. cerevisiae***	60 62.8	(Chemler et al., 2007)
Flavanone Flavone Flavonol	***p-coumaric acid***	pCDF-LE4CL-1 pRSF-ACC pET-CHS/CHI pACYCDuet-1 pACYC-FNS pACYC-F3H/FLS	***E. coli***	87 84 33	(Katsuyama et al., 2007b)

Table 1. Contd.

Pinocembrin Naringenin Eriodictyol	Cinnamic acid ***p***-Coumaric acid Caffeic acid	4CL, CHS, CHI, ACC, biotin ligase	***E. coli***	429 119 52	(Leonard et al., 2007)
Pinocembrin Naringenin Eriodictyol	Cinnamic acid ***p***-Coumaric acid Caffeic acid	4CL, CHS, CHI	***E. coli***	710 186 54	(Leonard et al., 2008)
Pinocembrin Narigenin	4-coumaric acid or cinnamic acid	ScCCL , CHS, CHI	***S. venezuelae***	6.0 4.0	(Park et al., 2009)
Naringenin Genistein Kaempferol Quercetin	(***p***-coumaric acid, Phenylalanine) (Naringenin, ***p***-coumaric acid, Phenylalanine)	pESC-URA-PAL-C4H pESC-HIS-4CL pESC-LEU-CHS-CHI pESC-TRP-CPR pESC-HIS-4CL-IFS pESC-HIS-4CL-FLS pESC-TRP-F3H-CPR pESC-TRP-F3H-F30H	***S. cerevisiae***	15.6, 8.9 7.7, 0.14, 0.1 4.6, 0.9, 1.3 0.38,0.26,ND	(Trantas et al., 2009)
Naringenin Eriodictyol	phenylpropanoic acid		***E. coli***	270 150	(Fowler et al., 2009)
Flavones Exopolysaccharides	ND_1	ND_2	***P. igniarius***	11.43 1.33	(Zhu et al., 2010)

ND indicates the compound was not found after extraction.
ND_1 indicates that there is no precursor molecule.
ND_2 indicates that there is not an artificial biosynthetic gene cluster of flavonoids in the ***P. igniarius***.

assimilation of an exogenous carbon source to directly synthesize malonyl-CoA. This strategy bypassed the natural metabolism of malonyl-CoA from glucose, and directly increased the production of malonyl- CoA (Leonard et al., 2008).

Construction of artificial P450 enzymes

The membrane-bound cytochrome P450 enzymes preclude the use of industrially relevant prokaryotes such as ***E. coli***. Koffas et al. have constructed an artificial plant cytochrome P450 enzyme for synthesis of isoflavones. The production catalyzed by P450 in vivo was improved to 20-fold higher than that achieved by the native enzyme expressed in a eukaryotic host and up to 10-fold higher than production by plants. It is an innovative method for the utilization of laboratory bacteria to robustly manufacture high-value plant P450 products (Leonard and Koffas, 2007).

Synthetic biology

Synthetic biology is a research field that designs a biological system which behaves predictably and functions superior to the natural counterpart by applying engineering tools (Liang et al., 2011). The practical applications of synthetic biology are in the

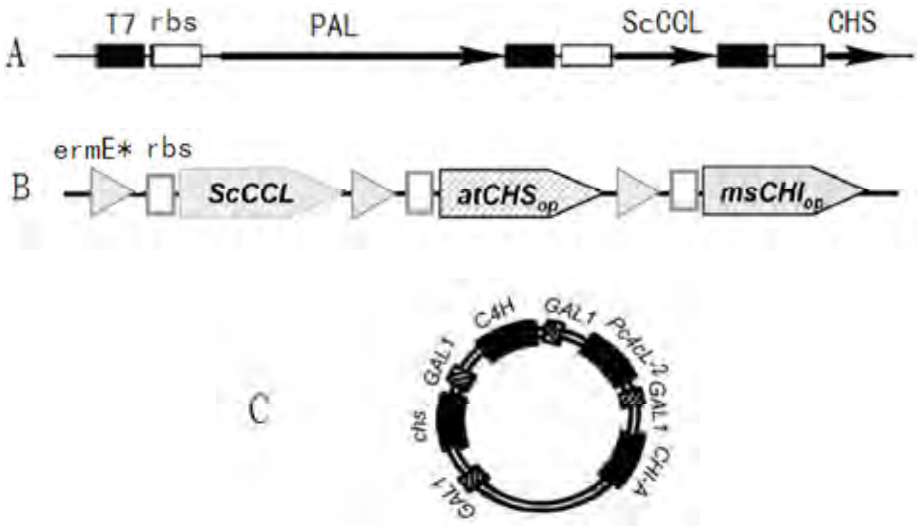

Figure 4. Schematic representation of the cloning strategy used for different assembling plasmid. A The PAL, ScCCL, CHS genes were placed under the control of the T7 promoter. B Construction of expression plasmids carrying flavanone biosynthetic genes and ermE*promoter. C The plasmids carrying four flavanone biosynthetic genes and GAL1 promoter.

areas of bioremediation (Gilbert et al., 2003), biosensing (Rajendran and Ellington, 2008), and biofuel production (Steen et al., 2008; Waks and Silver, 2009) and even with potential clinical applications (Anderson et al., 2006; Khosla and Keasling, 2003; Ro et al., 2006). Although we are still very far from rationally assembling a living cell from scratch, and far from understanding all of the design principles which biological networks operate (Mukherji and Van Oudenaarden, 2009), now this emerging novel technology has been explored for use in higher production of flavonoids by the following designs.

Minimum genome factory

The Ikeda team of Kitasato University has constructed a versatile model host for the heterologous expression of secondary metabolites. They deleted a region of more than 1.4 Mb nonessential genes from the 9.02-Mb industrial microorganism ***S. avermitilis*** linear chromosome, which generates a series of defi ed deletion mutants that do not produce any of the major endogenous secondary metabolites found in the parent strain (Komatsu et al., 2010). In addition, to create a “minimum genome factory” where some nonessential genes are deleted from the bacterial genomes is also a national project in Japan. Target microorganisms are ***B. subtilis, E. coli, C. glutamicum*** and ***yeasts*** which include ***S. cerevisiae*** and ***Schizosaccharomyces pombe*** (Horinouchi, 2008). Using these model hosts, it will be beneficial for the extraction and purification of targeted products from fermentation, and will lay the foundation for large-scale synthetic flavonoids.

Multivariate-modular pathway

Multivariate-modular pathway is an innovative approach and effective strategy for assembling modules into intricate and customizable larger scale systems (Purnick and Weiss, 2009). Ajikumar et al. (2010) have reported a multivariate-modular system, which partitioned the taxadiene metabolic pathway into two modules: a native upstream methylerythritol-phosphate pathway forming isopentenyl pyrophosphate and a heterologous down-stream terpenoid–forming pathway. By the approach, they succeeded in increasing titers of taxadiene. The pathway of flavonoids synthesis may also be divided into several modules, and people can explore which step is the limiting factor in metabolic pathways such as the supply of UDP-glucose. And then unlock the potential of the phenylpropanoid pathway for higher production of flavonoids natural products.

Conclusion

The above studies clearly show that the artifi ial gene cluster containing enzymes of flavonoids synthesis converts phenylalanine to pinocembrin and tyrosine to naringenin in microbial. This proves that ***E. coli, S. venezuelae***, ***P. igniarius*** and ***S. cerevisiae*** are excellent expression systems for reproducing the phenylpropanoid pathways of plant. To optimize the fl vonoid production, some researchers have been exploring new strategies such as looking for the best flavonoids synthase gene, choosing the suitable promoter, constructing new carbon flow etc.

Nevertheless, the heterologous production of flavonoids in microorganism usually requires transferring the multi-gene of the whole pathway into the host strain, which limits its large-scale production as well as its product. Moreover, development of efficient recombinant production platforms for natural product biosynthesis is often limited by the availability of precursors and cofactors derived from the host's native metabolism. Another difficulty must also be addressed: the conditions of strains growth, the toxic and concentration of products in fermentation, byproducts also must be considered.

With the progress of microbiology and enzyme engineering technology, multi-enzyme systems would be constructed ***in vitro*** which contain the main enzymes of phenylpropanoid pathway, and synthesize flavonoids. This will overcome some kinds of restrictions in micro-organism fermentation production, such as the content of oxygen for aerobic organisms' growth in the fermentation, the transport of flavonoids compounds through the membrane. In addition to using enzyme catalysis, it will be more efficient and fast to produce flavonoids, and it has a high fi al concentration in the reaction solution. Further-more, immobilization of multi- enzymes would decrease the difficulty in the separation of enzyme and reaction mixture

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Full Length Research Paper

A novel poly (L-lactide) degrading thermophilic actinomycetes, *Actinomadura keratinilytica* strain T16-1 and *pla* sequencing

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An actinomycete strain T16-1 demonstrated the maximum poly (L-Lactide)-degrading activity when cultured in basal liquid medium at 50°C. According to 16S rDNA sequence analysis, chemotaxonomic and DNA-DNA hybridization revealed that strain T16-1 belong to the family Thermomonosporaceae, genus *Actinomadura*. On the basis of phenotypic and phylogenic data, strain T16-1 which is a novel PLA-degrading thermophilic actinomycete was identified as *Actinomadura keratinilytica*, but the color of its colony on ISP plates, NaCl tolerant and utilization of mannitol, raffinose and arabinose were different. Partial sequence of poly (L-lactide) depolymerase gene from strain T16-1 was demonstrated. The gene consisting of 222 amino acids was related to serine protease from *Streptomyces* sp. with 43 to 46% identity.

Key words: ***Actinomadura keratinilytica,*** cloning, identification, poly (L-lactide).

INTRODUCTION

Abundant quantities of plastic wastes are serious problems for global environment and have stimulated the improvement of biodegradable plastics. Poly (L-lactide) (PLA) aliphatic polyester is synthesized from L-lactic acid, which can be produced from farm and agricultural products such as cassava, rice, corn and corncob by fermentation methods (Miura et al., 2004; Wee et al., 2006). Recently, there has been interest in using plastic composting by microbes as a method for treating plastic waste (Tomita et al., 2003).

Several thermophilic bacteria such as ***Bacillus brevis***, ***Bacillus stearothermophilus and Geobacillus thermocatenulatus*** have been reported to possess PLA-degrading activity (Tomita et al., 1999, 2003, 2004). Some actinomycetes, belonging to members of the family Pseudonocardiaceae were also able to produce enzymes that decompose PLA, such as ***Amycolatopsis*** sp., ***Lenzea waywayandensis*** and ***Kibdelosporangium aridum*** (Jarerat et al., 2002). Recently, PLA-degrading enzyme production by ***Actinomadura*** sp. using statistical method was reported by Sukkhum et al. (2009a). Unculturable microorganisms were identified from the compost consisting of PLA by using metagenomic method including ***Paecilomyces***, ***Thermomonospora*** and ***Thermopolyspora*** (Sangwan and Wu, 2008).

In order to screen novel microorganism that are suspected to possess PLA-degrading activity at high

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temperature for composting application and recycling of biodegradable plastic wastes, PLA-degrading actinomycetes were isolated at 50°C. The strain that produced the highest PLA degrading activity was selected and identified.

This is the first finding of a strain belonging to the species ***Actinomadura keratinilytica*** with the capacity to degrade PLA, producing appropriate enzyme at 50°C. The ***pla*** sequencing of a new PLA-degrading strain was investigated in this study.

MATERIALS AND METHODS

Isolation of PLA-degrading actinomycetes and their enzyme production

Soil samples (0.1 g each) were suspended in 9 ml sterile distilled water mixed well and then plated on an emulsified PLA agar plates. The plates were prepared as follows. 1 g PLA pellet was dissolved in 40 ml dichloromethane. To 1,000 ml of a basal medium (containing per liter, $(NH_4)_2SO_4$, 4 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; yeast extract, 1 g; agar, 15 g, pH 7.0; sterilized at 121°C for 15 min), 40 ml of the PLA solution was added. The medium was sonicated with an ultrasonic processor model VCX 500 (Sonic and Materials, INC., Newtown, USA) for 5 min. The colonies forming clear zones were selected as PLA-degrading strains and were sub-cultured and purified on an emulsified PLA agar plate.

The ability of isolates on the enzyme production was determined in culture broth. The strains were cultured in 250 ml Erlenmeyer flask containing 100 ml of basal liquid medium consisting per liter, PLA film, 1 g; $(NH_4)_2SO_4$, 4 g; K_2HPO_4, 2 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; extract, 1 g, pH 7.0; with shaking speed of 150 rpm for 4 days at 50°C. The culture broth was centrifuged and the obtained supernatant was used to analyze the PLA-degrading activity. PLA-degrading enzyme activity was assayed based on decrease in turbidity by a modified method of Nakamura et al. (2001) as described by Sukkhum et al. (2009a). One unit of the PLA-degrading activity was defined as a 1 unit decrease in optical density at 630 nm per min under the assay condition described.

16S rDNA sequencing and G+C content analysis

For biomass preparation, strain T16-1 was grown in shake flask of tryptone soya broth (TSB) at 50°C for 2 to 3 days and harvested by centrifugation. Cells for the chemotaxonomic analysis were washed twice in distilled water and freeze-dried. Cells for the molecular systematic analyses were washed in NaCl-EDTA buffer (0.1M EDTA, pH 8.0, 0.1M NaCl) and stored at -20°C until required.

Genomic DNA was extracted from strain T16-1 as described by Hopwood et al. (1985). Further purification steps including RNase treatment were carried out according to the method of Saito and Miura (1963). The G+C content of the DNA was determined by the HPLC method of Tamaoka and Komagata (1984). A gene fragment specific for 16S rRNA gene-coding region was amplified by means of PCR. Two primers, 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') were used. All primer positions were specified by the ***Escherichia coli*** numbering system (Brosius et al.**,** 1978). The amplified and purified 16S rRNA genes were sequenced directly with a Big Dye terminator V3.1 Cycle sequencing Kit on an ABI 3100 automated DNA sequence (Applied Biosystems, Foster City, Califonia, USA).

Multiple alignments of the sequences obtained were performed using program CLUSTAL X (version 1.81) (Thompson et al., 1997) Gaps and ambiguous bases were eliminated. Comparison of the aligned sequences was made for 1,254 bases of 16S rRNA gene sequence in constructing phylogenetic trees by the neighbor-joining method of Saitou and Nei (1987) using the MEGA 3 program (Kumar et al., 2004). Distance matrices for the aligned sequences were calculated by the two-parameter method of Kimura (1980). Robustness for individual branches was estimated by bootstrapping of 1,000 replications (Felsenstein, 1985).

DNA-DNA hybridization

DNA-DNA hybridization experiments were used to resolve the taxonomic relationships between representatives of these closely related species. Levels of DNA relatedness of strain T16-1 and related organisms was determined with a method modified from that of Ezaki et al. (1989), using the -galactosidase/4-methylumbelliferyl- -galactoside system.

Chemotaxonomic characterization

The isomer of diaminopimelic acid in the cell wall and the whole-cell sugar pattern were determined as described by Staneck and Roberts (1994). Menaquinones were analysed by LC/MS (Shimadzu LC-MS application data sheet No. 010). Phospholipids were extracted by the method of Minnikin et al. (1979) and identified using two dimensional TLC. Cellular fatty acid methyl esters were prepared and analysed according to the protocol of the MIDI Sherlock Microbial Identification System (Agilent Technologies 6890N Network GC System) (MIDI, 2002; Sasser, 1990).

Cultural and morphological characterization

To examine the extent of growth, pigmentation and color of colony, strain T16-1 was grown on inorganic salt-starch agar (International Streptomyces Project (ISP-4) as described by Shirling and Gottlieb (1966). Morphological structures were studied using 20 days of strain on humic vitamin agar (HV) and observed by a scanning electron microscope (model JSM6060, JEOL, Ltd., Tokyo, Japan).

Phenotypic characterization

In addition to PLA degradation, strain T16-1 was also examined for the ability to degrade cellulose (cellulose medium), gelatin (glucose-peptone-gelatin medium), skim milk (skim milk medium) and starch (ISP-4) as described by Hamada (2000), in addition to PLA. NaCl tolerance and growth temperature were assessed using yeast extract-malt extract medium (ISP-2). Utilization of carbon sources was tested by using carbon utilization medium (ISP-9) modified from Pridham and Gottlieb (1948). Each carbon source was added to ISP-9 medium to give a final concentration of 1% (w/v). Lactic acid utilization was also tested. The basal medium (Tomita et al., 2003) with 0.1 % lactic acid was used in this test. The results were taken by measuring dry cell weight.

Sequencing of *pla* from strain T16-1

Bacterial strains and plasmid

Strain T16-1 was used as chromosomal DNA sources. Genomic DNA was extracted from strain T16-1 as described by Hopwood et al. (1985). ***E. coli*** JM109 was used as the host for recombinant

plasmid. Plasmid pUC19 was used as vector containing ***pla***.

Preparation of oligonucleotide primers for cloning of *pla* from strain T16-1

N-terminal amino acid sequence (NPPSAGLDR) of purified PLA-degrading enzyme was used for design forward primer, plaF (5'-CCSWCSGCSGGSCTSGACCG3-'). According to Blast p search, reverse primer was obtained from the most closely related amino acid sequence, plaR (5'-GGSGTSGCCATSGWSGTSCC-3'). In the sequences, S represent C or G and W represent A or T. After PCR amplification, a ca. 650 bp fragment was obtained from genomic DNA of strain T16-1 using Takara Ex Taq polymerase.

Cloning of *pla* and Its DNA sequencing

Vector (pUC19) was digested by ***Sma***I and ligated with ***pla***. The plasmid containing ***pla*** was designed as pUCpla. Plasmid pUCpla was introduced into protoplast of ***E. coli*** JM109, a recombinant ***E. coli*** strain containing ***pla*** was selected by blue/white colony on Luria-Bertani (LB) medium containing 10 mg/ml ampicillin, IPTG and X-gal. Recombinant plasmid (pUCpla) was extracted according to nucleospin plasmid kit and direct sequence of multiple containing site (MCS) by using two oligonucleotide primers, M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-GGAAACAGCTATGACCATG-3').

Protein and nucleotide sequences were compared with those on databases using FASTA (version 3.0) and BLAST (version 1.49) programs implemented at the EMBL/GenBank/DDBJ nucleotide sequence databases. Multiple-sequence alignment was done using a GENETYX program (Software Development Co., Tokyo, Japan).

RESULTS

Isolation of PLA-degrading actinomycetes and their PLA degrading activity

Eighty samples of surface layer soil were taken from Huai kha khaeng wildlife sanctuaries, Uthaithani province, Thailand. However, among these soil samples, only 10 samples were found 11 active strains isolated at 50°C and 2 isolates at 40°C. This indicates that PLA-degrading microorganisms could be regarded as having a lower population in the soil as reported by Pranamuda et al. (1997) and Ikura and Kudo (1999).

Among these, strain T16-1 produced the highest PLA-degrading activity at 22 U/ml, while, strains T16-4 and T9-1 produced 15 and 10 U/ml, respectively. The clear zone formation on emulsified PLA agar and enzyme activity in culture broth did not show a strong correlation, but some strains which had high activity might have a larger clear zone. Beside the size of clear zone, strains T16-1, T16-4, T9-1 and T7-1 demonstrated high clearness on the plate, which could associated with the extent of their activity in culture broth.

The GenBank accession number for the 16S rRNA sequence of strain T16-1 is FJ199994. The strain T16-1 was deposited in culture collection as BCC 28970 and NBRC 104111.

Genotypic properties

For classification, strain T16-1 was subjected to a polyphasic investigation. In a phylogenetic tree based on 16S rRNA gene sequences for 1,254 nucleotides (nt), strain T16-1 clustered with the members of the genus ***Actinomadura*** (Figure 1). The highest levels of 16S rRNA gene sequence similarity was found to be ***Acinomadura keratinilytica*** WCC-2265^T (100%), ***Actinomadura rubrobrunea*** NBRC 15275^T (98.0%) and ***Actinomadura viridilutea*** NBRC 14480^T (97.9%).

In the present investigation, it was evident that DNA–DNA relatedness values in reciprocal hybridizations were much lower than 70%, indicating that bacterial strain represents a separate genomic species. In the case of strain T16-1 and related species: [***A. keratinilytica*** WCC-2265^T (80.1 to 86.5%), ***A. viridilutea*** NBRC 14480^T (30.3 to 33.2%) and ***A. rubrobrunea*** NBRC 15275^T (34.4-36.6%)], strain T16-1 should be identified as ***A. keratinilytica***. The G+C content of the DNA was 72.2 mol%.

Chemotaxonomic characterization of Strain T16-1

Strain T16-1 contained ***meso***-diaminopimelic acid. Galactose, glucose, madurose, mannose and ribose were detected in whole-cell hydrolysates indicating a chemotype IIIB cell wall (Lechevalier and Lechevalier, 1970). Polar lipids of strain T16-1 included diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol and phosphatidylinositol mannosides. 14-Methyl-pentadecanoic acid (16:0 iso; 23% of total fatty acids) and 15-methyl-hexadecanoic acid (17:0 iso; 30% of total fatty acids) were the major fatty acids (Table 1).

WCC-2265^T also produced a relatively large proportion of iso-branched fatty acids, predominantly 16:0 iso and 17:0 iso. The major menaquinones of strain T16-1 were MK-9(H_6) (61%), MK-9(H_4) (18%) and MK-9(H_8) (17%). In addition, a small amount of MK-9(H_2) was detected. Fatty acid, DAP type and menaquinones of strain T16-1 was the same as strain WCC-2265^T (Puhl et al., 2009). The results summarized in Table 1 suggest that strain T16-1 belonged to the genus ***Actinomadura*** sp.

Cultural and morphological characteristics

The phenotypic properties of strain T16-1 and WCC-2265^T was shown in Table 2. Strain T16-1 exhibited good growth on ISP-2, ISP-3 and ISP-4. The substrate mycelium of the strain T16-1 was cream-yellow. The aerial mycelia were rare but when present green on ISP-4. This was different from substrate mycelium of WCC-2265^T which was yellow-orange on ISP-2 and gray-white on ISP-3 and ISP-4. Morphological observation of a 20-d-

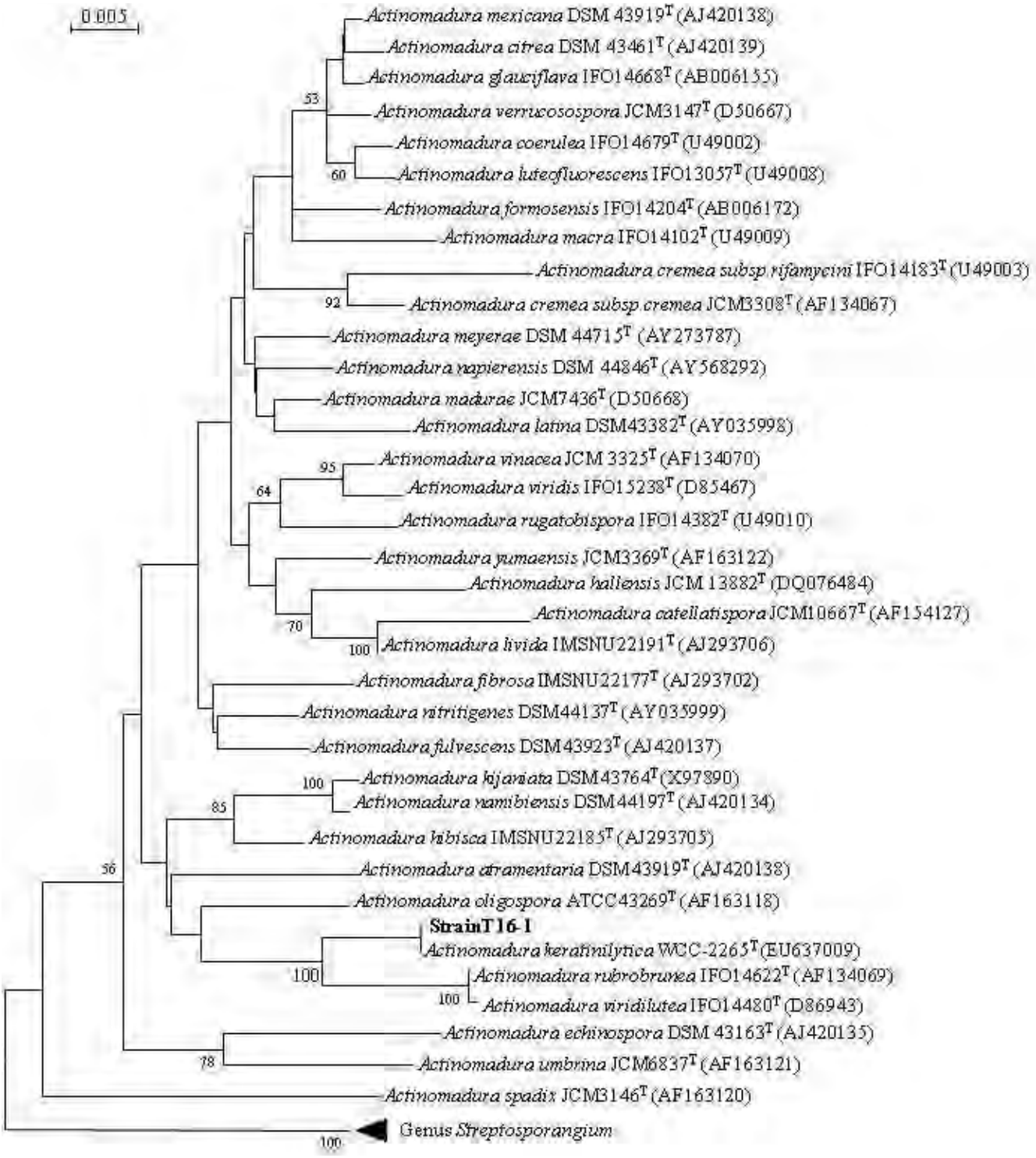

Figure 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing relationships of strainT16-1 to representatives of validly described species of ***Actinomadura.*** Bootstrap values at branching points are expressed as percentages from 1000 replications (only values greater than 50% are indicated).The scale bar indicates 0.005 substitution per nucleotide position. T, type strain.

old culture of the strain grown on Humic vitamin (HV) agar revealed the presence of oligosporic curved chains of spiny spores.

Phenotypic properties of strain T16-1

Strain T16-1 utilized D-glucose, inositol, raffinose,

Table 1. Chemotaxonomic characteristic of strain T16-1.

Characteristic	Strain T16-1
DAP type	***meso***-DAP
Sugar type	Type IIIB (Galactose, glucose, madurose, mannose and ribose)
Phospholipid	Diphosphatidylglycerol, phosphatidylinositol, phosphatidylglycerol, phosphatidylinositol mannosides
Menaquinone	
MK-9(H_6)	61%
MK-9(H_4)	18%
MK-9(H_8)	17%
MK-9(H_2)	small amount
Fatty acid*	
15:0 iso	14.8%
15:0	6.7%
16:0 iso	22.8%
16:0	7.3%
17:0 iso	29.6%
17:0	13.6%
17:0 10 methyl	5.3%

*Abbreviations for fatty acids: 15:0 iso, 13-methyltetradecanoic acid; 15:0, pentadecanoic acid; 16:0 iso, 14-methylpentadecanoic acid; 16:0, hexadecanoic acid; 17:0 iso, 15-methyl hexadecanoic acid; 17:0, heptadecanoic acid; 17:0 10-methyl, 10-methyl heptadecanoic acid.

sucrose, D-fructose, L-rhamnose, D-xylose, L-arabinose, D-mannose and L-lactic acid. Strain T16-1 was positive for cellulose, gelatin, skim milk and PLA degradation, nitrite from nitrate and starch hydrolysis. The temperature range for growth was 30 to 60°C. Strain T16-1 tolerates 3% NaCl in the medium (Table 2). Optimum temperature and pH, NaCl tolerant and utilization of mannitol, raffinose and arabinose of strain T16-1 as shown in Table 2 were different from type strain of ***A. keratinilytica*** WCC-2265^T (Puhl et al., 2009)

Cloning and sequencing of *pla*

The PCR product of 650 bp was ligated with pUC19/***Sma***I and transformed to ***E. coli*** JM109. Blue/white colony selection on LB+ampicillin+X-gal+IPTG plate was performed and white colony was selected as recombinant strain. The recombinant plasmid was extracted from the recombinant strain and cleaved by ***EcoR***I and ***Hind***III. The DNA fragment containing DNA sequences from plasmid pUC19 (vector) was obtained. Recombinant plasmid was extracted from recombinant strain and directly sequence based on the described method.

The DNA sequences of ***pla*** was translated to amino acid sequences, alignment (Figure 2) and blast searched for the most closely related gene from DDBJ/GenBank/EMBL amino acid sequence databases. The ***pla*** (222 amino acids) from ***A. keratinilytica*** T16-1 was the most closely related to ***Streptomyces griseus*** subsp. griseus NBRC 13350 putative secreted subtilisin-like serine protease of 43% identity and alkaline serine protease from ***Streptomyces pristinaespiralis*** ATCC 25486 of 46% identity which is different from the reported ***pla*** sequences.

DISCUSSION

The clear zone method is easy and significantly enhanced the isolation of PLA-degrading microorganisms from the environment. ***Amycolatopsis*** sp. strain HT-32 and strain K104-1 were isolated from soil samples by using plate count and clear zone method on an emulsified PLA agar plate for at least 14 days at 30°C (Pranamuda et al., 1997). In this work, clear zone formation was observed from the isolate T16-1 within 7 days on the plate at 50°C, indicating that the strain produced PLA-degrading enzyme at higher temperature. The maximum enzyme activity, 44.0 U/ml was obtained by using response surface methodology after 4 days cultivation in shake flask and 150 U/ml in an airlift fermenter at 0.5 vvm, initial pH 7.0 and 50°C (Sukkhum et al., 2009a).

In previous studies, several strains of ***Amycolatopsis*** sp. were reported as potent strains in their ability of PLA

Table 2. Comparison of phenotypic properties of strain T16-1 and WCC-2265^T.

Characteristic	T16-1	*WCC-2265^T
***meso*- DAP type**	+	**+**
Conidia		
Chain arrangement	Flexous	Flexous-straight
Number in chains	10	5-15
Shape/ornamentation	Globose/spiny	Globose/aculeate
Color of colony		
ISP-2	Cream-yellow	Yellow-orange
ISP-3	Cream	Gray-white
ISP-4	Cream-yellow	Gray-white
Aerial hyphae		
ISP-2	Green-white	Trace
ISP-3	White	-
ISP-4	Greenish gray	-
Growth at/in		
Temperature	30-60°C, optimum at 50°C	30-55°C, optimum at 45°C
NaCl conc.	0-3% NaCl	>6% NaCl
pH	pH 6-8, optimum at pH 7	pH 4-10, optimum at pH 6-9
Utilization of		
Arabinose	+	+/-
Fructose	+	+
Glucose	+	+
Inositol	+	+
Mannitol	+	-
Raffinose	+	-
Rhamnose	+	+
Sucrose	+	+
Xylose	+	+

+, positive or present; -, negative or absent; +/-, not positive but not negative. *Puhl et al. (2009).

degradation (Pranamuda et al., 1997; Pranamuda et al., 2001; Tokiwa and Jarerat, 2004). Furthermore, other actinomycete strains such as ***L. waywayandensis*** and ***K. aridum*** also exhibited PLA degrading activity (Jarerat et al., 2004). Un-cultural strain such as ***Paecilomyces***, ***Thermomonospora*** and ***Thermopolyspora*** were found to be predominant strains in compost that consist of PLA (Sangwan and Wu, 2008). Phenotypic and genetic data obtained from strain T16-1 lead to its identification as ***A. keratinilytica***, which is a novel PLA-degrading thermophilic actinomycete strain.

Although, 16S rDNA sequencing and DNA-DNA hybridization indicated that strain T16-1 was the same as type strain (WCC-2265^T), some phenotypic characterizations, such as color of colony on ISP plates, optimum temperature and pH, NaCl tolerance and utilization of mannitol, raffinose and arabinose were different.

The gene sequencing demonstrated that ***pla*** is related to serine protease secreted from ***Streptomyces griseus*** subsp. ***griseus***. This result correlates with the N-terminal of the purified enzyme which showed the similarity with serine protease from ***Streptomyces avermitilis*** MA-4680 as described by Sukkhum et al. (2009b).

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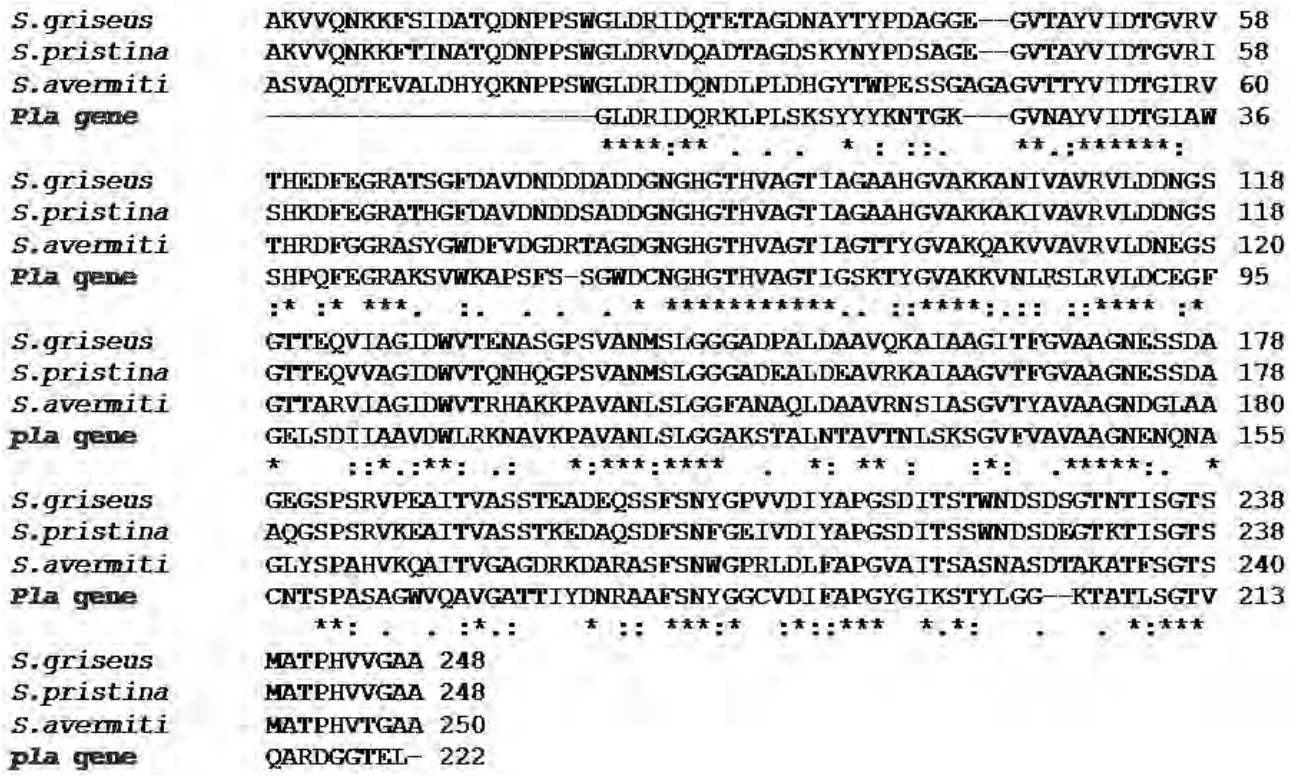

Figure 2. Alignment of ***pla*** with the most closely related amino acid sequences of serine protease.

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Full Length Research Paper

RepC as a negative copy number regulator is involved in the maintenance of pJB01 homeostasis

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The plasmid pJB01 contains a single operon consisting of three orfs, *copA, repB* and *repC* cistrons. The operon, also called *repABC* operon, starts transcription at T695 or A696 on the pJB01 genetic map. CopA (called RepA in pMV158 family) or ctRNA (counter-transcript RNA) of this plasmid play roles as a repressor of RepB, a replication initiator, on the transcriptional and translational level, respectively. RepC did not bind 73 bp PCR product including three tandem repeats (5′-CAACAAA-3′), the binding sites for RepB and any other regions on pJB01. However, when RepB and RepC were added simultaneously in the reaction mixture for gel mobility shift assay, unexpectedly, three kinds of retarded bands were observed. It suggests that RepC can interact with RepB by protein-protein interaction. In addition, the copy numbers of RepC-deleted pJB01 *ermC* (erythromycin-resistant methylase C) plasmids are increased 1.37-1.45 folds when compared with that of parent pJB01 *ermC*. From these, it could be proposed that RepC plays a role as a negative regulator to modify RepB function in the initiation of pJB01 replication, and therefore, the copy number of pJB01 is maintained via mutual global regulation of various replication factors, such as CopA, ctRNA, RepB and RepC.

Key words: pJB01, ***repABC*** operon, replication initiator, RepC, global regulation.

INTRODUCTION

Genetic analysis of replication control mechanisms had first been attempted for plasmid R1 through isolation of mutants to increase copy number (Nordstrom et al., 1972). The determinants of copy number control had been discovered in plasmid itself via characterization of these mutants, in which negative regulators (inhibitors) acting at the initiation step were involved in this control. A model of replication control by negative effectors was first proposed and described in quantitative terms by Pritchard et al. (1969). When a plasmid colonizes a new host, these negative regulators will be negligible owing to a little accumulated concentration of the inhibitors at initial stage. This seems desirable for successful establishment, since uninterrupted plasmid replication permit the normal copy number in a short time. Once the characteristic copy number is reached, maintenance of the average copy number in the population will then require adjustments to fluctuations in this value in individual cells. The control systems constantly maintain copy number by either

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Abbreviations: ctRNA, Counter-transcript ribonucleic acid; ***ermC,*** erythromycin-resistant methylase C; **RT-PCR,** reverse transcription polymerase chain reaction; **RNA,** ribonucleic acid; **EDTA,** ethylenediaminetetraacetic acid; **DNA,** deoxyribonucleic acid; **TBE,** Tris/Borate/ ethylenediaminetetraacetic acid; **NCBI,** national center for biotechnology information; ***sso,*** single strand origin; ***dso,*** double strand origin; **RC,** rolling-circle.

increasing or decreasing the rate of replication per plasmid copy and cell cycle. Although mechanisms to counter-select newly replicated-plasmid molecules exist, for example, hemimethylation and supercoiling (Abeles et al., 1993; Nordstrom et al., 1984), individual plasmid copies are selected for replication at random from a pool including replicated and non replicated copies.

The inhibition of plasmid replication associated with an increase in the gene dosage of copy number control genes has been used to identify these genes (Pritchard, 1978; Nordstrom, 1985; Novick, 1987; Austin and Nordstrom, 1990; Kittell and Helinski, 1992; Chattoraj and Schneider, 1997). Control of replication by inhibitors requires measurement of the concentration of plasmid copies within the cell. This is probably achieved by an unstable inhibitor expressed constitutively or by a stable inhibitor synthesized shortly after each initiation event (Pritchard et al., 1969). The regulators via these alternatives modulate the initiation frequency after each initiation event and lead to increase or decrease in the rate of initiation of replication when the average copy number is, res-pectively, lower or higher than required. Otherwise, when the frequency of initiation is determined by the level of an initiator protein, one mechanism for controlled plasmid replication (unlike phage replication) includes inactivation of the initiator protein after each replication event (Rasooly and Novick, 1993; Wojtkowiak et al., 1993). In this study, in order to examine RepC function of pJB01, we performed RT-PCR (reverse transcription-PCR), gel mobility shift assay and constructs of ***repC***-deleted mutants to demonstrate a single operon consisting of ***copA, repB*** and ***repC*** genes, protein-protein interaction between RepB and RepC, and copy number control of pJB01 by RepC, respectively.

MATERIALS AND METHODS

Bacterial strains and plasmids

A pathogenic ***Enterococcus faecium*** JS2 (Amp^s, Erm^s, Tet^s, and Van^l), a strain non-harboring pJB01 plasmid, was isolated from patients in Samsung Biomedical Research Institute in Korea. In order to clone pJB01, ***Escherichia coli*** TG1 strain was employed. Cloned plasmids were transformed to ***E. faecium*** JS2 by the electroporation method (Dunny et al., 1991; Bensing and Dunny, 1993). Transformed plasmids were isolated from ***E. faeium*** JS2 by a modified alkaline lysis method (Sambrook et al., 1989; O'Sullivan and Klaenhammer, 1993). For selection of a host harboring target plasmids from ***E. coli*** TG1 and ***E. faecium*** JS2, an ***ermC*** (Khan et al., 2002) gene of plasmid pGKV21, after amplification by PCR using the portion of pE194 from this plasmid (van der Vossen et al., 1985**)**, was transferred into pJB01 and named pJB01 ***ermC***.

RT-PCR (reverse transcription polymerase chain reaction) - RT-PCR

Total RNAs were prepared from exponentially growing ***E. faecium*** JS2 cultures harboring pJB01 ***ermC*** (RNeasy Mini Kit, Qiagen). For the 1st strand of three cDNAs containing ***copA*** and a part of ***repB*** genes, ***copA***, ***repB*** and a part of ***repC*** genes, and ***copA***, ***RepB*** and ***RepC*** genes, each of oligonucleotides such as the 3′-end of RepB as 5′-AATTTCATAAAAGCTTCCCC-3′, the 3′-end of E104Y as 5′-GTCTTTTGAATAATGCGTTAAATAC-3′ and the 3′-end of RepC as 5′-AAGCTTTAGTTATCCGCCCTTTCAAC-3′ was annealed to total Ribonucleic acid (RNA) (15 μg) at 65°C for 5 min, and continuously, the reactions were done as described by a Superscript™ $RNaseH^-$ RTase catalogue of Invitrogen. The next PCR was performed by the above three kinds of PCR products used as templates, and the 5′-end of CopA [5′-GGATCCATGGCTAGAGAAAAATCAGA-3′] as the 5′-end primer and the same oligonuclotides used for cDNA syntheses as the 3′-end primers.

Purification of repC and repB and gel mobility shift assay

Each of ***repB and repC*** genes was amplified by PCR and subcloned to the pQE30 vector for over expression. Subcloned ***repB*** and ***repC*** genes were over expressed by T7 promoter in ***E. coli*** JM109, and then, purified according to manufacturer's directions (Invitrogen, Korea). A 73-nucleotide stretch from nucleotides 583 to 655, the three tandem repeats-containing region of plasmid pJB01, was amplified by PCR. One pmol of each PCR product was incubated with various concentrations of RepB and RepC in 20 μl of reaction buffer [NB buffer (20 mM Tris›HCl, pH 8.0; 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0; 100 mM KCl; 5 mM dithiothreitol), 50 μg/ml calf thymus Deoxyribonucleic acid (DNA) and 50 μg/ml bovine serum albumin] for 30 min at room temperature. Reactions were stopped by adding non-denaturing loading buffer [20% (v/v) glycerol, 0.01% (w/v) Bromophenol Blue in Tris/Borate/ Ethylenediaminetetraacetic acid (TBE) buffer]. Reaction products were separated on 5% native polyacrylamide gels. The resolved bands were stained with Ethidium Bromide (0.5 μg/ml) in TBE buffer for 30 min, and destained with distilled water for 30 to 120 min.

Site-directed mutagenesis of the pJB01 repC region and copy-number calculation

All mutants were obtained by inverse PCR (Mcpherson et al., 1993) using pJB01 ***ermC*** as a template. To remove ***repC*** gene from the parent plasmid pJB01 ***ermC***, the 3′-end of RepB as 5′-AATTTCATAAAAGCTTCCCC-3′ and the 5′-end of Em 5′-GCTAGCATCGATTCACAA AAAATAGG-3′ oligonucleotides were used as primers. The desired mutant plasmids were harvested by using ***E. coli*** TG1, and then, transformed to ***E. faecium*** JS2. To calculate their copy numbers, cultural broths of ***E. faecium*** JS2 harboring plasmids were grown to mid-exponential phase, and adjusted to 0.5 optical densities at 600 nm in wavelength. Equal aliquots of each culture were withdrawn. Cells were collected by centrifugation and plasmids were prepared by an Accuprep™ plasmid extraction kit (Bioneer, Korea). Isolated plasmids were separated on agarose gels and stained by Ethidium Bromide (0.5 μg/ml). Monomer covalently closed circular plasmids were quantified by Gel doc 2000 (Bio-Rad, USA) and covalently closed monomers of mutant plasmids were compared with that of parent pJB01 ***ermC***.

RESULTS AND DISCUSSION

Demonstration of a single repABC operon and its surrounding structure

The plasmid pJB01 (GenBank accession number

AY425961) was isolated originally from ***E. faecium*** JC2 (Kim et al., 2006), but it was not harbored by ***E. faecium*** JS2. It was assumed that this plasmid should have a single operon which consists of three orfs as ***copA, repB*** and ***repC*** by the sequencing analysis using a Translation of ExPASy tool. This operon begins transcription at T695 or A696 on the pJB01 genetic map (Kim et al., 2008). CopA (as RepA in pMV158 and pE194)), the product of ***copA*** gene, binds to the promoter (or operator) region of the operon and regulates it at transcriptional level, as in case of pMV158 (del Solar et al., 1995, 1997) and pE194 (Kwak and Weisblum, 1994). RepB, as a product of ***repB*** gene and a replication initiator, performs nicking and nick/closing reactions on the nick site in the ***dso*** region (Kim et al., 2006). However, the corresponding function of RepC, the product of ***repC*** gene, has not been identified yet, through primary sequence analysis using the BLAST search of NCBI (National Center for Biotechnology Information). The transcription of ***copA*** (171 nts), ***repB*** (666 nts), and ***repC*** (462 nts) genes begin at the 721st, 964th and 1661st nts and terminate at the 891st, 1629th and 2122nd nts, respectively. Since consensus sequences of the promoter region were not existed in the intergenic regions of 72 nts (***copA***-***repB***) and/or 31 nts (***repB***-***repC***), it was thought that these genes are a single operon. As shown in Figure 1, RT- PCR results using each oligonucleotide set also revealed that these genes consist of a single operon.

Binding on three tandem repeats by protein-protein interaction of RepB and RepC

Following termination of the leading strand replication of pT181 plasmid, repC as an initiator protein is released with a short oligonucleotide attached to one subunit of homodimer repC, which prevents it from being recycled, as an essential feature of the plasmid's replication control system. It was reported that the oligonucleotide probably results from the passage of the replication fork past the RepC nick site after one round of replication, the subsequent cleavage and re-ligation of the DNA by repC (Khan, 1997; Zhao and Khan, 1997). Although repC can bind to the recognition sequence in the leading strand origin, repC*, as a modified one with a short oligonucleotide, cannot induce cruciform extrusion, which is an essential structure for replication initiation. RepC* is defective in its ability to oligomerize on the DNA for the next round replication. DNA binding and replication activities of RepC* is greatly decreased, implying that it may play only a minor regulatory role in pT181 replication ***in vivo*** (Zhao et al., 1998).

On the contrary, Rep proteins of pMV158 family do not lead to a covalent binding with nicked DNA in itself (Moscoso et al., 1997). Therefore, it should be existing in other mechanism for deactivation of generated Reps in its family including pJB01. Since in this work the pJB01 ***repC-RepC*** gene was co-expressed as a component of the polycistronic mRNA of ***copA*** and ***repB*** gene, as shown in Figure 1, it could not be excluded from the possibility that RepC might play a role as a regulator of pJB01 replication through modification of RepB. In Figure 2, RepC did not show binding on the DNA segment containing three tandem repeats, known as DNA binding sites for RepB (as an initiator). However, simultaneous addition of RepB and RepC, particularly in higher concentrations of RepC, showed three extra retarded bands compared to the case of RepB only. It suggests that RepC interacts with RepB by protein-protein interaction. Interestingly, plasmid pLC2 (Vogel et al., 1991) which belongs to pMV158 family seems to be very similar to plasmid pJB01, in the aspect of the organization of ***sso*** (single strand origin), ***dso*** (double strand origin) and genes encoding for replication initiation factors. Particularly, unidentified pLC2 orf 3, which composed of 153 amino acid residues and a weakly acidic protein as a theoretical pI of 5.11, is homologous up to 96% to corresponding pJB01 RepC (Figure 3). Based on the similar organization and amino acid sequence homology, it could not be excluded from the possibility that pLC2 orf 3 also regulates the initiation of replication via protein-protein interaction with orf 2.

Increase of the copy numbers of the repC-deleted mutants

In order to examine the role of RepC in the regulation of pJB01 copy number, three mutants of RepC-deleted pJB01 ***ermC*** were created by inverse PCR (Figure 4a). All RepC-deleted pJB01 ***ermC*** mutants increased the copy numbers of 1.37-1.45 times when compared with that of parent plasmid pJB01 ***ermC*** (Figure 4b). In our previous work (Kim et al., 2008), the copy numbers of ctRNA mutants showed 1.78-5.43 times higher than that of parent pJB01 ***ermC***. Based on these, it is assumed that RepC inhibits the initiation of replication weakly by modification of RepB, on the contrary to ctRNA does as a main repressor on the translation level.

A hypothetical model for the copy number control of pJB01

Based on all the data obtained in this work, RepC seems to be involved in the regulatory system of pJB01 DNA replication, in addition to various factors such as CopA, ctRNA and RepB. In some detail, according to our study it was demonstrated that CopA represses transcription of a ***repABC*** operon by interaction on partial palindromic sequence formed near the -35 box consensus sequence in the promoter region (unpublished data). In addition, pJB01 ctRNA, a 54 nucleotide transcript encoded on the opposite strand from the ***copA/repB*** intergenic region and

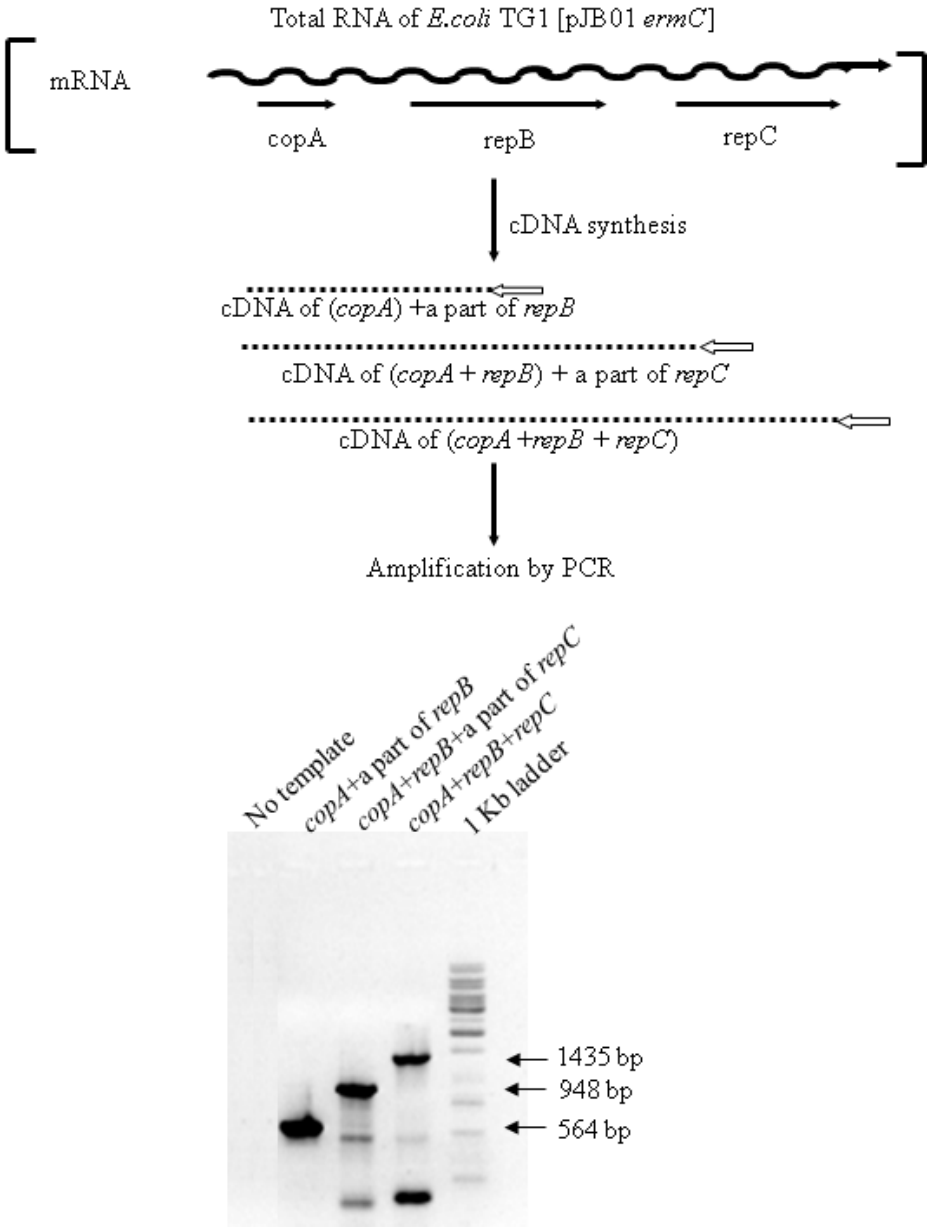


Figure 1. Demonstration of a single operon system consists of ***copA***, ***repB*** and repC cistrons. Each primers marked by open arrows at the experimental strategy in the upper panel was annealed to total RNA isolated from ***E. coli*** TG1 culture harboring the pJB01 ***ermC***, and extended by reverse transcriptase. The RT-PCR products consist of 564, 948 and 1435 bps as indicated on the right side of the gel, respectively. A full-length mRNA transcript of the ***repABC*** operon was represented by a thick-waved arrow. Each cistrons of a putative operon was marked below the ***repABC*** mRNA, primers for the first cDNA synthesis were represented in open arrows, and their reverse transcripts were shown in dotted lines. PCR products were separated on a 1% agarose gel and those products corresponding to three parts of a putative operon were marked by arrows on the right of the gel image.

also negatively regulates RepB expression on the translational level by partially overlapping an ARBS (Kim et al., 2008). Interestingly, RepB promotes replication activity on appropriate concentrations, but under overexpressed conditions, it not only decreases the replication activity of plasmid pJB01 but threatens viability of its host (unpublished data). Since the initiators of many rolling-circle (RC) plasmids do rate-limiting for replication, these proteins seems to be usually inactivated after accomplishment of one round of replication. In the pT181 plasmid, inactivation of the initiator RepC protein occurs by the attachment of an oligonucleotide to its active

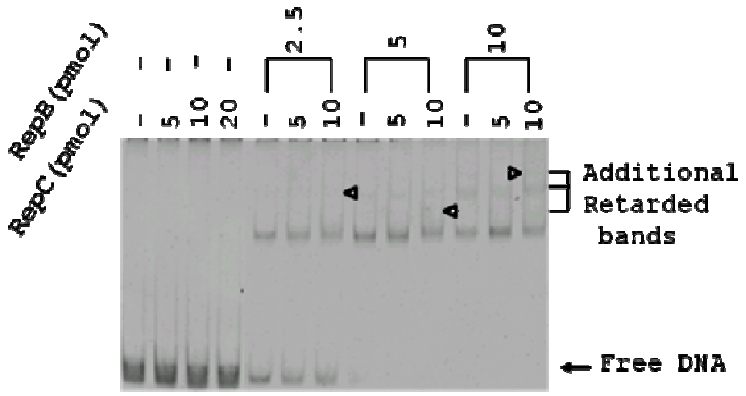
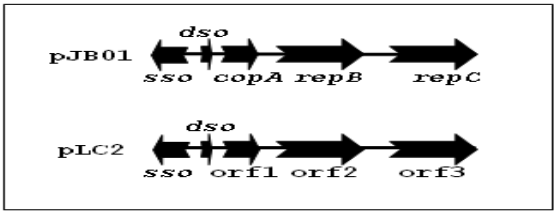


Figure 2. Confirmation of the protein-protein interaction between RepB and RepC by gel-shift assay. Free DNA indicates 73 bps PCR product containing three tandem repeats. Three additional retarded bands were produced by simultaneous addition of RepB and RepC to the reaction mixture for gel-shift assay. Concentrations of RepB and RepC added in pmol are indicated on the top of the gel image.

A



B

Name	pJB01	pLC2	Description
Origin	*Enterococcus faecium* JC1	*Lactococcus curvatus*	
Family Of RCR	pMV158	pMV158	
Full length	2235 bp	2489 bp	
Direct/tandem Repeats	CAACAAA	ACAAACCC (or CAACAAA)	pJB01, 3 tandem repeats; pLC2,2 direct (or 3 tandem) repeats
CopA	56 aa	56 aa	Homology up to 64%
RepB	221 aa	221 aa	Homology up to 86%
RepC	146 aa	153 aa	Homology up to 96%

C

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                10         20         30         40         50         60
pJB01       MKFEETLLPEKSDVMTLQNMIRKYNKQNFETANQTDFAIYIKDDSENVMGGISGEIFGNW
pLC2        MKFEETLLPEKSDVMTLQNMIRKYNKQNFETANQTDFAIYIKDDSENVMGGISGKIFGNW
Consensus   MKFEETLLPEKSDVMTLQNMIRKYNKQNFETANQTDFAIYIKDDSENVMGGISGeIFGNW

                70         80         90        100        110        120
pJB01       MDIEYLVIHESVRRNGLGRDLLKKAEELAIRSNCKNIFLYTFDFQGKDFYPKFGFKEVYV
pLC2        MDIDYLVIHESLRKNGLGRDLLKKAEDLAIRSNCKNIFLYTFDFQGKDFYPKFGFKEVYV
Consensus   MDI#YLVIHES1RrNGLGRDLLKKAE#LAIRSNCKNIFLYTFDFQGKDFYPKFGFKEVYV

                130        140        150
pJB01       KRQYPLTGTEHFFVKKLSNEIDKEAGEILEETE
pLC2        KRQYPLTGTEHFFVKKLSNEIDKETE--------
Consensus   KRQYPLTGTEHFFVKKLSNEIDKEae
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Figure 3. Comparison of replication factors between pJB01 and pLC2. (A) A schematic model of their gene organizations. Plasmid pJB01 is the same as pLC2 in view of gene organization, for example, orientations and arrangements of ***dso***, ***sso***, and three orfs. (B) Some features of their replication factors. Various features of replication factors in two plasmids were compared such as direct/tandem repeats, and orfs including origin of plasmids and prototype of RCR. (C) Alignment of the amino acid sequence of RepC in pJB01 with that in pLC2. Two RepCs show about 96% homology. Upper-case letters are residues conserved more than 90%, Lower-case letters are residues conserved 50 to 90%, and # indicates the NDQE conserved positions.

tyrosine residue (Rasooly and Rasooly, 1996; Jin et al., 1996; Zhao et al., 1998). However, since Rep proteins as a replication initiator of pMV158 family do not bind covalently with an oligonucleotide after one round replication, its inactive forms are not observed (Moscoso et al., 1997), on the contrary to pT181 RepC.

Therefore, pMV158 family may need a new system for preventing accumulation of active Rep proteins, in other

A

Mutant name	Deleted region	Description
23-2	pJB01Δ1667-2167/GCTAGC(Em5′)	PCR primer : RepB 3′ end-Em 5′, total synthesized size : 2746 bp
23-3	pJB01Δ1669-2167/CTAGC(Em5′)	Deletion of G residue in *Nhe*I site (5′ -GCTAGC-3′), total synthesized size : 2747 bp
23-5	pJB01Δ1669-2167/GCTAGC(Em5′)	total synthesized size : 2748 bp

B

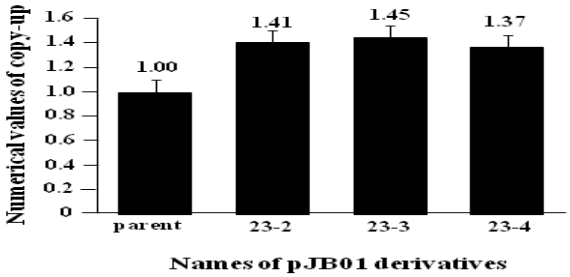

Figure 4. Changes of copy numbers of a few pJB01 ***ermC ΔrepC*** mutants. (A) Constructions of three pJB01 ***ermC ΔrepC*** mutants. The mutants were constructed by deletion of most parts of the ***repC*** gene, remaining some amino acid residues encoded at start and end portions. (B) Copy number ratios of three ***repC*** mutants. X-axis represents names of pJB01 ***ermC*** and its ***repC*** mutants as 23-2, -3 and -4, and Y-axis indicates the copy-up ratios of three mutants against parent plasmid pJB01 ***ermC***.

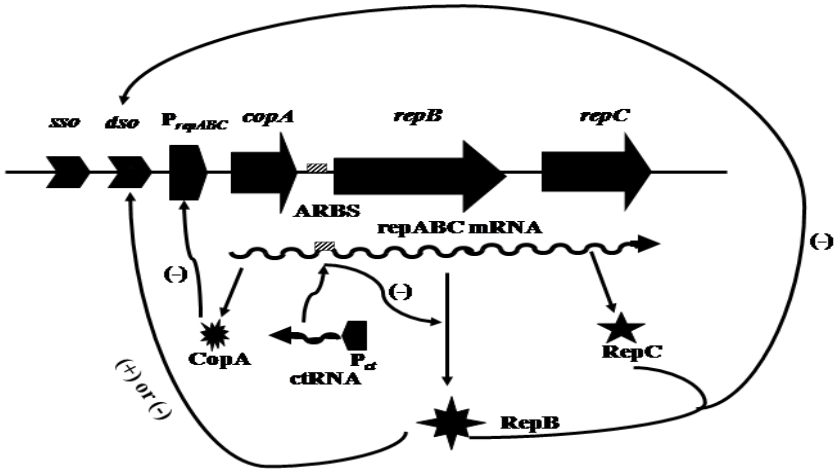

Figure 5. A hypothetical model for copy number control of pJB01. RepB is a positive regulator in the initiation of pJB01 RC replication at a normal level ***in vivo***, whereas it functions as a negative regulator at an overexpressed level. The ***copA***, ***repB*** and ***repC*** genes consist of a single operon, repABC and its transcription is autoregulated through CopA's binding on the putative operator region (P_{repABC}). Furthermore, the translation of ***repB*** is regulated by binding of ctRNA to the ARBS positioned between ***copA*** and ***repB***. On these points of view, including co-expression of ***copA*** and ***repB*** with ***repC*** as a polycistronic mRNA, it could be assumed that RepC plays negative roles in the initiation of plasmid pJB01 replication through RepB-RepC interaction.

words, for rate-limiting of replication. Based on our data in this work, it could be hypothesized that RepC modifies RepB by protein-protein interaction and this leads to lessen the optimal initiation activity of RepB replication. It is supported in that the ***repC***-delteted mutants show a few copy-ups by 1.37 -1.45 times against parent pJB01 ***ermC*** plasmid (Figure 4). The copy number control of pJB01 might be hypothesized as shown in Figure 5. The ***dso*** is

controlled positively or negatively depending on the expressed amounts of RepB. CopA binds a putative operator and then, auto-regulates transcription of the ***repABC*** operon. The ctRNA inhibits translation of RepB by binding on ARBS. RepC interacts with RepB and may play a role as a putative negative regulator at the ***dso***. In this study, it was demonstrated that ***copA, repB and repC*** genes consist of a single operon, and that RepC interacts with RepB by protein-protein interaction. Moreover, a few copy-ups of its ***repC***-deleted mutants against parent pJB01 ***ermC*** plasmid were observed. Taken all, it was suggested that the maintenance of the coy number of pJB01 is accomplished by mutual interactions of various replication factors, such as CopA, ctRNA, RepB and RepC.

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Full Length Research Paper

Cloning and characterization of a female gametophyte-specific gene in *Gracilaria lemaneiformis* (Gracilariales, Rhodophyte)

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In this study, forward and reverse suppression subtractive hybridization (SSH) libraries were constructed between female and male gametophyte of *Gracilaria lemaneiformis* to isolate genes differentially expressed between gametophytes. Dot-blots were performed to screen 384 colonies randomly selected from each subtracted libraries. Partial gene (designated as GMF-01) which is female gametophyte-specific was selected to isolate its cDNA full length sequences and to be characterized. Then we got its cDNA full length sequences using SMART-RACE technic. Sequence analysis showed that the open reading frame (ORF) of GMF-01 is 1002 bp long with a GC content of 47.7%, encoding 333 amino acids. GMF-01 does not have significant match in the databases when examined on NCBI website. Amino acid sequence analysis showed that the theoretical pI of this translated protein is 7.92, while the instability index is computed to be 43.61, which has classified the protein as unstable. Results of prediction showed 45.05% of its secondary structure has random coil and sub-cellular location prediction indicated that it is probably a extracellular protein.

Key words: ***Gracilaria lemaneiformis,*** suppression subtractive hybridization (SSH), sex determination, gametophyte.

INTRODUCTION

Gracilaria lemaneiformis is a commercially important agarophyte that can be used to produce agar, a major ingredient of dairy products, surgical jellies, ointments, cosmetics and healthcare products (Tseng, 2001). ***Gracilaria***, along with ***Porphyra***, ***Laminaria*** and ***Undaria*** has a bulk production by farming in China. It is not only an economically important algal species, but also a good material for genetical studies (Chen et al., 2009). Thus, it is important to pursue basic studies on ***G. lemaneiformis***.

Gracilaria has a life history involving three distinct stages: gametophytes, carposporophytes and tetrasporophytes. Even though the phases and sexes of ***Gracilaria*** look identical before sexual maturation, there are physical differences between them (Kain and Destombe, 1995), such as growth rate in phases of ***G. lemaneiformis*** (Zhang and van der Meer, 1988), levels of polyamines in sexes and phases of ***G. cornea*** (Guzman-Uriostegui et al., 2002), and lipid composition among different developmental stages of ***Gracilaria verrucosa*** (Khotimchenko, 2006). Owing to their particular life history, differentiation of phase and sex in red algae has already attracted researchers' attention. Researchers have been engaged in the study of the mechanisms of phase formation since 1976 (Ren and Zhang, 2008). However, no satisfactory results were obtained due to the limitation of applicable methods in the past. With the development of molecular biology, great progress has been made recently, such as that made by Ye et al. (2006). 6 ISSR primers, which had proved previously to

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be able to yield clear bands in ***Gracilaria***, were used to distinguish the phases and sexes of ***G. lemaneiformis*** (Sun et al., 2003)***.***

Until now, several phase-specific and sex-specific genes have been identified. Eight unique cDNAs for the sporophyte and seven specific for the gametophyte, including elongation factor alpha and lipoxygenase encoding genes have been isolated from ***Porphyra purpurea*** (Liu et al., 1996). A heat-shock protein encoding gene, which might be involved in the differentiation of female gametophyte, has been identified from ***Griffithsia japonica*** (Lee et al., 1998). An ubiquitin gene of ***G. lemaneiformis*** during phase formation is identified and characterized (Ren et al., 2009). GlRab11, the first functional Rab-like protein identified in ***G. lemaneiformis*** was isolated and the cDNA full-length of GlRab11 was obtained (Ren et al., 2008). cDNA subtracted hybridization was employed to study ***Porphyra purpurea*** phase-specific genes (Liu et al., 1994) and suppression subtractive hybridization (SSH) was deve-loped by Diatchenko et al. (1996), which turned out to be a successful tool for rapid screening of differentially expressed genes (Shim and Dunkle, 2002; De la Vega et al., 2007). SSH had been applied to study differential expression of genes in developmental stages (Brun et al., 2003; Zhu et al., 2003; Singh et al., 2007) and under stress conditions (Bahn et al., 2001; Caturla et al., 2002). Sun et al. (2002) reported an analysis of 180 ESTs of the ***G. lemaneiformis*** tetrasporophyte cDNA library. Suppression subtractive hybridization (SSH) was employed between RNA extracted from female gametophyte and tetrasporophyte. Fourteen cDNAs were identified, among which ***SSH466*** was a putative tetrasporophyte-specific gene (Ren et al., 2006).

In this study, we constructed SSH libraries between female and male gametophyte of ***G. lemaneiformis*** and isolated the cDNA full-length of ***GMF-01,*** which is a female gametophyte-specific Gene.

MATERIALS AND METHODS

Algae materials and cultivation

G. lemaneiformis used in this study were collected from Zhanshan Bay (Qingdao, China). The healthy and mature fronds were used. Tetrasporophytes with released tetraspores were separated from female and male gametophytes under the microscope. Then one female gametophytes and one male gametophytes developed from tetraspores were picked out. The separated algae materials were brushed and rinsed in sterilized seawater until they were completely divorced from epiphytes. The materials were cultivated in Provasoli medium (Provasoli, 1966) under a light intensity of 50 $^{-}$mol photon $m^{-2}s^{-1}$ with a 12:12 (L: D) cycle at 15±1°C. The thallis were used for RNA extraction.

SSH library construction

To isolate sex-relative genes, suppression subtractive hybridization (SSH) was performed between RNA isolated from male and female gametophytes of ***G. lemaneiformis***. Total RNA was extracted from each sample with RNeasy Plant Mini Kit (Qiagen, China), reverse-transcribed and amplified using a SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, USA). Both forward (female gametophyte as tester and male gametophyte as driver) and backward (male gametophyte as tester and female gametophyte as driver) SSH were performed using a PCR-select cDNA subtraction kit (BD Biosciences Clontech) according to the manufacturer's instructions. In order to confirm differential expressions of the clones, cDNA dot-blots were performed. Based on the results of cDNA dot-blots, clones that expressed differentially between female and male gametophyte were sequenced and aligned with the BLAST algorithms at the National Center for Biotechnology Information (NCBI). The sequences were also analysed with ContigExpress (Vector NTI Suite 6.0) to find contigs.

Cloning of the cDNA full-length and sequence analysis of GMF-01

According to the partial sequence previously obtained and to get the full length of ***GMF-01***, 5' and 3' cDNA ends were amplified with a BD SMART^{TM} RACE cDNA amplification kit (BD Biosciences Clontech). Primers B5 (5'-GCGCTACCGTTGCTCCATAATCCAC-3') and B3 (5'-CATCCAGTTCTACTCGCTTCTTATACC-3') were designed and respectively used in the 5′ and 3′ RACE.

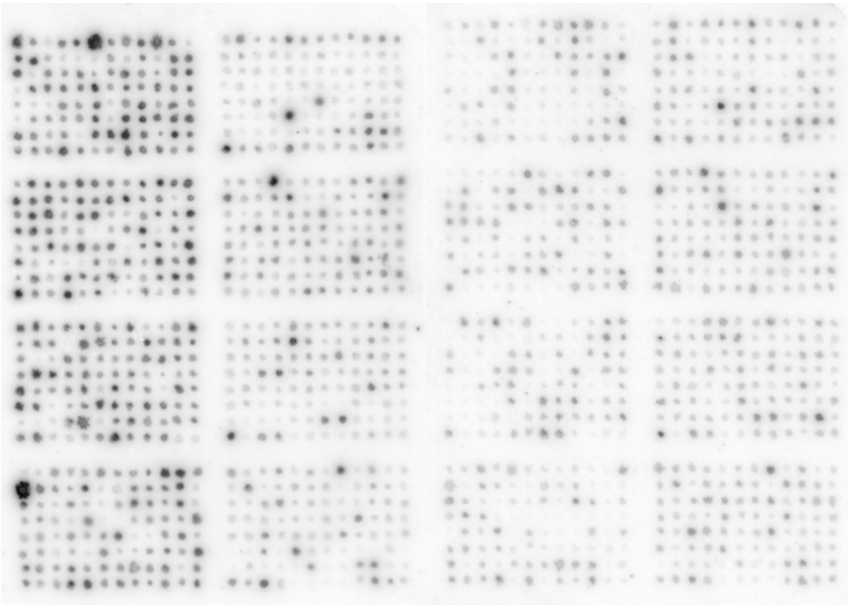
Sequence searches were performed by the BLAST algorithm on NCBI databases. Theoretical isoelectric point (pI) and molecular weight were predicted using ExPasy ProtParam tools (http://us.expasy. org/tools/protparam.html). Protein analysis was performed with ProtComp (www.softberry.com), TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and PSORT (http://psort.nibb.ac.jp/form2.html).

RESULTS

Screening of the SSH cDNA libraries

A total of 768 clones were randomly selected from the SSH libraries (384 clones from each of the two SSH libraries) and screened by macro-array dot-blots. Both forward and backward subtracted radioactively labeled cDNA populations were then used as individual probes for identical blots (Figure 1). 411 clones were found to be positive clones. When analysing the sequencing results with ContigExpress (Vector NTI Suite 6.0), 136 contigs were found and one female gametophyte-specific sequence (480 bp) showed significantly differential expression between the two sexes. It was designated as ***GMF-01*** and was chosen to clone its full-length cDNA sequence. We are still analyzing the rest partial sequences and they will be published later. The partial sequence of ***GMF-01*** is as follows:

GCAGACTTCTACTATTCAGTATAGATGGGTTGAAGAA
TATAGGGCCACGTTTACCACCAAAGTGGAGATCGGA
GAAATTATTCGGACGCAGGACATCATCAACTCGCCT
GACTTTAACATGGGACAATCCGTTTCATTCGATGGAG
TCGAGTGGAGTCCTCCAGTCAGCGACCGGAAGCCG
CCGAACATTGGGGTAGCATACAAGGTTGACACGAAC
GCTCTGCATCCAGTTCTACTCGCTTCTTATACCATGA



A B

Figure 1. Dot blots of cDNA in subtractive. Two identical membranes blotted with PCR amplified cDNA sequences from subtracted libraries were probed with the forward subtracted probes (A) and the backward subtracted probes (B).

ACGGGCAAAAGAAATGGAGGCCGTTTTACTACGGCG
TTTCTGTAATTTCTGGAAACACTTTGGTAGGCTTCCC
CGTGATTAAGCTTCAGATCCAACTCGGTACTGCTATC
AAGGATAGCGCATTCGTAGGAAGTATAATCGGCAAC
AGTCTCGAAGTGGATTATGGAGCAACGGTAGCGCAA
ACAGTGTGCGTGTACTCCCGTGAACGTGAGACCACG
GTGAGGCT

Cloning of the full-length cDNA and sequence analysis of GMF-01

Based on the partial sequence obtained from the SSH library, two primers B5 and B3 were designed to amplify the 5' and 3' cDNA ends of ***GMF-01***. 1019 bp and 548 bp were amplified in the 5' and 3' SMART RACE reactions respectively. The full length of ***GMF-01*** cDNA had 1357 nucleotides. Sequence analysis showed that the open reading frame (ORF) of ***GMF-01*** is 1002 bp long with a GC content of 47.7%, encoding 333 amino acids. Searches from the public sequence databases using NCBI BLASTx showed that there was no significant match with ***GMF-01***. The predicted protein had a calculated molecular weight of 36.7 kDa and a theoretical pI of 7.92.

The instability index was computed to be 43.61 which classified the protein as unstable. The GRAVY (Grand average of hydropathicity) of this protein was -0.131, which indicated that the protein was hydrophilic. Results of prediction showed 45.05% of its secondary structure is random coil (Figure 2). Sub-cellular location prediction results with ProtComp, TMHMM (Figure 3) and PSORT all indicated that it's probably an extracellular protein. The cDNA and amino acid sequences are indicated in Figures 2 and 3.

DISCUSSION

As an agarophyte, components of ***G. lemaneiformis*** cells are extremely complicated. In the construction of SSH library, RNA isolated from ***G. lemaneiformis*** was too difficult to purify enough due to the polysaccharides. The SMART approach is a PCR-based amplification system that allows the creation of cDNA from a very small amount of total RNA (Cramer and Lawrence, 2004; Shary and Guha-Mukherjee, 2004; Pavan, 2011).

Thus, SMART strategy was taken before SSH library construction was carried out. That is the key point of the successfully construction of SSH library. We identified 3 practical sequences that are differentially expressed between female gametophytes and male gametophytes. One putative female gametophyte-specific gene ***GMF-01*** was selected to isolate the full-length of cDNA for further analysis.

In this study, we identified a female gametophyte-specific gene of ***G. lemaneiformis.*** The protein encoded by ***GMF-01*** may be a extracellular protein. It was not known yet whether GMF-01 was red-algae specific.

In the SSH library, we found some special gene

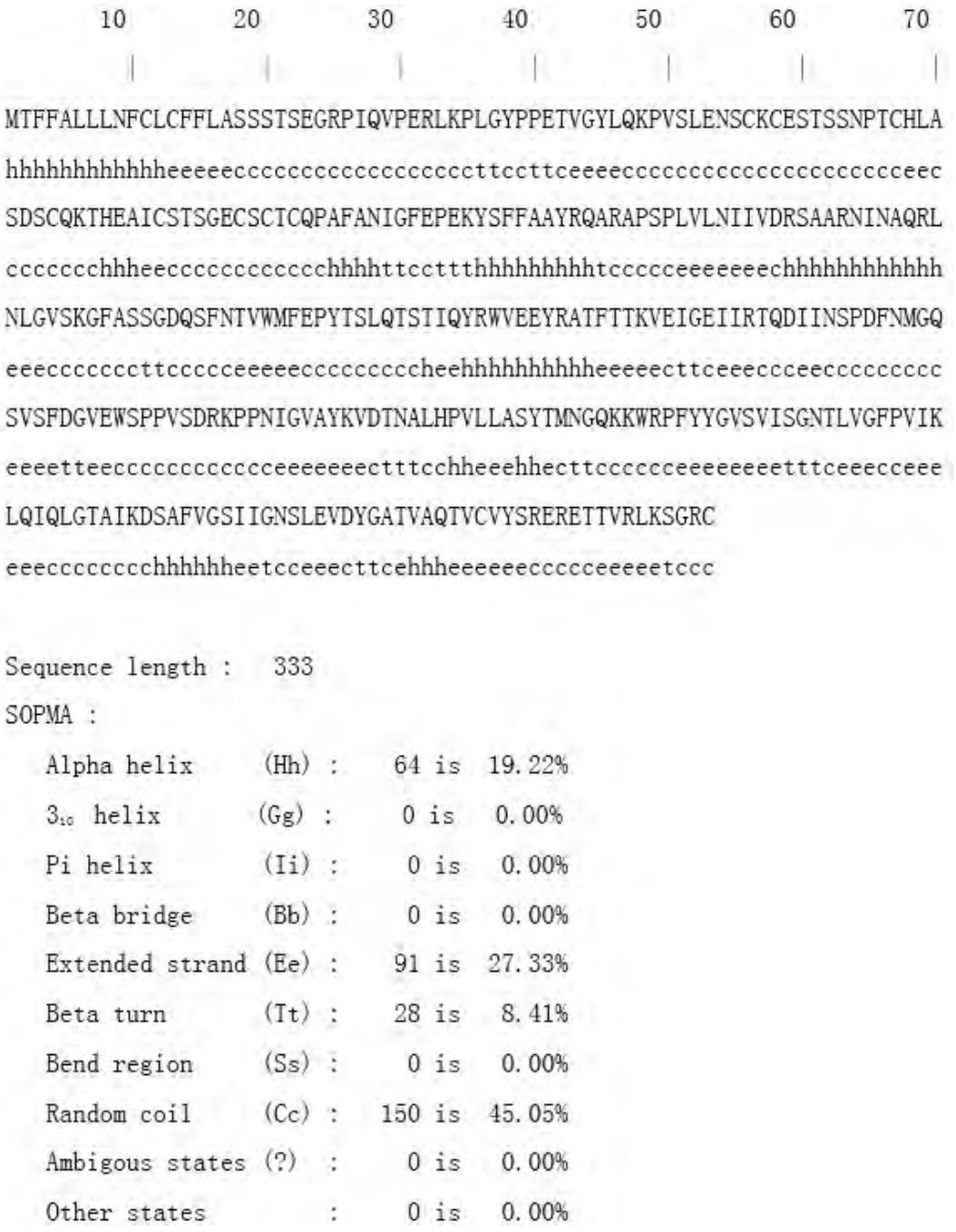


Figure 2. Prediction of secondary structure of GMF-01.

expression: (1) expression of rDNA and ribosomal protein, such as 16SrDNA, 23SrDNA, ribosomal protein S8, L23, L27. This may indicate that a number of genes are synthesized during sex differentiation, (2) expression of α-1,4-glucan lyase (AgII) which could degrade the floridean starch into glucose which can provide energy for sex differentiation, (3) expression of some protein genes related to cellular process, including aminotransferase, COP9 signalosome, Ran (one of GTP-binding proteins). These proteins play important roles in cell division and signal transduction. However, their function in sex regulation needs additional research.

The results in this study strongly suggest that ***GMF-01*** is a functional gene that may play important roles during the sex determination of ***G. lemaneiformis***. We are now committed to further studies of ***GMF-01***, such as gene expression analysis and semi quantitative determination by RT-PCR, more work are required to elucidate the

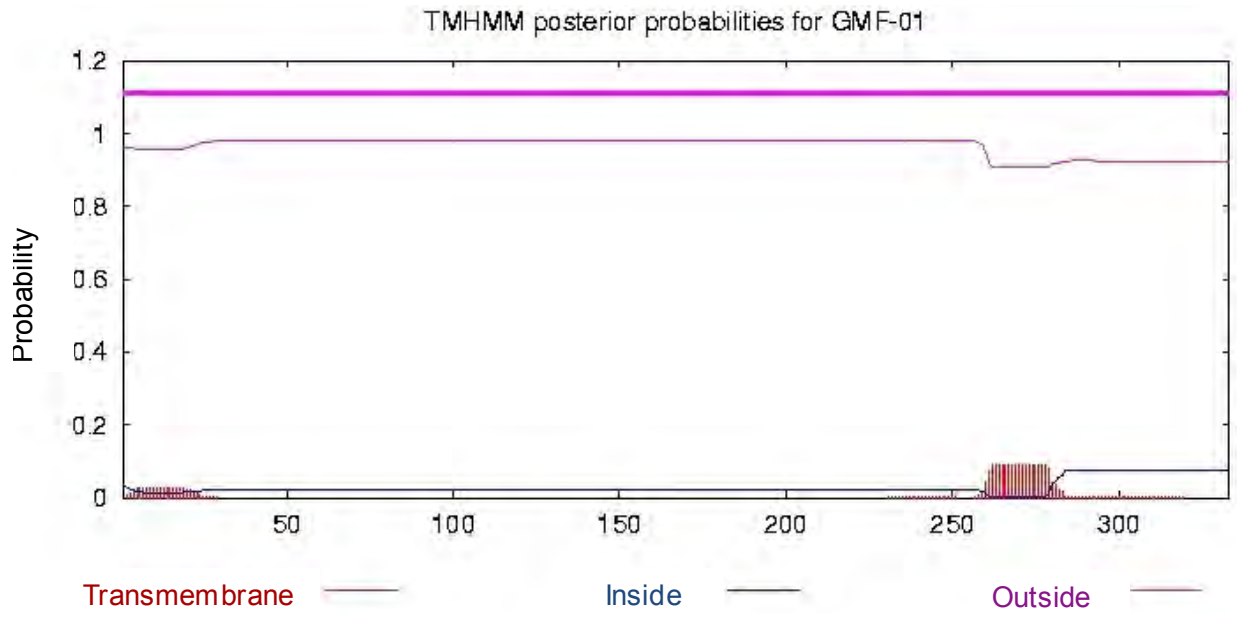


Figure 3. TMHMM posterior probabilities for GMF-01.

function of ***GMF-01***. The construction of additional transgenic clones in which ***GMF-01*** is knocked out should allow a better assignment of its function. Based on the SSH libraries constructed in this study, more differentially expressed genes could be found. The differentially expressed genes obtained in this study are closely related with gametogenesis of ***G. lemaneiformis***. Studies on these genes may play important roles in understanding sex determination mechanisms and will provide clues for red algal evolution pathways.

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Full Length Research Paper

Recombinant Clone ABA392 protects laboratory animals from *Pasteurella multocida* Serotype B

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In this study the potential of the previously contracted recombinant clone ABA392 derived from *Pasteurella multocida* serotype B to protect laboratory animal against haemorrhagic septicaemia was determined. After reconfirmation of plasmid DNA size, its stability and pathological effect of the clone, passive mice protection test and active immunization was carried out. Pooled serum sample from rats vaccinated with live and killed form of the clone was administered to mice and provided 66% protection while active immunization with the recombinant clone conferred 83% immunity to mice when challenged with lethal dose of *P. multocida*. ELISA results were positive for presence of antibody in serum of immunized mice. Sub-cloning of the insert ABA392 into an expression vector pQE32 was carried out to express its protein. It was found that the recombinant clone ABA392 is immunogenic and could be used as vaccine in future.

Key words: Recombinant clone, immunogenicity, *Pasteurella multocida* serotype B.

INTRODUCTION

Pasteurella multocida a Gram-negative coccobacillus commonly found in respiratory tract of many domestic and wild animals. *P. multocida* is causative agent of fowl cholera, atrophic rhinitis and haemorrhagic septicaemia (HS). The organism is capable of being pathogenic to human through animal bite. Most common human *P. multocida* infections are cellulites and abscess formation, however cases leading to meningitis, pneumonia, septic arthritis and endocarditis have been reported. HS is a highly fatal disease of cattle and water buffalo. In susceptible animals, the symptoms progress rapidly from dullness and fever to death within hours and recovery is rare (De Alwis et al., 1980). HS results from infection by *P. multocida* serotypes 6B or 6E (namioka-carter classification) or serotypes 2 or E2 (carter heddleston classification). HS is an important disease in Asia, Africa, some southern European countries and the Middle East. The highest incidence is in Southeast Asia causing great economical loss to the region (Bain et al., 1982).

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Pathogenicity and immunogenicity study of various components, derivatives, clones and mutants of *P. multocida* (Tabatabaei et al., 2007) has been carried out in order to find a suitable candidate for vaccine (Montserrat et al., 2004; Keith et al., 2007). In search of a promising vaccine, a recombinant vaccine would be an ideal choice of immunization. A recombinant clone ABA392 derived from HS isolate of *P. multocida* serotype B (PMB) (strain 202) constructed by Salmah (1997), carrying gene causing HS was used in this study as potential candidate for recombinant vaccine.

The aim of this study was to carry out the molecular characterization, histopathological analyses and immunological determination of the clone ABA392.

MATERIALS AND METHODS

Bacterial isolates

The bacterial strains and the recombinant clone ABA392 used in this study was constructed and provided by Dr. Salmah, Faculty of Medicine, University of Malaya, Malaysia. The clone ABA392 is derived from a *P. multocida* serotype B 202, carrying a recombinant plasmid. This clone was constructed by shotgun cloning method

using *E. coli* as host. Briefly, the *Pasteurella multocida* serotype B isolate, PMB202 genomic DNA was partially digested with the restriction endonuclease *Sau*3A 1, generating restriction fragments of approximately 500 to 1000 bp. were inserted at the unique *Bam*HI site in the vector plasmid pUC18 (Vieira and Messing, 1982). Of twenty selected recombinant plasmids, three of the clones obtained, ABA182, ABA282 and ABA392 were found toxigenic to mice. Only one clone, ABA392, showed mortality in mice. The recombinant plasmid previously sequenced harbours a sequence that code for a virulence factor to *P. Multocida.* The insert was found to be 921 bp. The strains were grown on brain heart infusion (BHI) blood agar incubated at 37°C overnight.

Plasmid stability testing

Plasmid stability testing was carried out as explained by Lanka and Barth with modification (Lanka and Barth, 1981). The cultures of ABA392 were grown overnight at 37°C in BHI broth. The clones were sub-cultured in BHI broth and BHI blood agar and were incubated overnight. The process of subculturing was repeated at least 10 times and after a final transfer into a fresh BHI broth and agar the clones were subjected to plasmid DNA extraction using promega wizard© miniprep plasmid DNA purification kit (USA).

Restriction endonuclease analysis (REA)

Restriction endonuclease analysis (REA) was performed as described previously by Salmah (2004). Three restriction enzymes *Eco*RI, *Pst*I and *Sma*I were used for single digestion and to release the insert gene of the recombinant clone for sizing. Double digestion of the recombinant clone plasmid DNA with restriction enzymes *Pst*I and *Sma*I was carried out. REA was done under conditions recommended by the supplier (New England Biolabs. Inc., U.S.A. and Bethesda Research Laboratories, U.S.A.). Restricted DNA (0.2 to 1.0 µg) was subjected to electrophoresis in 1.0% agarose gel.

Pathogenicity study

The cultures of ABA392 were grown overnight at 37°C in a 10 ml of BHI broth medium. The cultures were centrifuged for 5 minutes at 14000 r.p.m to pellet the cells. Cells were then resuspended in 5ml of phosphate buffered saline (PBS), pH 7.2 to achieve the concentration of 10^7 colony forming units CFU (Yazmin, 2003). In this study, 3-4 weeks old male Sprague Dawly's (SK/WDY) rats obtained from the animal house, Faculty of Medicine, University of Malaya were used. Rats were injected intramuscularly with 0.2 ml dexamethasone (0.33 mg/kg body weight) (Gardiner et al., 1996; Salmah et al., 2003) on the first day and thereafter the rats received 0.1 ml once daily. The rats were then challenged with 0.5 ml of bacterial inoculum intraperitoneally once on the day one only. Blood was collected from rats on daily basis for haematological assays and finally the rats were dissected at the end of the experiment and their internal organs used for histpathological studies and histo-slides were prepared.

Passive mice protection test (PMPT)

Pathogen free male SK/WDY rats weighing 150 to 250 g were used. Rats were divided in to different groups where each group was injected intramuscularly with 1ml of either killed or live strains of ABA392 or PMB202 prepared in Freund's complete adjuvant on 0, 2 and 4 weeks intervals. Pooled sera sample was prepared from each group of immunized rats and 0.5 ml of it was then injected into 6 to 8 weeks old ICR mice. Twenty four hours later, the mice were challenged with lethal doses of PMB and were kept under observation for 10 days (Bain et al., 1982).

Active immunization

Active immunization was carried out by immunization of 6 to 8 weeks old ICR mice as mentioned earlier. Mice were divided into different groups, where each group was injected intramuscularly with 1 ml (10^7) of either killed or live strains of clone ABA392 or *P. multocida* serotype B202. The bacterial strains were prepared in Freund's complete adjuvant. The vaccination was carried out either with a two dose or three dose schedules. In two dose schedule the vaccines were given on day zero followed by a booster dose two weeks later. Whereas the three dose schedule, vaccination was on day 0, week 2 and week 4 intervals. Serum was collected from all the rats before vaccination and one month post vaccination. The following day the mice were injected with lethal dose of PMB (Bain et al., 1982).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was developed based on the procedure described by Pati et al. (1996) with modifications. ELISA was performed on the hyper-immune sera obtained from the immunized mice to detect antibody. 100 µl of antigen was coated on the 96 well microtiter plate, sealed and kept overnight at 4°C. 100 µl of serum was then added and incubated for 1 hour at room temperature followed by 3 times wash with phosphate buffered saline with tween 20 (PBS-Tween 20) by using a multi-channel pipette. 100µl of diluted Anti-mouse IgG (H+L) Alkaline Phosphatase conjugate was then added followed by washing step as mentioned. 200 µl of the substrate was added and incubated for 30 min at room temperature and finally the reaction was stopped by adding 200 µl NaOH. The absorbance OD was read at 405 nm. All samples, including blanks, positive and negative controls were used in triplicates. The positive control used, was samples obtained from mice which had been hyper-immunized with *P. multocida* serotype B. The bacterial cells were grown in 500 ml of BHI broth at 37°C. The harvested culture were washed in PBS twice and then resuspended in fresh 0.2% formalinized PBS. This process inactivates the organism after 24 h of incubation. The density of the suspension was adjusted till a concentration of 10^7 CFU was achieved. The negative control used was serum, collected from healthy mice which were not exposed to *P. multocida* and injected with PBS as mentioned above. Cut-off value was determined using the two-graph receiver operating characteristic (TG-ROC), a Microsoft-Excel program. The ELISA results were analyzed based on the cut-off value including sensitivity and specificity obtained from TG-ROC.

RESULTS

Molecular analyses

The recombinant clone ABA392 was found to maintain its plasmid profile after it was subjected to plasmid DNA stability testing up to 10 generations. The clone carries 2 plasmid DNA, open circular plasmid (oc plasmid) with and approximate size of 5.6 kb and covalently closed plasmid (ccc plasmid) of 3.5 kb. RE analysis of the clone was carried out to determine the size of the insert. After single digestion of plasmid with *E*coRI, *P*stI and *Sma*I, 1

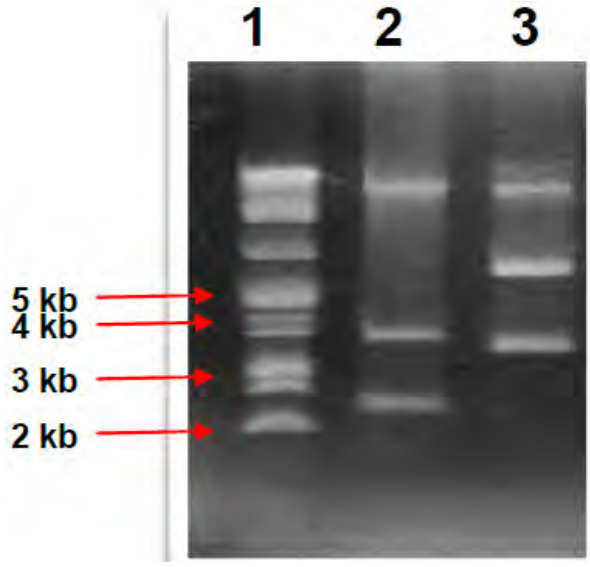

Figure 1. Agarose gel showing the insert size (ABA392). Lane 1: Supercoil DNA Ladder; Lane 2: pUC18; Lane 3: ABA392.

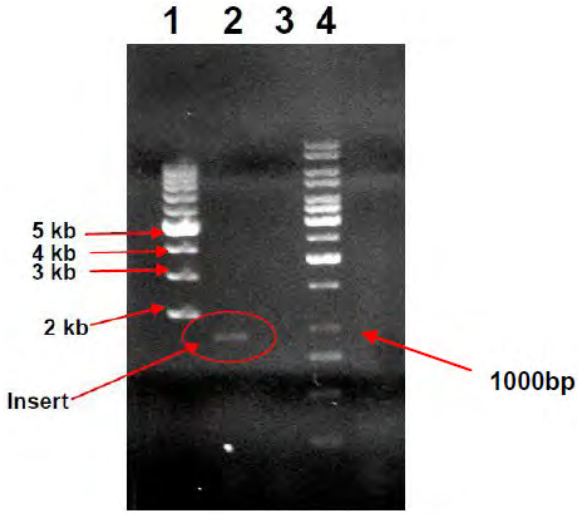

Figure 2. Plasmid Restriction Endonuclease Analysis was carried out, using the enzymes *Pst*I and *Sma*I. From the RE analysis the insert was recovered and found to be of the expected 0.9kb in size.
Lane 1: Supercoil DNA ladder; Lane 2: *Pst*I/*Sma*I; Lane 3: NA; Lane 4:1 kb DNA ladder.

fragment of 3.5 kb in size was observed (Figure 1). Two linearized fragments of 2.7 and 0.9 kb in size, corresponding to pUC18 and the inserted gene respectively was produced when the double digestion with *P*stI and *Sma*I was carried out (Figure 2).

Pathogenicity study

From histopathological examination it was observed that, when 0.5 ml of 10^7 CFU bacterial suspensions of the clone and PMB injected into immunosuppressed SK/WDY rats with dexamethasone (0.33 mg/kg body weight), typical HS symptoms are produced. Gross pathology showed severe multiple abscesses in parenchyma of lung. Upon examination of the histological slides, severe congestion of parenchyma of the lungs and infiltration of inflammatory cells were seen. Microscopic examination of histo-slides revealed severe haemorrhagic effect on spleen, lungs and liver (Figure 3).

Immunogenicity determination

On average, the hyper-immune sera obtained from vaccination of both killed and live form of the clone ABA392 (2 and 3 dose vaccination) provided 66% protection to mice challenged with *P. multocida* serotype B;2. On other hand the hyper-immune sera obtained from the parental strain PMB202 with similar vaccination, provided 100% protection. Mice that were actively vaccinated with ABA392 vaccine, when challenged with lethal dose of PMB showed 83% protection. The control group which had not received any hyper-immune sera died within 36 h. ELISA showed positive result for presence of antibody from hyper-immune sera of the rats immunized with the clone ABA392.

DISCUSSION

Haemorrhagic septicaemia is a disease which is acute, febrile and lethal. The condition sets in rapidly and is capable of killing susceptible animals in less than 36 to 48 h (Dawkins et al., 1990). Livestock, especially cattle and buffaloes are of significant economical importance to many countries in the world. In several Asian and African countries, significant foreign exchange and earning is from live stock (Kock et al., 2002). This study was based on the previously created clone ABA392 from *P. multocida* serotype B; 2, 202 (Salmah, 2000). This clone was shown to produce HS like signs and symptoms when injected into mice intra-peritoneally. Mice died within 36 h of injection. In order to further understand and study the immunogenic properties of this clone, this experiment was conducted. The clone ABA392 has shown promising results. In phase one of this study it was important to reconfirm the clone's stability although the clone has been proven to be stable up to 20 generations. The clone was found to be stable and the expected 3.5 kb plasmid was recovered. RE analysis showed the insert of 0.9 kb when subjected to electrophoresis. An effective treatment for HS is difficult, not only due to sudden onset of symptoms and death of animals but also due to the rising number of antibiotic resistance strains (Bindu et al., 2008;

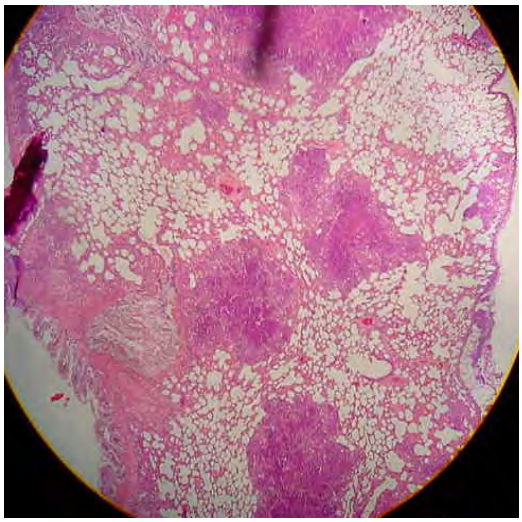

Figure 3. Histological section of Lung in animal treated with Dexamethasone and inoculated with ABA392 clone, showing severe congestion of parenchyma of the lungs and infiltration of inflammatory cells. H&E stain (40X).

Hussam et al., 2003). Hence vaccination would be a better and cheaper option in HS control. ABA392 when used as killed or live attenuated vaccine in 2 doses 2 weeks apart provided no protection to the vaccinated mice and serum of the mice showed a negative result based on the cut-off value obtained by TG-ROC analysis. The 3-dose vaccination provided 83% protection and ELISA results showed a high titer of antibody production. The serum from these mice used in PMPT study provided 66% protection as well. At the same time the similar vaccination regime was carried out with *P. multocida* serotype B:2, the parental strain. The two-dose vaccination was shown to be ineffective and the ELISA revealed a low titre of antibody while the 3-dose vaccination had provided almost a 100% protection to mice with a very high antibody titre. This study as well as previous studies (Salmah, 1997; Yazmin, 2003) has shown that the clone ABA392 is virulent. The histo-slides (Figure 3) results revealed the closeness of the lesions caused by the clone to that of the ones caused by parental strain PMB which causes HS, and the immunization tests shown that this clone not only can rise antibody when introduced as vaccine but also can provides protection when challenged with lethal doses of PMB in mice. These results provide a strong ground for creating a new, specific and a safe recombinant vaccine against HS caused by PMB. It is well known that a recombinant vaccine is the preferred choice of vaccination and this recombinant clone ABA392 seems to be a potential candidate.

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Full Length Research Paper

Improving the efficiency of involved bacteria in aeration tanks of waste water stations

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Advanced bioremediation in waste water and sewage treatments currently represents one of the important aspects of biotechnology. The removal of pathogenic microorganisms, complex hydrocarbons, heavy metals and nutrients were intensively investigated. The present work aims to improve the efficiency of involved bacteria in aeration tanks for maximizing mineralization process of organic substances and consequently reduce the time of the treatment process. Other target is the elimination of nutrients (N & P) to avoid their environmental and hazardous effects. In order to achieve these goals, isolation and identification of dominant microflora in aeration tanks were carried out and highly active strains were selected. Trails are newly done for hybridization or cloning one or more of dominant strains to increase their oxidizing efficiency. A pilot experiment was established in a green house to stimulate biological stage of municipal plant and to test the achieved genetically modified strains (Modell experiment). Also, monthly data were recorded of 20 parameters to highlight and controlling input and output of wastewater station of Taif city. Biological oxygen demand (BOD_5) and chemical oxygen demand (COD) clearly decreased in out fluent indicating lower organic load. The decrease of total organic carbon (TOC), total dissolved solids (TDS) and total suspended solids (TSS) assured the previous obtained data. Presence of large amount of dissolved oxygen (DO) in the out fluent means the efficiency of aeration pumping process. Nitrate content (NO_3^-) and nitrite content (NO_2^-) were sharply decreased in the out fluent indicating the higher requirement of H-acceptors. NH_3 (ammonia content) decrease, however total Kjeldahl Nitrogen (TKN) increase were due to intensive microbial bodies load. Total hardness (TH) decrease, which means lower conc. of Ca^{++} and Mg^{++} and better quality of output water. pH values were slightly decreased because of microbial acidic products. Turbidity was dramatically reduced because of different precipitation process. Phosphorous (P) content and Sulfate ($SO_4^{=}$) content decreased indicating consumption or fixion in microflora bodies. Otherwise, chloride (Cl^-) content were increased in outfluent because of chlorination process. Oil and grease were quite reduced in the outfluent. Finally, different heavy metals and hydrocarbons were found in the limit or lower than the permit levels globally. Total microbial count increased considerably in outfluent, especially in summer months; however, fermentative bacteria were very low because of enough O_2 present in outfluent. Only 15 strains of 280 isolates (about 5.4%) were found to be highly active in mineralizing organic substances which were completely identified. The most active one was used to modify the dominant strains by cloning technology and reinoculated in the pilot experiment

Key words: Waste water bioremediation, sewage microflora, mineralization of organic pollutants, eutrofication phenomena, microbial cloning, ligation reaction.

INTRODUCTION

Bioremediation of sewage wastes is considered the most effective, inexpensive and safe one from biotechnology views. The operations are based on different actions:

a) Mechanical remove of moderately sedimentable solids and scum.
b) Microbial mineralization of dissolved organic

pollutants in aeration tanks or in trickling filters.
c) Anaerobic digestion of sewage sludge to produce methane (Biogas).
d) Tertiary treatment to eliminate nutrient elements, that is, NO_3^-, NO_2^-, NH_3, PO_4, $SO_4^=$ to avoid Eutrophication phenomena.
e) Chlorination or ozonation to remove pathogenic microorganisms.
f) Biological chelating of heavy metals by Biomass of yeasts and algae, however, this removing is still temporary.

The paradox of sewage treatment depends essentially on:

1. Amount and type of easily decomposable organic matter, which considers the source of energy (H-donor) and supplies carbon for synthesis of microorganisms bodies (Burford and Bremner, 1975; Stanford et al., 1975; Reddy et al., 1982).
2. A sufficient oxygen concentration (pO_2) is necessary as H-acceptor for intensive mineralization. Moreover, bacteria could be switched to other acceptors such as nitrate or sulfate as O_2 alternative (Abou- Seada and Ottow, 1985; Ottow and Fabig, 1985).
3. pH: maximal mineralization usually occurred at neutral pH level (7.1-7.5). Highly acid or alkaline conditions lead to lower mineralization and less efficiency of biodegradation (Koskinen and keeney, 1982; Abou-Seada and Ottow, 1986).
4. The specific effect of quality of microorganisms in aeration tanks (Munch and Ottow, 1984, Abou-Seada and Ottow, 1987). With gene cloning technique, it could be managed and improved the efficiency of dominant bacterial strains in aeration tanks to optimize the treatment process (Oliver et al., 1977; Hickey and Smith, 1996).
5. Recently, Membrane Bioreactor (MBR) Technology, which provides a good alternative to conventional treatment by activated sludge followed by a setting tank in municipal waste- water plants. It combines the biological treatment with a membrane separation in one step, which 5 times smaller than activated sludge system. Moreover, the biomass concentration can be greater, which reduce reactor volume and also reduce operating coasts (Galil, 2003; Stephenson et al., 2002; Verma et al., 2006).

The target of this work is to improve the efficiency of involved bacteria in aeration tanks and so maximizing elimination of organic substances and also nutrient elements

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Abbreviations: BOD_5, Biological oxygen demand; **COD,** chemical oxygen demand; **TOC,** total organic carbon; **TDS,** total dissolved solids; **TSS,** total suspended solids; **DO,** dissolved oxygen; **TKN,** total Kjeldahl nitrogen; **TH,** total hardness; **MBR,** membrane bioreactor.

MATERIALS AND METHODS

This study was conducted during 16 months. Representative samples were monthly taken from influent and out fluent of municipal sewage plant in Taif city and analyzed bacteriologically and physico-chemically (20 parameters) to recognize the efficiency of treatment process.

Bacteriological analysis

Representative sewage samples were carefully and serially diluted in sterilized distilled water and densities of total microbial flora and fermentative bacteria were determined by plate count technique using Difco nutrient agar as described by Page et al. (1982). 280 pure cultures were picked up and further tested for their abilities to degrade the complex organic substances (cellulose, oil derivatives and phenols) under sterilized conditions.

The used synthetic mineral medium contains (per liter) 1.4 g KH_2PO_4; 2.2 g $NaHPO_4$; 0.5 g $(NH4)_2SO_4$; 0.2 g $MgSO_4$, 10 mg $CaCl_2$, 5.0 mg $FeSO_4$ 2.5 mg $MnSO_4$, 2.5 mg Na_2McO_4, 1.0 g KNO_3 and 5% source of C, trace element solution 10 ml, pH 7.2 as described by Sperl and Hoare (1971). The test was carried out in 5 replicates (tubes) for each strain. The tubes were inoculated and incubated at 30°C for 48 h. The active strains were able to degrade the organic matter (no turbidity) in all replicates. Control tubes without inoculation were simultaneously run.

To identify the most active strains, standard morphological, cultural and physiological tests were carried out as described by Naveke and Trepper (1979) and Sussmuth et al. (1987). Classification of isolates was followed according to Bergy,s manual of systematic bacteriology (Krieg and Holt, 1984; Sneath et al., 1986).

Chemical and physical determinations

1) Dissolved oxygen (DO) levels were determined by electrometric method using an oximeter basing on diffusion rate of molecular O_2 a cross membrane. The procedure was described in details by Tortora et al. (1986).
2) Biological oxygen demand (BOD_5) was detected by measuring dissolved O_2 in sewage samples before and after incubation at 20°C for 5 days. The difference is the amount of consumed oxygen (mg/L) in oxidizing decomposable organic pollutants by present microflora during 5 days. The higher organic C, The higher O_2 consumed (Jenkins, 1981).
3) Chemical oxygen demand (COD) is a rapid method for measuring consumed oxygen in oxidation of total organic compounds using concentrated dichromate digestion solution ($K_2CrO_7 + H_2SO_4$) under heating (Himebaugh and Smith, 1979).
4) Total organic carbon (TOC) and total dissolved solids (TDS) were measured as described by Waring and Gilliam (1983).
5) Nitrate, nitrite, ammonia and total nitrogen were measured as described in details by Page et al. (1982). Nitrate content was calorimetrically determined after reduction by cadmium and reaction with Na-salicylate, nitrite content was also calorimetrically evaluated by reaction with sulfanil acid and – naphthylamine, ammonia content was measured by Nessler's reagent, while total nitrogen was determined by Kjeldahl method.
6) Total hardness (TH) was measured by soap precipitation chiefly in presence of Ca^{++} and Mg^{++} ions. The procedure was described by Goetz and Smith (1959).

Figure 1. Pilot plant set-up.

7) Alkalinity is the sum of all titratable bases, that is, carbonate, bicarbonate, hydroxide, borates and silicates as reviewed by Jenkins and Moore (1977).
8) pH values were measured electrometrically with WTW-pH-meter.
9) Turbidity is caused by suspended and colloidal matter such as clay, silt, fine divided organic and inorganic matter as well as plankton and microscopic organisms that causes light to be scattered. The higher the intensity of scattered light, the higher the turbidity. The procedure was described by Hernandez et al. (1991).
10) Chloride ion (Cl^-) was quantitively detected by silver nitrate titration to precipitate silver chloride (white color) till red silver chromate is formed in range of pH 7 to 10 as referred by Cooper et al. (1982) and Paustian (1987).
11) Heavy metals (that is, Cad, Mo, Zn, Cr, M, Ag, Hg, Pb, Ni, Ba, Se) and hydrocarbons (phenols, pesticides, and detergents) were spectrophotometrically determined after filtration of sample using atom absorption as described by Page et al. (1982).
12) The samples were taken in July and August 2009 to represent summer season and December 2009 and Jan 2010 to represent winter season.

Pilot plant set-up

In order to optimize nutritional and environmental parameters to improve efficiency of wastewater treatment at Taif WWT plant, a set-up was designed and executed using local materials.

The location for the set-up was selected, inside Taif Univ. comps as an isolated piece of land constructed with walls and roof. This shall provide safety to surrounding environment and facilitate execution of the work under controlled conditions.

The set –up consists of several vessels connected to each other using 4-way valves (Figure 1). These type of valves provide flexibility and enable changing water flow forwards and backwards according to its composition, type of additives, degree of aeration and type of microorganisms to be used.

Experimental procedure

It is an attempt to divide biodegradation activities within several vessels such as accommodates certain microbial group, which is specific for degrading of particular component (s) present in wastewater. Therefore, the treated sewage coming out from certain vessel is pumped to the next vessel for further degradation of one or more of remaining organic pollutants. It believes that a number of feeding scenarios to the above-mentioned set-up have to be tested before to reach the optimal scenarios

Tested organisms

This work will be two- prolonged; either with naturally microflora exists in Taif wastewater plant or with the genetically modified strain (s) to confirm its highly improved capabilities in biodegradation of organic pollutants. For both directions, wastewater entering the system has to pretreat with manual removal of course and floating particles. A special vessel could be adapted for such task. A second vessel could be also used to optimize the conditions of biodegradation (temperature, O_2 potential, pH value, etc.) before pumping to the following vessels. Because of some technical problems and missing of peristaltic pumps, we hope, as soon to be finished and continue this work.

Cloning the EDTA and nitrilotriacetic acid (NTA) degradation gene *(emoB)* from the Bacterium BNC1 (Wild Type)

Used bacterial strains

Bacterium BNC1 (Wild Type) – *E. coli* BL21 (DE3) *E. coli* BL21 (DE3)

Table 1. Chemicals and enzymes.

No.	Item	Amount
1-	Taq-DNA polymerase + $Mgcl_2$ + 10Xbuffer	200 Units
2-	Pfu-DNA polymerase + $Mgcl_2$ +10Xbuffer	100 Units
3-	dNTP's mix	100 mM each
4-	T4-DNA ligase	50 Units
5-	NdeI	200 Units
6-	NotI	200 Units

Primers

EmoB (F): 5' - GAT GAC GAC GAC CAT ATG ACC TAC TCC - 3'
(Contained an additional *NdeI* recognition site).
EmoB (R): 5' - TCA AGT GAT GTG CGG CCG CGC GCG – 3'
(Contained an additional *NotI* recognition site).
Plasmids: pET30-LIC (expression vector) (Table 1)

Preparing the construction harboring the EDTA and nitrilotriacetic acid (NTA) degradation gene

Using PCR, two specific primers were used to amplify a 300 bp represent the coding sequence of the x gene from the genomic DNA of the strain of *E. coli* [Bacterium BNC1 (Wild Type)].
- The sequence of the specific primers was as the following:

F: 5' - GAT GAC GAC GAC CAT ATG ACC TAC TCC - 3'
R: 5' - TCA AGT GAT GTG CGG CCG CGC GCG – 3'

The PCR amplification reaction

The PCR was performed in a 50 ul reaction volume containing 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.01% (w/v) gelatine), 250 uM each of dGTP, dATP, dCTP and dTTP (dNTPs), 2.5 units of Taq DNA polymerases, 100 pmol of each primer and the DNA template, was released from the bacterial cells by boiling in a water bath for 5 min to lyses the cells and then the tubes were spun briefly to collect the condensate (Carozzi et al.,1991). PCR reaction conditions were: 94ºC for 3 min; 35 cycles of denaturation at 94ºC; annealing at 52ºC; and extension at 72ºC for 2 min each, followed by 7 min extension at 72ºC. After performing the PCR reaction, the amplified 300 pb fragment was eluted and purified from the gel and ligated to the pGEM T-easy cloning vector.

RESULTS AND DISCUSSION

Data recorded in Tables 2 to 5 Show the different parameters, which were measured to control the final product of municipal plant of Taif city and recognize the efficiency of treatment process. The following results were obtained:

1) Mineralization of organic pollutants indexed by BOD_5 (Biological oxygen Demand) and COD (Chemical Oxygen Demand) leads to removing ca 80% of organic load. The values of BOD^5 and COD (as means of 16 months, Table 2) clearly deceased from 353.3 and 759.1 mg O_2/L in influent to 1.43 and 6.82 mgO_2/L in out influent, respectively. Basing on the lower BOD^5 and COD, desto lower organic load

2) TOC (Total Organic Carbon%), TDS (Total Dissolved Solids%) and TSS (total Suspended Solids%) decreased from 148.9, 72.4 and 324.7 in influent to 6.89, 64.87 and 1.62 in outfluent, respectively. These results assured the previous obtained values of BOD and COD.

3) DO (Dissolved Oxygen) in plant influent was around Nell throughout the entire period (16 months) of the experiment; however, it increased in outfluent to 4.92 mg/L. It means that efficiency of O_2- pumping was enough

4) Mineral nutrients as indexed by NO_3^-, NO_2^- and NH_3 in the influent were (as means of 16 months measurements) 31.1, 19.4 and 64.9 ppm, respectively. Other side, in the outfluent, they are sharply decreased to 10.9, 0.0 and 5.8 ppm, respectively. It seems that intensive mineralization of organic pollutants enhanced the demand for H-acceptors and consequently leads to more reduction of nitrate and nitrite. The decrease of Ammonia may be due to highly assimilation in microflora bodies. Total Nitrogen relatively increased from 76.1% in influent to 91.9% in outfluent. This is due to of mineralization of protein of pollutants and N release.

5) Hardness decreased clearly from 154.1 mg $CaCO_3$/L (mean of 16 months) in influent into 135.3 mg $CaCO_3$/L in outfluent. It indicates lower concentration of Ca^{++} and Mg^{++} (ions), which improve the quality of output wastewater.

6) Alkalinity and acidity (pH values): Alkalinity means all of bases such as carbonate, bicarbonate, hydroxide, borates and silicates. It was noticed that alkalinity decreased from 362.7 influent to 224.3 mg/L in outfluent of the plant. Otherwise, pH values were slightly decreased from 7.67 influent to 7.56 in out fluent. It is easily to explain, because of acid production by microbial metabolisms.

7) Turbidity, which caused by suspended and colloidal substances, was dramatically reduced from 342.9 into 31.2. The main reason is various precipitation processes during the entire treatments.

8) Phosphorous and Sulfate: P and $SO_4^=$: P and S such like N compounds lead to enrich of photoplankton and microflora exhausting dissolved O_2 and lead to biological death of lakes and reveres (eutrophication phenomena).

Table 2. Determinations of Biological Oxygen Demand (BOD),Chemical Oxyge Demand (COD), Dissolved Oxygen (DO)And Total Dissolved Solids (TDS).

Month	Year	BOD5		COD		DO		TOC		TDS	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		input	output	Input	output	input	output	output	input	Output	input
1	2009	326.34	1.45	693.61	6.95	0	5.1	8.55	139.61	77.1	78.71
2	2009	332.02	1.48	702.18	7.1	0	5.9	8.1	179.04	75	80.28
3	2009	329.44	1.5	691.74	6.85	0	5.6	7.8	200.97	73.65	74.61
4	2009	336.06	1.53	694.37	6.73	0	4.85	7.65	189.07	70.9	73.9
5	2009	332.25	1.33	720	6.9	0.03	4.7	7.4	153.55	68.55	64.51
6	2009	348.48	1.35	769.2	6.65	0.01	4.4	7.1	150.23	64.6	74.93
7	2009	356.63	1.25	812.23	6.5	0.01	4.5	6.95	136.87	60.95	75.91
8	2009	360.09	1.44	818.1	7.35	0	4.35	6.7	134.45	60.7	71.81
9	2009	352.63	1.49	794.27	7.28	0.01	4	6.42	137	58.01	74.01
10	2009	418.12	1.49	922.83	6.88	0	4. 80	6.3	140.87	55.3	75.13
11	2009	407.08	1.45	817.3	6.4	0.01	4.9	6.15	143.33	54.15	94.17
12	2009	358.88	1.43	816.42	6.15	0.01	4. 75	6.01	189.45	60.45	62.73
1	2010	341.4	1.47	802.97	6.83	0.02	5.1	5.67	205.71	61.9	67.03
2	2010	340.12	1.44	665.04	6.86	0.01	4.98	5.71	164.14	66.24	56.69
3	2010	350.92	1.5	690.74	6.94	0.01	4.9	6.43	199.77	64.35	60.02
4	2010	362.17	1.37	735.9	6.7	0.01	4.83	7.29	205.8	66.1	65.27
	Mean values	353.3	1.43	759.1	6.82	nell	4.92	6.89	148.9	64.87	72.48

Table 3. Determinations of Total Suspended Solids (TSS%), Nitratecontent (NO_3^- ppm), Nitrite content (NO_2^- ppm), Ammonia (NH_3ppm) andTotal Kjeldahl Nitrogen (N_2%)

Month	Year	TSS		NO_3^-		NO_2^-		NH_3		TKN	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		Output	input	output	input	output	input	output	input	Output	input
1	2009	1.33	234.03	10.3	33.76	0	18	5.1	96.2	95.5	108
2	2009	1.15	249.61	12.5	28.28	0	14.7	4.88	83.8	88.4	98.7
3	2009	1.65	261.61	14.2	25.56	0.01	12.7	5.7	80	85.6	95.6
4	2009	1.5	258.13	14.6	23.29	0.01	10.6	5.04	60.4	88.4	75.3
5	2009	1.8	328.68	12.2	26.1	0.01	12.6	5. 75	57.8	89.9	68.9
6	2009	1.35	375.27	10.1	25.43	0	17.7	5.55	56.5	95.3	65.2
7	2009	2.28	370.48	8.87	23.41	0	16.9	6.35	63.7	92.2	73.2
8	2009	2.7	372.77	9.11	25.26	0	19.5	6.9	68.9	94.5	76.3
9	2009	1.93	351.63	6.45	34.18	0	26.6	6. 80	66.3	95.8	75.4
10	2009	1.77	404.17	8.75	41.18	0	32.5	6.68	61.6	99.4	73.3
11	2009	1.93	386.93	9.9	30.39	0	24	5.85	54.4	98.7	67.6
12	2009	1.58	341.13	9.57	32.21	0	24.1	5.1	58.1	90.6	70.7
1	2010	1.52	334.77	9.45	32.44	0	24.1	6.46	58	88.7	70.2
2	2010	1.75	287.82	15.3	39.9	0	23.6	6.8	57.2	90.1	65.5
3	2010	1.65	305.58	12.9	38.8	0	17.1	5.61	57.4	88.9	65.5
4	2010	1.5	331.6	11.4	36.78	0	15.5	4.7	58.7	87.4	67.3
	Mean values	1.62	324.7	10.9	31.1	0	19.4	5.8	64.9	91.9	76.1

Therefore, The clear decrease of P and $SO_4^=$ content from 24.6 and 50.8 ppm influent to 5.1 and 7.76 mg/L in outfluent, respectively. It may be due to consumption or fixion in microflora bodies. These results has another benefit to avoid eutrophication.

9) Chloride Ion Cl^-: This parameter was found to be increased from 146.2 ppm in influent into 170.9 ppm in outfluent because of chlorination treatment to remove

Table 4. Determinations of Total Hardness (TH), Alkalinity, pH values, Turbidity and Phosphorous compounds (P).

Month	Year	Total Hardness		Alkalinity		PH		Turbidity		P	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		output	Input	output	input	output	input	output	input	output	input
1	2009	122.3	142.3	315.4	348.4	7.55	7.69	24.6	254.8	4.9	20.94
2	2009	111.2	141.2	323.6	339.8	7. 60	7.7	25.8	265.7	5.64	19.43
3	2009	118.5	138.8	303.4	333.2	7.75	7.78	29.4	274.9	5.37	20.37
4	2009	126.1	146.9	310.8	334.8	7.65	7.77	21.5	274.7	5.55	24.5
5	2009	129.8	146.1	301.5	331.1	7.65	7.7	38.4	348.6	5.8	22.91
6	2009	130	150.1	312.4	343.8	7.6	7.88	32	392.8	4.46	22.16
7	2009	147.5	167.9	326.6	366.4	7.62	7.88	33.8	389.3	6.43	22.49
8	2009	145.5	155.6	343.5	362.5	7.64	7.9	30.5	390.8	5.86	22.66
9	2009	140.9	150.3	330.8	374.5	7.72	7.92	38.8	372.8	5.64	23.69
10	2009	134.7	154.2	333.5	346.5	7.61	7.91	45.4	425.4	5.7	23.73
11	2009	139.8	159.8	324.6	354.5	7.64	7.88	42.6	402.4	5.75	25.22
12	2009	143.5	163.5	355	385.5	7.52	7.82	36.2	460.2	5.25	25.35
1	2010	147.7	170.4	239.8	420.5	7.56	7.77	30.6	352.9	5.13	26.73
2	2010	149.8	169.2	242.1	385.4	7.3	7.43	37.5	309.6	5.24	30.09
3	2010	141.4	152.2	248.9	385.3	7.42	7.5	38.7	326.4	5.45	30.51
4	2010	137.1	157.5	250	390.6	7.35	7.65	39	351.6	5.9	32.71
Values	mean	135.3	154.1	244.3	362.7	7.56	7.76	31.2	342.9	5.1	24.64

Table 5. Determinations of oil-grease content, sulfate content (SO4=), Chloride ion (Cl-) , Setting solids and temperature degrees.

Month	Year	Oil and Grease		$SO_4^=$		Chloride		Settling solid		Temperatue	
		Plant fluent		Plant fluent		Plant fluent		Plant fluent		Plant fluent	
		output	input	output	input	output	input	output	input	output	input
1	2009	0	28.78	7.1	61.2	171.3	139.8	ND	4.07	21.6	22.8
2	2009	0	26.36	7.22	57.9	172.6	134.6	ND	3.73	21.8	22.9
3	2009	0	28.77	7.96	54.2	170.6	132.4	ND	3.66	22.5	23.3
4	2009	0	28.67	7.64	48.7	168.8	140.5	ND	4.01	22.9	23.5
5	2009	0	28.84	7.13	47.9	173.4	135.4	ND	4.29	25	25.9
6	2009	0	27.77	8.15	47.1	175.6	140.9	ND	4.34	26.4	27.4
7	2009	0	27.74	9.56	44.6	174.2	150.9	ND	4.39	26.6	28
8	2009	0	27.06	7.68	51.8	164.6	147.9	ND	4.39	27.7	28.3
9	2009	0	34.93	7.6	49.8	173.5	144.9	ND	4.25	28	29.1
10	2009	0	37.33	7.6	47.4	174.6	139.3	ND	7.68	27.5	28.7
11	2009	0	34.83	9.06	49.8	168.6	190.1	ND	4.44	26.4	27.8
12	2009	0	32.45	6.6	51.1	164.6	148.1	ND	5.01	22.4	25.4
1	2010	0	29.65	7.1	49.8	173.3	146.2	ND	5.58	21.8	24.2
2	2010	0	29.14	7.16	49.2	172.6	141.9	ND	3.99	22.4	24.9
3	2010	0	28.74	9.96	51.7	172.6	142.8	ND	3.12	23.3	25.5
4	2010	0	27.5	6.64	50.1	164.6	163.6	ND	3.07	24.2	26.3
Values	Mean	0	29.9	7.76	50.8	170.9	146.2	ND	4.37	24.4	27.5

pathogenic flora.

10) Oil and Grease content: The removal efficiency of oil-grease was quit completely, which it was 29.9 mg/L in influent and reached Nell in the outfluent.

11) Temperature values of treated wastewater ranged between 27.5°C at the begin to 24.4°C at the end of treatment process, being clearly higher in summer months than winter months.

12) The contents of different heavy metals (that is, Cad, Mo, Zn, Cr, M, Hg, Ag, Pb, Ni, Ba, Se) and hydrocarbons (phenols, pesticides detergents) were found throughout the investigation period in the limit or lower than the

Table 6. Determinations of total microbial flora and fermentative microorganisms (Cell /L).

Month	Year	Total microorganisms Cell /ml		Fermentative bacteria Cell / ml	
		Output	input	Output	Input
1	2009	$4.5x10^6$	$3.5x10^5$	$2.1x10^2$	$4.9x10^2$
2	2009	$4.1x10^6$	$2.8x10^5$	$3.3x10^2$	$4.5x10^2$
3	2009	$5.9x10^6$	$3.9x10^5$	$3.5x10^2$	$4.2x10^3$
4	2009	$8.4x10^6$	$4.1x10^5$	$3.1x10^2$	$5.3x10^3$
5	2009	$9.1x10^6$	$5.5x10^5$	$4.1x10^2$	$6.3x10^3$
6	2009	$6.3x10^7$	$6.1x10^6$	$5.3x10^3$	$8.5x10^4$
7	2009	$8.5x10^8$	$9.2x10^6$	$7.6x10^3$	$9.0x10^4$
8	2009	$8.8x10^8$	$9.9x10^6$	$8.1x10^3$	$8.2x10^4$
9	2009	$7.9x10^7$	$7.4x10^6$	$7.2x10^3$	$6.8x10^4$
10	2006	$7.4x10^6$	$6.9x10^5$	$2.3x10^3$	$6.5x10^3$
11	2009	$5.9x10^6$	$5.4x10^5$	$6.8x10^2$	$4.6x10^3$
12	2009	6.5x105	$5.3x10^4$	$5.3x10^2$	$3.4x10^3$
1	2010	$5.4x10^5$	$4.7x10^4$	$3.1x10^2$	$4.4x10^2$
2	2010	$3.9x10^5$	$2.4x10^4$	$1.8x10^2$	$5.1x10^2$
3	2010	$4.5x10^5$	2.3x105	$2.4x10^2$	5.8x103
4	2010	$6.3x10^6$	$3.3x10^5$	$4.1x10^2$	$6.8x10^3$
	Mean	$121x10^6$	$2.3x10^6$	$2.2x10^3$	$23.1x10^3$

permit levels globally. It may be due to those sources of influent introduced to Taif municipal plant mainly urban sewage or agricultural drainage or rainfalls, which rarely contain heavy metals or complex hydrocarbons in comparison with industrial wastewater.

Microbiological densities and selection of highly active strains

Data given in Table 6 show that densities of total microbial flora present in Tarif WWW plant increased con-siderably in out fluent ($121x10^6$ cell/ml liquor as mean of 16 tested months) in comparison with influent (only $2.3x10^6$ cell/ ml liquor). Moreover, the total count was clearly noticed higher in summer months than in winter months. This result could be attributed to suitable conditions in aeration tanks for bacterial growth beside effect of high temperature in summer. Other side, fermentative bacteria were lower in outfluent ($2.2x10^3$ cell/ml as a mean of 16 months) in comparison with influent ($23.1x10^3$ cell /ml), being also higher in summer months . This result may be due to negative effect of residual O^2 present in outfluent comparing with absent O_2 in influent as recorded before (Table 2). This result is in agreement with the findings of Abou-Seada and Bardtke (1989).

Selection test of active strains:

Only 15 strains of 280 isolates were found to be highly active in degradation of organic substances and fate residuals in pure cultures within 2 days incubation at 25°C. They constituted a very small fraction (only 5.4%) of total isolates. This may be attributed to the difference between biochemical properties of mixed and pure cultures, which reflects the importance of microbial association to increase their efficiency. The active strains were completely identified according to Watanabe et al. (1981) Bergey,s manual (Krieg and Holt, 1984; Sneath et al., 1986). They were found to belong to two groups:

Group I: Gram negative, short rods, motile, oxidase negative and fermentative of glucose (*Enterbacter* spp).
Group II: Gram positive endospores forming single or in chain long rods, which are motile, oxidase positive and non fermentative (*Bacillus* spp.).

Cloning the EDTA and nitrilotriacetic acid (NTA) degradation gene (emoB) from the Bacterium BNC1 (Wild Type)

Elution and purification of PCR fragments

The 300 bp DNA band was excised from ethidium bromide –stained agarose gel with a razor blade, weighted and transferred to a plastic tube. Three volumes of binding buffer was added to agarose gel piece, the tube placed in a 45 – 55°C water bath and incubated for 5 min or until the agarose is melted. The High pure filter tube was combined to the collection tube and the sample was pipted to the upper one. Centrifugation was

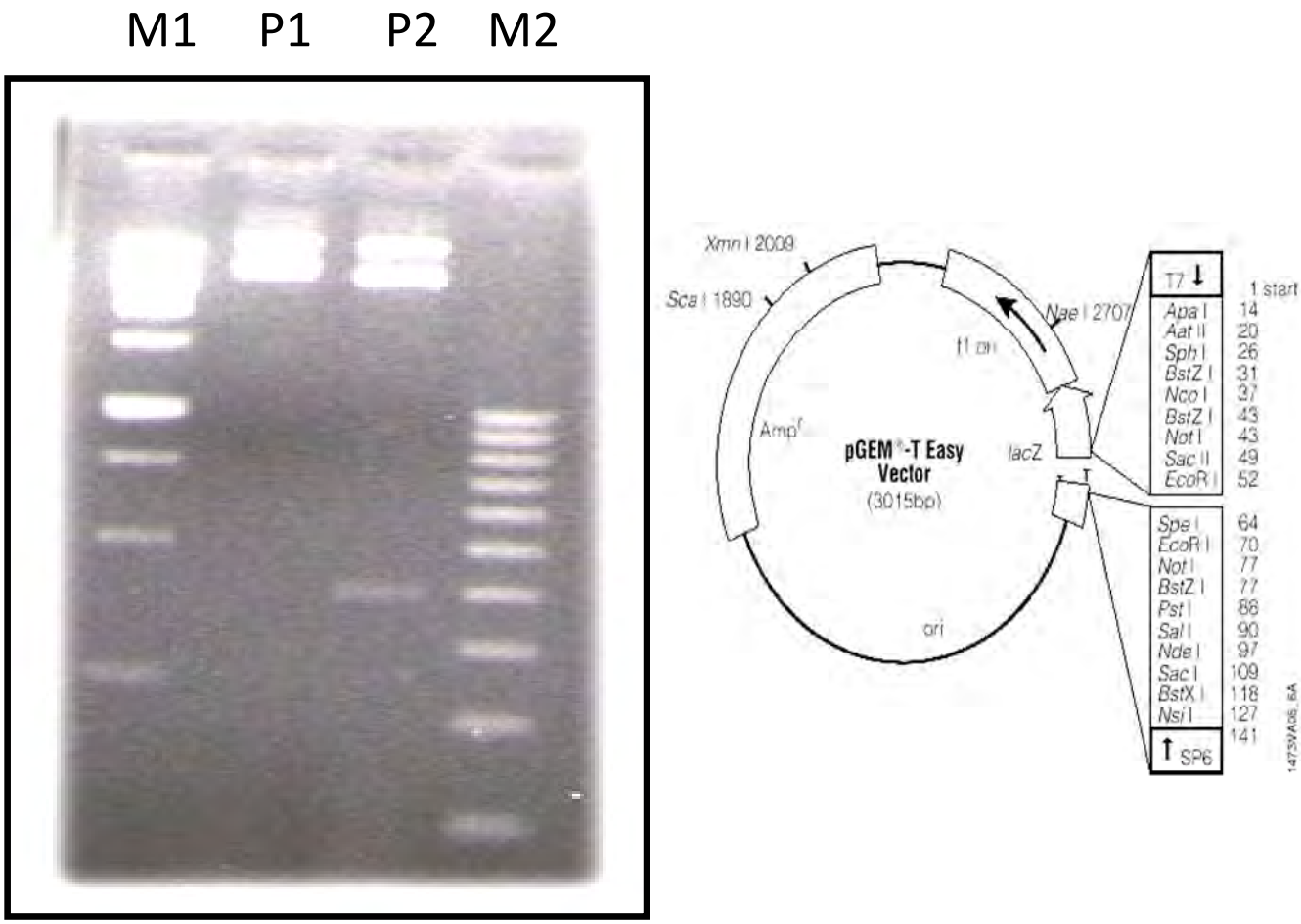

Figure 2. Ligation reaction.

performed for 30 s at maximum speed (approx. 13,000 g) in a standard tabletop centrifuge. The flow through was discard and the filter tube combined again with the same collection tube. A 500 µl binding buffer added into the upper reservoir, and incubated at room temperature for 1 min and centrifugation again for 30 s. The flow through was discard and the filter tube combined again with the same collection tube. A 500 µl wash buffer added to the upper reservoir and centrifugation for 30 s (Biospin Gel Extraction Kit).

The wash buffer flow was discarding and the filter tube combined again with the same collection tube. A 200-µl wash buffer was added, and centrifugation for 30 s. The collection tube was discarding, and the filter tube inserted in a clean 1.5-ml reaction tube. 50 –100 µl elution buffer or sterile distilled water (pH 8-8.5) used for the elution of the DNA.

Ligation reaction

In a clean tube the following components were add: 1 µl 10x ligation buffer, 2 µl PCR vector (50 ng), 1 µl T4 DNA ligase, 3 µl purified PCR product and sterile distilled water up to 10 µl.

The ligation tube was mixed briefly and spined down to collect the contents in the tube. Incubation was performed at 14°C for 16 h. The ligation was checked and part of this mixture was used to transform high efficiency competent cells of *E. coli* strain JM109 (Figure 2).

Genetic modification of dominate strains

The group team will introduce this construct to the genetic background of the most dominant strains. Thereafter, the modified strains will be checked in the laboratory for their containing of the desired gene using specific PCR primers. The final step will be moving the genetically engineered bacteria to the pilot experiment.

The pilot experiment

The genetically modified microorganisms were inoculated to the treated wastewater in the designed vassals and

samples were analyzed till finishing the mineralization process. In comparison with the control (uninoculated) vassals, the processing time will be reduced from 36 to 28 h, which represent 22% time downgrading. This result is very important, which means increasing capacity and efficiency of treatment station and so decreasing treatment cost.

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Full Length Research Paper

A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii* T30C

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Microbial degradation of Tapis crude oil contaminated soil by *Acinetobacter baumannii* T30C was conducted to evaluate the efficiency of the selected potential hydrocarbon degrader in stimulating bioremediation of crude oil-contaminated soil with different treatment units in microcosms with 2.5 kg soil. Previously, *A. baumannii* T30C was isolated from a Tapis crude oil-contaminated soil of oil refinery plant, Terengganu, Malaysia. The reduction of residual hydrocarbons in the soil was observed for a period of about 35 days. The study showed that amendment of nutrients was needed for stimulating the growth of *A. baumannii* T30C and indigenous microorganism in assisting the degradation of residual hydrocarbons in the soil.

Key words: Bioremediation, bioaugmentation, aliphatic hydrocarbons, amendment of nutrients.

INTRODUCTION

Environmental damage due to the oil spills in the past and recent time has focused on the need for the environment friendly strategies for remediation of the contaminated site. For instance, contamination of the environment with crude oil results in pollution, in particular presents a chronic problem to commercial fisheries, recreational resources and public health. Bioremediation is suggested for remediation of contaminated soil sites because of its low cost and its ability to convert contaminants to harmless end products (Rahman et al., 2002; Sathishkumar et al., 2008).

Other physical and chemical processes have been used to remove spilled oil from environment; however the use of these technologies has not always been successful (Aldrett et al., 1997). Bioremediation, the use of microorganisms or microbial process to degrade environmental contaminants is among these technologies (Boopathy, 2000). Numerous microorganisms, including bacteria, fungi, and yeasts are known for their ability to degrade hydrocarbons (Swannell and Head, 1994). Recently, bioaugmentation which involves the addition of microorganisms to enhance specific biological activity has been applied in attempts to remediate numerous environmental problems (Vogel, 1996). The potential of using microorganisms for degradation of crude oil and its constituents to minimize contamination have prompted a number of researchers to study the process in laboratories. For instance, augmenting the contaminated site with appropriate bacterial inoculum is a promising technique to enhance the biodegradation of hydrocarbons. The aim of this study is to evaluate the efficiency of *A. baumannii* T30C in the remediation of Tapis crude oil-contaminated soil to degrade petroleum hydrocarbons.

MATERIALS AND METHODS

Bacterial culture and maintenance

The strain of *A. baumannii* T30C was isolated from the crude oil contaminated soil in Terengganu, Malaysia, and maintained on

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Nutrient Agar slant. The subculture was done every month to maintain its survival.

Soil preparation

The soil selected for this study had approximately six years of exposure to petroleum Tapis crude oil in Terengganu, Malaysia. The crude oil contaminated soil together with some of its physicochemical characteristic information was provided by Petronas Research Sdn. Bhd., Malaysia.

Inoculum preparation

In order to obtain a standard inoculum, a bacterial isolate *A. baumannii* T30C was grown in nutrient broth (NB) at 37°C in an orbital shaker at 200 rpm to yield an absorbance reading of 0.5 at 600 nm. The cells were harvested by centrifugation, rinsed with sterile distilled water before being re-suspended in sterile mineral salt medium (MSM), which consisted of $(NH_4)_2SO_4$, 3 g; KH_2PO_4, 4 g; Na_2HPO_4, 7 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.001 g; $FeSO_4 \cdot 7H_2O$, 0.001 g; Tween 80, 4.0 g/L in 1 L of distilled water. When used as an inoculum at 10% (v/w), the resulting colony forming unit (CFUs/g) was approximately 1×10^7 CFUs/g of soil.

Biodegradation experiments

The ability of bacterial isolate to remediate the petroleum crude oil contaminated soil sample was performed by carrying out the biodegradation experiment in soil for 35 days under room temperature (30 ± 3°C). The experiments were carried out in rectangular aluminium trays of 30 cm × 21 cm × 6 cm (Length × Width × Height) containing 2.5 kg soil. Prior to starting the experiment, the water-holding capacity (WHC) and pH of the soil were determined according to Alef and Nannipieri (1995). Determination of WHC of soil is based on the calculation as follows:

$$\% \text{ Water-holding capacity} = [(100 - W_p) + W_i] / dwt \times 100$$

where; W_p is the weight of the percolated water in grams, W_i is the initial amount of water in grams contained in the sample, and *dwt* is the soil dry weight in grams.

Experiments were conducted with the following treatment combinations:

Treatment A: Soil + bacterial isolate + nutrients
Treatment B: Soil + nutrients
Treatment C: Soil + bacterial isolate
Treatment D: Soil + sterile distilled water (abiotic control)

For each experimental unit, 10% (v/w) of bacterial inoculum which made up of approximately 1×10^7 colony forming units (CFUs)/g of soil and nutrients from optimized MSM were seeded by maintaining the moisture level of 60% WHC of soil. Initial pH of the soil ranged from pH 6.0 to pH 7.0. The trays were covered with sterile aluminium foil. The contents of the trays were mixed thoroughly every alternate day to provide adequate aeration. Every five days, the samples were taken out from the respective experimental unit and analyzed for viable cell count and the extent of biodegradation was determined. The bacterial counts in different treatment units were determined with standard dilution plating technique using sterile nutrient agar and the colony forming units were counted after 24 to 48 h of incubation at 37°C. The extent of biodegradation was determined by extracting the extractable hydrocarbons according to the method described previously by Mishra et al. (2001a) and Das and Mukherjee (2007). Total petroleum hydrocarbon (TPH) was extracted from 10 g of soil by sequentially extraction with 100 ml of hexane, dichloromethane and chloroform. All the three extracts were pooled and dried at a rotary evaporator (LABOROTA 4000, Heidolph, UK). All determinations were carried out in triplicates. After drying, the amount of extractable hydrocarbon was determined gravimetrically.

Gas chromatographic analysis

For analysis of crude oil sample, dodecane (Fluka Chemika) was used as an internal standard and O-terphenyl was used as a surrogate standard (Aldrich, USA). Calibration standards at 7 concentration levels were prepared at a range of 0.2 to 1.4 µg/µl, respectively. Before extraction, O-terphenyl was added to each sample as surrogate standard to give a final concentration of 1.0 µg/µl for calculating the efficiency of recovery. The internal standard with the final concentration of 0.7 µg/µl was used for quantitative purposes and checking the deviation from injection error.

The analysis for aliphatic hydrocarbons was performed on a Perkin Elmer Clarus 500 gas chromatograph equipped with a flame-ionization detector (FID). A high-temperature capillary column (DB-5HT, 30 m × 0.32 mm i.d., 0.1 µm film thickness; J&W Scientific) was used. The carrier gas was helium (2.5 ml/min). The injector and detector temperatures used were set at 290 and 300°C, respectively. The following temperature program was used: 2 min hold at 50°C; ramp to 300°C at 6°C /min; and 16 min hold at 300°C. One microliter aliquot was injected in the split less mode with a 1 minute purge-off. This method was according to Wang et al. (1994). The overall percentage of oil biodegradation was determined from the chromatogram by calculating the average of peak height area of each compound on the chromatogram of a biodegraded sample to the control sample.

RESULTS

Initial residual hydrocarbons concentration in the soil was around 4200 mg/kg of soil as determined by gravimetric analysis. Figure 1 shows the profile of viable cell count of augmented *A. baumannii* T30C in different treatment of crude oil contaminated soil. Treatment D refers to the control soil sample which was not amended with any addition of bacterial inoculum or nutrient showed a viable cell count of 2.29×10^3 CFUs/g of soil on the first day of inoculation. The bacterial cell numbers continued to increase after 10 days of inoculation. Then, the numbers decreased until 15 days of incubation and remained at viable cell count of approximately 1.55×10^5 CFUs/g of soil until the study was terminated. For Treatment A and Treatment B, the initial viable cell counts were 2.19×10^7 CFUs/g of soil and 3.24×10^4 CFUs/g of soil, respectively. Treatment A with the addition of bacterial inoculum and nutrient showed slightly increment of viable cell count of 2.23×10^8 CFUs/g of soil after 5 days of incubation. Then, the viable cell count decreased slightly to 1.29×10^8 CFUs/g of soil until the end of the incubation period. For Treatment B which was added with nutrient, the results showed the effect of treatment to stimulate the growth of the indigenous microbial population. The viable cell count was dramatically increased to 4.17×10^8 CFUs/g of soil after 5 days of incubation. The viable cell count was

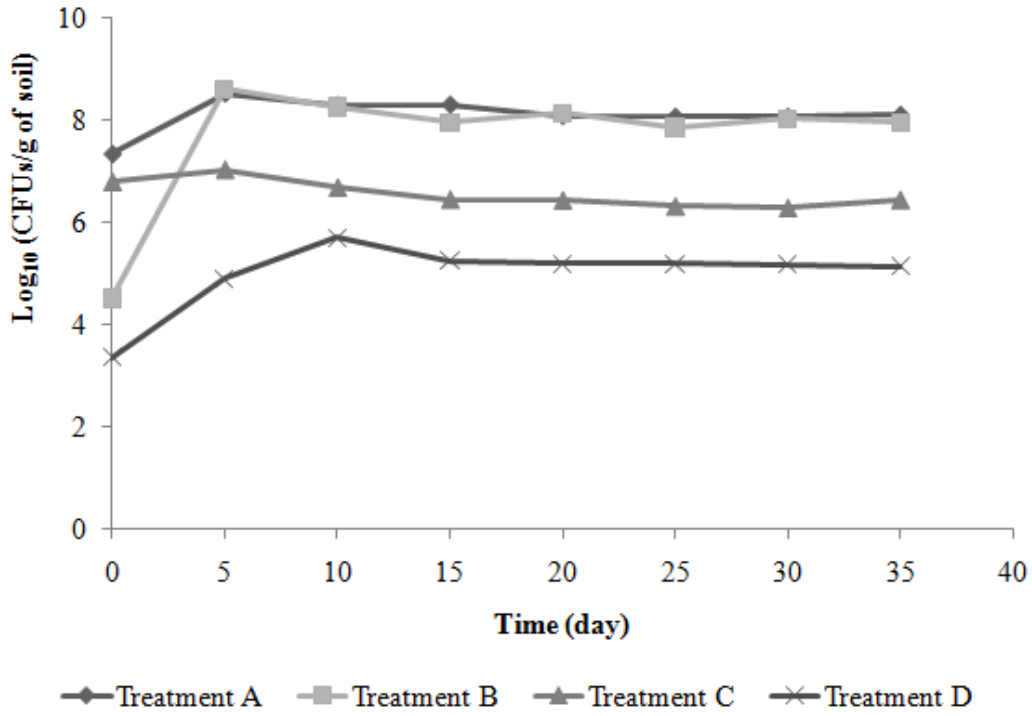

Figure 1. Profile of viable cell count in different treatment of crude oil contaminated soil. For each experimental unit, 10% (v/w) of bacterial inoculum which made up of approximately 1 × 10^7 colony forming units (cfus)/g of soil and nutrients from optimized MSM were seeded by maintaining the moisture level of 60% water holding capacity (WHC) of soil. Treatment A: Soil + bacterial isolate + nutrients; Treatment B: Soil + nutrients; Treatment C: Soil + bacterial isolate; Treatment D: Soil + sterile distilled water (abiotic control).

decreasing after that and achieved about 9.12 × 10^7 CFUs/g of soil at the end of the experiment (35 days). Treatment A and Treatment B showed almost the same amount of reduction in residual hydrocarbons concomitantly by the end of the 35 days of treatment, which decreased to around 2605 mg/kg (42%) and 2423 mg/kg (43%), respectively (Figure 2). Thereby, it was established that the augmentation of *A. baumannii* T30C into the soil was not playing a part in reduction of residual hydrocarbons. This was supported by the result of Treatment C in which only bacterial isolate T30C was augmented without the addition of nutrient in the oil contaminated soil. Results showed that there was a delay in the reduction of residual hydrocarbons and limitation of bacteria to consume the contaminant in the soil without amendment of nutrient. For Treatment C, the residual hydrocarbons decreased to 3448 mg/kg, which was approximately 15% of degradation. The viable cell count decreased from 6.31 × 10^6 CFUs/g of soil to 2.82 × 10^6 CFUs/g of soil. Amendment of nutrients was needed for stimulating the growth of bacteria and assisting the degradation of residual hydrocarbons in the soil. Treatment D however was used as a biotic control in which the lost of residual hydrocarbon was not significant and could be neglected. Gas chromatographic analysis revealed that most of the aliphatic hydrocarbons extracted from soil have been utilized by augmented *A. baumannii* T30C together with indigenous microorganisms Treatment A, 77%; Treatment B, 88% (Figure 3).

DISCUSSION

For soil bioremediation, suitable microorganisms are necessary for an optimal treatment of soils contaminated with petroleum hydrocarbons (Vasudevan and Rajaram, 2001). Mishra et al. (2001a) reported that bioremediation by using microbial inoculants has been a common practice, since it could enhance the rate of biodegradation. On the other hand, environmental factors, such as oxygen level, temperature, nutrients, pH, moisture content, and so forth, may influence the biodegradation of hydrocarbons in soil which have been reported by previous studies. These factors can substantially affect the microbial growth and biodegradation of organic contaminants if not properly governed (Balba et al., 1998). Whereas, Freijer et al. (1996) reported that environmental conditions may influence the mineralization rate of petroleum hydrocarbons and biomass production in determining the successfulness of bioremediation study.

Over the 35 days incubation of *A. baumannii* T30C in

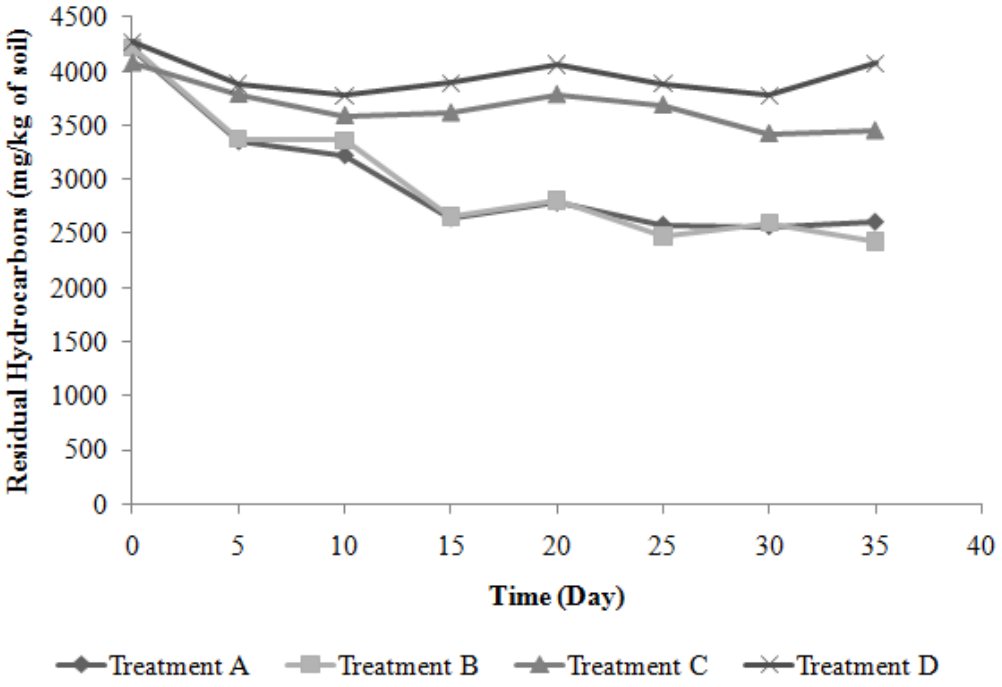

Figure 2. Profile of residual hydrocarbons reduction in different treatment of crude oil contaminated soil. For each experimental unit, 10% (v/w) of bacterial inoculum which made up of approximately 1 × 10^7 colony forming units (cfus)/g of soil and nutrients from optimized MSM were seeded by maintaining the moisture level of 60% water holding capacity (WHC) of soil. Treatment A: Soil + bacterial isolate + nutrients; Treatment B: Soil + nutrients; Treatment C: Soil + bacterial isolate; Treatment D: Soil + sterile distilled water (abiotic control).

an aluminium tray, the soil was tilled and sterile distilled water was added to maintain 60% of WHC of the soil for every interval of 5 days sampling. Tillage is a mechanical manipulation of soil to improve soil conditions (Sabaté et al., 2004). Molope et al. (1987) reported that tillage may alter physical and chemical properties of soil in such a way that it stimulates microbial activity. Tillage redistributes carbon, nitrogen and water and reduces spatial distribution within the soil (Rhykerd et al., 1999). In general, biodegradation of contaminants in soil systems is optimal at soil moisture content between 30 and 80% of WHC (Dibble and Bartha, 1979). Bioremediation of hydrocarbon contaminated soils requires careful control of moisture in which 20% to 60% water saturation of the soil is optimum (Rosenberg, 1993). Failure to appreciate this fact would lead to the maintenance of excessive water levels in the soil and concomitant reduction in oxygen availability (Baker, 1994). Too much water may cause the soil to become anaerobic as reported by Rosenberg (1993).

The soil selected for this study which was obtained from an oil refinery at Terengganu, Malaysia possessed the pH value ranging from 6.0 to 7.0. Biological activity in the soil is greatly affected by the pH, through the availability of nutrients and toxicants and the tolerance of organisms to pH variations (Eweis et al., 1998). According to Eweis et al. (1998), the growth of most microorganisms is usually greatest within a pH range of 6 to 8, although some fungi have optimal growth regions at pH levels of less than 5. Hence, it was expected that *A. baumannii* T30C may exhibit an optimal growth in the soil pH range of 6.0 to 7.0.

The soil treatments were conducted in the room temperature of 30 ± 3°C over the 35 days incubation in aluminium trays. Since the optimal growth temperature of *A. baumannii* T30C was 37°C, it was classified as mesophiles. This group of bacteria has optimal growth in the range of 25 to 35°C in the soil. Baker (1994) reported that soil temperature influences the extent of biodegradation in related to microbial activity with rates of metabolic reactions generally increasing with increasing temperature. This fact was also supported by Zekri and Chaalal (2005) who found that increasing the temperature increases the rate of oil degradation by thermophilic bacteria of *Bacillus* sp.; increasing temperature increases and accelerates the growth of bacteria that resulted in increasing the degradation process of the crude oil at high temperature.

In the present study, the initial indigenous population of oil-degrading bacteria was found to be 2.29 × 10^3 CFUs/g of soil on Treatment D which the control soil sample was not amended with the addition of bacterial inoculum and

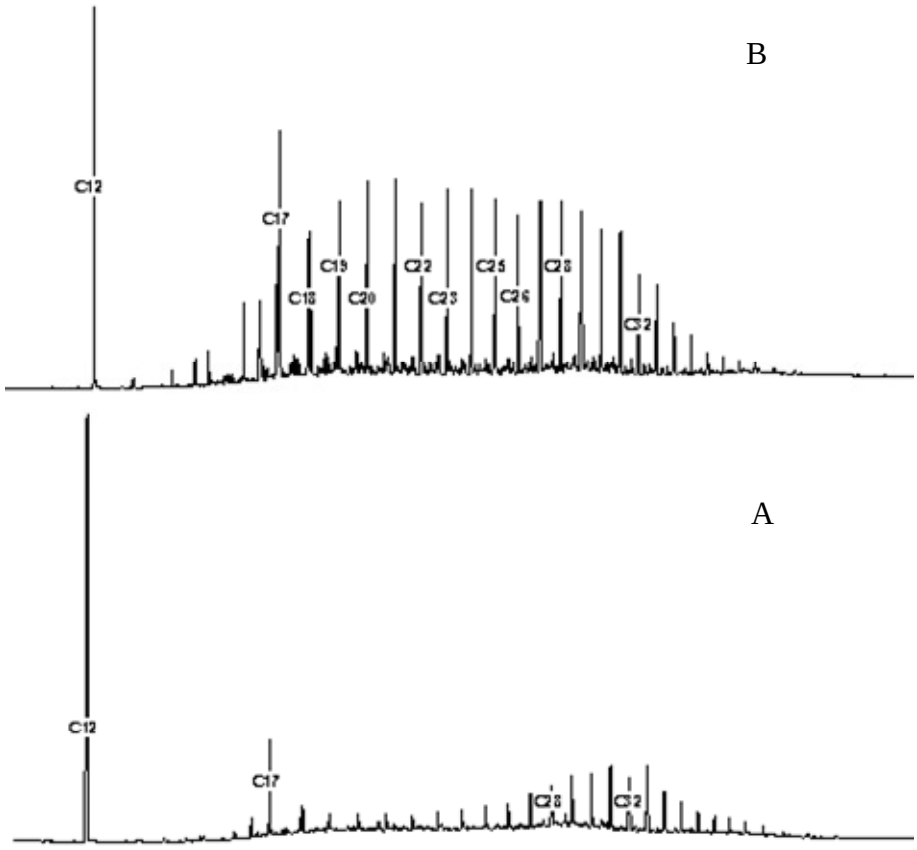

Figure 3. GC-FID profile for Tapis petroleum crude oil in soil bioremediation study. (A) GC profile at Day=35. (B) GC profile at Day=0.
IS refers to internal standard.

nutrient. Previous researches reported that bioremediation is negligible if the population of hydrocarbon-degrading microorganisms is less than 10^5 CFUs/g of soil (Mishra et al., 2001b). The bacterial inoculum needs to be added. However, lower crude oil degradation has been observed as shown on Treatment C which only *A. baumannii* T30C was augmented without addition of nutrient in the oil contaminated soil. This was supported by the result presented on Treatment A (soil + bacterial isolate + nutrients) which the nutrients stimulated the bacterial growth and enhanced biodegradation of crude oil in the soil. This may be attributed to the limitation or deficiencies of nutrient in the soil. Smith et al. (1998) reported that nutrient deficiencies can occur due to the enrichment of carbon sources caused by the pollution events. Addition of nutrients has been reported to enhance the degradation process (Leahy and Colwell, 1990; Barathi and Vasudevan, 2003; Sabaté et al., 2004). Sabaté et al. (2004) reported that the treatment of soil which involved nutrient addition, such as nitrogen and phosphorus sources may lead to a large decrease in total petroleum hydrocarbons by accelerating the growth of microbial population. This was also supported by Leahy and Colwell (1990) in which additional of nitrogen and phosphorus sources are essential for microbial growth. On the other hand, Treatment B (soil + nutrients) showed similar capacity in the degradation of crude oil even though *A. baumannii* T30C was not augmented into the soil. This phenomenon indicated that indigenous microorganisms were capable of degrading crude oil in the soil. Similar result was also observed by Barathi and Vasudevan (2003) who reported that the indigenous microorganisms were capable of degrading alkanes and aromatics existed in crude oil contaminated soil without the addition of bacteria into the soil. Similar findings have been reported by Ghazali et al. (2004) for the diesel-polluted soil that was not inoculated with any microbial consortium which resulted in an increase in numbers of naturally occurring microorganisms in the soil. Venosa et al. (1996) also showed that microbial inoculation did not enhance the removal of hydrocarbons from soil contaminated with crude oil due to other environmental parameters (Dibble and Bartha, 1979).

A few studies have been reported on the effect of bioaugmentation application to the degradation of petroleum hydrocarbons. Hanson et al. (1997) reported

that an increase in the number of colony forming units and crude oil degradation in crude oil contaminated with *Acinetobacter* sp. A3, which was capable of degrading Bombay High crude oil, than in unamended soil. On the other hand, higher rate of degradation of alkanes in the soil by *Pseudomonas fluorescens* was achieved as reported by Barathi and Vasudevan (2003). However, Sabaté et al. (2004) reported that the additional supplementation of nutrients and an inoculation of bacterial consortium had no effect on hydrocarbons degradation.

In terms of crude oil biodegradation study by gravimetric analysis, it is difficult to compare gravimetric data with those reported by other authors. Viñas et al. (2002) reported that there was great variability in the concentration of crude oil used, the type of crude oil, the incubation time and in the methodologies used to quantify degradation. A number of different solvents, fractioning and analytical techniques have been used causing the difficulties in evaluation and comparison of data obtained from research works. Sabaté et al. (2004) reported that the treatment of soil without addition of nutrients showed the reduction of petroleum hydrocarbons about 55% by gravimetric analysis, whereas the treatment containing nutrients and glucose was the most efficient, which represented a biodegradation of 79%. It showed higher reduction of petroleum crude oil compared with the present study of Treatment A (42%) and Treatment B (43%). As described in other studies, Sabaté et al. (2004) reported that the degradation of organic chemicals in soil usually shows a rapid initial phase of descent followed by a period of little or no change in concentration. This kinetics is known as the “hockey stick” phenomenon (Alexander, 1999). Similar pattern was also found on the reduction of petroleum hydrocarbon in Treatment A and Treatment B. A depletion of nutrients, a decrease of microbial populations, lower availability and higher recalcitrance of residual contaminants explain this kind of dynamic (Heusemann, 1997; Alexander, 1999).

For a soil with high organic-carbon content, the age of the soil was related to the biodegradation. Trindade et al. (2005) reported that the longer the soils are exposed to the environment, the higher would be the resistance of the compounds to be degraded, which would decrease the bioavailability of pollutants to microorganisms. This was attributed to the weathering process including evaporation, dissolution, dispersion, photochemical oxidation, water-oil emulsification, microbial degradation and absorption onto suspended particulate materials, further complicating the already complex oil mixture (Wang and Fingas, 1997). Weathering refers to the result of biological, chemical and physical processes which selectively depletes lower molecular weight n-alkanes and volatile aromatic compounds (Readman et al., 1996). The oil-contaminated soils which has been exposed about 6 years to environment has shown a lower biodegradation by gravimetric analysis (Treatment A, 42%; Treatment B, 43%). This could be due to the occurrence of weathering processes causing the lower bioavailability of hydrocarbons. Nevertheless, a higher degradation of aliphatic hydrocarbons was obtained regarding to chromatogram analysis (Treatment A, 77%; Treatment B, 88%). Studies concerning the relationship of chemical composition and biodegradability of crude oil have shown that crude oil containing higher concentration of n-alkanes was found to be more susceptible to microbial attack (Westlake et al., 1974). Of the various petroleum fractions, n-alkanes of intermediate length (C_{10}-C_{20}) are the preferred substrates and tend to be most readily degradable, whereas shorter chain compounds are rather more toxic (Klug and Markovetz, 1971).

Conclusion

Though the weathering process was responsible for the low levels of biodegradation and residual hydrocarbon removal after 35 days of experiments, the well adapted indigenous microbial population of degraders was responsible for better decontamination results compared with the augmentation of *A. baumannii* T30C into the oil-contaminated soil. Addition of nutrient is mandatory for enhancing bacterial growth and degradation activity. However, in situations where the indigenous population of degraders is small, inoculation with an active strain of bacterial isolate or population of degrading strains is an option for enhancing the biodegradation rate and reducing acclimation period.

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Full Length Research Paper

Seasonal variation in the diversity and abundance of phytoplankton in a small African tropical reservoir

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Phytoplankton species composition and seasonal changes were investigated in the shallow reservoir of Adzopé. Taxonomic composition, diversity and abundance of phytoplankton were studied at 4 stations from May 2008 to February 2009, in relation to physical and chemical factors (temperature, conductivity, dissolved oxygen, transparency, pH, nutrients) and climatic factor (rainfall). The phytoplankton comprised 144 taxa, mainly Chlorophyta (29.45% of taxa), Euglenophyta (29.45%) Bacillariophyta (23.97%) and Cyanobacteria (10.27%). Phytoplankton density values were greatest during the transition season (short rainy season), lower during the dry seasons, and extremely lower during the long rainy season (mean value 356 10^5 cells/l, 44 10^5 cells/l and 35 10^5 cells/l respectively). Abundance was dominated by *Anabaena constricta* Szafer (Geitler) and *Microcystis aeruginosa* (Kütz.) Kütz. The diversity index varied between 2.1 bits/cells in long rainy season and 4.6 bits/cells in short dry season. The redundancy analysis (RDA) demonstrated a separation between the long rainy season and the other seasons due to the influence of the flood pulse.

Key words: Phytoplankton, shallow reservoir, species dominance, West Africa.

INTRODUCTION

Phytoplankton is usually at the base of aquatic food web and is the most important factor for production of organic matter in aquatic ecosystem. Most reservoirs will require significant amount of phytoplankton to have productive and sustainable fisheries. The interplay of physical, chemical and biological properties of water most often lead to the production of phytoplankton, while their assemblage (composition, distribution, diversity and abundance) is also structured by these factors. The importance of phytoplankton in tropical reservoir ecosystems include its use in estimating potential fish yield (Hecky and Kling, 1981), productivity (Park et al., 2003), water quality (Walsh et al., 2001), energy flow (Simciv, 2005), trophic status (Reynolds, 1999) and management (Beyruth, 2000). These reservoirs are increasingly threatened by human activities (Cecchi, 2007; Descy and Sarmento, 2008).

In Côte d'Ivoire, about 500 small reservoirs have been constructed on most river systems for water supplies and are presently considered to be threatened (Aka et al., 2000). In spite of this, little is known about the ecology of small reservoirs in Côte d'Ivoire, even though many aquatic habitats are being degraded by pollution, siltation and other human activities (Cecchi, 2007). Water resources in these reservoirs are utilized for drinking, washing, bathing, and irrigation purposes. They receive run-off and wastewater discharges from agriculture and domestic uses. The only published account of Ivorian small reservoirs, a survey of the reservoir of Agboville, clearly showed the impact of river regulation, pollution and emphasized the need for further investigations (Gone et al., 2010). Therefore, the present investigation has attempted to study the water quality in relation to the phytoplankton in the reservoir of Adzopé. This reservoir was selected because it is surrounded by Adzopé city and there are major activities in the vicinity on its catchment.

The seasonal fluctuations in phytoplankton abundance and species composition in any water body is due to differential response of different species to changing

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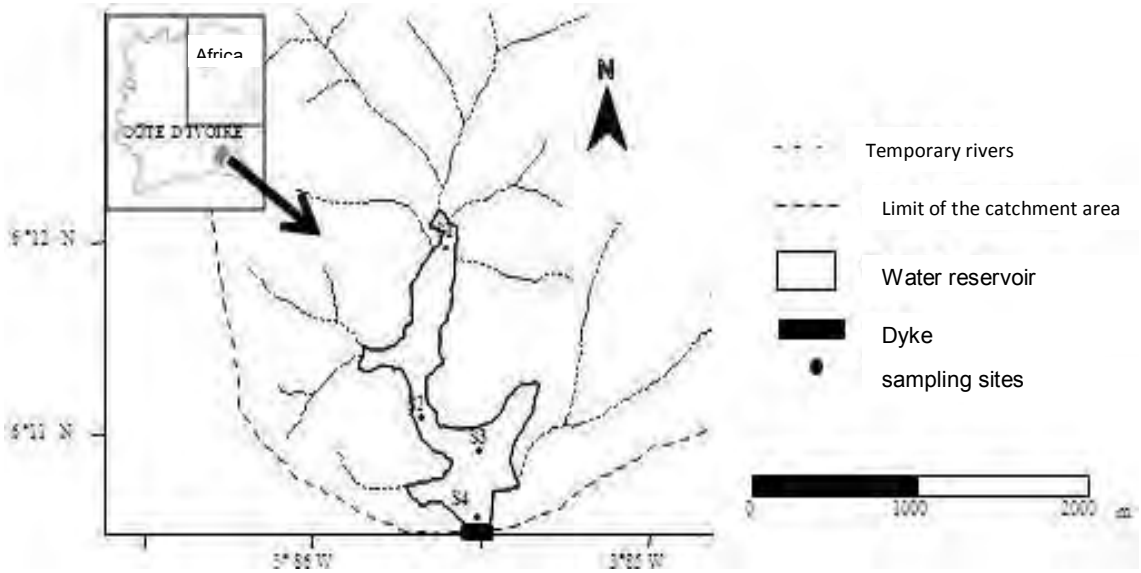

Figure 1. Adzopé reservoir showing the sampling sites (Universal transverse mercator, (UTM))

levels of light, temperature, nutrients, grazing pressure, onset of parasitic infection, extracellular metabolites of plants and animals with a change in season during a course of year (Agrawal, 1999). The seasonal fluctuations in number and kind of phytoplankton are more pronounce in temperate or polar lakes and reservoirs than those in tropical regions (Reynolds, 1988). According to several studies (Abbas, 2009; Figueredo and Giani, 2009), the tropical lakes and reservoirs do not experience marked seasonal fluctuations and thus do not exhibit variations in stocks of phytoplankton species composition. The phytoplankton abundance is regulated by fairly equal influences of any factor like nutrients supply, water turbulence, grazing pressure etc. throughout year.

The aim of this study was to examine the phytoplankton variability throughout year in terms of distribution, abundance and diversity in relation with the physico-chemical variables in Adzopé's reservoir.

MATERIALS AND METHODS

Study area and sampling stations

Adzopé Reservoir (6°10'52" and 6°12'15" N and 3°85'65" and 3°86'73" W) is located in the south-east of Côte d'Ivoire that belongs to the subequatorial zone (Iltis and Lévêque, 1982), characterized by four climatic seasons: long rainy season (April to July); short dry season (August to September); short rainy season (October to November); long dry season (December to March). It is led by the temporary rivers inflow and direct run-off during the rainy season. The dwelling sewage flows though permanently into the reservoir. The total area of the reservoir is estimated at 61.44 ha. The reservoir has a mean depth of 4.91 m and a length of about 2 km. Bank around the reservoir is often occupied by dwelling and market gardening. The hydrological regime of this reservoir depends on precipitations.

Four representative stations (S1, S2, S3, S4) were sampled according to the longitudinal gradient (Figure 1). S1 was located in the upper zone of the reservoir (close to 2.7 m average depth), S2 (4.1 m) and S3 (6.3 m) in the central zone, and S4 (6.5 m) in a down zone, near the dyke.

Sampling and study of phytoplankton community

At each station, four samplings were carried out in order to represent the four climatic seasons: May 2008 for long rainy season (LRS), September 2008 for short dry season (SDS), November 2008 for short rainy season (SRS) and February 2009 for long dry season (LDS) (Figure 2).

Water samples were taken between 06:00 and 09:00 am with 2.5 L Van Dorn bottle from the surface of water. For each sample, the Aqualitic CD24 was used to assess water temperature and conductivity. Dissolved oxygen was measured with the Aqualitic OX24, and pH with the Aqualitic, pH24. For nutrients (nitrates and soluble reactive phosphorus), subsamples of 30 ml were collected and refrigerated for later analysis following the spectrometric method (AFNOR, 2005). The transparency of the water was estimated by Secchi disk.

For phytoplankton analysis, sub-samples of 50 ml were gathered and preserved with 200 µl Lugol's solution. These samples were examined in the laboratory using an Olympus BX40 microscope equipped with tracing and measuring devices. Before microscopic identifications, organic substances on the samples were removed using HNO_3^- for diatoms (Leclercq and Maquet, 1987). The species were identified under microscope Olympus BX40 and classification was done with standard works (Compère, 1989; Ouattara et al., 2000) and more specific literature (Couté and Iltis, 1981; Komárek and Fott 1983; Compère 1986; Krammer and Lange-Bertalot, 1991; Uherkovich, 1995; Da et al., 1997; Komárek et al., 2003; Komárek and Anagnostidis, 2005). Aliquots were settled in 5 or 10 ml settling chambers and density of phytoplankton was estimated using the Utermöhl (1958) method as modified by Laplace-Treyture et al.

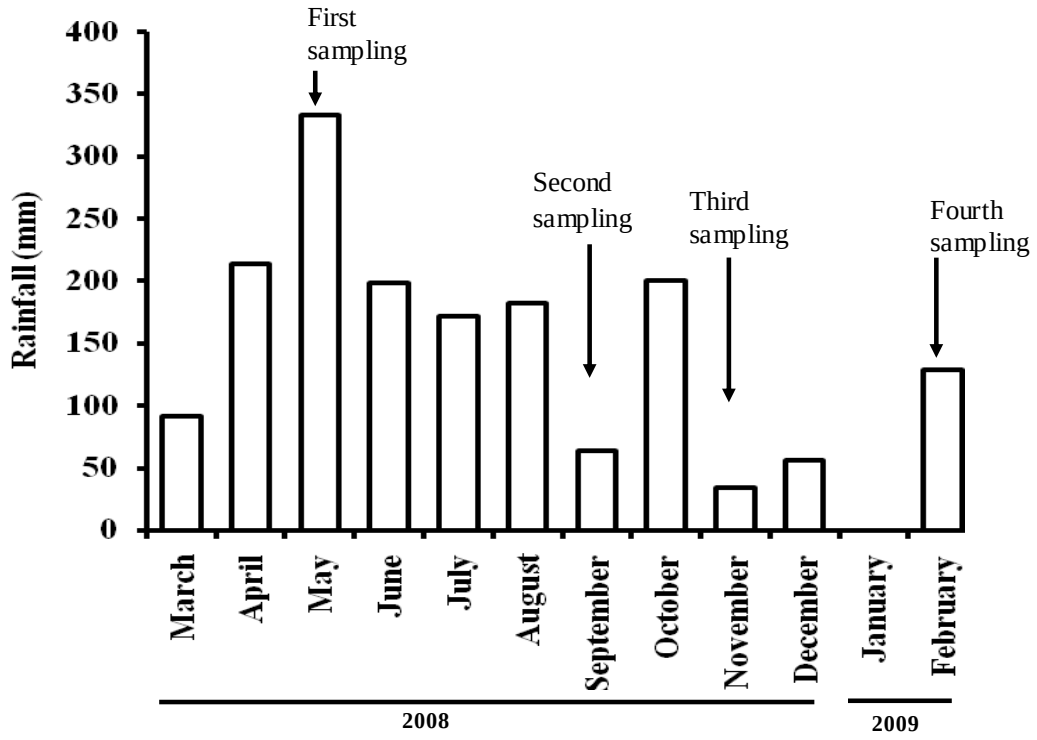

Figure 2. Monthly rainfall data from May 2008 to March 2009 recorded at the study area obtained from the SODEXAM. The arrows indicate the sampling periods

(2007), with a DIAVERT inverted microscope. To analyze phytoplankton community structure, species richness (total number of species recorded after counts), population density (cells.l^{-1}), Shannon-Wiener diversity index (Shannon and Weaver, 1949) and Pielou evenness (Pielou, 1966) were used. Both evenness and diversity (bits/cell) were based on abundance data.

Statistical analyses

Differences of physical-chemical parameters, phytoplankton density, Shannon diversity and evenness among sampling seasons and stations were tested using ANOVA Kruskal-Wallis (significance level 0.05). However, means and standard deviation of all measurements were recorded for each parameter. The coefficient of variation (CV%) was computed according to Zar (1999):

$$CV(\%) = \frac{\sigma}{\mu} \times 100$$

Where σ is the standard deviation and μ is the mean of the measurements of each parameter.

To investigate the phytoplankton community environment relationship, a ReDundancy analysis (RDA) (ter Braak and Smilauer, 2002) was performed on the dominant species (contribution > 2%) and on eight environmental variables: temperature; conductivity; pH; dissolved oxygen; nitrate; soluble reactive phosphorus (SRP); transparency and depth. In this analysis, we used a linear model, because the gradient length of the first axis obtained by detrended correspondence analysis was 0.39. The significance of the first four axes was tested using a Monte Carlo analysis with 499 permutations. ReDundancy analysis was computed with the program CANOCO for Windows 4.5 (ter Braak and Smilauer, 2002). The others were performed with STATISTICA software 7.1 (StatSoft, 2005).

RESULTS

Physical and chemical variables

Physical and chemical variables at all four sampling sites are presented in Table 1. Average values of the conductivity (182.68 μS/cm) and dissolved oxygen (6.40 mg/l) were slightly higher at the site 2, located at the middle stretch of the reservoir, than those at the others sites and Secchi transparency depth presented the highest values at site 4 (62.50 cm), close to the dyke of the reservoir. Dissolved oxygen and transparency showed high coefficient of variation values (CV > 25%). Concerning conductivity, only station S2 presented high variability. In such variables as water temperature, pH, nitrate and SRP, the four sampling sites were similar. Their coefficients of variation values were relatively low (CV < 25%) except nitrate that displayed a high variability (CV > 68%). No significant differences in physical and chemical variables occurred between the four sampling sites (Kruskal-Wallis test; $p > 0.05$).

Precipitation data showed the characteristic of local seasonal patterns (Figure 1), with four periods during this study: long rainy season (March to August), transition period rainy to short: short dry season (September), short

Table 1. Means and coefficient of variation (CV) of physical and chemical variables in Adzopé impoundment at different stations (S1 to S4). SRP: soluble reactive phosphorus, ns = non significant.

Variables	Units	S1		S2		S3		S4		Kruskal-Wallis test
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	
Conductivity	µS/cm	166.78	11.31	182.68	25.65	176.40	15.38	178.35	17.97	ns
Temperature	°C	28.65	10.22	28.45	10.60	25.95	6.93	26.93	11.80	ns
Dissolved oxygen	mg/L	5.63	36.17	6.40	37.91	4.69	33.84	4.79	50.35	ns
pH		7.07	8.95	6.86	13.07	6.84	13.15	7.10	12.45	ns
Nitrate	mg/L	0.15	98.55	0.11	68.58	0.32	113.65	0.17	148.04	ns
SRP	mg/L	1.17	0.06	1.17	0.02	1.17	0.06	1.17	0.02	ns
Transparency	cm	52.75	26.92	56.25	33.99	50.50	42.98	62.50	31.53	ns

rainy season (October to November) and long dry season (December to February). Water temperature showed a variation within a small amplitude (Table 2). Water transparency was higher at the beginning of the short season. Nitrate and soluble reactive phosphorus presented higher values during the transition period from the rainy to the short season and lower concentrations during the long rain season for soluble reactive phosphorus and during the short rainy and long dry seasons for nitrate. Conductivity decreased during long rainy season, increased during the transition period, decreasing again during the next rainy season and reaching higher values during the long dry period. Values of dissolved oxygen increased regularly from long rainy to short rainy season and decreased during long dry season. pH values oscillated abruptly from long rainy to short rainy season and were higher during the last period, with clear seasonal pattern.

In this study, there were significant differences in conductivity between the long dry season and the others seasons ($p < 0.05$). Water temperature showed significant seasonal variation between long rainy season and short rainy season, between long rainy season and long dry season and between short rainy season and short dry season ($p < 0.05$). Dissolved oxygen concentrations, pH, soluble reactive phosphorus concentrations and water transparency also showed significant differences between the long rainy season and the others seasons ($p < 0.05$). For nitrate concentrations, significant variation was observed between short dry season and long dry season.

Phytoplankton community

A total of 144 phytoplankton taxa were identified belonging to 6 phylums. The chlorophyta and euglenophyta were the most diversified with 29.45% of total species, followed by the bacillariophyta (23.97%) and the cyanobacteria (10.27%). The xanthophyta and the pyrrhophyta were the least diversified group with 4.11% and 2.74% of total taxa, respectively.

Overall, species richness not differed signify-cantly between sampling sites. S1 (95 taxa) and S3 (92 taxa) were similar, but S2 (88 taxa) and S4 (117 taxa) have lower and higher total richness respectively than the two sites.

There are marked seasonal differences in the quantitative and qualitative composition of the phytoplankton communities at each site (Figure 3). In terms of total cellule numbers for each species in all the algal groups, the highest maximum counts were recorded in S1 (332 10^5 cells/l), S2 (221 10^5 cells/l) and S4 (356 10^5 cells/l) during the short rainy season whereas the lowest densities occurred during the long rainy season and long dry season in S1 (35 and 48 10^5 cells/l, respectively), S2 (23 and 104 10^5 cells/l, respectively) and S4 (89 and 44 10^5 cells/l, respectively). Contrary to these sampling sites, the highest maximums cells counts were observed during the long rainy season (171 10^5 cells/l) and long dry season (214 10^5 cells/l) at site 3.

Most of the abundance was represented by only 3 of the 6 phylums: the cyanobacteria, the chlorophyta and the euglenophyta (Figure 4). Phytoplankton analysis indicates that cyanobacteria were dominant in 56.25% of the samples, whereas chlorophyta and euglenophyta were dominant in 31.25% and 12.5% of them, respectively. In samples where the latter were dominant, cyanobacteria were generally subdominant.

Table 2. Means of physical and chemical variables and their coefficient of variation (CV) in Adzopé impoundment at different periods. CV: variation of coefficient; SRP: soluble reactive phosphorus. The letters indicate the significant difference between variables (Tukey test, $p < 0.05$). There is no significant difference between the mean having an alphabetical letter in common along the same row (Tukey test, $p > 0.05$); LRS: long rainy season; SDS: short dry season; SRS: short rainy season; LDS: long dry season.

Variables	Units	LRS		SDS		SRS		LRS	
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Conductivity	µS/cm	157.50^{a}	3.79	179.65^{a}	2.42	148.50^{a}	1.17	217.05^{b}	13.13
Temperature	°C	24.68^{a}	7.66	26.23^{a}	1.37	31.70^{b}	3.19	28.83^{ba}	4.35
Dissolved oxygen	mg/L	2.60^{a}	36.52	5.73^{b}	5.02	8.04^{b}	24.23	6.17^{b}	15.61
pH		8.10^{a}	1.17	6.40^{b}	1.43	6.66^{b}	2.93	6.86^{b}	8.55
Nitrate	mg/L	0.15^{ab}	81.91	0.44^{a}	68.55	0.02^{ab}	40.0	0.05^{b}	35.35
SRP	mg/L	1.170^{a}	0.05	1.171^{b}	0.009	1.171^{b}	0.01	1.171^{b}	0.06
Transparency	cm	28.75^{a}	27.44	66.75^{b}	19.28	64.75^{b}	5.26	61.50^{b}	4.30

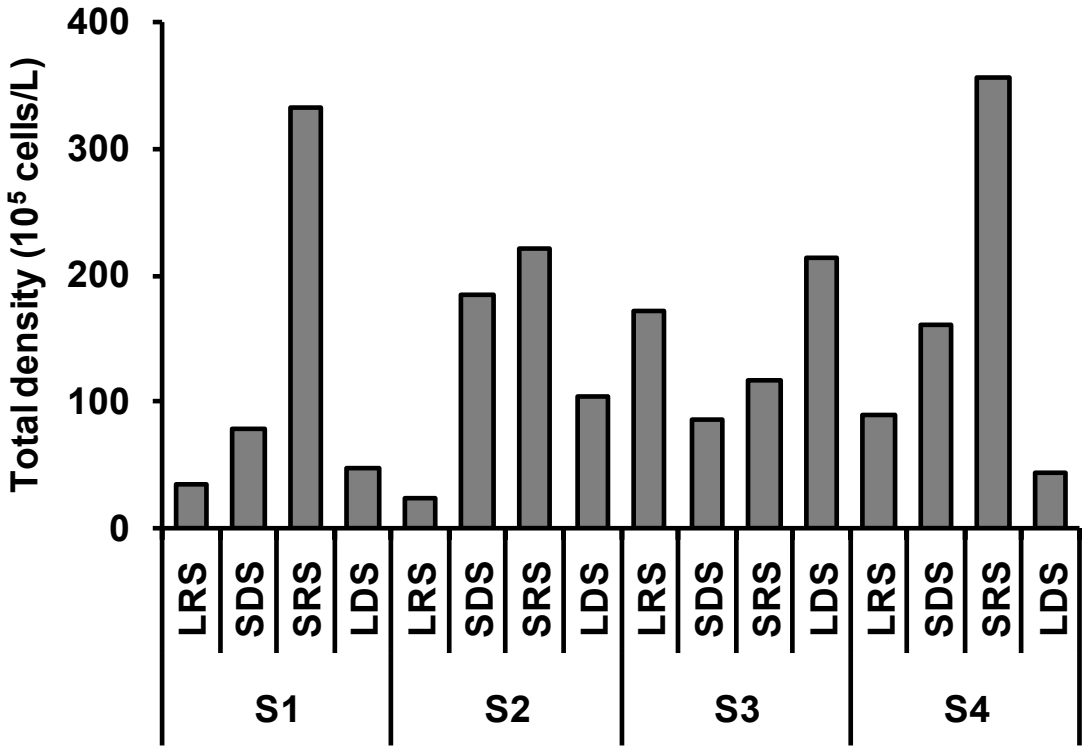

Figure 3. Temporal patterns of total density in four stations of Adzopé Reservoir. LRS: long rainy season; SDS: short dry season; SRS: short rainy season; LDS: long dry season.

Seasonally variation of phytoplankton density in Adzopé reservoir is comprised between 35 10^5 cells/l and 356 10^5 cells/l (Figure 5). Minimum and maximum of phytoplankton density were recorded during long rainy season and short rainy season respectively.

Table 3 shows that cyanobacteria were more frequent than the other groups. Altogether, the 17 dominant taxa composed up to 78% of total phytoplankton abundance. In terms of taxa relative abundance, Cyanobacteria dominated most of sampling periods throughout the impoundment and comprised between 51 to 75% of total phytoplankton by a high contribution of the filamentous *Anabeana constricta* Szafer (Geitler) and the colonial form *Microcystis aeruginosa* (Kütz.) Kütz.. Cyanobacteria dominated community of the impoundment declined to less than 27.93% during the long dry season at all sites, with significant decrease of 9.58% at site 1 (short dry season) and 12.99% at site 4 (long dry season) and replacement by a chlorophycean (33.04 to 34.1%) and an euglenophycean (51.1 to 38.96%) dominated community. *Dictyosphaerium pulchellum* Wood, *Oocystis borgei* Snow and *Crucigeniella crucifera* (Wolle) Kom.

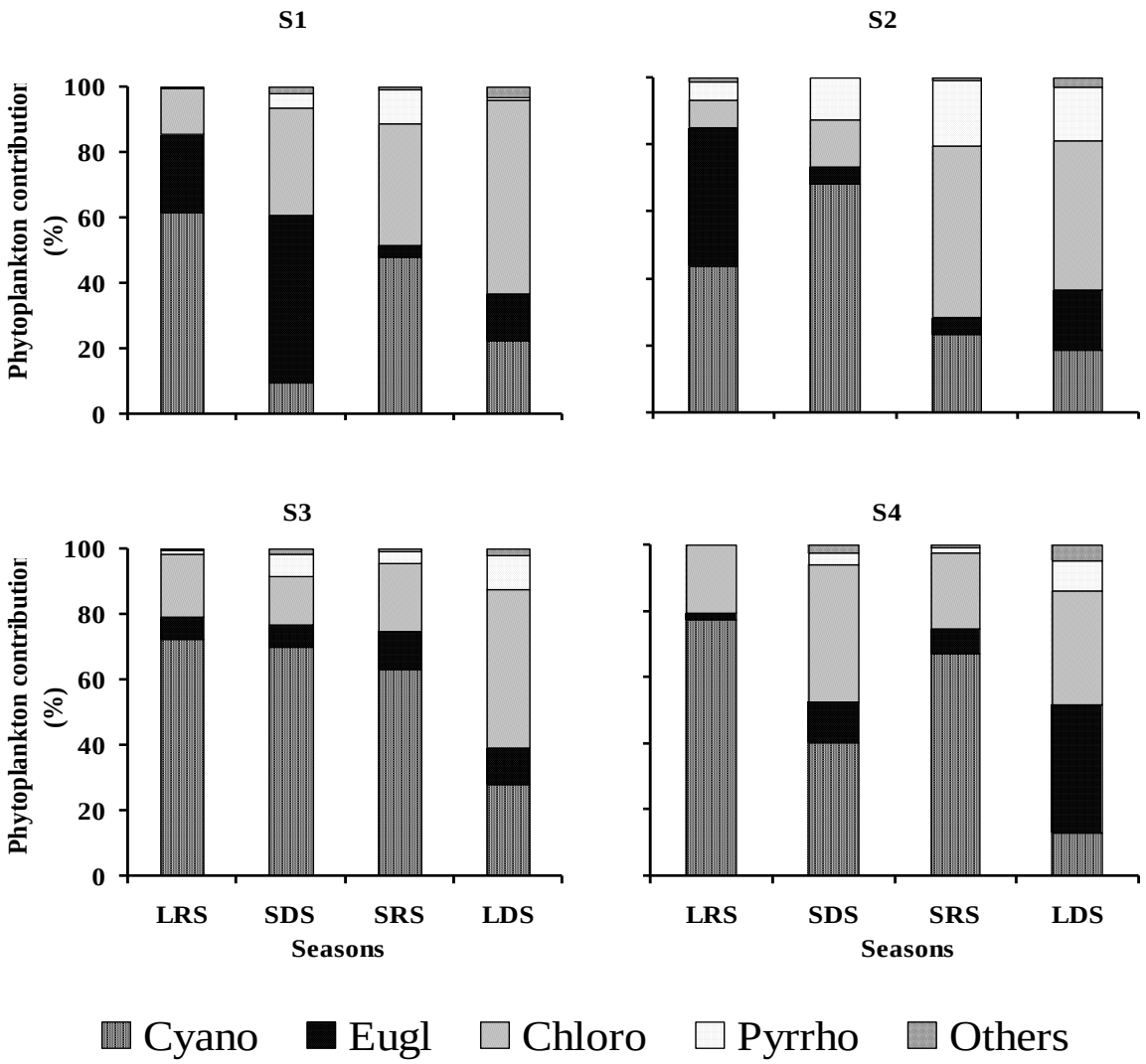

Figure 4. Community succession in Adzopé Reservoir. S1; S2; S3; S4: samplings stations. Cyano: Cyanobacteria; Eugl: Euglenophyta; Chloro: Chlorophyta; Pyrrho: Pyrrhophyta; LRS: long rainy season; SDS: short dry season; SRS: short rainy season; LDS: long dry season.

contributed the largest proportion to chlorophyta abundance in the impoundment. Other important phytoplankton species observed in sampling sites were the euglenophyta *Trachelomonas volvocina* Ehrenbg. and the Pyrrhophyta *Peridinium cinctum* (Müller) Ehrenbg.

There was no statistically significant differences in phytoplankton abundance among sampling sites (ANOVA; $p = 0.962$). In the impoundment, total density showed statistically significant differences between long rainy season and short rainy season (Tukey test; $p = 0.039$). Values of diversity (H') and evenness (E) indexes are shown in Figure 6. Their seasonal and spatial variations did not show a defined pattern. During SRS, H' and E values increased in S1 and S3, whereas in S2 they diminished; during SDS and LDS they increased in S2 and in S4. Values of E index varied between 0.4 and 0.82; minimum value was registered in S4 during LRS, when the taxa *Coelomoron* sp., *A. constricta* and *C. crucifera* reached the 81% of total cells; the maximum value was registered in the same site (S4), during LDS. The diversity index showed the same trend with E index, with high values observed during the SRS (4.39 bits/cells) at S1and SDS and LDS in S4 (4.63 and 4.60 bits/cells, respectively) and lowest values at S1 (2.1 bits/cells) and S4 (2.2 bits/cells) during the LRS.

Significant differences in Shannon-Wiener's diversity index were recorded between LRS and LDS (Tukey test; $p < 0.05$) whereas evenness index was not significantly different between the seasons. Both diversity and evenness indexes were not statistically significant different among sampling sites (Kruskal-Wallis test; $p > 0.05$).

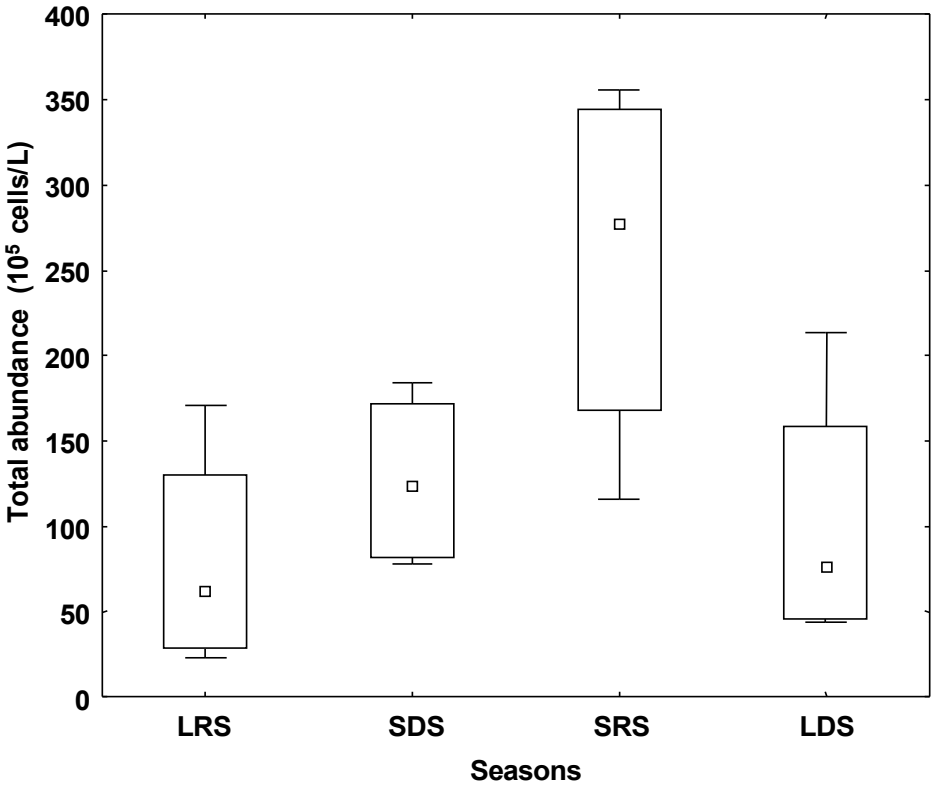

Figure 5. Seasonal patterns of total abundance in of Adzopé Reservoir. LRS: long rainy season; SDS: short dry season; SRS: short rainy season; LDS: long dry season

Relationship of phytoplankton with environmental variables: Redundancy analysis (RDA)

A RDA tested phytoplankton variations in relation to physical and chemical variables in the impoundment. The first two components accounted for 51.1% of the variance. Figure 7 shows that the first axis (31.1% of the variance) was positively correlated with rainfall and pH and negatively correlated to water transparency and soluble reactive phosphorus. In sample ordering according to the first two axes, no completely homogeneous groups were found.

A general gradient from right to left, starting from samples from long rainy season towards those from the other seasons was observed. High water transparency values and soluble reactive phosphorus concentrations during the periods of low rainfall, especially during the transition period from the long rainy to the long dry season, coincided with the dominance of majority of phytoplankton taxa, such as *Aphanothece* sp., *C. crucifera, Scenedesmus acutiformis* Schröd., *D. pulchellum*, *Karenia* sp. *Merismopedia punctata* Lemm. and *Komvophoron* sp.

The second axis (20% of the variance) is characterized by variables that determine the algal metabolism with high oxygen production. According to this axis, high abundances occurred, in general, during higher water temperature.

DISCUSSION

Analysis of the phytoplankton community in the small reservoir of Adzopé reveals some similarities with the situation observed in reservoirs in Central and North Côte d'Ivoire (Bouvy et al., 1998; Arfi et al., 2001), Lake Guiers in Senegal (Bouvy et al., 2006) and Lake Tana in Ethiopia (Wondie et al., 2007). These authors described the occurrence of many species of cyanobacteria. This group is commonly encountered in nutrient-rich shallow reservoirs (Pearl, 1988; Komárek and Anagnostidis, 2005). The absence of relationships between abundances of these dominant cyanobacteria and dissolved nutrient levels is notable. Most environmental variables (water transparency, dissolved oxygen, temperature, conductivity and nutrient concentrations) were similar between seasons, except for the rainfall and pH. According to the catchment's morphology and the characteristics of the surrounding area it can be assumed that Adzopé reservoir receives a high nutrient input. Despite the high external nutrient loads, nitrate was present in much lower concentrations than phosphorus

Table 3. Dominant phytoplankton species (contribution > 2% to total density) in Adzopé reservoir at each station during study period. F: percentage of occurrence, P: proportion in terms of abundance of the species in the community.

Taxa	Acronymes	S1		S2		S3		S4	
		F (%)	P (%)	F (%)	P (%)	F (%)	P (%)	F (%)	P (%)
Cyanobacteria									
Aphanothece sp.	Aphp	100	1.09	100	3.81	0	4.34	0	0
Microcystis aeruginosa (Kütz.) Kütz.	Mism	0	0	50	12.46	75	3.53	75	9.45
Aphanocapsa sp.	Apsp	25	4.23	25	1.08	0	0	25	4.63
Merismopedia punctata Lemm.	Mepu	0	0	0	0	50	7.42	25	8.74
Coelomoron sp.	Cosp	25	1.31	0	0	0	0	75	7.69
Pseudanabaena cf. *limnetica* (Lemm.) Kom.	Psli	25	1.37	25	4.21	75	10.05	50	4.84
Komvophoron sp.	Kosp	25	4.72	25	0.70	0	0	0	0
Anabaena constricta Szafer (Geitler)	Anco	100	26.04	75	15.04	75	28.14	75	20.71
Euglenophyta									
Trachelomonas volvocina Ehrenbg.	Trvo	100	7.61	75	0.92	100	4.26	100	2.53
Chlorophyta									
Pediastrum biradiatum var. *longecornutum* Gutw.	Pebl	75	3.98	25	0.38	50	0.31	50	1.30
Dictyosphaerium pulchellum Wood	Dipu	75	11.25	50	4.90	75	3.79	50	3.08
Oocystis borgei Snow	Oobo	0	0.00	25	13.98	25	0.05	0	0
Crucigeniella crucifera (Wolle) Kom.	Crcr	75	7.27	75	7.81	100	12.07	100	11.03
Crucigenia quadrata Morr.	Crqu	50	2.36	50	1.15	100	1.87	25	0.26
Scenedesmus acutiformis Schröd.	Scac	75	2.11	75	1.17	100	2.23	100	1.70
Pyrrophyta									
Karenia sp.	Kasp	0	0	75	0.89	100	3.79	75	1.12
Peridinium cinctum (O.F.Müller) Ehrenbg.	Peci	50	6.90	100	15.04	100	1.90	75	1.10

and might have a limited effect on phytoplankton total densities.

Furthermore, results showed no significant phosphorus decrease in either during the rainy or the dry seasons, probably because of the prevailing high nutrient concentrations and the low phosphorous requirements of the cyanobacteria species dominating the phytoplankton community. The capacity of some dominating cyanobacteria (for example, *Anabaena constricta*) to fix atmospheric nitrogen and the presence of terminal heterocystes is also a determining factor for their proliferation in various aquatic systems (Padisák, 1997; Walsby, 2001; Oberholster et al., 2004).

All the sampling sites were quite similar in community composition and were dominated by species like *A. constricta* and *M. aeruginosa*. An important reason for the absence of difference between sites is probably that this reservoir examined in this study is too shallow to stratify thermally and is all well mixed system. Furthermore, the small amplitude changes occurring in the reservoir is probably due to the fact that sampling sites are connected to small rivers

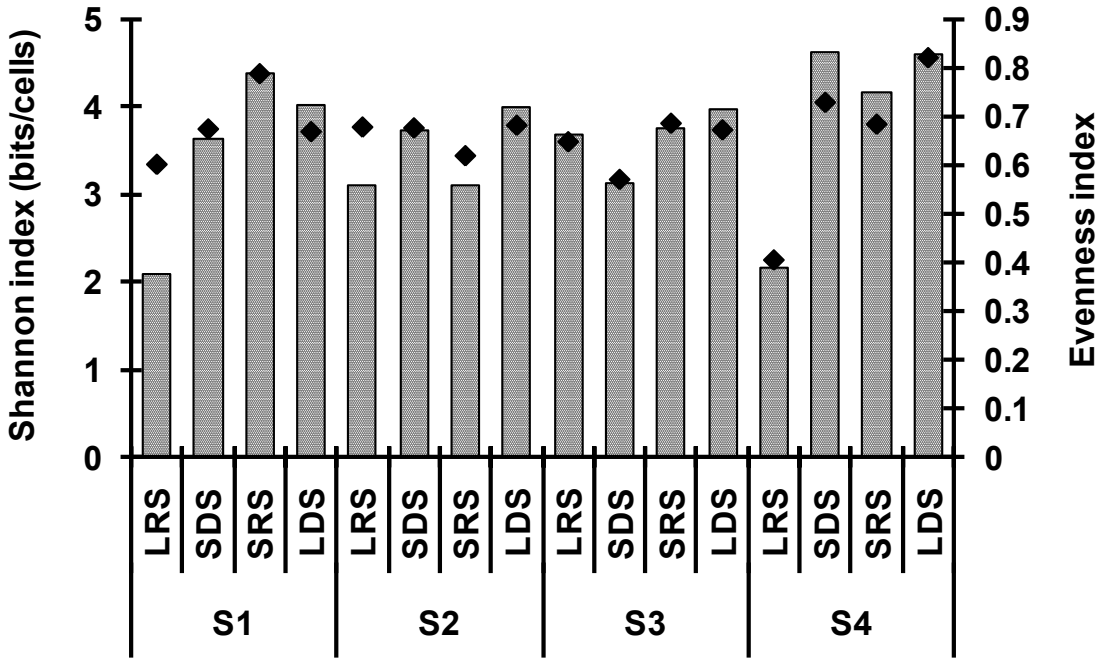

Figure 6. Temporal variation of Shannon and evenness index in the four stations of Adzopé reservoir. H': Shannon index; E: Evenness index; LRS: long rainy season; SDS: short dry season; SRS: short rainy season; LDS: long dry season.

which regularly contribute to mixing and to homogeneous water quality. The development of the dominating cyanobacteria during the short rainy season in the reservoir increases the phytoplankton abundances. The colonial and filamentous cyanobacterium were present during this period, characterised by conditions transitory from short dry season to short rainy season, thus exploiting the homogenization of the water column to grow quickly. Some species have gas vacuoles (*A. constricta* and *M. aeruginosa*), giving them an excellent capacity for vertical migration (Walsby, 1994; Brookes and Ganf, 2001; Komárek and Anagnostidis, 2005).

There is little relationship between nutrients and phytoplankton abundances. All the variables in this data set are not significantly correlated. It is likely that phytoplankton densities at this reservoir is limited by a combination of variables, involving light and possibly grazing, but rainfall seems to be most important to explain the seasonal variation observed, acting as a possible disturbing factor.

According to Talling (1986) more generally in Africa, annual patterns of phytoplankton seasonality are usually either dominated by hydrological features (water input-output) or by hydrographic ones (water column structure and circulation), which influences the chemical dynamics of the water column and ultimately their biota. Changes in the community structure through the association of precipitation events and general hydrological budget is one of the characteristics key of shallow lakes (Garcia de Emiliani, 1997). These changes are not only a result of the water level changes owing to the lakes' filling and drying phases, but also the accompanying input of allochthonous nutrients and suspended solids.

Data obtained in the present study also point to the importance of the small reservoirs' hydrology for structuring the phytoplankton community. However, the effect of grazing, one of the main factors shaping phytoplankton structure was not tackled in this study. Probably, grazing by zooplankton on phytoplankton was not significant because of filamentous cyanobacteria species dominating the phytoplankton community which are inedible for the abundance of rotifers dominating the zooplankton community in this impoundment (Ahizi, 2010).

According to Bouvy et al. (2006) eutrophic tropical systems are often characterised by opportunist piscivores and planktivores; predominant rotifers are considered to be inefficient grazers but the phytoplankton population could be regulated by fish, especially by tilapias, regularly captured by fishermen in this impoundment. Diversity index (H') showed similar mean values in all sampling sites (between 2.1 and 4.6 bits/cells) although a reductional tendency was observed during the long rainy season. Low diversity values were strongly affected by the most abundant species. Very low richness values were detected during the development of cyanobacteria. Other phytoplanktons, such as the 3 chlorophytes species (*D. pulchellum*, *O. borgei* and *C. crucifera*) were only observed when densities of total phytoplankton community were low. Conversely, Figueredo and Giani

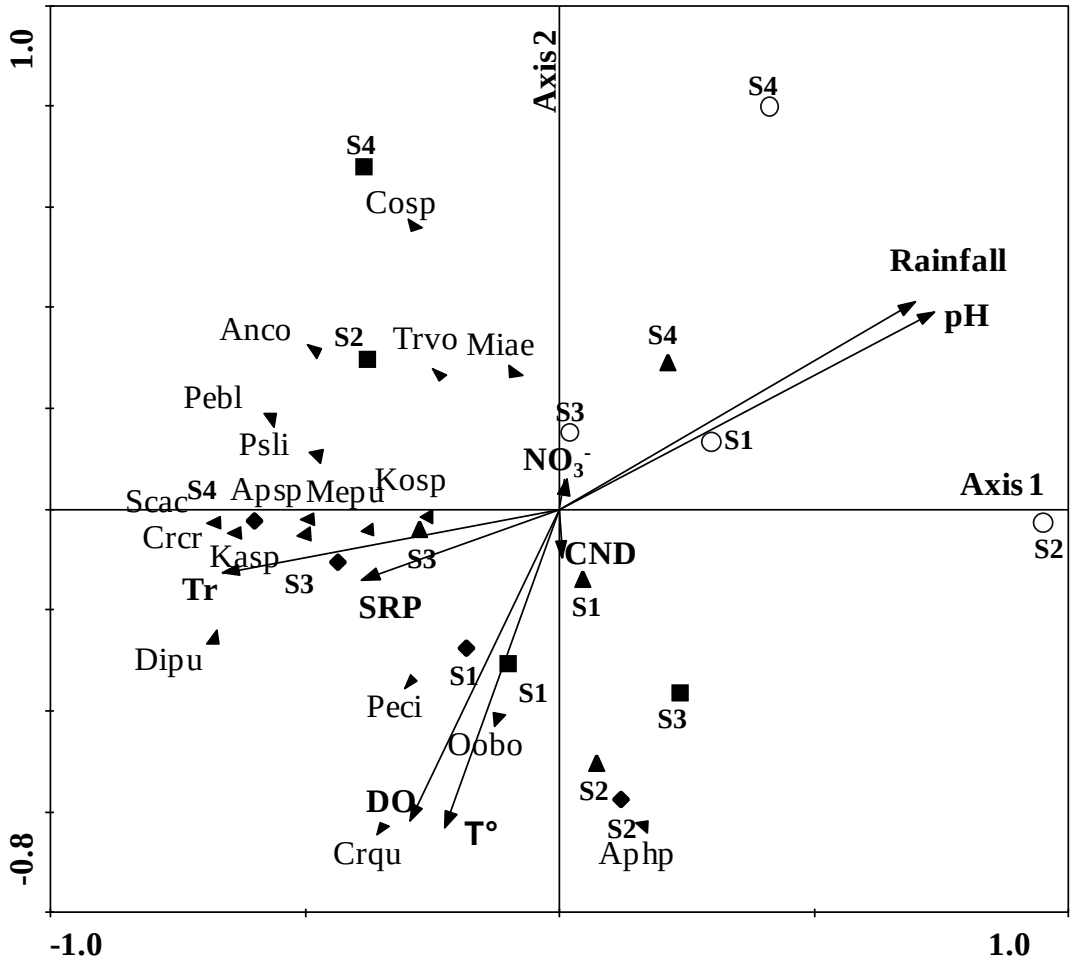

Figure 7. Scores derived from the ReDundancy analysis (RDA axis 1 and 2) applied to the dominant species and environment data. For the acronyms (Table 3). T: temperature, CND: conductivity; DO: dissolved oxygen; NO_3^-: nitrate; SRP: soluble reactive phosphorus; Tr: transparency; station (S).

(2001) and Kemka et al. (2004) in their study of Pampulha Reservoir in Brazil and Yaoundé municipal Lake (Cameroon) respectively reported higher phytoplankton diversity during the rainy seasons. They argued that the onset of rainfall destabilises the hitherto established conditions, creating a new environment conducive to other phytoplankton for exploitation.

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Full Length Research Paper

Phytochemical and antibacterial properties of *Combretum mucronatum* (Schumach) leaf extract

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The cold extraction method was used to obtain the methanol extract of the leaf of *Combretum mucronatum.* The extract was analyzed for antibacterial activities, using some pathogenic bacteria namely: *Klebsiella pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Bacillus cereus, Salmonella typhi* and *Bacillus subtilis*. The antibacterial bioassay was carried out *in-vitro* and it revealed that the methanol leaf extract inhibited the growth of the tested organisms at a concentration of 25.0 mg/ml except *K. pneumoniae* and *S. pyogenes* which were resistant. The extract exhibited the highest inhibitory potential on *S. aureus* with a zone of inhibition value of 35.0 mm at a concentration of 25.0 mg/ml. This was followed by *E. coli and P. aeruginosa* which were inhibited with zones of inhibition values 30.0 mm and 25.0 mm respectively. *B. cereus* was the least inhibited with a zone of inhibition of 16.0 mm. Result of the phytochemical screening tests revealed that the extract contains saponin, tannins, anthraquinone and cardiac glycoside. The rate at which the extract was able to kill the test organisms showed that the organisms decreased with increased time of exposure to the extract. *P. aeruginosa* decreased to zero at the 24th hour. The minimum inhibitory concentration (MIC) of the leaf extract ranged from 25.0 to 3.12 mg/ml. The result of the antibiotic sensitivity test compared well with the commercial antibiotics.

Key words: Antibacterial, zone of inhibition, phytochemical screening, extracts, rate of killing.

INTRODUCTION

Man and plants

The generality of the life of man is 100% dependent on the plants in our environment (Selby, 1998). This means that the diversity of plant species in the world is so useful for our adaptation to the environment and existence.

A medicinal plant is defined by the World Health Organization (WHO) consultative group as any plant in which one or more of its organs contains substances that can be used for therapeutic purposes or which are precursors for the synthesis of useful drugs (World Health Organization, 2003).

The medicinal plants with high therapeutic and prophylaxis value are mostly from the plants that are herbaceous and the majority of the world's population uses such herb products as a primary source of medicine.

Traditional medicine

The term traditional medicine (Indigenous medicine or folk medicine) describes medical knowledge systems, which developed over centuries within various societies before the era of modern medicine. WHO defines traditional medicine as the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (World Health Organization, 2003).

The use of traditional medicine is now spreading in most countries of the world with over 80% of the primary

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health care needs based on the extract from these medicinal plants. The WHO also notes, though, that "inappropriate use of traditional medicines or practices can have negative or dangerous effects" and that "further research is needed to ascertain the efficacy and safety" of several of the practices and medicinal plants used by traditional medicine systems (World Health Organization, 2003).

Modern medicine and herbal medicine

Modern medicine has provided many breakthrough treatments for serious diseases. Some conditions, however, have eluded the healing grasp of contemporary western medicine, which emphasizes rigorous scientific investigation of therapies. In addition, rising costs of some treatments have placed modern healthcare beyond the reach of many. The drugs that routinely fill pharmacy shelves of post-industrialized nations remain inaccessible to the majority of the people in the world. At the same time, more antibiotic pathogens are emerging by the day. Now, populations in many areas of the globe use herbal medicine also called botanical medicine or phytotherapy, as the principal means of healthcare.

Herbal medicine is the use of natural plant substances to treat illness. Based upon hundreds, even thousands of years of experience, herbal medicine provides an alternative to modern medicine, making healthcare more available. In fact, the majority of the world's population uses herb products as a primary source of medicine. While some regulating authorities fear the consequences of unrestricted herbal remedy use, herbal medicine offers a degree of hope to some patients whose disease states do not respond favorably to modern pharmaceuticals. More often, however, herbal remedies are used to treat the common ailments of daily living like indigestion, sleeplessness, or the common cold. Resurgence in interest in herbal medicine has occurred in the United States as medical experts have begun to recognize the potential benefit of many herbal extracts. So popular has herbal medicine become that scientific clinical studies of the effectiveness and proper dosing of some herbal medicines are being investigated.

Herbal medicine recognizes the medicinal value of plants and plant structures such as roots, stems, bark, leaves, and reproductive structures like seeds and flowers. To some, herbal medicine may seem to be on the fringes of medical practice. In reality, herbal medicine has been in existence since prehistoric time and is far more prevalent in some countries than is modern healthcare.

The use of herbs ground into powders, filtered into extracts, mixed into salves, and steeped into teas has provided the very foundation upon which modern medicine is derived (Barney, 1996). Indeed, herbal medicine is the history of modern medicine. Many modern drugs are compounds that are derived from plants whose pharmacological effects on humans had been observed long before their mechanisms of action were known.

A common example is aspirin. Aspirin, or acetylsalicylic acid, is a compound found in the bark of the willow tree belonging to the taxonomic genus Salix. Aspirin, now sold widely without prescription, is an effective analgesic, or pain reliever, and helps to control mild swelling and fever. While aspirin is synthetically produced today, willow bark containing aspirin was used as an herbal remedy long before chemical synthesis techniques were available. Similarly, the modern cardiac drug digitalis is derived from the leaves of the purple foxglove plant, ***Digitalis purpurea.*** Foxglove was an herbal known to affect the heart long before it was used in modern scientific medicine.

Description of plant

Combretum mucronatum is a usually dark green plant that clings to other plant around for support and exposure to sunlight for its photosynthetic activities. It is widely distributed in West Africa and could be found in the Savanna forest of the region. The plant is mainly found in Western Nigeria especially during the rainy season.

The plant has been used extensively in traditional medicine for the treatment of a variety of diseases. The leaves and roots are used in traditional medicine for treatment of wounds, cough, dysentery, and as anthelmintic, antimicrobials, and antipyretic (Sofowora, 1982). The decoction from the leaves and root is given for the treatment of venereal disease in woman (Wallis, 1967).

This work is intended to assay for the antibacterial and phytochemical properties of the leaf extract of ***C. mucronatum*** with a view to verifying its traditional use as a medicinal plant and to examine whether its action on the said bacteria are cidal or static.

MATERIALS AND METHODS

Leaves of ***Combretum mucronatum,*** conical flasks, spatula, forceps, Bunsen burner and tripod stand, stirring rod, beakers, measuring cylinder, test tubes, test tube rack, cotton wool, filter paper, petri dishes, aluminum foil, nutrient agar, sterile water, methanol and nutrient agar.

Collection and preparation of plant leaf

The plant leaves used for this project (***Combretum mucronatum***) were collected on January 13, 2008 from the forest and wild life reserve of Federal University of Technology, Akure, Nigeria, where they were found growing naturally. They were identified at the department of Crop Science and Production of the Federal University of Technology, Akure, Nigeria. A voucher specimen was submitted at the departmental herbarium.

Table 1. Antibacterial activity of the methanol extract of ***Combretum mucronatum***.

Organisms	Zone of inhibition(mm)/at the concentration of 25.0 mg/ml
Staphylococcus aureus	35.0
Escherichia coli	30.0
Pseudomonas aeruginosa	25.0
Streptococcus pyogenes	–
Salmonella typhi	18.0
Klebsiella pneumoniae	–
Bacillus subtilis	25.0
Bacillus cereus	16.0
Proteus mirabilis	20.0

Collection and identification of bacterial samples

Bacterial isolates were collected at Don Bosco Catholic Medical Centre, Araromi Street, Akure, Ondo State, Nigeria. The bacterial isolates used for this research work included: ***Salmonella typhi, P. mirabilis, E. coli, S. aureus, B. subtilis, B. cereus, Klebsiella pneumoniae, P. aeruginosa*** and ***S. pyogenes.***

Extraction of leaves

The leaves were air dried for six weeks and crushed into powder. Exactly 600 g of the powder was soaked in methanol for 72 h, after which it was first sieved with a muslin cloth, then filtered using No 1 Whatman filter paper. The filtrate was collected in a beaker and dried ***in vacuo.***

Pytochemical screening

The pytochemical screening was done according to the method described in Trease and Evans (1996).

Determination of antibacterial activities of leaf extract

The antibacterial activity of the plant extracts was assayed using agar dilution method described by Olutiola et al. (1991). The concentration of the extract used is 25 mg/ml. The plates were incubated at 37°C for 24 h. Clear zones around the bored holes are indicative of the inhibition of the organisms by the extract.

Determination of minimum inhibitory concentration

Five concentrations (25, 12.5, 6.25, 3.12 and 1.56 mg/ml) of the methanol extract of ***C. mucronatum*** leaf were assayed, the method of (Trease and Evans, 1996) was used. The plates were incubated for 24 h at 37°C.

Concentration of the crude extract that showed zone of inhibition, below which there was no inhibition was recorded as the minimum inhibitory concentration.

Antibiotics sensitivity test

The disc diffusion method described by (Khan et al., 2002) was used.

Standardization of bacterial samples

A 10 ml of 18 h old broth culture was centrifuged at 2000 rpm for 10 min. It was decanted to retain the residual cells and 10 ml of normal saline was added. It was then centrifuged to wash the cells. The supernatant was decanted and this process was done four times to wash the cells. The washed cells were stored with 15 ml of normal saline.

Determination of the rate of killing of organisms by the extract

A 5 ml of 25 mg/ml of the methanol extract and 5 ml of the standard culture was added together in a sterile test tube. The solution was allowed to stand for 24 h, for interaction between the organism and the extract. At intervals of one hour, 1 ml of the solution was pour plated using nutrient agar. The microbial load was determined, after incubation at 37°C for 24 h.

RESULTS AND DISCUSSION

C. mucronatum leaves are extensively used to treat wounds, cough, dysentery, helminthic infections, bilious, pyretic and generally used as antimicrobials. Results from this work corroborates its use as antimicrobial. Table 1 shows that the methanol extract of ***Combretum mucronatum*** inhibited all the tesed bacteria except ***S. pyogenes*** and ***Klebsiella pneumonia***. ***S. aureus*** showed the highest susceptibility with the highest zone of inhibition value of 35.0 mm, hence its traditional use in the treatment of wounds (Wallis, 1967) seems rational. This was followed by ***E. coli*** and ***P. aeruginosa*** with zones of inhibition values of 30 and 25mm respectively. Studies have implicated ***S. aureus, E. coli***, and ***P. aeruginosa*** among others as leading causative agents of nosocomial and community infections (Branger et al., 2005; Oteo et al., 2005)

The high susceptibility of these organisms is a clear indication of the effect of the leaf extract as a good treatment for the bacterial infections caused by the trio. This further confirms its traditional use in the treatment of dysentery.

Table 2. Phytochemical groups in dried leaf extracts of ***Combretum mucronatum***.

Phytochemical groups	Presence/absence
Saponin	+ve
Tannins	+ve
Phlobatannin	-ve
Alkaloids	-ve
Anthraquinone	+ve
Cardiac glycoside	
Legals Test	+ve
Salkowski Test	+ve
Keller Killian Test	+ve
Liebermans Test	+ve

+ = Present; - = Absent.

Table 3. Minimum inhibitory concentration of the methanol extract of ***Combretum mucronatum*** against the bacterial isolates.

Organisms	Concentration of methanol extract (mg/ml)
Staphylococcus aureus	3.12
Escherichia coli	3.12
Bacillus cereus	3.12
Proteus mirabilis	6.25
Salmonella typhi	12.5
Bacillus subtilis	3.12
Psedomonas aeruginosa	3.12

Klebsiella pnuemoniae and ***S. pyogenes*** were not susceptible to the extract. It is however possible that an increase in the concentration of the extract, or its purification may cause the extract to inhibit these two organisms. Again, if the extract from this plant is used in synergy with the extract from another medicinal herb, an inhibition of these two organisms may be effected. Oloke (1997) demonstrated the effectiveness of synergism between tetracycline, bacterim and seed extract of ***Aframomum memegueta.***

Antimicrobial activity in plants have been reported to be as a result of bioactive components present in the plants, such as alkaloids, saponins, tannins, anthraquinones, steroids, flavonoids etc (Harbone, 1984; Odugbemi, 2006). Table 2 shows the presence of various bioactive com-ponents in the extracts. These are most likely responsible for broad spectrum inhibitory effect shown on Table 1.

The minimum inhibitory concentration (MIC) tests of the leaf extract on the test organisms as shown on Table 3 indicated that the MIC of the methanol extract of ***C. mucronatum*** is at 3.12 mg/ml for all the test organisms except ***S. typhi*** and ***P. mirabilis***, which were 12.5 and 6.25 mg/ml respectively. This low MIC values also show that the extract has a strong antibacterial effect on the test organisms.

The effect of the commercial antibiotics on both gram positive and gram negative bacteria compares well with that of the crude extracts used in this study (Table 4). ***S. pyogenes*** and ***K. pnuemoniae*** were resistant to all the antibiotics except to gentamycin and tetracycline. This duo also showed resistant to the extract in this study.

The effect of the extract on the test organisms can be considered to be of a broad spectrum action because both gram positive and gram negative bacteria were inhibited. This effect is also seen from the result of the rate of killing of the organisms by the extract (Figures 1 and 2). The number of organisms present at each hour declined till the 24th hour. The extract had a bactericidal effect on ***P. aeruginosa*** which got totally killed within the 24 h of exposure.

This research was conducted on crude extract; it is believed that if the extract is further purified, stronger inhibitory results will be achieved and the structure of the active phytochemical components can also be determined. Furthermore, an assay of the toxicological analysis will assess its safety and level of tolerance in mammalian body.

Herbal medicine has proven to be of great importance to the treatment of basic human diseases from time

Table 4. Antibiotic sensitivity test on bacterial isolates.

Gram positive organisms	Zones of inhibition (MM)							
	GEN	PEN	STR	TET	AMP	CHL	CXC	ERY
Staphylococcus aureus	25.0	_	20.0	13.0	_	13.0	_	26.0
Streptococcus pyogenes	15.0	_	_	15.0	_	_	_	_
Klebsiella pneumoniae	14.0	_	_	8.0	_	_	_	_
Bacillus subtilis	13.0	_	11.0	_	_	8.0	_	_
Bacillus cereus	15.0	_	13.0	_	_	10.0	_	_
Gram negative organisms	**GEN**	**NAL**	**NIT**	**COL**	**STR**	**TET**	**AMP**	**COT**
Escherichia coli	17.0 mm	22.0	15.0	11.0	10.0	_	_	_
Pseudomonas aeruginosa	10.0	_	_	10.0	9.0	_	_	_
Salmonella typhi	14.0	19.0	14.0	10.0	13.0	_	_	_
Proteus mirabilis	12.0	_	_	_	10.0	18.0	_	16.0

GEN-Gentamycin PEN-Penicillin; STR-Streptomycin; TET-Tetracycline; AMP-Ampicillin; ERY- Erythromycin; CHL-Chloramphenicol; NAL-Nalidixic acid; NIT-Nitrofurantoin; (COT-Cotrimazole.

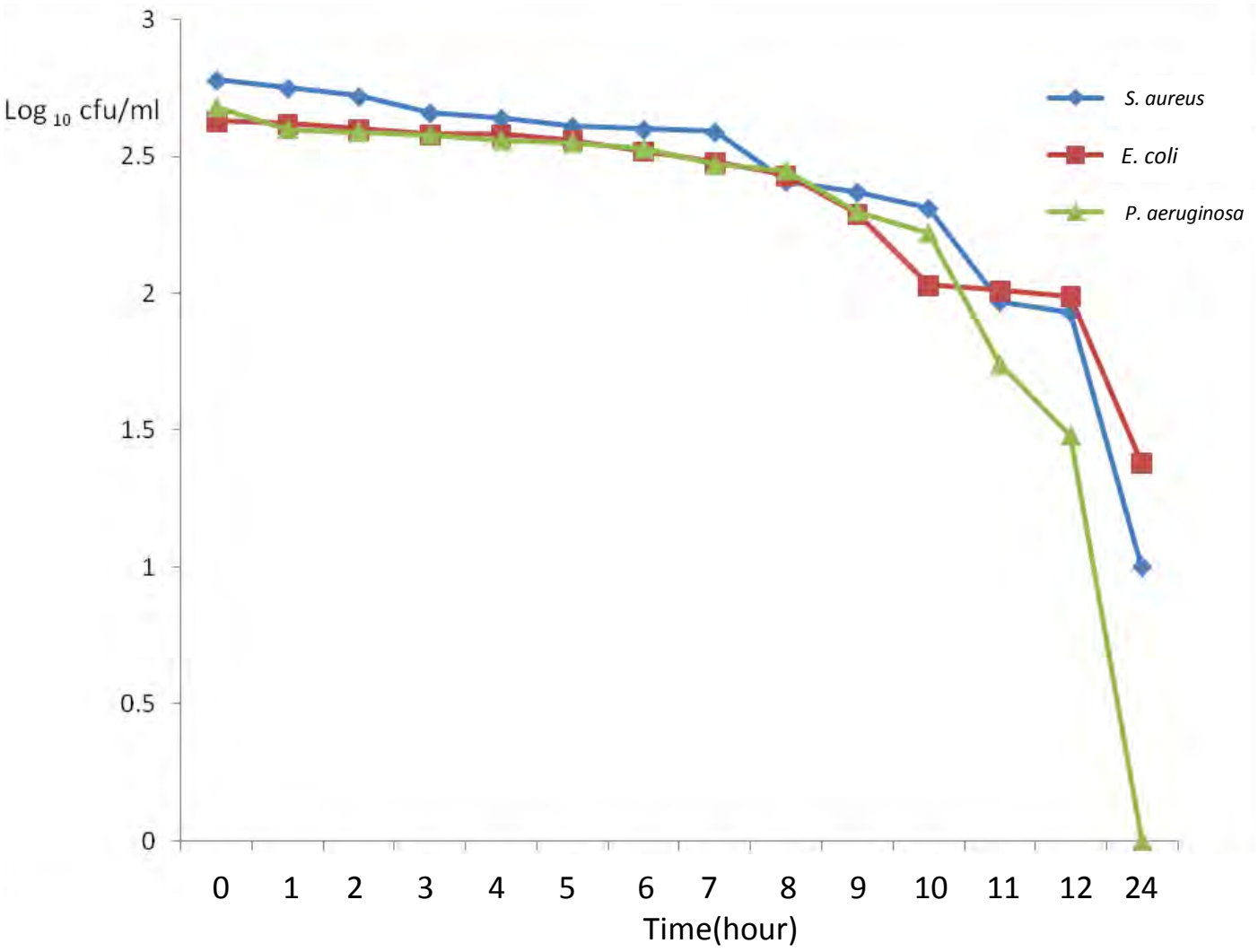

Figure 1. Rate of killng of some bacterial isolates by ***Combretum mucronatum*** leaf extract.

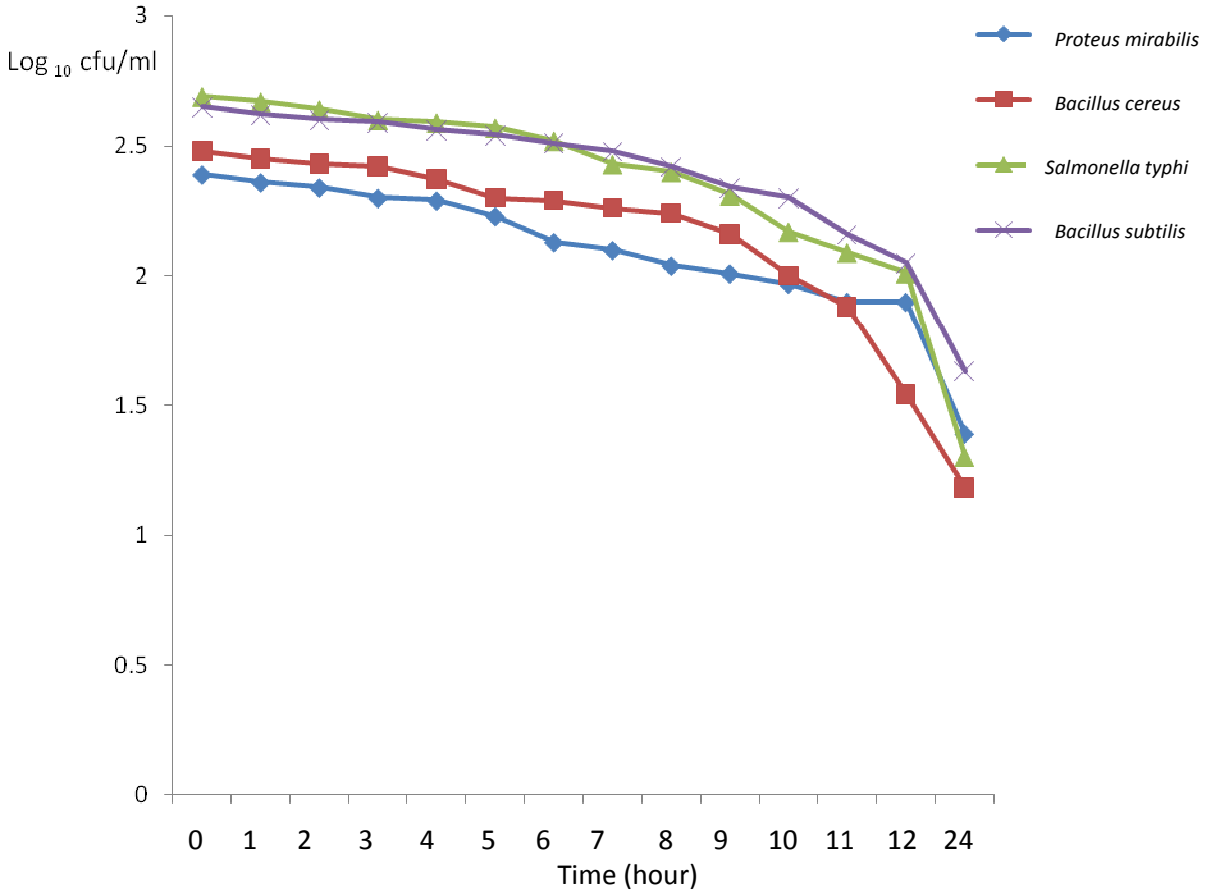

Figure 2. Rate of killng of of some bacterial isolates by ***Combretum mucronatum*** leaf extract.

immemorial, however, this natural endowment (plants) should be exploited scientifically to its full potential so as to give answers to our health problems.

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Full Length Research Paper

"Bacteriological analysis of drinking water sources"

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The quality of potable water and treatment of waterborne diseases are critical public health issues. Bacterial contamination of drinking water sources is the most common health risk. The research determines bacteriological quality of drinking water sources in Serbo town, south west Ethiopia. A Cross-sectional study design on bacteriological analysis of drinking water was conducted in Serbo town from September to October, 2010. 100 ml of water specimen was collected from each water sources and transported for testing to the department of medical laboratory sciences and pathology laboratory by cold chain. The water samples were tested using the multiple tube technique on OXOID MacConkey Broth, (Oxoid Ltd, Basingstoke, Hampshire, England) for presumptive coliform count followed by *Escherichia coli* confirmation. A total of twenty four drinking water samples were analyzed. Eighteen (75%) were from unprotected wells and the remaining six (25%) were from protected wells. Twenty three out of the total (87.5%) have presumptive bacteria count above the permissible limits for drinking water. Majority of the water sources were not safe for drinking. Hence, regular disinfection of drinking water sources needs to be run.

Key words: Potable water, most probable number, fecal coliform, protected well and unprotected well.

INTRODUCTION

Water is one of the most important elements for all forms of life. It is indispensable in the maintenance of life on earth. It is also essential for the composition and renewal of cells. Despite of this, human beings are continuing to pollute water sources resulting in provoking water related illnesses (Ethiopian Federal MOH, 2004, WHO, 2008).

Diseases related to contamination of drinking-water constitute a major burden on human health. The most common and widespread health risk associated with drinking-water is microbial contamination. Up to 80% of all sicknesses and diseases in the world are caused by inadequate sanitation, polluted water or unavailability of water. As to 2006 report of world health organisation (WHO) approximately three out of five persons in developing countries do not have access to safe drinking water and only about one in four has any kind of sanitary facilities. Water may also play a role in the transmission of pathogens which are not faecal excreted. Contamination of drinking water with a type of ***Escherichia coli*** known as O157:H7 can be fatal. Many microorganisms are found naturally in fresh and saltwater (WHO, 1996; Amira, 2011). The microbiological quality of drinking water has attracted great attention worldwide because of implied public health impacts (Amira, 2011). Total and fecal coliform have been used extensively for many years as indicators for determining the sanitary quality of water sources. Water born outbreaks are the most obvious manifestation of waterborne disease.

Microbiological examinations have several roles in the investigation of waterborne outbreaks (http://www.who.int/water_sanitation_health/dwq/9241546301/en/, 2003).

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In Ethiopia over 60% of the communicable diseases are due to poor environmental health conditions arising from unsafe and inadequate water supply. Frequent examinations of faecal indicator organisms remain the most sensitive way of assessing the hygienic conditions of water. Fecal coliform have been seen as an indicator of fecal contamination and are commonly used to express microbiological quality of water and as a parameter to estimate disease risk. Most portable number (MPN)) is a typical test for fecal coliform (Mengesha et al., 2004).

In 2007, 74% of Ethiopia's population had lack of safe drinking water. Although urban coverage is around 80%, the majority of the population (89%) live in rural areas, where most reports suggest that fewer than 12% have access to potable water. Only 19% of the rural populations have access to safe drinking water supplies (Government of Ethiopia, 2007).

The provision of safe and adequate water supply for the population has far reaching effects on health, productivity and quality of life, as well as on the socio-economic development of the nation. Therefore, this study determines the quality of water sources and the extent of contamination at study area which will help in the intervention actions to be taken by the concerned bodies and will provide baseline information for further study.

MATERIALS AND METHODS

Study design and period

A cross sectional study was conducted on drinking water sources to assess the extent of bacterial contamination from September to October, 2010 in Serbo town, south west Ethiopia.

Study area

The study was conducted in Serbo town. Serbo is found in Jimma zone, Kersa woreda; the town is located 325 km southwest of capital Addis Ababa and 19 km from Jimma town. Jimma is the largest city in southwestern Ethiopia, located in the Jimma zone of the Oromia region with 17 woredas. Based on figures from the central statistics agency (CSA, 2007) the zone has an estimated total population of 2,495,795, of whom 1,255,130 are men and 1,240,665 are women; 141,013 (5.6%) of its population are urban dwellers (CSA, 2007).

Data collection and processing

From individual water sources, 100 ml sample of water was collected. The water was collected using sterile bottles and transported for testing immediately to the department of medical laboratory science and pathology laboratory by ice cold containers within 50 min of collection. All communal public water source and twenty randomly selected private owned water sources were included. The water samples were tested by multiple tube technique using OXOID MacConkey Broth (Oxoid Ltd, Basingstoke, Hampshire, England). First 100 ml of water specimen was collected for each sample and distributed five tubes with 10 ml of water and one 50 ml amount of water in bottles of sterile selective culture broth containing lactose and an indicator were incubated in an incubator at 44°C for 24 h. After incubation, the number of bottles in which lactose fermentation with acid and gas production has occurred was counted. Finally, by referring to probability tables the MPN of coliform in 100 ml water sample has been estimated (Cheesbrough, 2006).

Ethical consideration

Permission from municipality of the town for public water source samples and consent from private water source owners were obtained before water sample collection.

RESULTS AND DISCUSSION

Twenty four water samples were collected from the study area. Six were from protected wells and eighteen were from unprotected wells. From the six protected wells, four of them were public owned and the rest two of them were owned by private. All the water sources had no regular treatment. From these water sources 87.5% (21/24) have presumptive bacteria count MPN above the permissible limits for drinking water. Analysis of protected wells which demonstrated three of the six samples had total coliforms count of more than 10 per 100 ml of water and all these three had ***E. coli*** (Table 1). On the other hand, analysis of unprotected wells revealed that all eighteen of the samples had total coliform count greater than 10 per 100 ml. In all of the unprotected well ***E. coli*** was confirmed. However, from the total samples only one sample had fecal coliform count of zero (Table 1). Both protected and unprotected wells were contaminated by fecal coliform, which is particularly ***E. coli***. Totally, there was only one water source with excellent type, two with acceptable, nine unacceptable and twelve grossly polluted (Table 1).

Out of twenty four analyzed wells, seventeen of them were located downhill and the rest of the water sources were located above hill. All the wells located below hill had total coliform count of more than 10 per 100 ml of water (Table 1). Of the total analyzed samples, only three had acceptable fecal coliform count (less than 10 MPN per 100 ml of water), from these one source was in an excellent range and two of them were within an acceptable range. All the three of these samples were collected from protected wells (Table 1).

In relation to distance of water source from latrine, 79.2% of water sources were found at a distance of less than 30 m which is below WHO recommendation for minimum distance that should be exist between latrine and water source. On top of this majority (54.2%) of water sources were without cover. Out of eleven water sources owing cover, 27% of them were safe for drinking and on the other hand, all the wells without cover had fecal coliform count of more than 10, hence unsafe for drinking.

Table 1. Indicator bacteria count and possible factors of water source contamination in Serbo town, Jimma zone, Ethiopia 2010.

Well type	MPN				Total (%)
	0	1-10	11-50	>50	
Protected well	1	2	2	1	6 (25)
Unprotected well	0	0	7	11	18 (75)
Total	1	2	9	12	24 (100)
Distance of wells from latrine (m)					
<30	0	0	8	11	19 (79.2)
>30	1	2	1	1	5 (20.8)
Total	1	2	9	12	24 (100)
Presence of cover on the wells					
Yes	1	2	3	5	11 (45.8)
No	0	0	4	9	13 (54.2)
Total	1	2	7	14	24 (100)
Location of wells					
Above hill	1	1	2	3	7 (29.2)
Down hill	0	0	7	10	17 (70.8)
Total	1	1	9	13	24 (100)

Supply of water that owes no threat to the consumer's health depends on continuous protection. Because of human frailty associated with protection, priority should be given to selection of the purest source. Polluted sources should not be used unless other sources are economically unavailable. Ensuring bacteriological quality of drinking water sources is vital to public health function. On the other hand regular examination of water quality for the presence of organisms, chemicals, and other physical contents should provides information on the level of the safety of water. Frequent examinations of fecal indicator organisms remain the most sensitive way of assessing the hygienic conditions of water (World Health Organization 2003).

This research measures only microbial water quality by using ***E. coli*** as an indicator for fecal pollution. As a limitation, the physiochemical analysis was not done due to logistics constraints. However, we believe that the information obtained about fecal contamination of the water sources at Serbo town is the first in its kind and revealed the hygienic condition of water sources which are used by the community.

In this study 87.5% of wells have MPN of ***E. coli*** above the allowable limit**.** This indicates that majority of the water sources of Serbo town were fecally polluted. In comparison with a study conducted in Uganda, 2002 which showed that 90% samples had exceeded the WHO guideline (Haruna et al., 2005), the finding of this study was consistent. However as compared with a study conducted in North Gondar 2000 on unprotected wells and springs, the finding of this study was a little bit higher. This might be associated with the majority of water sources included in this study were unprotected (Mengesha et al., 2004). On the other hand as compared with a study done in Sudan Darfur 2011 to investigate drinking water quality, our finding showed higher percentage of MPN above allowable limit. This might be associated with the type of water sources difference in two communities (Amira, 2011).

If we compare the finding of this study with a study conducted in Jimma town in 2005, it showed that 95.8% of samples were unacceptable or grossly contaminated. The finding of this study (87.5%) was lower. This difference in percentage might be due to variation in methods used. The presence of fecal coliforms and ***E. coli*** in almost all of water sources were demonstrated in this study. Accordingly the potability and safety of these sources was questionable. As it is shown in a study conducted in Lesotho Highlands, adequate protection of water sources could improve the hygienic quality of water sources (Kravitz et al., 1999).

In our study from total analyzed twenty four samples, there were three water sources with MPN less than 10 per 100 ml of water. Three of them were from protected well whereas there is no water source with this MPN less than10 per 100 ml from unprotected sources, showing that protected wells are safer than unprotected sources.

According to a research conducted in south western Saudi Arabia, 2009 (AlOtaibi, 2009) and in Tamil Nadu, 2006 (Rajendran et al., 2006), all well water sources were

positive for coliforms using MPN method whereas in our study, one well was free of total coliform. The gap might be due to the protection of wells. The appropriate location of wells with respect to latrine needs to be above hill (Ethiopian Federal MOH, 2004). From a total of twenty four analyzed water sources, Seventeen (70.8%) of the wells were located below hill and seven (29.1) of them were located above hill. This greater percentage of wells, which were located bellow hill, might have contributed for larger number of water sources for not to be safe as a result of having a chance to leak to the well.

Conclusion

In conclusion, majority of the water sources had unacceptable total coliform count and all the water sources which were positive for presumptive coliform count had ***E. coli*** showing fecal contamination of water sources, and we recommend regular disinfection of drinking water sources, periodic bacteriological appraisal of drinking water sources, and construction and distribution of piped water.

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Full Length Research Paper

Purification and characterization of bacteriocin isolated from *Streptococcus thermophilus*

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***Streptococcus thermophilus* is used primarily as starter cultures to counter the harmful bacteria grown in cheese and yogurt making/preservation processes. These bacteria produce some exogenous toxins called bacteriocins having the antimicrobial activities against both Gram positive and Gram negative bacteria. In our study *S. thermophilus* growth was obtained at pH 5.5 and temperature 40°C. Bacteriocin activities were checked after their treatment with different enzymes, organic solvents, sodium chloride (NaCl) and detergents as well as their heat stability and effect of pH was studied. Bacteriocin activity was found heat stable at 100°C for 30 min and was found stable in the 3-10 pH range but lost the activity after the treatment with proteinase-K and protease enzymes. Activity was lost in treatment with lipase and amylase which shows the presence of lipo-glycolated peptide. Bacteriocin activity was lost on the presence of Dithiothreitol (DTT) and -mercaptoethanol which showed the presence of disulphide bond present in bacteriocin and essential for its activity. Urea and ethylene diamine tetraacetic acid (EDTA) also affected the bacteriocin activity but found stable to survive in the presence of 6% NaCl. Antibacterial assay showed the strong growth inhibition of test bacteria. Bactericidal activity was further purified to homogeneity by ammonium sulphate precipitation and different chromatographic techniques. Molecular weight was calculated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as 2.7 kDa.**

Key words: ***Streptococcus thermophilus***, bacteriocin, antimicrobial peptide, yogurt culture bacteriocin, disc diffusion test.

INTRODUCTION

Microbial defense system carries extraordinary armament in form of bacteriocins. These peptides are now well recognized for their property to inhibit the wide spectrum of Gram negative and Gram positive bacterial growth (Abdelkader et al., 2009; Mezaini et al., 2009; Tuncer and Ozden, 2010). Bacteriocins are produced by almost every bacterium and within a species tens or even hundreds of different kinds of bacteriocins are produced (Line et al.,2008). Bacteriocins are produced in both Gram-negative and Gram-negative bacteria and can be divided into different subgroups on the basis of structural and amino acid sequence similarities. Colicins (Parret et al., 2003) and the microcins (Kemperman et al., 2003) are major subgroups of Gram negative bacteriocins. Gram-positive
bacteria are sub-grouped in five major classes on the basis of their internal cross linking, post translational structures and primary structures (Nes et al., 1996; Nissen-Meyer and Nes, 1997; Kemperman et al., 2003). Subclasses include class one lantibiotics or lanthionine containing bacteriocins (McAuliffe et al., 2001; Guder et al., 2000), class two, small (<10 kDa) heat-stable, non-lanthionine-containing peptides bacteriocins (Van-Kraaij et al., 1999; Yi et al., 2010; Netz et al., 2001), Class three**,** large (>30 kDa) heat-labile proteins that are of lesser interest to food scientists (De-Jong et al., 2010; Chen et al., 2004), class four, complex proteins requiring

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the association of carbohydrate or lipid moieties (Alpay et al., 2003) and Class five, circular, head-to-tail ligated bacteriocins (McAuliffe et al., 2001; Kemperman et al., 2003).

Bacteriocins from Gram-positive organisms, such as lactic acid bacteria (LAB), have attracted much attention and have been the subject of intensive investigation due to their extensive incorporation as bio-preservatives ingredients into model foods particularly in the dairy industry (Diop et al., 2007) and also in human therapeutics (Martin-Visscher et al., 2008). Normally the cells producing the bacteriocins are immune to its antagonistic action and therefore might enjoy a competitive advantage over sensitive bacteria inhibiting the same ecological niche (Joerger, 2003).

Yogurt starter cultures commonly contains ***Streptococcus thermophilus*** which have been well studied for the production of the bacteriocins, is a heterogeneous group of peptide which have wide spectrum of activity against bacteria (Ivanovaa et al., 1998; Kabuki et al., 2007; Iyera et al., 2010). ***S. thermophilus*** bacteriocins have been reported to have a broad inhibitory spectrum against several bacteria ***Listeria monocytogenes***, ***Salmonella typhimurium***, ***Escherichia coli***, ***Yersinia pseudotuberculosis*** and ***Yersinia enterocolitica*** (Ivanovaa et al., 1998), ***Clostridium tyrobutyricum*** (Mathot et al., 2003).

These bacteriocins are mostly heat (Kabuki et al., 2007) and pH stable (Vinderola et al., 2002; Mezaini et al., 2009). As the LAB have now achieved the status of generally recognized as safe (GRAS), these bacteria or their bacteriocin can be used as probiotic or food preservative in food to keep the food product safe from spoiling bacteria (Buriti et al., 2007). In our study, we have reported isolation and purification of heat and pH stable bacteriocin activity from ***S. thermophilus*** isolated from yogurt. This activity may be further used as a preservative of food products as it showed strong growth inhibition of both Gram-negative and Gram-positive bacteria.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used during the research were molecular biology grade and were purchased from Oxoid, Riedel Dehaen, Merck, ICN, BBI, BDH Chemicals, Panerac, Schwan, Sigma and Fermentas Different culture media like Nutrient Agar/broth, M 17 agar/broth and Muller Hinton Broth media were used. ***E. coli*** and ***Staphylococcus aureus*** were used as test bacteria. ***S. thermophilus*** isolated from yogurt and maintained in protein molecular biology labortory (PMBL), Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad was used as starter culture.

Screening of bacteriocin activity

Inoculum with $1x10^6$ – $2x10^8$ CFU/mL culture in selective broth (M17) was incubated at 37°C for 24 h and then centrifuged at 10,000 rpm (4°C) for 20 min. The residue was discarded and cell free extract was used to screen the bacteriocin activity against ***Bacillus substilis*** and ***S.aureus*** by using disc diffusion method (CLSI, The Clinical and Laboratory Standards Institute, 2007). Nutrient agar (Oxoid, UK) was prepared and test bacteria was added to the medium and poured in sterilized Petri plates. Small filter paper discs were laid flat on growth medium containing 100 µL of cell free supernatant. The Petri plates were then incubated at 37°C for 24 h, for the growth of bacteria. Antibacterial activity was measured by clear zones formation.

Production of bacteriocin

Broth culture (500 mL) with inocula was incubated to obtain a large repertoire of bacteriocin at 37°C and pH 5.0 for 18 h. After the incubation cell free supernatant was obtained by centrifugation of media at 12,000 x***g*** for 30 min, the cell free supernatant was used further for ammonium sulphate precipitation.

Bacteriocin characterization

Sensitivity of bacteriocin to pH, temperature and enzymes

To determine the pH stability of bacteriocin, pH values were adjusted within the range of 3 to 10 by hydrochloric acid (HCl) or sodium hydroxide (NaOH), and each sample was held for 1 h at 37°C. Sensitivity of bacteriocin to heat was checked by heating the culture supernatant for 15 min at 65°C, 80°C, 100°C and 121°C. Sensitivity to proteolytic enzymes was checked when bacteriocin sample was treated with Proteinase-K (1 mg/mL), Trypsin (0.5 mg), Protease (1 mg/mL) and Lipases (1 mg/mL). 1 mL of crude bacteriocin was added to 1 mL of enzyme and incubated at 37°C for 2 h and then boiled at 100°C for 5 min. The disc diffusion assay was performed after treatment to detect activity against test organism peptide (Mathot et al., 2003).

Effect of organic chemicals and detergents

The effect of organic solvents on bacteriocin was tested with organic solvents including dithiothreitol (DTT) (5%), -mercaptoethanol (50 mM), ethylene diamine tetraacetic acid (EDTA) (10 mM), and organic solvents; acetone, chloroform, ethanol and methanol at final concentration of 5.0% (Todorov et al. 2006). The surfactants tested were sodium dodecyl sulphate (SDS), Tween 20, Tween 80, Triton X-100 and urea at final concentrations 1%. Controls consisted of either active precipitates or detergents used. All samples and controls were incubated at 37°C for 5 h and tested for activity.

Effect of sodium chloride (NaCl)

The effect of NaCl on bacteriocins activity was checked by growing the bacteriocin producing strain in agar medium with 2, 4 and 6% NaCl and assaying the activity by disc diffusion method (Larsen et al., 1993).

Bacteriocin purification

The partial purification of bacteriocin was conducted by 80% ammonium sulphate precipitation and centrifuged at 10,000 x***g*** and 4°C for 10 min to separate residues and supernatants from crude precipitated extracts; the supernatants were stored at 4°C in 100 mL sterilized bottles (pH; 8.0). Dialysis was followed in a tubular

cellulose membrane (1000 cut off) against 2 L distilled water for 24 h, from which the bacteriocin titer was performed. Protein concentration of the bacteriocin in supernatant was determined by the Bradford method of protein estimation (Bradford, 1976), using bovine serum albumin as the standard.

Gel filtration and ion exchange chromatography

The dialyzed samples were purified by gel filtration using Sephadex G-200. The elution of sample was performed by 10 mM Tris-HCl buffer (pH 8.0). The fractions with maximum protein contents were pooled out and applied for antimicrobial assay. The active factions obtained from the gel filtration were further purified by ion exchange chromatography. DEAE-Sephadex was used as resin in ion exchange chromatography column equilibrated with the 10 mM phosphate buffer (pH, 7.0). Elution was done by the gradient of 10 mM phosphate buffer and 0.1-1 M NaCl buffer (pH, 7.0). All samples of crude extract and partially purified extracts were then subjected to antibacterial assay.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The characterization of the bacteriocin (peptides/proteins) was performed by SDS-PAGE (Rehman et al., 2009). The SDS-PAGE was carried out for the crude and purified protein samples possessing antimicrobial activity. The experiment was carried out on Mini-PROTEIN, mini vertical electrophoresis apparatus (BioRad, UK) using the 12% gel. The samples were prepared by mixing the protein 2:1 ratio with SDS-sample buffer of Sigma. The gel was stained for protein with coomassie brilliant blue, along with the marker proteins (Lewus and Montville**,** 1991). The apparent molecular mass of the sample was calculated by comparison with the mobility of the standard markers.

RESULTS AND DISCUSSION

Screening and production of bacteriocin activity

The zones of inhibition showed the presence of bactericidal activity against ***B. Substilis*** and ***S. aureus*** in initial screening. Bacteriocin production was strongly dependent on pH, time and temperature as claimed by Todorov and Dicks (2004). Cell free supernatant showed the bacteriocin activity as 2600 AU/mL obtained after centrifugation.

Characterization of bacteriocin

Sensitivity of bacteriocin to pH, temperature and enzymes

Bacteriocin activity was remained stable at the 100°C for 30 min but it lost its activity after its incubation at 121°C (Table 1). These findings are similar to the Thermophilin 110 which remained stable when held at 100°C for 60 min but lost its activity against ***Pediococcus acidilactici*** after 90 min or longer exposure. Contradictory observations were made for ***S. thermophilus*** 580 bacteriocin, where its heat instability was due to a heat labile peptide (Mathot et al**.**, 2003). Bacteriocin activity was also found stable at wide range of pH 3-10. Khali (2009) reported the ***S.thermophilus*** bacteriocin which was pH resistant over the wide range of 3-9 (Table 2). Proteinase-K and pepsin were strongly inhibited the bacteriocin activity showing the bacteriocin protein in nature. This bacteriocin activity was similar to Thermophilin 110 Gilbreth and Somkuti (2005), Thermophilin A (Ward and Somkuti, 1995) Thermophilin T (Aktypis et al., 1998) and the bacteriocin of ***S. thermophilus*** 580 (Mathot et al., 2003) in this manner. The inactivation of the bacteriocin by treatments with lipase enzymes (Table 3) suggests that activity was dependent on the presence of either a carbohydrate or lipid moiety as described by Maurad and Meriem (2008).

Table 1. Temperature sensitivity of bacteriocin activity.

Temperature	Bacteriocin activity
Control	2600 (AU/mL)
04°C (30 min)	-
65°C (30 min)	+
80°C (30 min)	+
100°C (30 min)	+
121°C (15 min)	-

Table 2. pH stability of bacteriocin activity.

pH	Bacteriocin activity
Control	2600 (AU/mL)
2.0	-
3.0	+
4.0	+
5.0	+
6.0	+
7.0	+
8.0	+
9.0	+
10.0	+

Effect of organic solvents, sodium chloride and detergents

Bacteriocin activity was lost after treatment with DTT and -mercaptoethanol. This may be due to the reduction of disulphide bonds (Table 4). Our findings were in agreement with Todorov et al. (2006) and Khalil et al. (2009). Bacteriocin activity remained unaffected in the presence of all detergents (Table 5). Similar results were observed by Ivanova et al. (2000) and Ogunbanwo et al. (2003). Bacteriocin activity was found stable in the presence of 2, 4 and 6% NaCl (Table 6).

Table 3. Effect of enzymes on bacteriocin activity.

Enzyme	Bacteriocin activity
Control	2600 (AU/mL)
Proteinase-K	-
Trypsin	+
Protease	-
Lipases	-
Amylase	+

Table 4. Effect of organic solvents on bacteriocin activity.

Organic solvent	Bacteriocin activity
Control	2600 (AU/mL)
Dithiothreitol (DTT)	-
-mercaptoethanol	-
Acetone	+
Chloroform	+
Ethanol	+
Methanol	+

Table 5. Effect of detergents on bacteriocin activity.

Treatment	Bacteriocin activity
Control	2600 (AU/mL)
Sodium Dodecyl Sulphate (SDS)	+
Tween 20	+
Tween 80	+
Triton X-100	+
Urea	-
EDTA	-

Table 6. Effect of NaCl on bacteriocin activity.

Treatment	Bacteriocin activity
Control	2600 (AU/mL)
2% NaCl	+
4% NaCl	+
6% NaCl	+

Purification of bacteriocin

Crude of the recovered proteins were applied to ammonium precipitation and dialysis. Protein recover was 26.0 fold after ammonium sulphate precipitation. Dialysis removed the salt and concentrated the protein with 15 mL volume. Dialyzed protein sample was then fractionated by gel filtration and ion-exchange chromatography, using DEAE-Sephadex. Gel filtration using Sephadex G-200 active fractions (Figure 1) were pooled out and applied to ion exchange chromatography (Figure 2). Bacteriocin activity was purified up to 597 folds chromatographic techniques (Table 7). The overall yield and activity are summarized in Table 7.

Antibacterial assay

Bacteriocin activity was found active against Gram-positive bacteria; ***S. aureus***, ***B. subtilis*** and Gram-

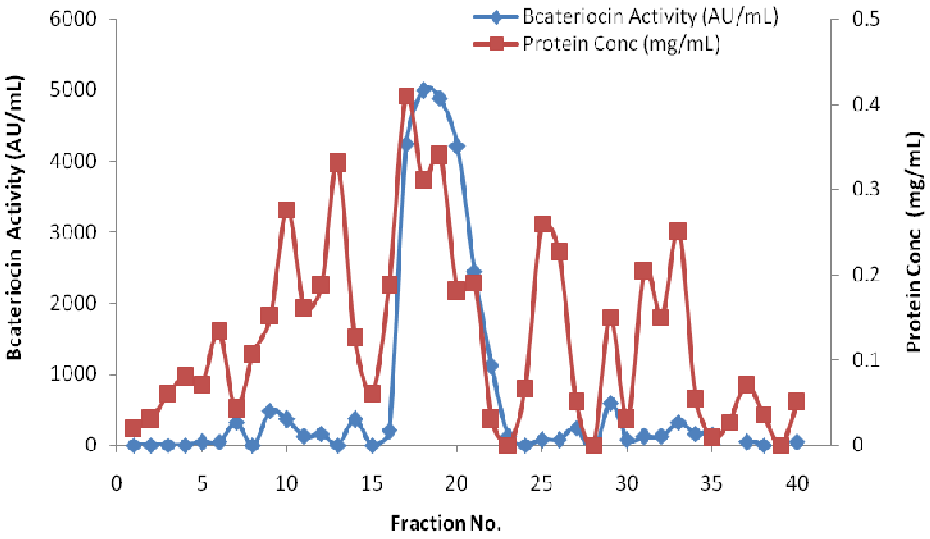

Figure 1. Gel fitration of bacteriocin activity showing the protein concentration and the bacteriocin activity in the active fractions.

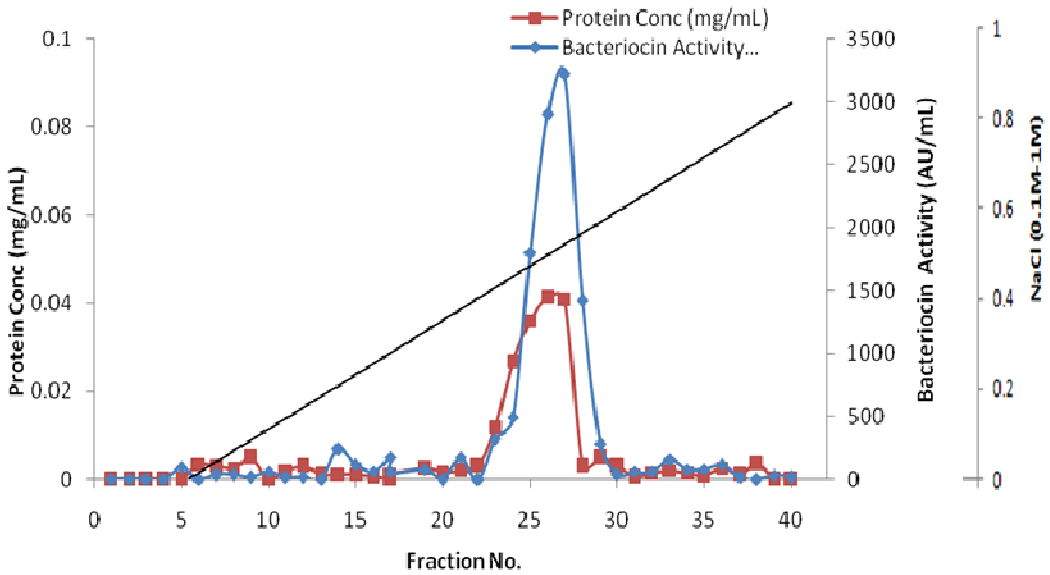

Figure 2. Ion exchange chromatography of bacteriocin activity.

Table 7. Purification fold bacteriocin activity purification steps.

Purification Level	Volume (ml)	Activity (AU/ml)	Total act. (AU)	Protein (mg/ml)	Specific activity	Recovery	Purification fold
Culture supernatant	500	2600	1300000	13.5	192.6	100.0	1.0
Ammonium sulphate precipitation	20	10500	210000	2.1	5000.0	16.2	26.0
Dialysis	15	10200	153000	1.9	5368.4	11.8	27.9
Gel filtration chromatography	4	4600	18400	0.4	11500.0	1.4	59.7
Ion exchange chromatography	2	3450	6900	0.03	115000.0	0.5	597.1

Activity unit (AU/mL), Reciprocal of the highest dilution x1000/volume of bacteriocin added; Specific activity (AU/mg), total activity of the subsequent purification step/total protein of the same step; Recovery (%), total activity of subsequent step x 100/total activity of crude preparation; Purification fold, Specific activity of subsequent step/ Specific activity of crude preparation.

Table 8. Antimicrobial activity of bacteriocin against Gram negative and Gram positive bacteria.

	S. aureus	*B. substilis*	*E. coli*	*P. multocida*
Bacteriocin activity (mm inhibition zone)	24.5 ± 05	9.5 ± 03	10.7± 05	8.7± 03
Rifampin	26.25 ± 08	12.5 ± 03	10.77 ± 01	10.26 ± 07

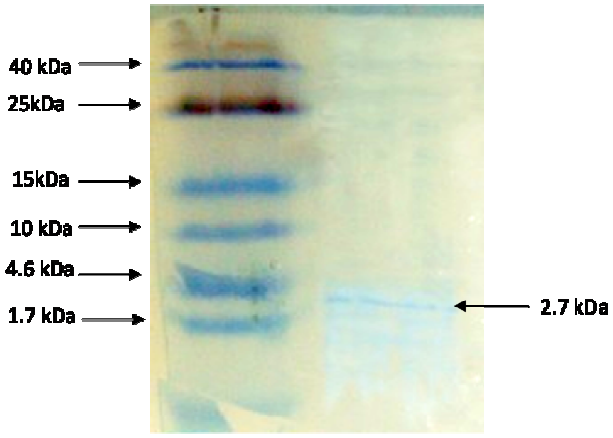

Figure 3. SDS-PAGE showing the molecular weight of the bacteriocin activity as 2.7kD.

negative bacteria; ***E. coli***, ***Pasteurella multocida***. Results are shown in Table 8.

Molecular weight determination

Molecular weight of the bacteriocin was determined by SDS-PAGE (Figure 3). The molecular weight of the purified bacteriocin activity was 2.7 kDa. Aktypis et al. (1998) reported that SDS-gel electrophoresis of partially purified thermophilin T showed that bacteriocin activity was associated with a protein band of approximately 2.7 kDa molecular mass (Figure 3).

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Full Length Research Paper

Incidence and antibiotic susceptibilities of *Yersinia enterocolitica* and other *Yersinia* species recovered from meat and chicken in Tehran, Iran

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The objective of this study is to investigate the prevalence and determine the antimicrobial susceptibility pattern of *Yersinia* species in beef meat and chicken meat samples in different seasons. In this study 379 pieces of beef and chicken meats were examined for the presence of *Yersinia* species between April 2007 and March 2008. 25 g of homogenized food samples was pre-enriched in PBS then it was cultured on cefsulodin-irgasan-novobiocin (CIN) agar. Susceptibility testing of bacterial strains was performed at 28°C by the agar diffusion method. *Yersinia* spp. was isolated from 60 of 379 (15.8%) beef meat and chicken meat samples. *Y. entrocolitica* was found in 48 of 60 (80%) positive samples. The rate of other 3 *Yersinia* spp, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, were 7(11%), 4(6%) and1 (0.01%) out of 60 isolates, respectively. 98% of isolates were susceptible to choloramphenicol and gentamicin. The most antibiotic resistance belongs to cephalothine (98%). Our results showed that isolation ratio of *Y. enterocolitica* and the other species is higher in cold climates. The majority of isolates were resistant to cephalothine. The most active pharmacologic agents were chloramphenicol, gentamicin and trimetoprim.

Key words: *Yersinia* enterocolitica, antimicrobial susceptibility, beef meat, chicken.

INTRODUCTION

Yersinia enterocolitica, a Gram-negative, urease positive and facultative anaerobic species, is highly heterogeneous microorganism and it can be divided into several bioserotypes and serotypes (Bottone, 1997; Robins-Browne, 1997). *Yersinia* contamination represents a significant problem in food supplies, since this bacterium needs long period to grow (Fredriksson-Ahomaa and Hannu Korkeala, 2003). *Y. enterocolitica* is known as a psychotropic waterborne and foodborne enteropathogen. This microorganism can grow to large numbers at refrigeration temperatures, so meat, chicken, milk, cheese contaminated with that organism could become a significant health risk for consumers (Black and Jackson, 1978; Stern and Pierson, 1979; Soltan-Dallal et al., 2004; Hudson et al., 2008). This microorganism is primarily a gastrointestinal tract pathogen with a strong propensity for extra intestinal spread under defined host conditions (Soltan-Dallal and Moezardalan, 2004a). It causes a broad range of diseases from acute bowel disease to

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Abbreviation: CIN, Cefsulodin-irgasan-novobiocin.

Table 1. Percentage number of contaminated samples.

Food type	Total number of samples	Number of contaminated samples	
		n	%
Fresh meat	189	19	31.7
Chicken meat	190	41	68.3
Total	379	60	100

extra intestinal manifestation such as reactive arthritis , uveitis and sepsis (Jacobs et al., 1989; Chandler and Parisi, 1994). Systemic and extraintestinal infections and enterocolitis in immune-compromised patients require antibiotic therapy, and the agents used most commonly include chloramphenicol, gentamicin, tetracycline, cotrimoxazole and ciprofloxacin (Butler, 1990; Hoog, 1987). Although there is data concerning the incidence of *Y. enterocolitica* and related species in foods are reported in some countries (Fredriksson-Ahomaa and Hannu, 2003; Siriken, 2002; Dominique et al., 1981; Soltan and Moezardalan, 2004b), but compared with other bacterial enteropathogenes, there are a few studies about the antimicrobial susceptibility of *Yersinia* spp, which are isolated from staff foods and human. The aims of this study are to investigate the prevalence of *Y. enterocolitica* and other *Yersinia* species in meat and chicken samples in different seasons and to determine the antimicrobial susceptibility pattern of *Yersinia entrocolitica* and other *Yersinia species* isolated from meat and chicken in Tehran, Iran**.**

METHODS AND MATERIALS

Sample collection

189 pieces of beef meat and 190 pieces chicken meat were purchased from 28 different local butcher's shops and supermarkets in Tehran, Iran, and were examined for the presence of *Yersinia* species between April 2007 and April 2008**.**

Cold enrichment, Bacterial isolation and identification

25 g of each sample was added to plastic bag containing 10 ml phosphate buffered saline (pH 7.2) and homogenized by Stomacher (400 circulator Seward-England) and incubated at 4°C for one, two, three and four weeks. At weekly intervals, a suspension aliquot was cultured linearly on Cefsulodin Irgasan Novobiocin Agar (CIN) (Merck-Germany) with Yersinia selective supplement. Cultured plates were incubated at 26°C, between 24 - 48 h. The colonies were considered macroscopically and microscopically after incubation. Suspected colonies were examined to motility test by SIM medium both at 25 and 37°C. In addition, biochemical test including urease activity, KIA and Simon citrate were used for the bacterial identification (Johnson, 1998).

Antimicrobial susceptibility

Susceptibility testing of bacterial strains was achieved at 28°C on Mueller-Hinton agar (Merck-Germany) by the agar diffusion method. The sensitivity spectrum of each of the isolates to ten different antibiotics was determined according to CLSI (2005). (Clinical and Laboratory Standard Institute) protocol and zone size interpretative chart explained by High Media Company. Antibacterial agents included ampicillin (10 mcg), trimethoprim (5 mcg), chloramphenicol (30 mcg), tetracycline (30 mcg), streptomycin (10 mcg), cephalotin (30 mcg), ciprofloxacin (5 mcg), cephotaxime (30 mcg), nalidixic acid (30 mcg), and gentamicin (10 mcg). Antibiotic disks were provided by High Media Company.

Data analysis

Statistical analysis of results was performed with SPSS/PC 12 software (SPSS Chicago, IL). A P value<0.05 was used for statistical significance.

RESULTS

Out of 375 pieces including 189 beef meat and 190 chicken meat samples, 16% (n=60) *Yersinia* species were isolated (Table 1). 70% (n=42) of isolates were achieved from chicken meat and 30% (n=18) from beef meat. The prevalence of *Y. enterocolitica* with the most incidence was 80% (n=48), that were found in chicken meat and beef meat with rate of 62.5% (n=30) and 37.5% (n=18) respectively. The occurrence of *Y. enterocolitica* was slightly higher in chicken than meat. The frequency of the other *Yersinia* spp. including *Y. frederiksenii*, *Y. intermedia* and *Y. kristensenii* were 11.6% (n=7), 0.06% (n=4), and 0.01% (n=1). Except for *Y. frederiksenii* (n=1), none of *Y. intermedia* and *Y. kristensen* were not found in meat (Table 2).

As we stated above, all of samples were cultured at the end of week for four weeks. The highest isolation rate was 33, 75 and 42% in 4 °C/3-week enrichment for *Y. entrocolitica, Y. intermedia* and *Y. frederiksenii* respectively. The only isolated *Y. kristensenii* accrued in 2-week enrichment. The rates of Yersinia isolated in various seasons were 20, 16 and 13% in November, April and March respectively. The prevalence of *Yersinia entrocolitica* and other species in various month was mentioned in Table 3.

All isolates were subjected to antimicrobial resistance testing by the disc diffusion method. An overview of the antibiotic susceptibility of the strains is shown in Table 4. Our results indicated that among *Y. entrocolitica*, the sensitive rate for gentamicin and chloramphenicol was 97% (n=46) and for trimethoprim and ciprofloxacin was

Table 2. Percentage distribution of *Yersinia* spp in tested samples.

Food	Number of contaminated samples			
	Yersinia			
	Y. enterocolitica	*Y. intermedia*	*Y. frederiksenii*	*Y. kristensenii*
	n (%)	n (%)	n (%)	n (%)
Fresh meat	18(30)	1(1.7)	0	0
Chicken meat	30(50)	3(5)	7(11.7)	1(1.7)
Total	48(80)	4(6.7)	7(11.7)	1(1.7)

Table 3. Frequency distribution of *Yersinia Spp* in different months of year.

month of sampling	Type of isolate				Total
	Y. kristensenii	*Y.intermedia*	*Y.frederiksenii*	*Y. enterocolitica*	
	n:1	n:4	n:7	n:48	
April	0(%)	0	1	9	10
May	0	0	1	4	5
June	0	0	0	5	5
August	0	0	0	1	1
September	0	0	0	2	2
October	0	0	0	2	2
November	0	1	1	10	12
December	0	2	3	3	8
January	0	1	1	2	4
February	0	0	0	3	3
March	1	0	0	7	8
	1	4	7	48	60

Table 4. Antibiotic resistance patterns of isolated *Yersinia* spp.

Antibiotic	*Y. entrocolitica*			*Y.frederiksenii*			*Y. intermedia*			*Y. kristensenii*		
	n=48			n=7			n=4			n=1		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin (10 mcg)	17	4	27	3	0	4	4	0	0	1	0	0
Trimethoprim (5 mcg)	45	0	3	7	0	0	4	0	0	1	0	0
chloramphenicol (30 mcg)	47	1	0	7	0	0	4	0	0	1	0	0
Tetracycline (30 mcg)	36	6	6	7	0	0	3	0	1	1	0	0
Streptomycin (10 mcg)	28	15	5	6	1	3	4	0	0	0	1	0
Cephalotin (30 mcg)	1	0	47	0	0	7	0	0	4	0	0	1
Ciprofloxacin (5 mcg)	45	3	0	7	0	0	4	0	0	1	0	0
Cephotaxime (30 mcg)	40	8	0	7	0	0	4	0	0	1	0	0
nalidixic acid (30 mcg)	37	0	11	5	0	2	4	0	0	0	0	1
Gentamicin (10 mcg)	47	1	0	7	0	0	4	0	0	1	0	0

93.7% (n=45). Among ß-lactam antibiotics including ampicillin, cephalotin and cephotaxime the most resistant rate belongs to cephalotin (97%/n=59), followed by ampicillin (n=31), whereas none of *Yersinia* spp. was resistant to cephotaxime. All of *Y. intermedia*, *Y. kristensen* and Y*. frederiksenii* isolates were sensitive to trimethoprim, chloramphenicol, ciprofloxacin, cefotaxim and gentamicin.

In total, 15/8% (n=60) of this isolates exhibited a resistant phenotype, and most of these strains showed resistance to more than one antimicrobial test. 34 (56%) isolates were resistant to two or more antibiotic test

The highest co-resistance rate was seen for the ampicillin, cephalotin with rate of 28% (n=17), followed by 11% (n=7) for ampicilin, nalidixic acid and cephalotin. Four isolates (6%) were resistant to five antibiotics including ampicillin, nalidixic acid, cephalotin, tetracycline and streptomycin.

DISCUSSION

Yersinia spp. was reported from 9 to 99.5 percent in different sources such as meat, grandbeef, pork, environment, water and human (Kapperud, 1977; Ostroff, 1995; Soltan, 2001; Okwori et al., 2005). In our study, we analyzed two kinds of meat, chicken meat and beef meat, which are broadly consumed in Iran. Our results indicated *Y. entrocolitica* has a high incidence among of other *Yersinia* spp in meat and chicken. Similar findings had been achieved in Mexico (Ramirez and Vazquez-Salinas; 2000). The prevalence of *Y. frederiksenii* (11.6%) in present study was similar to results of Capita (Capita et al., 2002). Ibraham examined 50 beef samples for *Yersinia* spp. in Turkey and the isolation rate was 20% including of 9 (18%) *Y. enterocolitica* and 2 (4%) *Y. frederiksenii* (Ibraham and Mac Rae, 1991). Also in another study in Spain, *Y. enterocolitica* and *Y. frederiksenii* were detected in 22 (55%) and 6 (15%) samples, respectively (Capita et al., 2002). The various results between these different studies and our study probably can be due to several factors such as isolation methods, season of isolation and geographical location regarding to various temperatures and number of analyzed samples. These factors play an important role in isolation of the *Yersinia spp*. For instance, in this study the most of *Yersinia* spp. isolated in cold months such as November, April and December. It is known that the isolation rate of *Y. enterocolitica* is higher in cold climates (Warnken and Nunes, 1980). According present finding the highest rate of *Yersinia* spp, exception of *Y. kristensenii*, was isolated in 4 °C/3-week enrichment (Jiang and Kang, 2000).

Although *Y. entrocolitica* has been known as a most principal cause of human infections, however there are some reports about coursing gastroenteritis in human by other species such as *Y. intermedia*, *Y. kristensen* and Y*. frederiksenii* (Brenner and Bercovier, 1980; Hamama and Marrakchi, 1992). Strains of these pathogenic biotypes contain marker associated with virulence and these are located on the chromosome (ail) and on the virulence plasmid (PYV) (Goverde et al., 1993; Khorramizadeh et al., 2007). In USA the Center for Diseases Control and Prevention (CDC, 1982), conducts investigations of outbreaks of yersiniosis to control them and to learn more about how to prevent these infections. According with others authors, it can be assumed that further clinical studies are needed to assess the epidemiological importance, the occurrence and the possible etiological relevance of *Y. enterocolitica* (Ramirez and Vazquez-Salinas, 2000; Hoffmann et al., 2002).

Therefore it seems the investigation of susceptibility pattern and antibiotic resistance can be necessary for treatment of strains clinically. Antimicrobial resistance in foodborne pathogens and therapeutical intervention has always been an important issue in public health (Soltan et al., 2010). Using of antimicrobial agent in veterinary as a growth promotion, treatment or prophylactic can develop the antibiotic resistance in food animals. It can be a reason for antibiotic resistance transfer to humans via the food chain (Mayrhofer and Paulsen, 2004; Ezekiel et al., 2011). According to our results, the majority of isolates were resistant to cephalothine that may be due to presence of Enzyme B. The first report about B lactamase production by *Y. entrocolitica* was in 1973 (Cornelis et al., 1973). Soon afterwards, the presence of two types of chromosomal β-lactamase was described in some clinical isolates of *Y. enterocolitica*, a non-inducible broad-spectrum β-lactamase, enzyme A, and an inducible cephalosporinase, enzyme B (Cornelis and Abraham, 1975). The presence of two types of chromosomal B-lactamase was described in some clinical isolates of *Y. entrocolitica*, Enzyme B as Inducible cephalosporinase is one of them (Jeannette et al., 2000; Pham and Bell, 2000) The antibiotic susceptibility of *Y. enterocolitica* to gentamicin and chloramphenicol, ciprofloxacin and streptomycin are similar to reports of Okwori et al. (2005), who documented sensitivity of *Y. enterocolitica* strains of animal origin to ciprofloxacin and floxavid. The results indicated most active pharmacologic agents were chloramphenicol, gentamicin and trimethoprim. Regarding to high sensitivity of *Yersinia* spp. to gentamicin and chloramphenicol, these agents should be effective in the treatment of *Yersinia* spp when clinically indicated. It is suggested from results that the presence of *Yersinia* in beef meat and chicken meat represent a health risk for consumers. The education of people who involve in production, processing and final preparation of animal products is required to avoid cross-contamination.

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Full Length Research Paper

Screening of *Saccharomyces cerevisiae* for high tolerance of ethanol concentration and temperature

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The fermentation process of sugar feedstock materials at industrial scale requires the utilization of microorganisms capable of working at high ethanol concentration and high temperatures. The selection of *Saccharomyces cerevisiae* strains, able to ferment sugars obtained from different material at temperatures above 35°C with high ethanol yield, has become a necessity. Three yeast strains were irradiated with gamma ray and screened for their ability to grow and ferment molasses in a temperature range of 35-45°C. The yeasts were placed in a liquid medium, and irradiated at different doses (0.1, 1, 2, 3, 4, 5 and 10 KGy/h). Although all the isolated strains had growth (in agar plates) at 35 and 40°C, but just two strains showed growth at 42°C, and there was no growth at 45°C. Two pure yeast strains were isolated (PTCC[5269] M_3 and Areni M_7). The efficiency of temperature and high concentrations of ethanol tolerant strains were more than double of ethanol production compared with using the initial strains of yeast. All resistant strains were tested on liquid medium of molasses, and nutrients with 30% (v/v) ethanol had significant difference (P>0.01) for growth intensity at same condition with initial strains.

Key words: Bioethanol, gamma radiation, ***Saccharomyces cerevisiae***, thermotolerant.

INTRODUCTION

Saccharomyces cerevisiae is an important micro-organism in bio-industry and its tolerance to temperature and ethanol concentration is one of the main characteristics used for deciding whether it can be used as a bio-fermentation resource (Osho, 2005). Thus, in the industrial ethanol production, there are many important factors which should be considered such as ethanol or sugar tolerance of strains, and enzymatic activities for good operation (Furukava et al., 2004). One of the problems associated with fermentation of sugar is the high temperatures (35-45°C) and high ethanol concentration (over 20%). Tolerance to high temperatures and ethanol concentrations are important factors of microorganisms for increasing efficiency at industrial scale. The fermentation efficiency of ***S. cerevisiae*** at high temperatures is very low due to increased fluidity in membranes to which the yeast responds by changing its fatty acids composition (Mager and Siderius, 2002; Schuller et al., 2004).

Stress or environmental stimuli can cause structural changes and/or metabolic changes in an organism acting as expression activator for genes involved in the synthesis of specific compounds that protect the organism (Lieckefeldt et al., 1993). The factors triggering the expression of this type of genes can be biotic or abiotic. Biotic factors induce changes in the gene expression of the guest, giving rise to the synthesis of specific compounds that generate resistance to the strange organism. Abiotic stresses can be temperature, osmotic stress, anaerobic conditions, heavy metals, growth regulators, ultraviolet or gamma radiation, metabolic repressors, and pH (Brosnan et al., 2000). Stress due to temperature has been the most studied abiotic factor, where both heat and cold induce the synthesis or storing of a group of proteins that increase stress resistance (Steensma and Linde, 2001). Some successful attempts to adapt yeasts to high temperatures have been described. ***S. cerevisiae*** yeasts, capable of

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fermenting at 40 and 45°C have been obtained using progressive cultures (De Barros et al., 1998). Additionally, thermo-tolerant yeasts have been obtained by selecting survivors after a shock process at relatively high temperatures. El Sheikh and Berry (1980) demonstrated that 15 min of incubation at 55°C resulted in yeasts tolerant to higher temperature than non-incubated controls. The objective of this work was to select ***S. cerevisiae*** strains capable of fermenting glucose at temperatures above 35°C with an ethanol yield of at least 70% of theoretical value in Fermentation process.

Excess amount of ethanol has been reported to cause mitochondrial DNA damage and degrades bio membranes in yeast cells (Swiecilo et al., 2000). Ethanol can dissolve fatty acid constituents of the cell membranes; disrupt cytoplasmic membrane rigidity (Osho, 2005). Many reports have accented a relationship between the fatty acid compositions of lipid membranes and ethanol stress tolerance (You et al., 2003) which stop mitochondrial bio molecules translocation and proton motive force (Ekunsanmi and Odunfa, 1990) and finally cause cell death. According to these phenomena, resistant strains to ethanol have many mechanisms to overcome ethanol perils.

Invertase enzyme activity propriety in the yeast strain is very important. Invertase is one of the important extracellular enzymes in ***Saccharomyces*** that is responsible for converting sucrose to its subunits, glucose and fructose (Sengupta et al., 2000).

Approaches to measure ethanol tolerance involve determination of ethanol effects on cell growth, fermentation ability, viability and batch culture performance (Ekunsanmi and Odunfa, 1990). High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol (Swiecilo et al., 2000). Resistant strains to ethanol stress have other abilities like resistance to other stresses such as osmotic pressure and oxidative and heat (Swiecilo et al., 2000). Mc Kee and Lawrence (1979) found that partially different sets of gene functions are required for the production of different kinds of mutations induced by ^{60}Co gamma rays in ***S. cerevisiae***. This observation was very similar to others made previously with respect to UV mutagenesis (Lawrence and Christensen, 1976, 1978, 1979).

Induction of mutation in ***S. boulardii*** was carried out by 1, 2, 3, 4 and 5 KGy exposure of γ irradiation. Results revealed that the survival percentages were decreased by increasing the doses of γ rays whereas the survival percentage was 2.67% at exposure dose of 5 KGy. On the other hand, the mutant percentages were increased by increasing the radiation intensities, that is, doses. The highest numbers of mutants were induced as a result of 4 KGy dose of γ rays applications, which gave the highest mutants percentage (14.29%) (Abosereh et al., 2006).

To-on et al. (2007), with the yeast fermentation performance reviews collected from various sources, were able to isolate a particular strain capable of producing 15% ethanol at temperature of 40°C in 48 h. It was called thermo-tolerant.

Benton et al. (2006), with DNA analysis in different strains of yeast exposed to different doses of gamma rays (1, 10 and 100 Gy), observed significant differences between their DNA. Akachaa et al. (2007) suggested that low doses of γ-ray (10 and 20 Gy) significantly increased the enzyme activity on ***S. cerevisiae***. They also described the impact of irradiation on immobilization efficiency of biocatalyst entrapped on to alginate gel beads. When yeast irradiated to a dose of 20 Gy was immobilized, alcohol-dehydrogenase stability improved up to 1.4 times at 45°C compared to the immobilized non-irradiated cells (Ben Akachaa et al., 2007).

This study attempted to screen ***S. cerevisiae*** isolates for high tolerance of ethanol concentration and high temperature ranges.

MATERIALS AND METHODS

S. cerevisiae strains

One of the original types of ***S. cerevisiae***, PTCC^{5269} (Persian type culture collection) was obtained from the Persian Type Culture Collection of Yeast Cultures which is used in the alcohol industry building, Tehran, Iran (Type NO. 1). Another strain, TTCC^{1323} (Turkish type culture collection) was kindly provided by Turkish Type Culture Collection, Ankara, Turkey (Type NO. 2) and third strain was isolated from commercial indigenous wine yeast strains in Areni, Armenia (Type NO. 3). All these were used in the study.

Media

The yeast strains were kept in solution of 10% molasses, 1 g urea, 0.3 g magnesium sulphate, 0.3 g ammonium phosphate and 0.3 g potassium sulphate, all resolved in 1 L of distilled water, with pH solution set at 5.6. Then the medium was autoclaved at 121°C for 15 min (El Sheikh and Berry, 1980). When required, the yeast culture was activated by transferring to yeast extract peptone agar (YEPA) medium as a complete medium plates (Santangelo, 2006). Inoculum was prepared by putting 0.1 ml of yeast with sampler and added to 100 ml of the above medium. After 24 h incubation at 35°C the culture contained approximately 7.2×10^2 cells per ml determined by the method of Camacho-Ruiz et al. (2003). The cells were grown before irradiation up to the stationary phase of growth on a solid growth medium.

Radiation

S. cerevisiae culture was radiated with ^{60}Co as a source of Gamma ray at different doses (0.1, 1, 2, 3, 4, 5 and 10 KGy/h) such that about minimum 10% of the cells defunct (Abosereh et al., 2006). The dried sample of ***S. cerevisiae*** (10 g) was milled with the blender for 30 s to yield a powder about 50%. Cultures were irradiated in air at ambient temperature and atmosphere pressure. ***S. cerevisiae*** cells were grown in YEPA broth medium with shaking at 15°C. For irradiation, the dried samples were packed in polyethylene bags and irradiated (Justin et al., 2001). Mutagenesis of yeast strain was carried out at the Centre of Biotechnology Research, Tabriz, Iran. Cultures were irradiated at ambient temperature and atmosphere

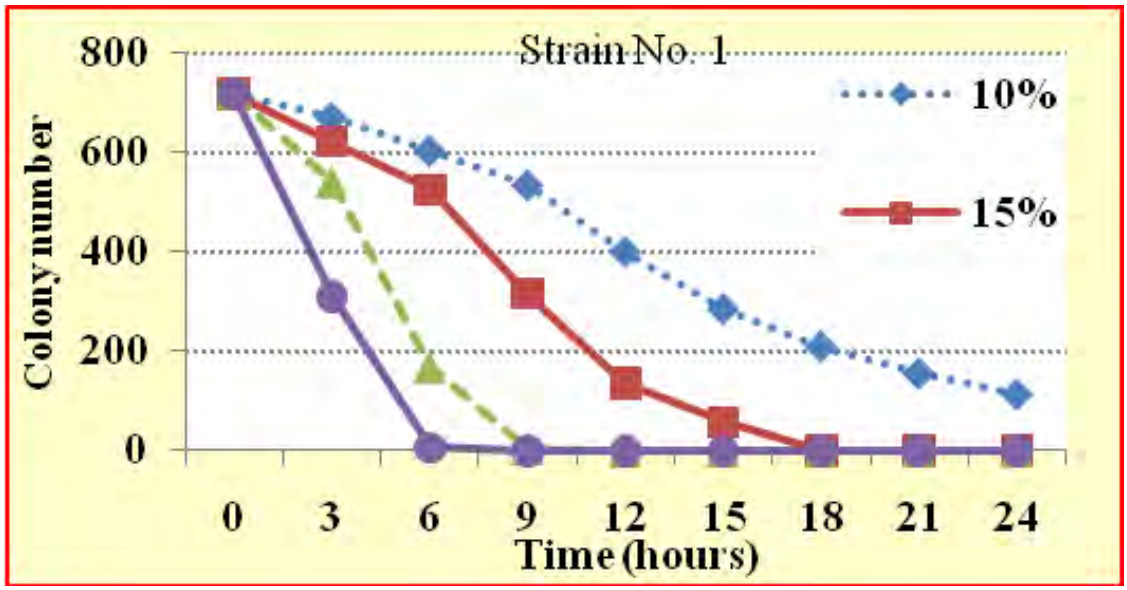

Figure 1. Grown chart of strain No.1 in different concentrations of ethanol.

pressure by shaking with gentle agitation.

Mutant cells selection

Serial dilution was prepared from each strain and 1 ml of solution containing 7.2 × 10^2 cells of yeast suspension was added to medium (10% molasses and 2 g/L; urea, 1.00 g; $MgSO_4$, 0.3 g; NH_4PO_4, 0.3 ml; K_2SO_4 sterilized at 121ºC for 15 min) with different concentrations of ethanol- 15, 20, 25 and 30% (Sherman et al., 1986). Test tubes containing mentioned medium were incubated at different temperatures (35, 38 and 42ºC). Survival cell density was evaluated by plating 0.1 ml of each treatment onto the surface of malt agar medium once every 3 to 72 h. Plates were then incubated for 48 h at 35°C and colonies were counted. Survival yeast percentages were estimated for each treatment.

Survival yeast colonies in medium containing 20% alcohol incubated at 38°C were transferred to medium containing 25% alcohol and incubated at 38 and 42°C for 48 h. Survival colonies in 25% alcohol incubated at 38°C were cultured at complete medium for 48 h. Then 0.1 ml of this isolates inoculated to 30% alcohol in medium mentioned above was incubated at 38 and 42°C for 48 h.

Colonies from irradiated strains which had growth at 42°C (from each treatment of three tested strains) were isolated in a maintenance medium, and evaluated by measuring the CO_2 displacement in a growth medium in a standard tube saccharimeter at 42°C for 24 h.

Irradiated solution of yeast with the gamma-ray was transferred in medium (CM) and yeast survival was compared in different dose of gamma rays with three different initial populations. Also their tolerance to temperatures and different concentrations of alcohol was investigated.

Few colonies grown in 17% alcohol concentration on 40°C were incubated after mixing for 48 h and then their tolerance to alcohol concentrations sundries (15, 17, 20, 25 and 30%) and temperature (35, 40, 45, 50 and 55°C) was investigated. Then the colonies grown in 20% alcohol concentration and temperature of 45 degrees, and also grown in CM for 48 h were assessed again at high concentrations of alcohol and high temperatures. It is done in order to get a particular strain of yeast that has a maximum temperature tolerance and alcohol.

RESULTS AND DISCUSSION

Evaluation of ethanol production

A sample of each strain (0.1 mol) with approximately 7.2×104 cells per ml was placed in test tubes containing different ethanol concentrations (10, 15, 17.5 and 20%) and maintenance medium for incubating for 24 h (Camacho-Riz et al., 2003). Once in every two hours the samples were cultured in Petri dishes and incubated at 35°C for 48 h. Number of survival colony was significant between strains. Renaults showed that:

1. In 10% ethanol concentration, all the strains had growth but survival number for strain No.3 was more than the other two strains. Cell density decrease in strain No. 2 was more than the others. Statistically, no significant differences were observed in number of colonies between three strains (Figures 1, 2 and 3).
2. Growth in 15% ethanol concentration showed that strains No. 1 after 6 h was decreased and after 18 ho no colonies were formed. In strain No. 2 reducing of population was faster than the others, and after 15 h the population vanished. In strain No. 3 after 24 h only a few colonies were formed; therefore it showed that this strain had more ethanol tolerance than the others.
3. In 17.5% ethanol, decreasing of population density in all three strains was faster. Strains No. 1 and 2 after 9 h and strains No. 3 after 12 h of growth and biomass production ceased.
4. Growth in 20% ethanol and evaluation of survival cell

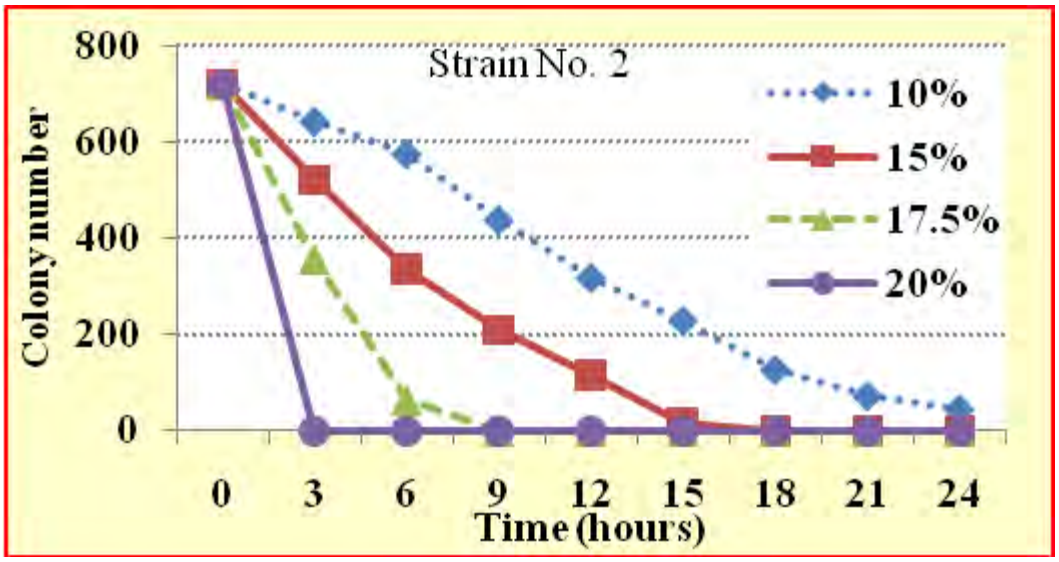

Figure 2. Grown chart of strain No.2 in different concentrations of ethanol.

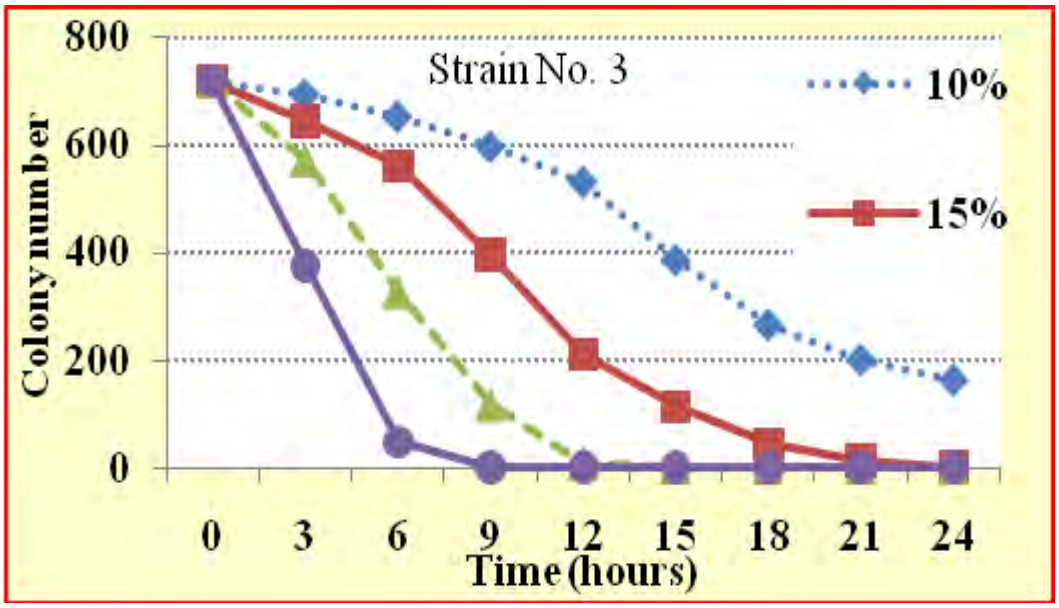

Figure 3. Grown chart of strain No.3 in different concentrations of ethanol.

showed that yeast No. 1 and 3 after six and yeast No. 2 after three hours had no growth.

Evaluation of ethanol production at different temperature conditions

All yeast strains exhibit growth and ethanol production at 25 and 42°C. As shown in Figure 4, yeasts No.1 and 3 had the highest activity with 110.1 and 98.6 g/L respectively at 35°C. But yeast No. 2 had the lowest activity and produced only 85.3 g/L at 30°C.

The yeasts (No. 1 and 3) which had growth at 35°C in the maintenance medium were incubated with shaker at 50°C for 6 h (Cakar et al., 2004) and then the growth medium and ethanol production were evaluated at 35, 38, 42 and 45°C. This thermal shock was repeated several times with different samples so it helped to reach the yeast with higher temperature tolerance. After thermal shock, tolerance range in yeasts No.1 and 3 increased to 42 and 38°C respectively (Figure 4).

The highest increase in ethanol production was

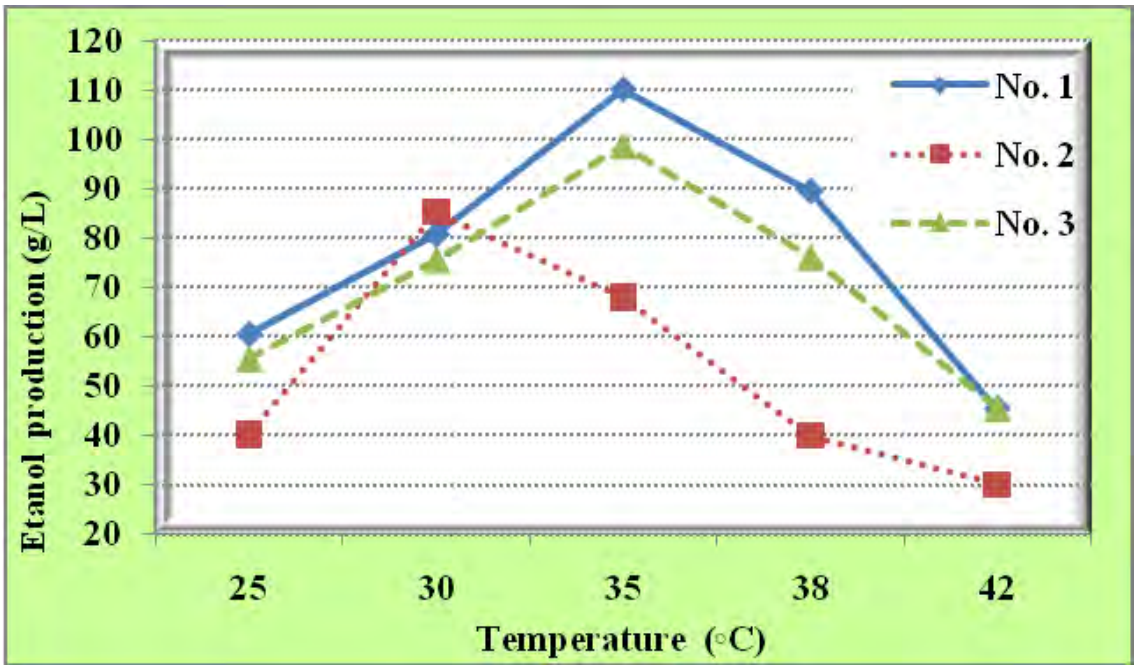

Figure 4. Ethanol production potential for three strains in different temperatures.

obtained by yeast No. 1 TS strain (Been exposed to thermal shock). After thermal shock treatment maximum 110.1 g/L ethanol production was observed at 35°C; the same amount at 38°C within 56 h produced by the same yeast. The yeast No. 3 TH ethanol production was 98.6 g/L during 60 h incubation at 38°C. Considering the range of tolerance to ethanol in strains No. 1 and 3 these two yeast strains can be selected for more study.

Effect of gamma ray irradiation on yeast

Solution of selected yeast strains was irradiated by different doses of gamma (γ) ray. Gamma irradiation, as a physical method, is known to cause injury to microorganisms and has been used widely for creating mutagenesis (Abosereh et al., 2006). Mutation as a result of gamma radiation was achieved by doses of 0.1, 1, 2, 3, 4, 5 and 10 KGy. The results showed that the gamma radiation higher than 1 KGy caused the death of a large number of yeasts in all yeast strains (Table 1). There was no growth at 10 KGy radiation dose. This result confirmed the work of Wang et al. (2001) on the characterization of ***S. cerevisiae*** mutant.

Evaluating the effect of gamma radiation on survival yeast enzyme activity, it was observed that the enzyme activity of ***S. boulardii*** was increased to 30-35% by radiation with low doses (0.1, 1 and 2 KGy) (Abosereh et al., 2006).

240 survived yeasts were obtained after γ irradiation treatments. Strains No. 1 and 2 were evaluated in terms of survival ability in 25 and 30% ethanol concentration and ethanol production capability in temperature of 38 and 42°C.

Strains tolerance to high concentrations of ethanol (25 and 30%) was incubated with shaker in sugar beet molasses of 20% (Cazetta et al., 2007) for 72 h at 38 and 42°C. Once in every three hours, 0.1 ml of each treatment was transferred to plates containing malt agar medium and colony formation represented survival (Reagan et al., 1995).

There was significant differences ($p<0.01$) between irradiated yeasts with the initial strains for traits of ethanol production yield and abilities to survive in high ethanol concentration at high temperatures. By Duncan mean comparison method two isolated strains were selected for maximum ethanol production in the high temperatures (Table 2). The isolated strains had two time efficiency of ethanol production more than the strain used in the alcohol industry. Also, they had the ability to survive and grow in 25-30% ethanol concentration.

Means comparison of ethanol production of the thermo-tolerant and ethanol tolerant yeasts with untreated strains of ***S. cerevisiae*** PTCC ^{5269} (No. 1) and ***S. cerevisiae*** Areni (No. 2) are shown in Table 2. Yeast fermentation medium contains molasses of 20% (w/v); yeast extract of 0.5% (w/v), pH 5.5 and starts with a 10% inoculum on a shaking incubator at 38 and 42°C with 80 rpm for 72 h average value of four replicates.

Of the 12 isolated yeast evaluated in the experiment at 38 and 42°C, only PTCC^{5269} M_3 and Areni M_7 had growth and produced highest bio-ethanol. Ethanol yield obtained 23.50% (v/v) and 22.60% (v/v) at 72 h. These two isolated strains had significant difference ($p<0.01$) with

Table 1. Effect of different doses of Gamma irradiation on survival of yeast strains.

Dose of γ ray (KGY)	Survival colonies					
	No. 1		No. 2		No. 3	
	N/ml	%	N/ml	%	N/ml	%
0	720	100	720	100	720	100
0.1	719	99.9	720	100	712	98.9
1	685	95.1	546	75.8	653	90.7
2	556	77.2	371	51.5	387	53.8
3	349	48.5	223	31.0	165	22.9
4	190	26.4	98	13.6	59	8.2
5	47	6.5	13	1.8	3	0.4
10	0	0.0	0	0.0	0	0.0

Table 2. Mean comparison of ethanol production for isolated tolerant yeast with initial strain of ***S. cerevisiae*** (p<0.01).

Isolated yeast	Ethanol % (v/v)	Ethanol % (v/v)
	38℃	42℃
PTCC^{5269} M_1	18.50 E	18.10 B
PTCC^{5269} M_2	21.97 B	19.12 B
PTCC^{5269} M_3	23.90 A	23.50 A
Areni M_1	20.30 CD	19.50 B
Areni M_2	21.37 BC	19.60 B
Areni M_3	20.10 CD	19.20 B
Areni M_4	21.20 BC	19.50 B
Areni M_5	20.30 CD	17.80 B
Areni M_6	18.80 E	17.10 B
Areni M_7	23.80 A	22.60 A
Areni M_8	20.30 CD	19.20 B
Areni M_9	19.37 DE	17.10 B
PTCC^{5269} (No. 1) Check	14.85 F	11.54 C
Areni (No. 3) Check	16.61 G	10.55 C

other treated and untreated strains of ***S. cerevisiae*** PTCC ^{5269} (No. 1) and Areni (No. 2). There was no growth and fermentation at 45°C by treated and untreated yeasts. Some of them were capable of growing and producing ethanol in stress conditions. The differences could be attributed to genotypic qualities of each microorganism. Similar effects were reported previously by Abdel-Fattah et al. (2000).

Edgardo et al. (2008) reported that thermo-tolerant strain of organosolv-pretreated ***P. radiata*** could produce 22 g/L ethanol (73% of the theoretical ethanol yield).

While the untreated strain can produce only 3.5 gethanol/L (12% of the theoretical ethanol yield).

The stability of the acquired stress resistance phenotype was also tested for all mutant clones by analyzing their relative level of stress resistance after five batch growth cycles (about 10 generations). All clones exhibited unaltered stress resistance (data not shown).

Conclusion

The gamma irradiation treatments made it possible to find two strains, PTCC^{5269} M_3 and Areni M_7, with high ethanol production yield at high temperature statement than the control strains. Employing these two gamma ray and temperature treated strains can cause increased ethanol concentration up to 25%. Thus fermentor evacuation and loading time can reduce three times and residual sugar well remain at minimum amount at any time of fermentation. In current study, the stress resistances of various individual clones selected from final mutant populations were determined. The results generally revealed heterogeneous populations. To understand the genetic background of these highly double-stress resistant mutant clones, which is the second step in inverse metabolic engineering (Bailey et al., 1996), their transcriptome and/or proteomic analysis would be necessary.

Achievement of thermal and high ethanol tolerant strains led greater ethanol yield in fermentation process than the control strains. By using these strains it is possible to convert pre-treated sugar material into ethanol at industrial scale more efficiently.

Kiransree et al. (2000) produced with molasses containing 14% sugar, maximum 53.2 and 45 (g/l) at 30 and 40°C respectively by thermo-tolerant strain. That strain showed 12% W/V ethanol tolerance. Isolated strain was also characterised for its ethanol producing ability using various starchy substrates in solid state and submerged fermentation.

The isolated strains ($PTCC^{5269}$ M_3 and Areni M_7) are now ready for being commercialised as a new fermentative strain for bio-ethanol production. So these strains have been deposited at the Department of Biotechnology, Urmia University, Urmia, Iran, and can be released for research and commercial purpose upon request. These cultures have economical importance for use in alcohol production during hot seasons.

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Full Length Research Paper

Genetic characterization of food-and-mouth disease virus WFL strain

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The complete genome of the foot-and-mouth disease virus (FMDV) strain WFL was cloned and sequenced. The results showed that the complete genome was 8155 nucleotides (nt) in length (including the poly(C) tract, but excluding poly(A) tail) and was composed of a 1059-nt 5'-untranslated region (UTR), a 6969-nt open reading frame, and a 127-nt 3'-UTR. *cre* region of 5'UTR was 55nt with 45.5% of G/C, and had a stem-loop. The stem-loops region of 3'UTR can fold into two stem-loops, SL1 and SL2. A phylogenetic tree was constructed based on complete amino acids sequences of WFL strain and reference strains. The strains were divided into 4 clusters. O/ES/2001, HKN/2002, LZ and WFL strain can be divided into one group. It was obvious that WFL strain had a close relationship to LZ strain, which indicated that the WFL strain was of serotype O. There were 16 different deduced amino acid residues between the WFL strain and the LZ strain.

Key words: Food-and-mouth disease virus, sequence, complete genome, untranslated region (UTR).

INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious and economically devastating disease of cloven-hoofed livestock, characterized by the appearance of vesicles on the feet and mouth (Marvin and Barry, 2004; Salguero et al., 2005; Sobrino et al., 2001).

The foot-and-mouth disease virus (FMDV) is a member of the family ***Picornaviridae***, genus ***Aphthovirus***. Seven serotypes (A, O, C, Asia 1, and South African Territories 1, 2, and 3) have been identifi d serologically based on their geographic origin (topotypes), e.g., the serotype O can be grouped into eight topotypes [Cathay, Middle East-South Asia (ME-SA), South-East Asia (SEA), Europe-South America (Euro-SA), Indonesia-1 (ISA-1), Indonesia-2 (ISA-2), East Africa (EA), and West Africa (WA)] based on nucleotide differences of up to 15% (Feng et al., 2004), and multiple subtypes occur within each serotype (Marvin and Barry, 2004). Viral infection or immunization with one serotype does not confer protection against the other serotypes (Grubman and Baxt, 2004).FMDV consists of a single-stranded, plus-sense RNA genome of approximately 8,500 bases surrounded by four structural proteins that form an icosahedral capsid. The genome contains 5' UTR (untranslated region), 3'UTR and a long open reading frame (ORF). The ORF can be translated into a single polyprotein, that can be cleaved into four structural proteins (VP4, VP2, VP3 and VP1), and 10 non-structural proteins (L, 2A, 2B, 2C, 3A, 3B1, 3B2, 3B3, 3C, and 3D) (Feng et al., 2004).

The main goal of the present study was to obtain the entire genome sequence of food-and-mouth disease virus WFL strain, including the 3′- and 5′-terminal non-coding regions of the genome.

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#Linzhu Ren and Lin Wang contributed equally to this work.

Table 1. Primers used for the amplification of the WFL strain.

Designation	Sequence of primers	Length	Location
A	5' TTGAAAGGGGGCGTTAGGGTCTC3'	46	1-19
B	5' TTGGCGCGCCACTAGTTTACCTCAGGGTACCT3'	27	921-941
C	5'TGCCCTTTAGGTACCCTGA3'	19	931-950
F	5' TGCGCGGCCGCATGCATGACAGGCGGCTC3'	26	4119-4139
G	5' TGCGAATTCTGTCATGCATGGCCGCTGT 3'	25	4124-4144
H	5' TTGCGGCCGCACTAGTCATATGTT3'	22	8140-8155
I_{RT}	5'TTGCGGCCGCACTAGTCATATGTTTTTTTTTTTTTTT3	34	

Table 2. Information on the foot-and-mouth disease virus referenced in this study.

GenBank ID	serotype	isolate	Genome size(bp)	5' NCR	3' NCR	Amino Acids
EF149009	Asia 1	Asia1/Jiangsu/China/2005	8189	1-1092	8083-8183	2329
EF149010	Asia 1	Asia 1/HNK/CHA/05	8187	1-1090	8080-8187	2329
FJ906802	Asia 1	Asia1/WHN/CHA/06	8239	1-1090	8080-8239	2329
DQ533483	Asia 1	ZB/CHA/58(att)	8193	1-1093	8084-8193	2329
AY390432	Asia1	YNBS/58	8163	1..1060	8051..8163	2329
AY686687	O	O/ES/2001	8163	1-1084	8054-8163	2322
AF511039	O	Akesu/58	8147	1..1039	8039..8147	2332
HQ009509	O	China/5/99(Fujian)	8231	1..1101	8101..8231	2332
DQ478937	O	OGBF15 derivative	8166	1-1058	8058-8166	2332
DQ248888	O	LZ	8104	1..1041	8011..8104	2322
AJ539138	O	Tibet/CHA/99	8183	1..1091	8088..8183	2332
AY317098	O	HKN/2002	8104	1..1042	8012..8104	2322
AF506822	O	China/1/99(Tibet)	8173	1..1081	8081..8173	2332
AY359854	O	OMIII	8083	1..1008	7963..8083	2317
AY333431	O	O/NY00	7731	1..712	7712..7731	2332
EF175732	O	WFL	8155	1-1059	8029-8155	2322

MATERIALS AND METHODS

Viral isolates, RT-PCR, and sequencing

Foot-and-mouth disease virus WFL strain was isolated from swine host in 1999 in Yunnan province, China, and adapted to BHK-21 cells. Total RNA was extracted using the RNeasy Mini kits (QIAGEN) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed into cDNA using the primer I_{RT} and ***SuperScript®*** III Reverse Transcriptase (***Invitrogen***).

The first-strand cDNA was then subjected to PCR amplification using primer pairs, A-B, C-F, and G-H (Table 1), to amplify 3 separate overlapping PCR products containing the complete genome of FMDV using ***LA Taq*** polymerase (Takara Biotechnology (Dalian) CO., LTD). The PCR products were purified and sequenced (Sangon Biological Engineering Technology and Service, Shanghai, China). The primers were designed based on the complete reference sequence obtained from GenBank.

Sequence analysis

The RNA structure was depicted according to the RNA-fold prediction program (Gruber et al., 2008). The reference sequences included in the analysis were obtained from GenBank (Table 2). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.1 (Tamura et al., 2007).

RESULTS AND DISCUSSION

Full-length genomic sequence of WFL

Here, we obtained the full-length genome of the WFL strain by RT-PCR. Using a total of 7 primers (Table 1), the complete genome sequence of the WFL strain was amplifi d as 3 separate overlapping PCR products. The result showed that the complete genome sequence of the WFL strain was 8155 nucleotides (nt) in length, including poly(C). The full-length sequence was submitted to GenBank (GenBank ID: EF175732).

The genomic organization of WFL was shown in Table 3. The complete sequence was divided into sixteen

Table 3. Architecture of the complete genome sequence of the WFL strain.

Genome segments	5'UTR			L	P1				P2			P3				3'UTR
	S	poly C	LF		VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D	
Nucleotide	371	17	671	603	255	660	654	639	48	462	954	429	213	639	1413	127
Amino acid				201	85	220	218	213	16	154	318	143	71	213	471	

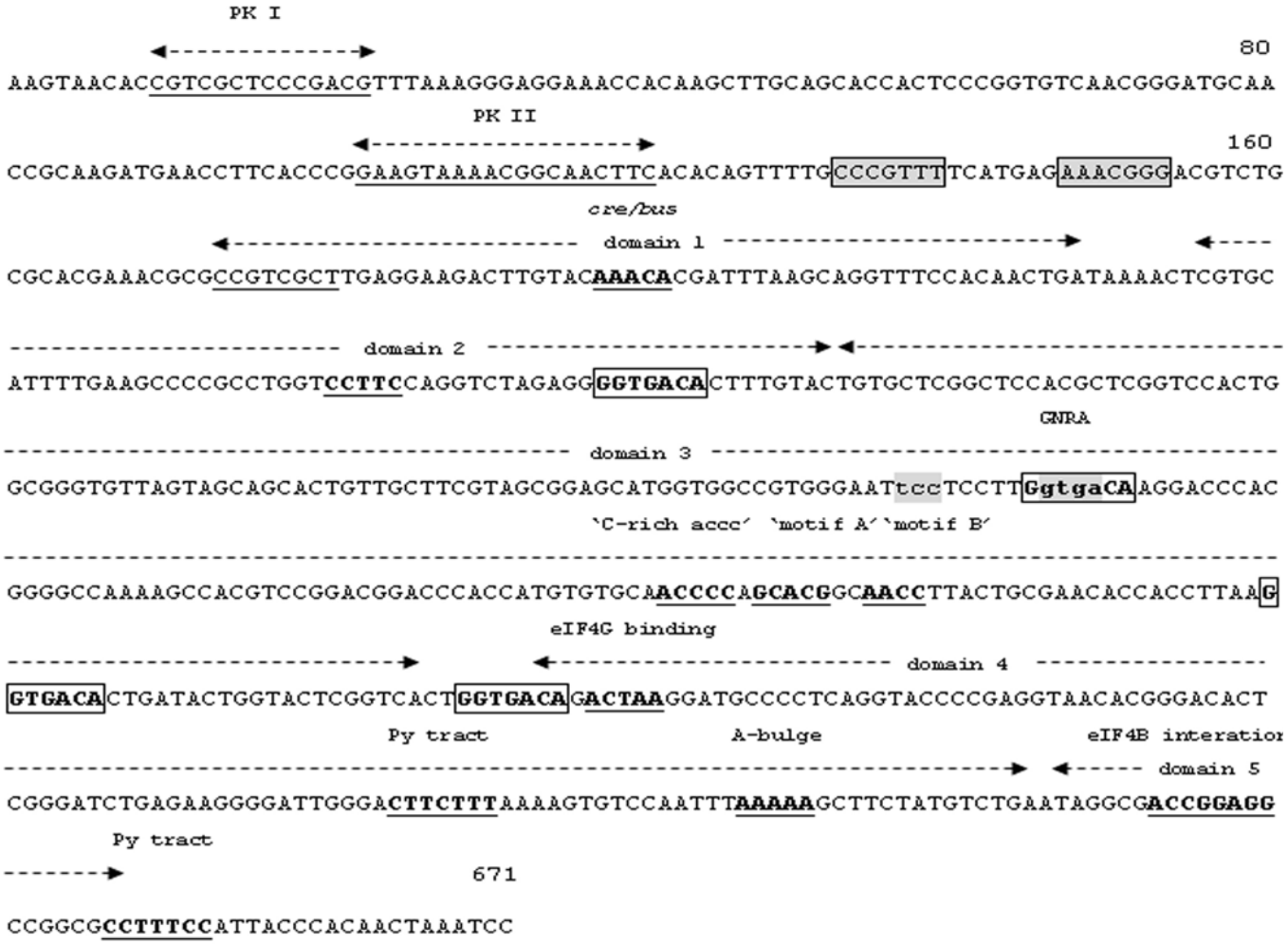

Figure 1. Architecture of the large fragment-5'UTR of FMDV transcriptional control region. Boundaries of different domains have been marked with dashed lines and arrows above the sequence. Conserved critical motifs were depicted in bold faced letter and underlined. Direct repeat motifs were depicted in bold faced letter and frame. Inverted repeats were depicted in frame and shadow. GNRA and $T_{377}CC$ were in low case and shadow eIF4C binding domain was GACTAA, and eIF4B interation domain was ACCGGAGG.

fragments except poly (A). 5' UTR (non-translated region) and 3'UTR were located in 1-1059 and 8029-8155, respectively (Table 3).

Characteristics of UTR

5'UTR played an important role in replication and selective translation of the viral RNA. The FMDV 5'UTR contains a short fragment called S-fragment, a poly (C) tract of variable length, followed by a large fragment (LF) of over 700 bases in length (LF-5' UTR) that can form a number of highly conserved secondary structures that include randomly repeated pseudoknots (PKs), a cis-acting replication element (***cre***) and an internal ribosome entry site (IRES) (Mohapatra et al., 2009; He et al., 2011). RNA helicase A (RHA) and $3C^{pro}$ specifically bind the FMDV S fragment. RHA interacts with the S fragment of the FMDV 5′ NTR (Lawrence and Rieder, 2009). The result showed (Figure 1) that the IRES element of the

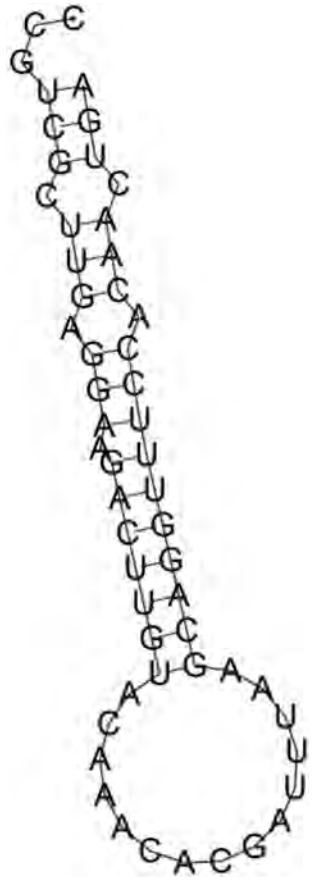

Figure2. Structure analysis of cre motif. The RNA structure was depicted according to the RNAfold prediction program (MFE plain structure).

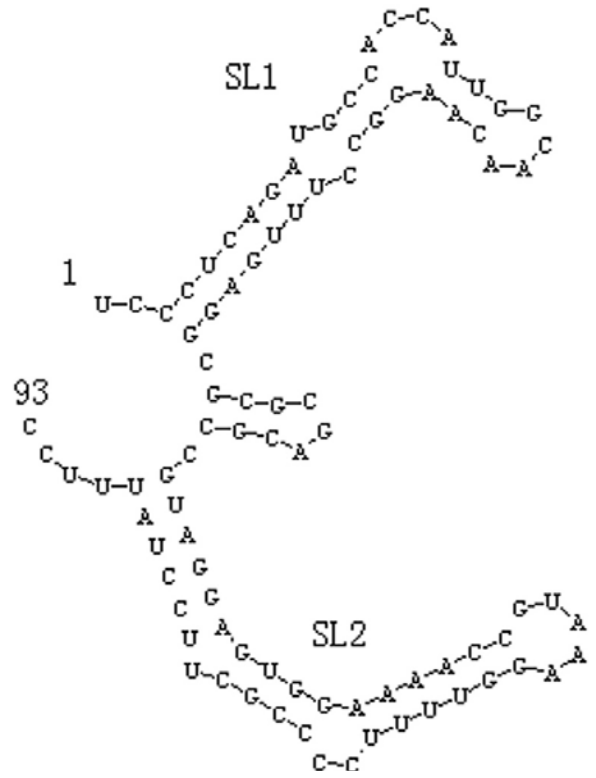

Figure 3. Secondary structure of the FMDV 3'UTR. The RNA structure was depicted according to the RNA-fold prediction program (MFE plain structure). SL1 and SL2 denoted conserved stem-loop structures.

WFL strain was about 458 nt in length and had five domains, which participated in the viral protein translation in a cap-independent manner. There were two PKs followed by an inverted repeats CCCGTTT/AAACGGG and ***cre*** (Figure 2).

The ***cre*** region was essential for RNA genome replication (Marvin and Barry, 2004). And a conserved 'AAACA' motif in the ***cre/bus*** region has been recently shown to be involved in VPg uridylylation (López et al., 2001; He et al., 2011). In this study, ***cre*** was 55nt with 45.5% of G/C, and had a stem-loop (Figure 2).

IRES including domain 2 to domain 5, and four direct repeat motifs, GGTGACA, were located in IRES region. It was reported that the conserved motifs and the structural domains in the IRES interact with an array of cellular factors involved in host translation initiation (Ramos et al., 1999; Pacheco et al., 2010) and some motifs were also crucial in maintenance of the tertiary structure of the IRES through RNA-RNA interaction (Fernández et al., 2006). Domain 4 followed by domain 5 in the IRES displayed highest degree of conservation (Mohapatra et al., 2009). The GNRA tetraloop was a thermostable tetraloop which can exist within a RNA structure solely on its own, or take place in an interaction with a receptor. The 'GNRA' tetraloop in domain 3, which plays critical role in determining the tertiary structural conformation of the IRES element (Mohapatra et al., 2009), was found to be 'GTGA' in the WFL strain. The cleavage site for RNase P within the 'GNRA' stem-loop was 'T_{377}CC' motif. In this study the conserved 'motif A', which interacts with 'GNRA' motif to maintain structural organization of the central domain of IRES (Nayak et al., 2006), was found to be 'G_{448}CACG' (Figure 1). The eIF4C binding domain was GACTAA, and the eIF4B interation domain was ACCGGAGG.

The 3' UTR, composed of two stem-loops and a poly(A) tract, was required for viral infectivity and stimulates IRES activity (Serrano et al., 2006). The 3' end established two distinct strand-specific, long-range RNA-RNA interactions, one with the S region and another with the IRES element (Serrano et al., 2006). The S region was recognized by each of the separate stem-loops. S-3'UTR interaction was dependent on a structural conformation induced by the presence of the poly(A) tract (Serrano et al., 2006). Here, it was found that 3' UTR of the WFL strain was 127nt, including 93nt stem-loops and poly (A). The 93nt stem-loops region can fold into two stem-loops, SL1 and SL2 (Figure 3).

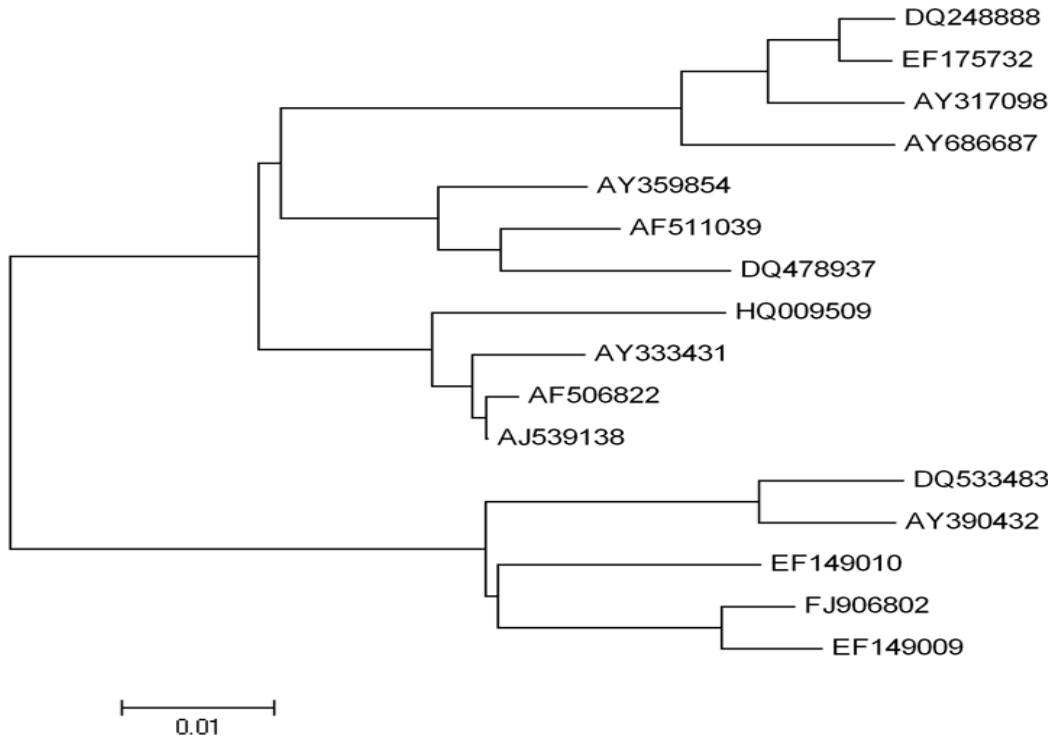

Figure 4. Phylogenetic tree constructed using the complete amino acids sequence of FMDV. A phylogenetic tree (Neighbor-Joining) was constructed using the software MEGA version 4.1 [15]. Four groups were categorized according to their evolutionary relationships. O/ES/2001, HKN/2002, LZ and WFL strain can be divided into one group.

Table 4. Different deduced amino acid residues between the WFL strain and the LZ strain.

Amino acid site	DQ248888	EF175732
37	K	R
57	R	Q
131	M	V
626	V	A
743	G	S
768	Q	K
1048	Q	E
1217	R	K
1405	E	K
1453	K	M
1533	V	A
1653	N	D
1667	A	V
2219	G	D
2258	A	T
2292	R	G

WFL belonged to serotype O

The open reading frame (ORF) of the WFL strain was 6969 nt, encoding 2322 amino acids (Table 2). A phylogenetic tree was constructed based on the deduced, complete amino acid sequences of the WFL strain and reference strains (Table 3) using MEGA 4.1 software. The four groups were categorized (Figure 4) according to their evolutionary relationships. The strains O/ES/2001, HKN/2002, LZ and WFL strain were grouped together. It was reported that both HKN/2002 and LZ were isolated from swine hosts in China, belonging to serotype O (Feng et al., 2004; Ma et al., 2006). And the strain O/ES/2001 was a recombinant of serotype O and Asia 1 (Wu et al., 2009). It was obvious that WFL strain had a close relationship to LZ strain (GenBank ID: DQ248888), indicating that the WFL strain belonged to serotype O. The close link between WFL and these three isolates was consistent with our previous finding using the VP1 sequence (data not shown).

Complete amino acids of the WFL strain and the LZ strain were compared. The results showed that there were 16 different deduced amino acid residues between WFL and LZ (Table 4). Detailed comparison of WFL with other strains is still doing.

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Full Length Research Paper

Nano theoretical studies of fMet-tRNA structure in protein synthesis of prokaryotes and its comparison with the structure of fAla-tRNA

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In this paper, we performed quantum mechanic calculations on structures of fMet-tRNA and fAla-tRNA. Comparing the results structure design was done by formalizing the amino acid and attaching in to the adenine nucleotide of tRNA. The performed calculations are Opt, SCRF, NBO and NMR.

Key words: Natural bond orbital (NBO), fMet-tRNA, NMR, Opt, SCRF, fAla-tRNA.

INTRODUCTION

It is generally accepted that initiation of protein synthesis in ***Escherichia coli*** starts with formyl-methionine, directed by the codons AUG or GUG. Protein synthesis proceeds by transfer of the growing polypeptide chain from the tRNA bound to the ribosomal P site to the incoming aminoacyl-tRNA in the adjacent A site. After translocation of the ribosome in the 30 direction of the mRNA, by the action of elongation factor G, the A site again becomes empty and the next codon exposed so that a new aminoacyl-tRNA ternary complex can be selected (Ogle and Ramakrishnan, 2005).

Synthetic polynucleotide containing AUG and/or GUG codons as well as natural mRNA have been used extensively in order to elucidate the mechanism of initiation of protein synthesis (Grunberg-Manago, 1977).

In all these studies it has been assumed that binding of fMet-tRNA to ribosome's is the polynucleotide in bacteria the start codon AUG is recognized by fMet-tRNA. This tRNA does not recognize internal AUG codons. Initiation of protein biosynthesis requires the correct positioning of charged initiator tRNA, fMet-tRNA in the ribosomal P-site of the mRNA-programmed 70S ribosome's (Gualerzi and Pon, 1990; La Teana et al., 1996; Monajjemi et al., 2008; Spurio et al., 1993; Forster et al., 1999).

The rapid development of molecular biology in recent years has been mirrored by the rapid development of computer hardware and software. This improvement leaded to the development of sophisticated computational techniques and a wide range of computer simulations involving such methods among the areas It is well observed that fMet-tRNA is the pharmacological targets of many of the drugs that are currently in clinical use or in advanced clinical trials. Therefore, the implication throughout this paper has been profound is the modeling of fMet-tRNA structure and function, the chemical behavior of fMet- tRNA within drug design and also understanding at a molecular level of the role of solvents in biotechnological applications (Agris et al., 1997; Monajjemi et al., 2010). We selected adenine of tRNA structure (first nucleotide in acceptor arm of tRNA structure) and then perform modeling of fMet-tRNA, during the process we formylized alanine and then attached it to the adenine of tRNA and then designed the structure of fAla-tRNA to compare it with fMet-tRNA, (Figure 1).

METHDOLOGY

At first, we have modeled the structure of fMet-tRNA and fAla-tRNA with Chem office package and then optimized at the B3LYP and HF levels of theory with 3-21G* basis set. After fully optimization of those structures , we have calculated NMR parameters and NBO analysis at the levels of HF/3-21G* and B3LYP/3-21G* theory and theoretically explored the solvent effects(GAS ,DMSO ,CHCL3,H2O) on structure of adenine + fMet and adenine + fAla and calculations of NMR parameters and NBO calculation have been Performed on a Pentium-4 based system using GAUSSIAN

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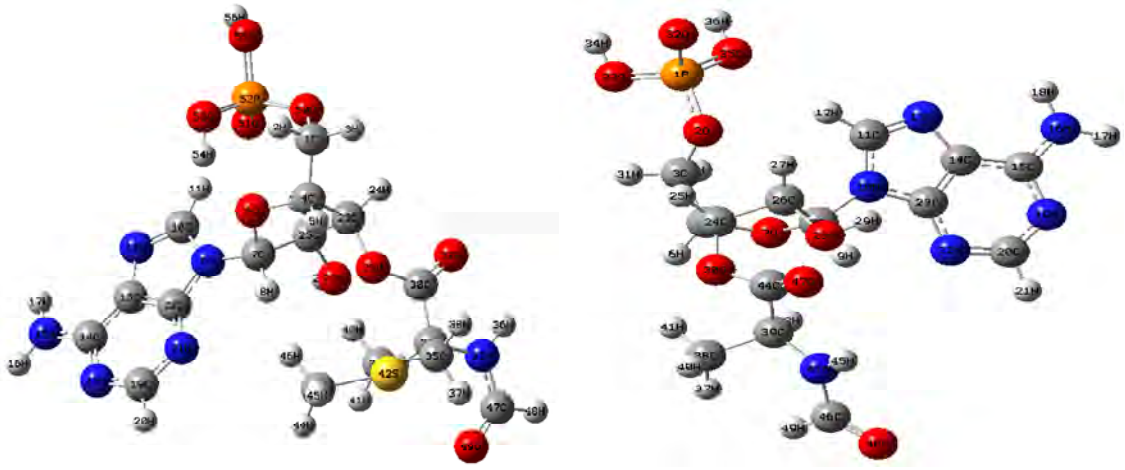

Figure 1. Structure of Adenine + fMet of fMet-tRNA and Adenine + fAla of fAla-tRNA

Table 1. Optimization energy for each method.

3-21G*	E(kcal/mol)			
	Gas	$CHCL_3$	DMSO	H2O
HF(fMet-tRNA)	-3738.1213366	-3738.1244432	-3738.1213031	-3738.1213011
B3LYP(fMet-tRNA)	-3755.0439622	-3755.0463986	-3755.0438037	-3755.0438020
HF(fAla-tRNA)	-1174948.9244	-1174951.5084	-1174952.5478	-1174952.6039
B3LYP(fAla-tRNA)	-1180837.1055	-1180839.6468	-1180840.7420	-1180840.8019

03 program (Gaussian et al., 1998).

RESULTS AND DISCUSSION

In this paper we performed quantum calculations on the structure of fMet-tRNA and fAla-tRNA and to perform the action, first we formilized (gave a formyl molecule to the structure) two amino acids methionine and alanin and then we attached them from tRNA to adenine nucleotide and then we performed the quantum calculations on the achieved structures (fMet-tRNA and fAla-tRNA).

In this paper HF and DFT/B3LYP methods with 3-21G* basis set were Employed for investigating the structure optimization and energy minimization of fMet-tRNA and fAla-tRNA (Figure 1) have been summarized in Table 1. The HF and DFT energies are of particular interest because they provide results for interactions appearing in solvent medium considered in this letter, which are in accord with biological behavior of fMet-tRNA and fAla-tRNA. Furthermore, recent papers often tend to ask about the role of water solvent effect on the stability of fMet-tRNA and fAla-tRNA structures. The detailed results of relative energy values for those structures in gas, DMSO, CHCL3 and water solvents optimized at the HF and B3LYP levels of theory with 3-21G* basis set are summarized in Table 1.

In the NBO analysis, in order to compute the span of the valence space, each valence bonding NBO (σ_{AB}), must in turn, be paired with a corresponding valence anti bonding NBO (σ^*_{AB}): Namely, the Lewis σ-type (donor) NBO are complemented by the non-Lewis σ^*-type (acceptor) NBO that are formally empty in an idealized Lewis structure picture. Readily, the general trans-formation to NBO leads to orbitals that are unoccupied in the formal Lewis structure. As a result, the filled NBO of the natural Lewis structure are well adapted to describe covalency effects in molecules. Since the non-covalent delocalization effects are associated with $\sigma \rightarrow \sigma^*$ interactions between filled (donor) and unfilled (acceptor) orbitals, it is natural to describe them as being of donor–acceptor, charge transfer, or generalized "Lewis base-Lewis acid" type. The anti bonds represent unused valence-shell capacity and spanning portions of the atomic valence space that are formally unsaturated by covalent bond formation. Weak occupancies of the valence anti bonds signal irreducible departures from an idealized localized Lewis picture, that is, true "delocalization effects". As a result, in the NBO analysis, the donor–acceptor (bond–anti bond) interactions are taken into consideration by examining all possible interactions between 'filled' (donor) Lewis-type NBO and 'empty' (acceptor) non-Lewis NBO and then estimating their energies by second-order perturbation theory.

These interactions (or energetic stabilizations) are referred to as 'delocalization' corrections to the zeroth-order natural Lewis structure. The most important interaction between "filled" (donor) Lewis-type NBO and "empty" (acceptor) non-Lewis is reported in Table 2, the level of HF/3-21G* and B3LYP/ 3-21G* basis set at the DFT theory. we observed interaction between Donor NBO, the LP(1,2) of O29, O32 and Acceptor NBO, the σ*(C30-O32), π*(C30 - O32), σ * (O29 - C30) of fMet-tRNA structure, and donor NBO, the LP(1,2) of O30, O47 and acceptor NBO, the σ * (C44-O47), π*(C44-O47), σ *(O30-C44) of fAla-tRNA structure and then we reported the energy and hybrid for C30-O29, C30-O32 and C30=O32 bonding of fMet-tRNA and O30-C44, C44-O47, and C44=O47 bonding of fAla-tRNA Table 3, by same level. The natural population analysis (NPA) was evaluated in terms of natural atomic orbital occupancies (Haser and Ahlrichs, 1989; Ahlriches et al., 1989). Table 4 show the molecular charge distribution on the O29, C30 and O32 atoms in structure of fMet-tRNA and O30, C44 and O47 atoms in structure of fAla-tRNA. These partial charges distribution on the atoms shows that the electrostatic repulsion or attraction between atoms can give a significant contribution to the intra- and intermolecular interaction.

Table 5 shows calculated natural orbital occupancy (number of electron, or ''natural population" of the orbital). It is noted that for σO29 - C30 of fMet-tRNA and σ O30-C44 of fAla-tRNA bond orbital, Decreased or increased occupancy of the localized σO29 - C30 of fMet-tRNA and σ O30-C44 of fAla-tRNA orbital in the idealized Lewis structure, and their subsequent impact on molecular stability and geometry (bond lengths) are also related with the resulting p character of the corresponding O29 natural hybrid orbital (NHO) of σO29 - C30 and O30 natural hybrid orbital (NHO) of σ O30-C44 bond orbital.

Nuclear magnetic Resonance (NMR) is based on the quantum mechanical property of nuclei (Benas et al., 2000). The chemical shielding refers to the phenomenon which associated with the secondary magnetic field created by the induced motions of the electrons that surrounding the nuclei when in the presence of an applied magnetic field. The energy of a magnetic moment μ , in a magnetic field, B, is as follow:

$$E = -\mu.(1- \quad)B \tag{1}$$

Where the shielding is the differential resonance shift due to the induced motion of the electrons (Magdalena and Sadlej, 1998). In general, the electron distribution around a nucleus in a molecule is more spherically symmetric. Therefore, the size of electron current around the field, and hence the size of the shielding, will depend on the orientation of the molecule within the applied field B0 (Melinda, 2003).

For chemical shielding (CS) tensor, which describes how the size of shielding varies with molecular orientation, we often use the following convention for the three principal components:

$$_{11} \leq \ _{22} \leq \ _{33} \tag{2}$$

The three values of the shielding tensor are frequently expressed as the isotropic value$(\ _{iso})$, the anisotropy$(\Delta \ )$, and the asymmetry $(\ )$ There quantities are defined as follows [Monajjemi et al., 2004]:

1. The isotropic value $_{iso}$:

$$_{iso} = \frac{1}{3}(\ _{11} + \ _{22} + \ _{33}) \tag{3}$$

2. The anisotropy shielding$(\Delta \ )$:

$$\Delta \ = \ _{33} - \frac{1}{2}(\ _{11} + \ _{22}) \tag{4}$$

3. The asymmetry parameter$(\ )$:

$$= \frac{|\ _{22} - \ _{11}|}{|\ _{33} - \ _{iso}|} \tag{5}$$

Instead of deriving $(\Delta \ _{ind})$from the difference of the PCM-optimized shielding and the PCM shielding of the molecule held at the geometry optimized in vacuum, it can be obtained from the shielding calculated in vacuum for a molecule that is geometry-optimized in solution [Monajjemi et al., 2007]. Thus,

$$\Delta \ _{ind} = \ _{Vac}(R_{sol}) - \ _{Vac}(R_{ref}) \tag{6}$$

Where $_{Vac}(R_{sol})$is the value of the nuclear shielding in vacuum but with the solute geometry optimized in solution $_{Vac}(R_{ref})$ are the corresponding parameters for calculation with reference solvent. In this case, we may suppose that optimization of solute molecule in solvent and then performing shielding calculations is similar to shielding calculations in the isolated system (Lynden and Rasaiah, 1997).

Self-Consistent Reaction Field (SCRF) method is based on a continuum model with uniform dielectric constant$(\ )$. The simplest SCRF model is the Onsager reaction field model. In this method, the solute occupies a fixed spherical cavity of radius a0 within the solvent field. A dipole in the molecule will induce a dipole in the medium, and the electric field applied by the solvent dipole will in turn interact with the molecular dipole leading to net stabilization.

The Gauge Including Atomic Orbital (GIAO) approach

Table 2. Second order perturbation theory analysis of NBOHF/3-21G* method.

Phase (fMet-tRNA)	Donor NBO (i)	Acceptor NBO (j)	E(2) (kcal/mol)	Phase (fAla-tRNA)	Donor NBO (i)	Acceptor NBO (j)	E(2) (kcal/mol)
Gas	LP (1) O29	σ* C30 - O32	9.57	Gas	LP (1) O30	σ* C44 – O47	9.83
	LP (2) O29	π* C30 - O32	60.32		LP (2) O30	π* C44 – O47	55.20
	LP (1) O32	σ* O29 - C30	0.58		LP (1) O47	σ* O30 – C44	0.78
	LP (2) O32	σ* O29 - C30	51.40		LP (2) O47	σ* O30 – C44	53.18
$CHCL_3$	LP (1) O29	σ* C30 - O32	9.62	$CHCL_3$	LP (1) O30	σ* C44 – O47	9.93
	LP (2) O29	π* C30 - O32	60.72		LP (2) O30	π* C44 – O47	56.26
	LP (1) O32	σ* O29 - C30	0.57		LP (1) O47	σ* O30 – C44	0.76
	LP (2) O32	σ* O29 - C30	51.15		LP (2) O47	σ* O30 – C44	52.58
DMSO	LP (1) O29	σ* C30 - O32	11.16	DMSO	LP (1) O30	σ* C44 – O47	10.00
	LP (2) O29	π* C30 - O32	70.17		LP (2) O30	π* C44 – O47	56.98
	LP (1) O32	σ* O29 - C30	1.64		LP (1) O47	σ* O30 – C44	0.74
	LP (2) O32	σ* O29 - C30	43.20		LP (2) O47	σ* O30 – C44	52.18
H2O	LP (1) O29	σ*C30 - O32	9.66	H2O	LP (1) O30	σ* C44 – O47	9.97
	LP (2) O29	π*C30 - O32	60.12		LP (2) O30	π* C44 – O47	56.66
	LP (1) O32	σ *O29 - C30	0.58		LP (1) O47	σ* O30 – C44	0.75
	LP (2) O32	σ *O29 - C30	51.56		LP (2) O47	σ* O30 – C44	52.35
B3LYP/3-21G* method							
Phase (fMet-tRNA)	**Donor NBO (i)**	**Acceptor NBO (j)**	**E(2) (kcal/mol)**	**Phase (fAla-tRNA)**	**Donor NBO (i)**	**Acceptor NBO (j)**	**E(2) (kcal/mol)**
Gas	LP (1) O29	σ* C30 - O32	7.25	Gas	LP (1) O30	σ* C44 –O47	7.06
	LP (2) O29	π* C30 - O32	55.35		LP (2) O30	π* C44 –O47	39.81
	LP (1) O32	σ* O29 - C30	0.72		LP (1) O47	σ* O30 –C44	0.75
	LP (2) O32	σ* O29 - C30	37.09		LP (2) O47	σ* O30 –C44	41.29
CHCL3	LP (1) O29	σ* C30 - O32	7.28	$CHCL_3$	LP (1) O30	σ* C44 –O47	7.15
	LP (2) O29	π* C30 - O32	55.66		LP (2) O30	π* C44 –O47	40.70
	LP (1) O32	σ* O29 - C 30	0.71		LP (1) O47	σ* O30 –C44	0.73
	LP (2) O32	σ* O29 - C 30	36.98		LP (2) O47	σ* O30 –C44	40.68
DMSO	LP (1) O29	σ* C30 - O 32	8.34	DMSO	LP (1) O30	σ* C44 –O47	7.17
	LP (2) O29	π* C30 - O32	50.45		LP (2) O30	π* C44 –O47	40.86
	LP (1) O32	σ* O29 - C30	1.55		LP (1) O47	σ* O30 –C44	0.73
	LP (2) O32	σ* O29 - C30	37.09		LP (2) O47	σ* O30 –C44	40.57
H2O	LP (1) O29	σ* C30 - O 32	7.25	H2O	LP (1) O30	σ* C44 –O47	7.15
	LP (2) O29	π* C30 - O32	55.35		LP (2) O30	π* C44 –O47	40.67
	LP (1) O32	σ* O29 - C 30	0.72		LP (1) O47	σ* O30 –C44	0.73
	LP (2) O32	σ* O29 - C 30	37.09		LP (2) O47	σ* O30 –C44	40.70

was used. The ab initio GIAO calculations of NMR chemical shielding tensors were performed using the DFT and HF method. The chemical shielding tensors were calculated with the GAUSSIAN 03 program. The isotropic chemical shielding $(_{iso})$, asymmetry parameter $( )$and anisotropy shielding (Δ) for O(29),O(32), C(30) atoms of fMet-tRNA ,and O(30),C(44) and O(47) atoms in structure of fAla-tRNA (Figure 1) have been summarized in Table 6 and O(29),O(32)and C(30) atoms are the connections of adenine of fMet and also O(30), C(44) and O(47) atoms are the connections of adenine to fAla , these atoms are so important to us.

Conclusion

In this work, we have summarized:

1. Optimization at the HF and DFT levels of theory

Table3. Calculated natural hybrid orbitals (NHOs) and the polarization coefficient for each hybrid in the corresponding NBO (parentheses) for the selected fMet-tRNA (1) and fAla-tRNA (2) using the selected methods.

Phase	Bond Hybrids (1)	C_{30}-O_{29}	C_{30} O_{29}	C_{30}-O_{32}	$C_{30}O_{32}$	C_{30}=O_{32}	C_{30} O_{32}
Gas	HF/3-21G* B3LYP/3-21G*	$sp^{2.66}$(0.5472) $sp^{2.64}$(0.5602)	$sp^{1.93}$(0.8370) $sp^{2.19}$(0.8283)	$sp^{2.07}$(0.5732) $sp^{2.12}$(0.5811)	$sp^{1.26}$(0.8194) $sp^{1.56}$(0.8138)	$sp^{1.00}$(0.5433) $sp^{99.99}$ (0.5660)	$sp^{1.00}$(0.8396) $sp^{99.99}$ (0.8244)
$CHCL_3$	HF/3-21G* B3LYP/ 3-21G*	$sp^{2.64}$(0.5484) $sp^{2.63}$(0.5610)	$sp^{1.94}$(0.8362) $sp^{2.20}$(0.8278)	$sp^{2.07}$(0.5735) $sp^{2.12}$(0.5815)	$sp^{1.26}$(0.8192) $sp^{1.57}$(0.8136)	$sp^{1.00}$(0.5448) $sp^{99.99}$ (0.5673)	$sp^{1.00}$(0.8385) $sp^{99.99}$ (0.8235)
DMSO	HF/3-21G* B3LYP/3-21G*	$sp^{2.66}$(0.5472) $sp^{2.66}$(0.5472)	$sp^{1.93}$(0.8370) $sp^{1.93}$(0.8370)	$sp^{2.07}$(0.5735) $sp^{2.07}$(0.5732)	$sp^{1.26}$(0.8192) $sp^{1.26}$(0.8194)	$sp^{1.00}$(0.5433) $sp^{99.99}$ (0.5660)	$sp^{1.00}$(0.8396) $sp^{99.99}$ (0.8244)
H2O	HF/3-21G* B3LYP/3-21G*	$sp^{2.66}$(0.5472) $sp^{2.64}$(0.5602)	$sp^{1.93}$(0.8370) $sp^{2.19}$(0.8283)	$sp^{2.08}$(0.5729) $sp^{2.12}$(0.5811)	$sp^{1.26}$(0.8196) $sp^{1.56}$(0.8138)	$sp^{1.00}$(0.5439) $sp^{99.99}$ (0.5660)	$sp^{1.00}$(0.8392) $sp^{99.99}$(0.8394)
Phase	**Bond Hybrids(2)**	**O30- C44**	**O30 C44**	**C44- O47**	**C44 O47**	**C44=O47**	**C44 O47**
Gas	HF/3-21G* B3LYP/3-21G*	$sp^{1.99}$ (0.8363) $sp^{2.40}$ (0.8277)	$sp^{2.67}$ (0.5482) $sp^{2.70}$ (0.5611)	$sp^{2.04}$(0.5730) $sp^{2.05}$ (0.5794)	$sp^{1.24}$(0.8196) $sp^{1.46}$ (0.8151)	$sp^{1.00}$(0.5506) $sp^{99.99}$ (0.5821)	$sp^{1.00}$(0.8348) $sp^{99.99}$ (0.8131)
$CHCL_3$	HF/3-21G* B3LYP/ 3-21G*	$sp^{2.02}$ (0.8351) $sp^{2.44}$ (0.8262)	$sp^{2.64}$ (0.5501) $sp^{2.67}$ (0.5634)	$sp^{2.04}$(0.5728) $sp^{2.05}$ (0.5792)	$sp^{1.24}$(0.8197) $sp^{1.45}$ (0.8152)	$sp^{1.00}$(0.5489) $sp^{99.99}$(0.5806)	$sp^{1.00}$(0.8359) $sp^{99.99}$ (0.8142)
DMSO	HF/3-21G* B3LYP/3-21G*	$sp^{2.03}$ (0.8343) $sp^{2.45}$ (0.8259)	$sp^{2.63}$ (0.5514) $sp^{2.66}$ (0.5638)	$sp^{2.04}$ (0.5726) $sp^{2.05}$ (0.5791)	$sp^{1.23}$ (0.8198) $sp^{1.45}$ (0.8152)	$sp^{1.00}$(0.5478) $sp^{99.99}$ (0.5804)	$sp^{1.00}$(0.8366) $sp^{99.99}$ (0.8144)
H2O	HF/3-21G* B3LYP/3-21G*	$sp^{2.03}$ (0.8346) $sp^{2.44}$ (0.8263)	$sp^{2.63}$ (0.5508) $sp^{2.67}$ (0.5633)	$sp^{2.04}$ (0.5727) $sp^{2.05}$ (0.5792)	$sp^{1.24}$ (0.8198) $sp^{1.45}$ (0.8152)	$sp^{1.00}$(0.5483) $sp^{99.99}$ (0.5807)	$sp^{1.00}$(0.8363) $sp^{99.99}$(0.8141)

provides a suitable computational model in terms of calculated NMR parameters and relative energies.

2. There was an increase in the relative stability of the interested structures through the improvement of basis set and including electron correlations , Hence, the most stable structures are perceived in the CHCL3 solution at the B3LYP/3-21G* level of theory.

3. We observed an increase in values of NMR chemical shielding around O29, O32 By increasing lone pair electrons contribution of oxygen (O29, O32) atoms in resonance Interactions, Hence, O29 atom has the highest chemical shielding among the oxygen atoms (fMet-tRNA).

4. We observed a decrease in the bond lengths of the O29-C30 of the structure by the increase of solvent dielectric constant (fMet-tRNA).

5. We observed an increase in the relative stability by increasing the LP Os (O29, O32) electrons contribution in the enhancement of π electron clouds (fMet-tRNA).

6. In many lab experiments it is proven that the real structure to start protein synthesis in prokaryotes is fMet-tRNA and we studied calculated and worked out the stability and the

Table 4. Atomic charge distribution described in terms of natural population analysis (NPA) for the compounds studied.

fMet-tRNA		Gas	$CHCL_3$	DMSO	H2O
O29	HF/3-21G*	-0.63608	-0.63768	-0.63608	-0.71482
	B3LYP/3-21G*	-0.49088	-0.49162	-0.49088	-0.5048
C30	HF/3-21G*	0.94389	0.94185	0.94389	0.981579
	B3LYP/3-21G*	0.75659	0.75427	0.75659	0.732509
O32	HF/3-21G*	-0.6402	-0.63594	-0.64019	-0.62108
	B3LYP/3-21G*	-0.55198	-0.54844	-0.55197	-0.51221
fAla-tRNA		**GAS**	**CHCl3**	**DMSO**	**H2O**
O30	HF/3-21G*	-0.64032	-0.63862	-0.63746	-0.63797
	B3LYP/3-21G*	-0.51736	-0.51386	-0.5132	-0.514
C44	HF/3-21G*	0.94048	0.94202	0.94294	0.94255
	B3LYP/ 3-21G*	0.75557	0.75507	0.75491	0.7551
O47	HF/3-21G*	-0.62227	-0.62765	-0.63107	-0.6296
	B3LYP/3-21G*	-0.50732	-0.51388	-0.51502	-0.51364

Table 5. Occupancy and Energy (kcal/mol) for between O29 - C30 atoms of -tRNA and σ O30-C44 of fAla-tRNA.

Phase	Method	NBO	Occupancy	Energy(kcal/mol)
Gas	HF/3-21G*	σ O29 - C30	1.99398	-773.7261
	B3LYP/3-21G*	σ O29 - C30	1.99305	-605.5910
$CHCL_3$	HF/3-21G*	σ O29 - C30	1.99399	-774.9309
	B3LYP/3-21G*	σ O29 - C30	1.99308	-606.6076
DMSO	HF/3-21G*	σ O29 - C30	1.99397	-773.5423
	B3LYP/3-21G*	σ O29 - C30	1.99305	-605.5910
H2O	HF/3-21G*	σ O29 - C30	1.99395	-773.3433
	B3LYP/3-21G*	σ O29 - C30	1.99305	-605.5910
Phase	**Method**	**NBO**	**Occupancy**	**Energy (kcal/mol)**
Gas	HF/3-21G*	σ O30 –C44	1.99342	-760.8998
	B3LYP/3-21G*	σ O30 – C44	1.9909	-568.1538
CHCL3	HF/3-21G*	σ O30 – C44	1.99343	-764.6523
	B3LYP/3-21G*	σ O30 – C44	1.991	-574.0712
DMSO	HF/3-21G*	σ O30 – C44	1.99343	-767.2251
	B3LYP/3-21G*	σ O30 – C44	1.99102	-575.2008
H2O	HF/3-21G*	σ O30 – C44	1.99343	-766.1019
	B3LYP/3-21G*	σ O30 – C44	1.991	-573.8453

real reasons that why this structure is produced in protein synthesis in prokaryotes and fAla-tRNA is not produced there and presented the results and finding in the charts.

As O(29), O(32) and C(30) atoms are the connections of adenine of fMet and also O(30), C(44) and O(47) atoms are the connections of adenine to fAla, these atoms are

Table 6. NMR parameters (ppm) of O(29) and C(30) and O(32) atoms of fMet-tRNA and O(30), C(44) and O(47) atoms of fAla-tRNA structures in gas phase at the level of HF/3-21G* and B3LYP/3-21G* theory.

NMR parameters	**GAS**
HF/3-21G* O(29) $_{iso}$	221.0980
HF/3-21G* O(29) Δ	229.6773
HF/3-21G* O(29)	-1.7280
B3LYP/3-21G*O(29) $_{iso}$	137.0772
B3LYP/3-21G* O(29) Δ	148.2147
B3LYP/3-21G* O(29)	-0.7952
HF/3-21G* C(30) $_{iso}$	44.2825
HF/3-21G* C(30) Δ	94.7091
HF/3-21G* C(30)	14.5365
B3LYP/3-21G* C(30) $_{iso}$	36.1343
B3LYP/3-21G* C(30) Δ	57.9762
B3LYP/3-21G* C(30)	16.9674
HF/3-21G*O(32) $_{iso}$	-50.7765
HF/3-21G* O(32) Δ	-52.6285
HF/3-21G* O(32)	-1.6899
B3LYP/3-21G* O(32) $_{iso}$	-46.0160
B3LYP/3-21G* O(32) Δ	-200.8172
B3LYP/3-21G* O(32)	-0.5470

NMR parameters	**GAS**
HF/3-21G* O(30) $_{iso}$	225.4508
HF/3-21G* O(30) Δ	162.0936
HF/3-21G* O(30)	0.3047
B3LYP/3-21G*O(30) $_{iso}$	159.0905
B3LYP/3-21G* O(30) Δ	96.9107
B3LYP/3-21G* O(30)	-0.1120
HF/3-21G* C(44) $_{iso}$	46.3914
HF/3-21G* C(44) Δ	23.04415
HF/3-21G* C(44)	2.9532
B3LYP/3-21G* C(44) $_{iso}$	39.8319
B3LYP/3-21G* C(44) Δ	113.81215
B3LYP/3-21G* C(44)	2.3026
HF/3-21G*O(47) $_{iso}$	-63.6974
HF/3-21G* O(47) Δ	-104.9796
HF/3-21G* O(47)	-1.6079
B3LYP/3-21G* O(47) $_{iso}$	-69.3989
B3LYP/3-21G* O(47) Δ	-138.4288
B3LYP/3-21G* O(47)	-1.3863

so important to us.

7. The largest $_{iso}$ value of mentioned nuclei of Adenine+ fMet of fMet-tRNA structure observed for O(29), whereas the smallest one belongs to O(32). It is interesting to note that the opposite trend have been observed for asymmetry parameters $( )$. This usual behavior may be readily understood in accord with biotechnological conceptions.

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Full Length Research Paper

Isolation and characterization of a potential biocontrol *Brevibacillus laterosporus*

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An antagonist bacterium ZQ2 was isolated from an apple rhizosphere at Mount Tai in China. The bacterium strongly inhibited the growth of numerous apple phytopathogens *in vitro*, such as *Rhizoctonia solani*, *Fusarium oxysporum*, *Fusarium solani*, and *Physalospora piricola*. The inhibition rates against the different fungi ranged from 55.26 to 88.17. The strain ZQ2 was identified as *Brevibacillus laterosporus* based on morphology, biochemical tests, and 16S rDNA sequence analysis. The antifungal metabolites produced by ZQ2 were thermally stable even after being maintained at 121℃ for 30 min. Meanwhile, the activity against the growth of *R. solani* was almost unchanged when the culture filtrate was irradiated under ultraviolet (UV) or at pH ranging from 1 to 11, and was only reduced under pH conditions from 12 to 14. When observed under a light microscope, the mycelia of *R. solani* inhibited by the antifungal metabolites appeared abnormal in growth. The strong antifungal activity and relatively stable active substances of *B. laterosporus* ZQ2 showed great potential for controlling fungal diseases in apples.

Key words: ***Brevibacillus laterosporus***, characterization, antifungal activity.

INTRODUCTION

Rhizoctonia solani, Fusarium oxysporum, Fusarium solani, and Physalospora piricola, common and damaging phytopathogenic fungi of apple trees, have a wide range of hosts and are major constraints to apple production. At present, chemical control remains the primary means of preventing the diseases caused by these fungi. Numerous synthetic chemicals comprise the major market share of fungicides (Imre et al., 2009; Jonssson et al., 2010). However, due to the increasing resistant mutants and environmental pollution, there is an increasing demand for more effective and safer fungicides with novel modes of action (Zhenzhen et al., 2010). Consequently, the selection of antagonistic microorganisms for biological control is considered as an alternative method (Li et al., 2009).

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In the last few years, various antifungal bacteria were investigated as potential biocontrol agents. Scientists have focused on the use of antagonistic bacteria and their active substances. Members of ***Brevibacillus*** clade, established as an independent genus from the reclassification of ***Bacillus brevis*** in 1996 (Shida et al., 1996), are well-known biocontrol microorganisms that produce structurally diverse secondary metabolites with broad antibiotic spectra. Some of these metabolites, such as chitinase and gramicidin oviet, have been extensively studied (Tatsushi and Kiyoshi, 2009). Numerous ***Brevibacillus*** species have potential as biocontrol agents in agricultural production, and some of these strains have become research hotspots.

In the present study, an antagonist ***B. laterosporus*** strain, ZQ2, was isolated from an apple rhizosphere via an ***in vitro*** screening technique and identified through morphologic observation, biochemical tests, and 16S rDNA sequence analysis. This strain produces stable metabolites that strongly inhibit numerous phytopathogenic

fungi of apple trees, including ***R. solani***, ***F. oxysporum***, ***P. piricola***, and so on. The present research aims to screen novel and effective antagonistic bacterium and hopes to provide an alternative resource for the biocontrol of fungal diseases in apples.

MATERIALS AND METHODS

Isolation and cultivation of the rhizosphere bacteria

Soil samples were collected from an apple rhizosphere at Mount Tai in Shandong Province, China and stored at low temperature at the College of Forestry in Shandong Agricultural University. One gram of soil was suspended aseptically with 100 ml of physiologic saline, agitated in a rotary shaker at 200 rpm for 30 min, and centrifuged. Afterward, serial dilutions of the sample supernate were prepared up to 10^{-6} with sterile water. Then, 0.1 ml of each gradient was spread on potato dextrose agar plates (PDA: 200 g potato, 20 g glucose, 5 g beef extract, 5 g sodium chloride, and 20 g agar in 1 L distilled water) and incubated for approximately 36 h at 28°C. The visible single colony was purified from the proper gradient by streak-plate method. The isolates were cultured on PDA plates and stored at 4°C.

Determination of antifungal activities

The antifungal activities of all bacterial isolates were determined in Petri dishes containing PDA medium using ***R. solani*** as the indicating fungus. The isolated bacteria were inoculated into Luria–Bertani medium (10 g peptone, 5 g yeast extract, and 10 g NaCl in 1 L distilled water) and incubated for 12 h at 30°C. After centrifugation, the filtrate was diluted with potato dextrose broth medium (PDB: 200 g potato, 20 g glucose, 5 g beef extract, and 5 g sodium chloride in 1 L distilled water) at 1:25 (v/v) and poured into Petri dishes. Six-millimeter disks of the tested fungi were placed at the center of the mixed PDA plate. Inhibition of fungal growth was evaluated as the percentage reduction of mycelial growth contrasted with that of the control plates without bacterial cell-free filtrate in the medium. The antifungal activities were calculated using the following equation: Growth inhibition (%) = [(mycelia length in the control plate – mycelia length in the treated plate)/ mycelia length in the control plate × 100] (Dake et al., 2007).

Identification of strain ZQ2

The bacterial isolate ZQ2, which exhibits high antifungal activity against ***R. solani***, was screened for strain identification. Cell morphology was observed via a light microscope (Nikon Eclipse E200, Japan), including Gram-staining and microexamination (shape and size of the cell, spore formation, and so on). Physiological and biochemical tests were performed according to literature (Xiuzhu and Miaoying, 2001), such as growth in pH 5.5 and 9.0, at temperature 15 and 50°C, and in NaCl concentration at 2 and 5%. Molecular technology was employed for exact identification of the strain. The bacterial genomic DNA was extracted and was purified using TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit (Takara, Japan). The 16S rDNA of the bacterium was amplified via polymerase chain reaction (PCR) and sequenced. The primers used for the amplification were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The PCR mixtures contained 5 KL 10 × buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 4 Kl of 2.5 mmol/L deoxynucleoside triphosphates, 1 Kl of each 10 Kmol/L primer, 2 Kl 2 U/Kl TaKaRa Ex Taq, and 20 ng template DNA. The thermal cycling conditions were as follows: 2 min denaturation at 94°C, then 25 cycles of denaturation for 0.5 min at 94°C, annealing for 1 min at 50°C, extension for 1 min at 72°C, followed by a final 10 min extension at 72°C. The products were examined and recovered by electrophoresis in a 1% (w/v) agarose gel and then sequenced.

Correlation between culture time and antifungal activity

A loop of single ZQ2 colony was inoculated from the PDA plate into a 250 ml Erlenmeyer flask with 50 ml of potato dextrose broth medium (PDB: 200 g potato, 20 g glucose, 5 g beef extract, and 5 g sodium chloride in 1 L distilled water) and incubated for 18 h at 28°C in a rotary shaker at 200 rpm to prepare the liquid seed. One milliliter of the seed culture was then transferred aseptically into a 250 ml flask containing 50 ml and fermented 200 rpm for 72 h at 30°C. Samples were taken every 6 h to test the antifungal activity against ***R. solani*** with the aforementioned technique.

Stability of the antifungal metabolites produced by strain ZQ2

The pH of the culture filtrate of strain ZQ2 was adjusted from 1.0 to 14.0 using 1 M HCl or 1 M NaOH, respectively. After maintaining at 4°C for 12 h, the pH levels of the samples were adjusted to 7.0 and antifungal bioassays were conducted to detect the strain activities. The effect of temperature was also assessed by maintaining the culture filtrate at 60, 80, 100, and 120°C for 30 min. The remaining activities against ***R. solani*** were tested after cooling to room temperature. A sample at room temperature and pH 7.0 was assayed as the control.

In the UV test, 10 ml of culture filtrate was poured into a 7.5 cm wide Petri dish, which was placed 30 cm under a 30 W UV lamp. Then, 1 ml sample was taken every hour for 6 h. The variations in the remaining activities were studied. All experiments were performed in triplicate, and the mean value was analyzed with SAS 9.0 software.

In vitro antifungal activities of the antifungal metabolites

Eight pathogenic fungi, namely, ***F. oxysporum***, ***F. solani***, ***P. piricola***, ***Aspergillus fumigatus***, ***Alternaria alternata***, ***Valsa sordida***, ***Colletotrichum gloeosporioides***, and ***Botrytis cinerea***, were kindly provided by the Plant Protection College of the Shandong Agriculture University (Taian, China) and maintained on PDA plates at 4°C prior to use. The inhibitory abilities of the antifungal substances produced by strain ZQ2 against the pathogens were determined as the percentage reduction in mycelial growth. As a control, the target fungi were grown on PDA plates without culture filtrate. The results were recorded after 72 h of incubation at 30°C. The morphology of the inhibited ***R. solani*** mycelia, along the edges, was then examined under a light microscope (Nikon Eclipse E200, Japan).

RESULTS

Isolation of antifungal strains

A total of 127 bacterial strains were obtained from the soil sample and bioassayed. Among the 12 strains that showed antifungal activity (data not shown), strain ZQ2 most strongly inhibited ***R. solani***. It exhibited high activity in the ***in vitro*** test, with an inhibition rate of up to 80.17%.

Table 1. Physiological and biochemical characterization of ***B. laterosporus*** ZQ2 and standard ***B. laterosporus***

Characteristic	ZQ2	Standard *B. laterosporus*
Shape	Rod	Rod
Size	0.83 × 3.6 Km	0.7–0.9 Km × 3–5 Km
Endospore forming	-	-
Gram stain	-	-
Motility	-	-
Oxidase	-	-
Voges-Praskauer (V-P) test	-	-
Starch hydrolysis	-	-
Glucose fermentation	-	-
Growth in pH 5.5	-	-
Growth in pH 9.0	-	-
Growth at 15°C	-	-
Growth at 50°C	-	-
Growth in 2% NaCl	-	-
Growth in 5% NaCl	-	-
Anaerobic growth	-	-

Identification of strain ZQ2

Strain identification results suggest that ZQ2 should belong to the ***Brevibacillus*** genus. The strain is Gram-positive, rod-like (0.8 to 1.5 Km × 1.2 to 2.0 Km), motile, facultative anaerobic bacterium with endospores formed in the cell. The strain can hydrolyze starch and gelatin, and produces catalase and oxidase. However, it did not grow at temperatures higher than 55°C or lower than 15°C. Detailed morphologic and biochemical characteristics are summarized and compared with the standard ***B. laterosporus*** (Xiuzhu and Miaoying, 2001) in Table 1. The 16S rDNA of this strain was then amplified and sequenced, consisting of 1444 nucleotides. The phylogenetic tree, established via the SAS 9.0 software using the data obtained from NCBI Genbank (Figure 1), also demonstrated that the strain is most likely to be ***B. laterosporus***. Therefore, ZQ2 was designated as ***B. laterosporus*** strain ZQ2, based on characterization and clustering similarity, and its 16S rDNA sequence was submitted to NCBI Genbank (Accession no. EU471747).

Correlation between culture time and antifungal metabolite production

The antifungal activity of strain ZQ2 was not detected until 12 h after inoculation (Figure 2), and then the active metabolites were continuously secreted along with the cell growth. The strongest activity against ***R. solani*** was observed at 60 h, after which the activity continuously declined until 72 h. This behavior demonstrates that the optimal time to harvest the antifungal metabolites produced by strain ZQ2 is 60 h after inoculation.

Stability of the antifungal metabolites

Figure 3a illustrates the thermal stability of the antifungal substances. The activity remained at more than 88% even when the culture filtrate was maintained at 120°C for 30 min. The same result was observed in the UV radiation test. The antifungal activity against ***R. solani*** was almost unchanged during the sampling period (Figure 3b), suggesting that the antifungal metabolites produced by ZQ2 are stable in open environments.

The antifungal activity was significantly reduced when the compounds were exposed to basic conditions from pH 12 to 14 (Figure 3c), but the active substances remained stable even after exposure to pH ranging from 1 to 11 (>80%).

In vitro antifungal test

The antifungal substances produced by ZQ2 displayed strong ***in vitro*** inhibition against ***F***. ***oxysporum***, ***F***. ***solani***, ***P***. ***piricola***, ***A***. ***fumigatus***, ***A. alternata***, ***V***. ***sordida***, ***C***. ***gloeosporioides***, and ***B***. ***cinerea*** after 3 days of incubation (Table 2). The maximum inhibition rate was observed against ***V***. ***sordida*** (88.17%), followed by ***A. alternata*** (79.02%), and ***A***. ***fumigatus*** (77.13%). When observed under a light microscope, the mycelia of ***R. solani*** along the edges of interaction zone appeared thick and opaque, their cytoplasmic contents became cumulate, and parts of the mycelia ruptured. There were no spores in the field of vision (Figure 4a). However, the fungal mycelia on the control plate showed normal thin and clear radial growth with visible spores (Figure 4b).

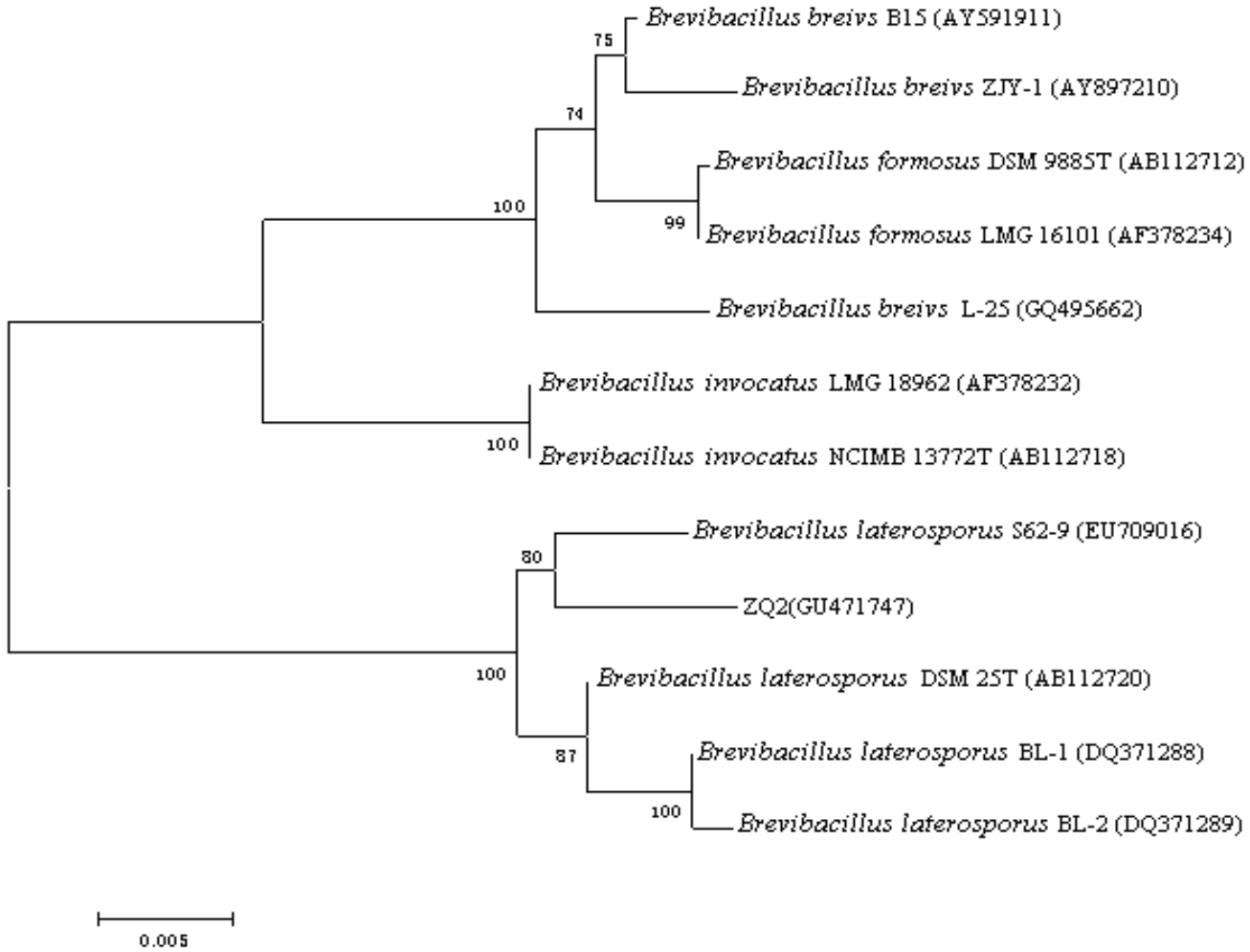

Figure 1. Phylogenetic tree of ***B. laterosporus*** ZQ2 based on 16S rDNA sequence analysis using the neighbor-joining method.

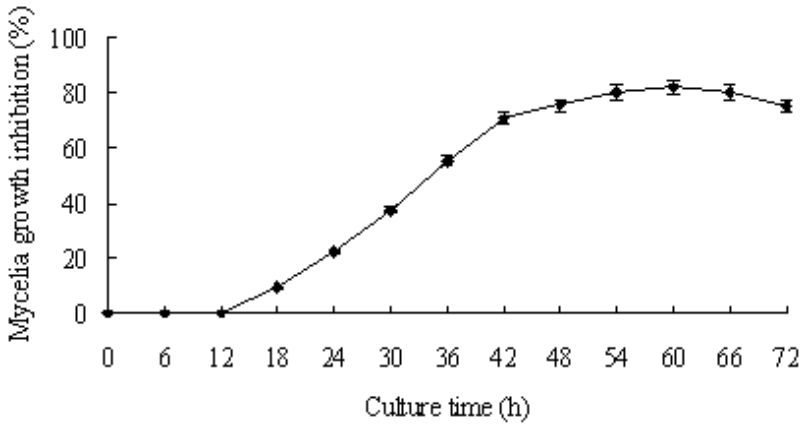

Figure 2. Correlation between culture time and antifungal activity.

DISCUSSION

Combined technologies were used in the present study to identify the bacterium strain ZQ2. According to its morphology, the bacterium is rod-shaped, Gram-positive, motile, and spore-forming (0.8 to 1.5 Km × 1.2 to 2.0 Km).

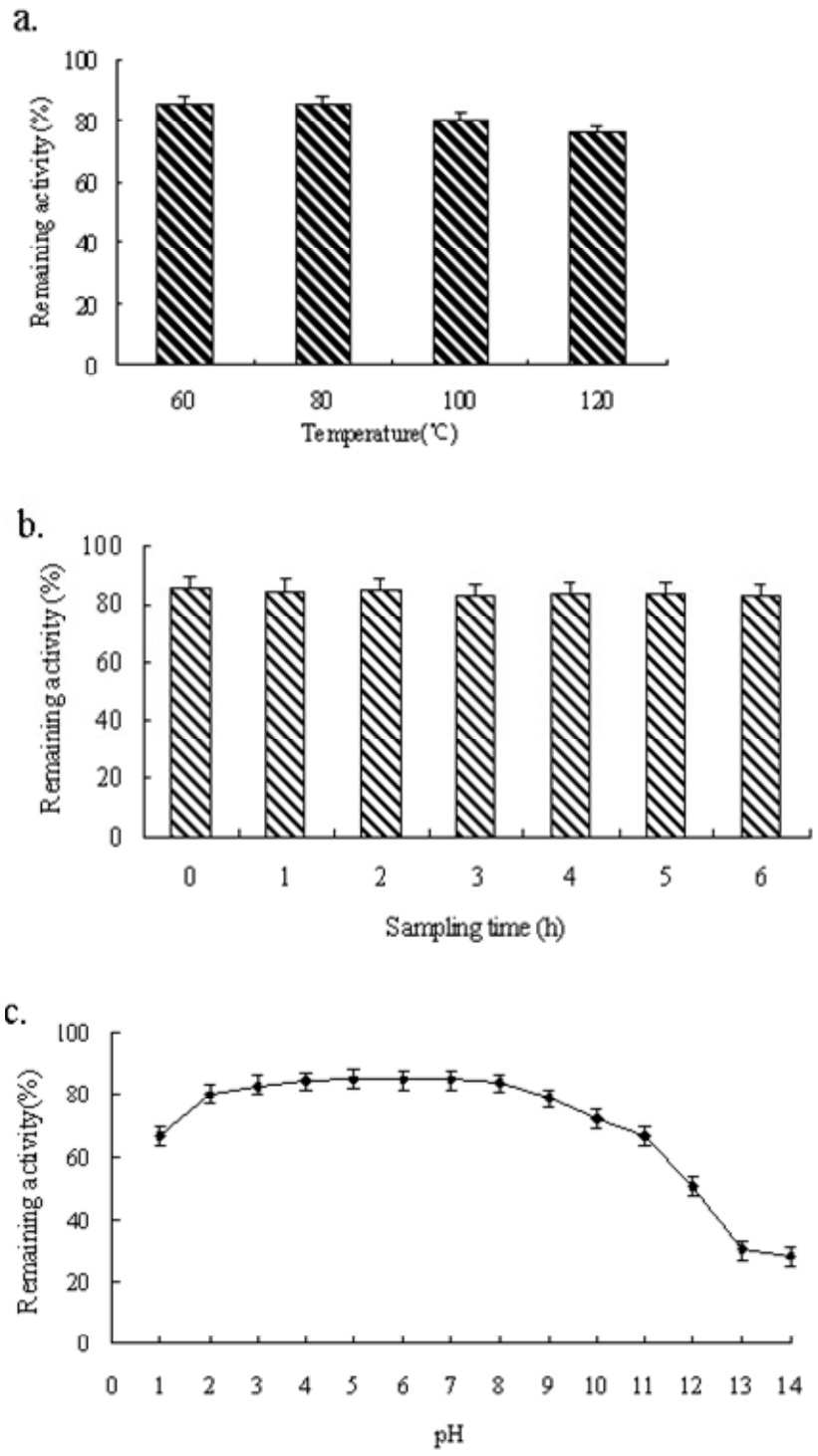

Figure 3. Effect of temperature (a), UV radiation (b), and pH conditions (c) on the stability of the antifungal substances from ***B. laterosporus*** ZQ2.

Table 2. Inhibition of phytopathogens using ***B. laterosporus*** strain ZQ2 antifungal metabolites.

Pathogenic fungi	*F. oxysporum*	*F. solani*	*P. piricola*	*A. fumigatus*	*A. alternata*	*V. sordida*	*C. gloeosporioides*	*B. cinerea*
Inhibition rates (%)	64.70	66.41	55.26	77.13	79.02	88.17	66.83	61.95

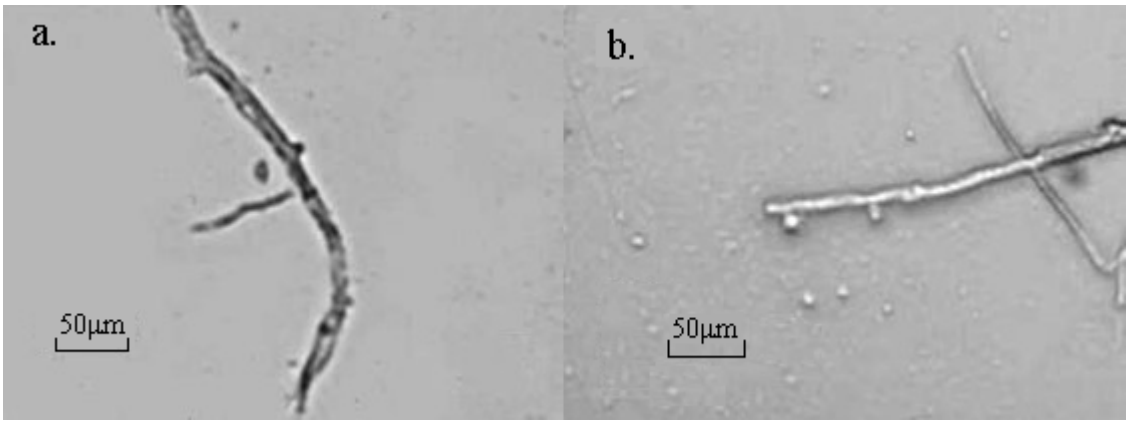

Figure 4. Effects of ***B. laterosporus*** ZQ2 active metabolites on the growth of ***R. solani***: (a) mycelia inhibited by the antifungal substances and (b) healthy mycelia on the control plate.

Biochemical indices showed positive oxidase production and glucose fermentation but negative in starch hydrolysis and V-P tests. Strain ZQ2 showed almost identical properties to the members of ***B. laterosporus*** (Saikia, et al., 2010), based on 16S rDNA sequence analysis. Homology also revealed the same result.

B. laterosporus bacteria are potent biocontrol agents against numerous insects belonging to orders lepidoptera and coleoptera (Oliveira et al., 2004) and can be used for nematode control due to their production of extracellular neutral protease (Baoyu et al., 2006). However, there are few reports on the use of ***B. laterosporus*** as a biocontrol agent for pathogenic fungi. To the best of our knowledge, this is the first report of the isolation of ***B. laterosporus*** with broad-spectrum antifungal activity from an apple rhizosphere.

Brevibacillus spp. can produce a wide variety of metabolites with antifungal activity, which can control plant diseases as biocontrol agents (Sunita et al., 2010). A number of the active metabolites are fungicidal or fungistatic peptides that are non-ribosomally synthesized by multi-enzyme–catalyzed systems. Most of these peptides are very stable because of their low molecular weight and specific structure. Some non-ribosomal peptides have already been purified from ***B. laterosporus***. Kelsey et al. (2007) isolated and elucidated the structure of the lipopeptide tauramamide from ***B. laterosporus*** Ren et al. (2007) purified an antimicrobial peptide, R-1, with a small molecular weight and composed of 9 kinds of amino acids from marine ***B. laterosporus***. In the present study, the active substances produced by ***B. laterosporus*** ZQ2 were relatively stable under different conditions and have broad and strong antifungal activity. These features show great potential in the application of strain ZQ2 and its metabolites in controlling pathogenic apple fungi.

The mechanisms of action of many antifungal peptides are still undetermined although investigations were conducted during the last few decades. Daniel et al. (2010) found that many short cationic peptides accumulate on the cell membrane of fungal hyphae and disturb sterol-rich membrane domains. Thicker hyphae and depolarized cells were observed in micrographs, and most spores did not germinate after antifungal peptide treatment. The phenomenon is very similar to the results in the present study, signifying that ***B. laterosporus*** strain ZQ2 likely secretes peptides with the same inhibitory mechanism on fungi.

The antifungal substances produced by ZQ2 are being purified from the PDB medium of ZQ2, and elucidation of their structures is in progress. In further research, the bioactive products will be characterized and the medium for production will be optimized. We hope to provide an alternative resource for controlling fungal diseases in apples using ***B. laterosporus*** ZQ2 as a novel biocontrol agent.

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Full Length Research Paper

Immunogenicity of envelope GP5 protein displayed on baculovirus and protective efficacy against virulent porcine reproductive and respiratory syndrome virus challenge in piglets

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In the present study, one recombinant baculovirus BacSC-GP5, expressing His6-tagged GP5 with the transmembrane domain (TM) and cytoplasmic domain (CTD) derived from baculovirus envelope protein gp64, was constructed and its immunogenicity and protective efficiency was evaluated in piglets. The results obtained show that, His6-tagged recombinant GP5 was expressed and anchored on the plasma membrane of Sf-9 cells, as revealed by Western blot and confocal microscopy. Immunogold electron microscopy demonstrated that, the GP5 glycoprotein was displayed successfully on the viral surface. Piglets immunized with BacSC-GP5 induced successfully GP5-specific enzyme-linked immunosorbent assay (ELISA) antibody, neutralizing antibody and lymphocyte proliferation response at 6 weeks after primary immunization. An *in vivo* challenge result indicated that piglets immunized with BacSC-GP5 did not show any obvious clinical signs and histological changes, and the quantitative real-time polymerase chain reaction (RT-PCR) also indicated that the porcine reproductive and respiratory syndrome virus (PRRSV) viral load from the serum in BacSC-GP5 group was significantly reduced at 14 and 21 days post-challenge compared to that in the negative control group. These results indicate that baculovirus-mediated gene delivery can be utilized as an alternative strategy to develop a new generation of vaccine against PRRSV infection.

Key words: Baculovirus surface display, porcine reproductive and respiratory syndrome virus (PRRSV), GP5 protein, immunogenicity, protective efficacy.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), characterized by severe reproductive failure in late term gestation in sows and respiratory disease in pigs of all ages, is considered as one of the most economically important diseases affecting the swine industry worldwide (Meulenberg, 2000). PRRSV, the causative agent, is an envelope, positive stranded ribonucleic acid (RNA) virus belonging to the family Arteriviridae (www.ictvdb.org/Ictv/index.htm). The viral genome is approximately 15 kb in length and contains nine open reading frames (ORF1a/1b, ORF2a/2b, ORF3-7) (Meulenberg et al., 1995). The GP5, encoded by ORF5 gene, is one of the key immunogenic proteins of PRRSV involved in the generation of PRRSV-neutralizing antibody and protective immunity and is one of the leading targets for the development of the genetic engineering vaccines against PRRS (Barfoed et al., 2004; Zheng et al., 2007). Several experimental vaccines expressing GP5 protein have been studied and tested in recent years, such as recombinant fowlpox virus (Shen et al., 2007),

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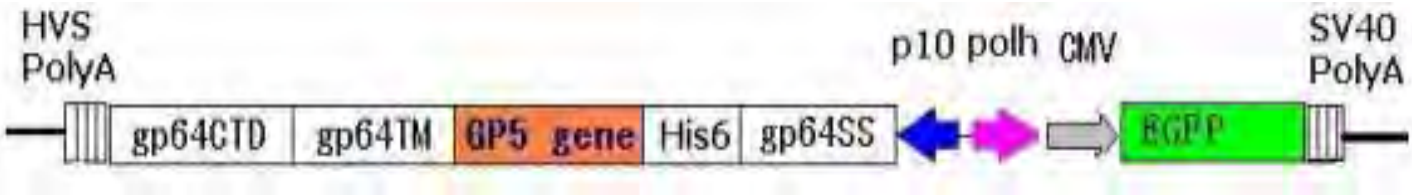

Figure 1. Schematic diagram of the recombinant baculovirus vector pBacSC-GP5. Plasmid pBacSC was constructed using pFastBac Dual (Invitrogen, Carlsbad, USA) as the backbone. The fusion gene fragments of baculovirus gp64 SS, His6, multiple cloning sites (*Xho* I, *Xba* I, *Pst* I, and *EcoR*I), baculovirus gp64 TM, baculovirus gp64 CTD were inserted into the corresponding site under the control of baculovirus p10 promoter. The immediate early promoter of human cytomegalovirus (CMV) and the enhanced green fluorescent protein (EGFP) coding sequences were cloned into the corresponding site under the control of baculovirus polyhedrin promoter (Ppoh). The GP5 gene was inserted into the pBacSC vector. The resultant plasmid was named as pBacSC-GP5.

recombinant adenovirus (Jiang et al., 2006a), and recombinant pseudorabies virus vaccines (Jiang et al., 2007; Qiu et al., 2005). Although partial protective immunity can be obtained with these experimental vaccines, one major problem in using these recombinant viruses as vaccines is the pathogenic potential of the vector virus.

Baculovirus has captured increasing interest as a vector due to its safety and abundant yields. Several reports have demonstrated that baculovirus carrying mammalian cell-active promoters is capable of transferring and expressing foreign genes in a variety of mammalian cells, as well as in animal models (Tani et al., 2003). Furthermore, it has also been reported that a pseudotyped baculovirus displaying the glycoprotein of vesicular stomatitis virus (VSV-G) on the envelope can extend the host range and increase the efficiency of transduction in mammalian cells (Pieroni et al., 2001). In recent years, several researchers have developed a baculovirus surface display vector, utilizing the baculovirus envelope glycoprotein-gp64 which is comprised of an N-terminal signal sequence and a mature domain including the transmembrane domain (TM) and cytoplasmic domain (CTD) (Kitagawa et al., 2005). And the heterologous gene was inserted between the SS and the mature domain. This method has been extended to develop various pseudotyped baculoviruses as potential vaccines, such as expressing avian influenza virus (AIV) hemagglutinin protein (Yang et al., 2007) and classic swine fever virus (CSFV) E2 protein (Xu and Liu, 2008). These researchers have demonstrated that direct vaccination with recombinant baculovirus could induce high-level humoral and cell-mediated immunity in mouse model or natural host.

The main objective of this study is to use the baculovirus surface display system expressing the PRRSV GP5 protein on the baculoviral envelope, in the hope that GP5 would retain superior immunogenicity upon *in vivo* immunization. Here, we constructed a baculovirus surface display vector expressing the GP5 protein on the baculoviral envelope and evaluated its immunogenicity and protective efficacy in piglets.

MATERIALS AND METHODS

Cells and viruses

The *Spodoptera frugiperda* 9 (Sf-9) cell lines were grown at 27°C in Grace's insect media (Invitrogen, Carlsbad, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Carlsbad, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin. The PRRSV Shaanxi strain (Wang et al., 2010), a highly pathogenic North American type isolate, was used in this study. PRRSV was propagated in MARC-145 cells, which were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, USA) supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C with 5% CO_2.

Recombinant baculovirus construction and preparation

The baculovirus surface display system, pBacSC plasmid, was constructed and provided by collaborators (Xu and Liu, 2008). To generate a recombinant baculovirus expressing the GP5 protein (BacSC-GP5), the DNA fragment of ORF5 (deleting the N-terminal signal peptide of 31 amino acid residues) was amplified from the plasmid pGEM-GP5 (containing the complete cDNA of ORF5 gene of the PRRSV Shaanxi strain, kept in our laboratory) by polymerase chain reaction (PCR) with forward primer (PRRSV-*Xho*I), 5'-GCTCTCGAGAGCAACAACAGCAG-3', and reverse primer (PRRSV-*Pst*I), 5'-GATCTGCAGGAGACGACCCCATTGTT-3'. The restriction sites are underlined. The amplified product was subcloned into the corresponding site in the pBacSC plasmid, resulting in the recombinant transfer plasmid pBacSC-GP5 (Figure 1). The recombinant baculovirus was subsequently generated by using the Bac-to-Bac® system (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. Recombinant baculovirus was further amplified by propagation in Sf-9 cells. Virus purification was performed as described previously (Abe et al., 2004), and the titer was determined by a plaque assay according to the manufacturer's manual (Invitrogen, Carlsbad, USA).

SDS-PAGE and Western blot analysis

The infected cell lysates were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene fluoride (PVDF) membranes (Amersham, New York, USA). Two primary antibodies were used to detect GP5 in the Western blot: mouse monoclonal His-tag antibody (1:3000 dilution,

Invitrogen, Carlsbad, USA) and porcine polyclonal PRRSV antibody (1:500 dilution, provided kindly by the Center of Animal Health and Epidemiology, Qingdao, China). The secondary antibodies were goat anti-mouse and rabbit anti-porcine IgG conjugated to HRP (1:5000 dilution, Invitrogen, Carlsbad, USA). The protein bands were visualized by the electrochemiluminescence (ECL) kit (Pierce, Rockford, USA). The purified virus particles were also detected by SDS-PAGE and Western blot analysis following the same protocol.

Confocal microscopy

The confocal microscopy was carried out as described previously (Yang et al., 2007). The cells were incubated with the primary antibodies (mouse monoclonal His-tag antibody and porcine polyclonal PRRSV antibody, 1:300 dilution) and the secondary antibodies (fluoresceine isothiocyanate (FITC)-conjugated goat anti-mouse and rabbit anti-porcine IgG, 1:1000 dilution, Invitrogen, Carlsbad, USA). The negative control cells were treated in the same way. Protein localization was visualized using a confocal microscope LSM 510 META (Zeiss, Berlin, Germany).

Immunogold electron microscopy

The immunogold electron microscopy was performed as described previously (Hu et al., 2003) with minor modifications. The grids were incubated with the porcine polyclonal PRRSV antibody (1:300 dilution) and the rabbit anti-porcine IgG conjugated with 10-nm gold particles (1:100 dilution, Sigma, St. Louis, USA). The grids were then examined under the transmission electron microscope H-7500 (Hitachi, Tokyo, Japan).

Immunization of piglets

To evaluate whether the pseudotyped baculovirus BacSC-GP5 can elicit immune responses, fifteen healthy 4-week-old piglets were obtained from a PRRS-free farm in Xi'an, China. The piglets were tested seronegative to PRRSV by ELISA (IDEXX, New York, USA). The animals were then separated randomly into three groups (five piglets each group), numbered, and housed in separate rooms. One group was inoculated intramuscularly twice at 2-week internals with 1 ml phosphate-buffered saline (PBS) containing 1.0×10^9 plaque forming units (PFU) of purified BacSC-GP5. The other two groups were injected respectively with 1 ml PBS containing 1.0×10^9 PFU of purified BacSC or PBS as the negative control groups. Serum samples were collected from the *V. jugularis* external at 6 weeks after primary immunization for serological tests. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with sodium heparin as an anticoagulant at 6 weeks after the primary immunization for lymphocyte proliferation assay.

Serological tests

GP5-specific antibody titers were tested using an indirect ELISA, with purified PRRSV as the coating antigen (Jiang et al., 2006a). The results were expressed as the ratio of OD490 produced by the serum samples compared with negative control serum. Sera with a ratio value higher than 2.1 were considered to be positive sera (Fan et al., 2008). The titers were expressed as the reciprocal of the highest dilution of sera producing ratio values of 2.1. Serum neutralization assays were performed as described previously (Jiang et al., 2006b). The neutralization titers were expressed as the reciprocal of the highest serum dilution in which no cytopathic effect (CPE) was observed. Each sample was run in quadruplicate.

Lymphocyte proliferation assay

Piglet PBMCs were isolated from the whole blood of immunized piglets with lymphocyte separation medium (TBD, Shanghai, China). The lymphocyte proliferation assay was performed as described previously (Jiang et al., 2006b). The stimulation index (SI) was calculated as the ratio of the average OD490 value of triplicate wells containing PRRSV stimulated cells to the average OD490 value of triplicate wells containing only cells with medium.

Challenge and histopathological analysis

At 6 weeks after primary immunization, all piglets were challenged intranasally with 1×10^5 50% tissue culture infection dose ($TCID_{50}$) of the PRRSV Shaanxi strain in 5 ml of PRRSV-infected-MARC145 culture supernatant fluid. After challenge, the animals were monitored daily for the presence of clinical signs, including inappetence, depression, cough, dyspnoea, and fever. Rectal temperatures were recorded from 2 days pre-challenge to 21 days post-challenge (dpc). At 21 dpc, all piglets were dissected and macroscopic lesions were examined among the different groups. Histological changes were further evaluated in the collected lungs from the different groups. After fixation with 10% formalin, the lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE).

Real-time RT-PCR analysis of viral replication in serum

After challenge, serum samples were collected at 7, 14 and 21 dpc. The viral copy numbers of serum samples were estimated by IQ™ 5 real-time PCR system (Bio-Rad, Hercules, USA) using IQ™ SYBR® Green qPCR Kit (Bio-Rad, Hercules, USA). Briefly, the primers Q-PRRSV-F 5′-CAAATAACAACGGCAAGCAG-3′ and Q-PRRSV-R 5′-AAACTCCACAGCGTAACTTA-3′ were used to amplify a 308 bp fragment from the ORF7 gene of PRRSV. The purified PCR product of PRRSV was cloned into pGEM-T easy vector and quantified by measuring OD_{260} using spectrophotometer (NanoDrop 2000, Wilmington, USA). A standard curve was obtained using serially diluted plasmid pGEM-ORF7 standards of $10^1\sim10^8$ copies/µl and was used to quantify the PRRSV viral genomic copy numbers. Viral RNA was extracted from 500 µl of serum sample by using the MiniBEST Viral RNA Extraction Kit (Takara, Dalian, China). Then, PRRSV cDNA synthesis was performed according to the PrimeScript™ RT-PCR Kit (Takara, Dalian, China) with slight modification using 5 µl of extracted RNA. The PCR amplification was carried out in a 25 µl reaction volume containing 12.5 µl IQ™ SYBR® Green supermix, 6 pM concentration of forward and reverse primers and 4 µl cDNA. All reactions were carried out in triplicate. Statistical analysis was performed by using the means of three replicate wells for each sample as compared with a standard curve. PCR data was presented as follows: mean log10 genomic copies/ml of serum ± standard deviation.

Statistical analysis

Student's t-test was used to evaluate the differences among groups in regard to humoral and cellular immune responses, body temperatures and viral copy numbers. P-values <0.05 were considered statistically significant.

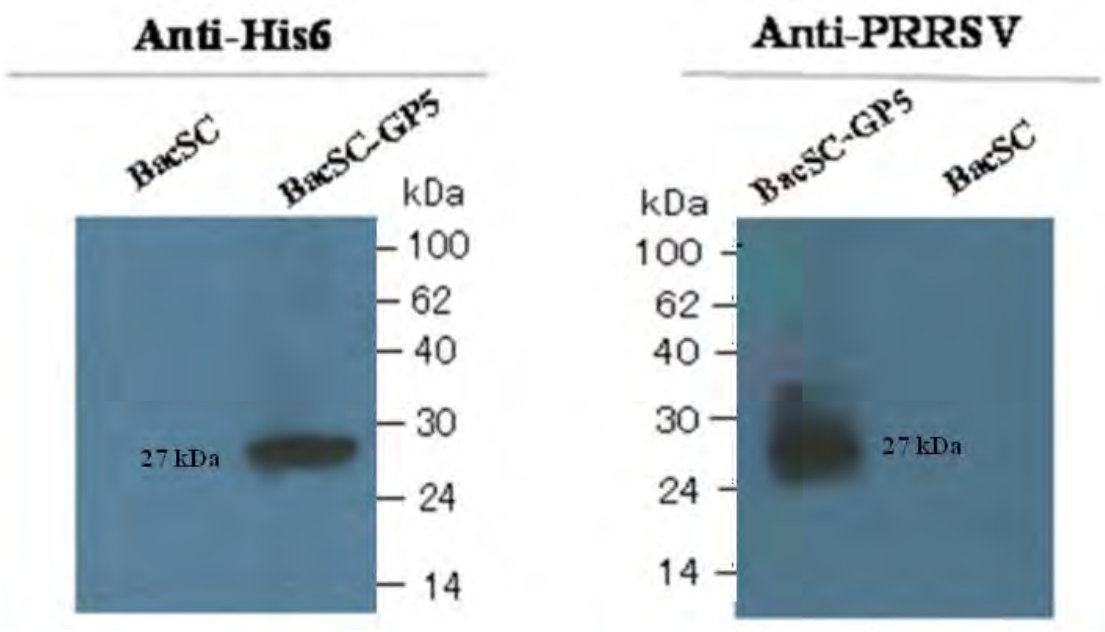

Figure 2. Western blot analysis of GP5 expression in insect cells. The Sf-9 cells were infected with BacSC-GP5 or BacSC at 10 MOI, harvested 3 days after infection, and subjected to Western blot assay. The 27 kDa fusion proteins (23 kDa of GP5 protein and 4 kDa of His6 fusion protein) were detected by His-tag and PRRSV antibodies. No proteins were detectable in the negative control (BacSC).

RESULTS

GP5 expression from recombinant baculovirus in insect cells

The Western blot analysis (Figure 2) showed that the cells infected by BacSC-GP5 expressed an approximately molecular mass of 27 kDa fusion proteins (23 kDa of GP5 protein and 4 kDa of His6 fusion protein) that were detected by both His-tag and PRRSV antibodies. This is in agreement with the predicted size from the GP5 protein. In contrast, there were no proteins recognized by His-tag and PRRSV antibodies in the BacSC.

The confocal microscopy result (Figure 3) revealed that there were no proteins recognized by His-tag and PRRSV antibodies in the negative control (Sf-9 cells only). In contrast, the GP5 protein could be detected by the His-tag and PRRSV antibodies in the BacSC-GP5 infected cells, whereas in the BacSC infected cells it could only be detected by the His-tag antibody. The protein localizes with the plasma membrane, thus demonstrating the anchoring of GP5 on the surface of Sf-9 cells.

Display of GP5 on recombinant baculovirus

To investigate whether the His6-tagged GP5 is incorporated into baculovirus, BacSC-GP5 and BacSC were purified by sucrose gradient ultracentrifugation and then subjected to SDS-PAGE and Western blot analysis. Figure 4 revealed that there was no target protein expression in the BacSC. In contrast, the protein expressed by BacSC-GP5 was detected by both His-tag and PRRSV antibodies. The purified viral particles were further analyzed by immunogold electron microscopy using porcine PRRSV primary antibody and anti-porcine IgG secondary antibody conjugated with 10-nm gold particles. Figure 5 revealed that gold particles displayed on the surface of BacSC-GP5 (A) but not on the surface of BacSC (B), indicating the GP5 was incorporated and displayed on the baculoviral envelope. Moreover, the incorporation of GP5 did not alter the virus morphology.

GP5-specific humoral and cellular immune responses in piglets

As shown in Table 1, piglets vaccinated with BacSC-GP5 produced ELISA antibody with titers of 1:83.2 and neutralizing antibody with titers of 1:11.2. No GP5 specific ELISA and neutralizing antibodies could be detected in piglets immunized with BacSC or PBS throughout this experiment.

At 6 weeks after primary immunization, PBMCs were isolated and restimulated *in vitro* with the purified PRRSV (20 μg/ml) to analyze the cellular immune responses. As shown in Table 1, significantly higher lymphocyte proliferative responses could be observed in piglets

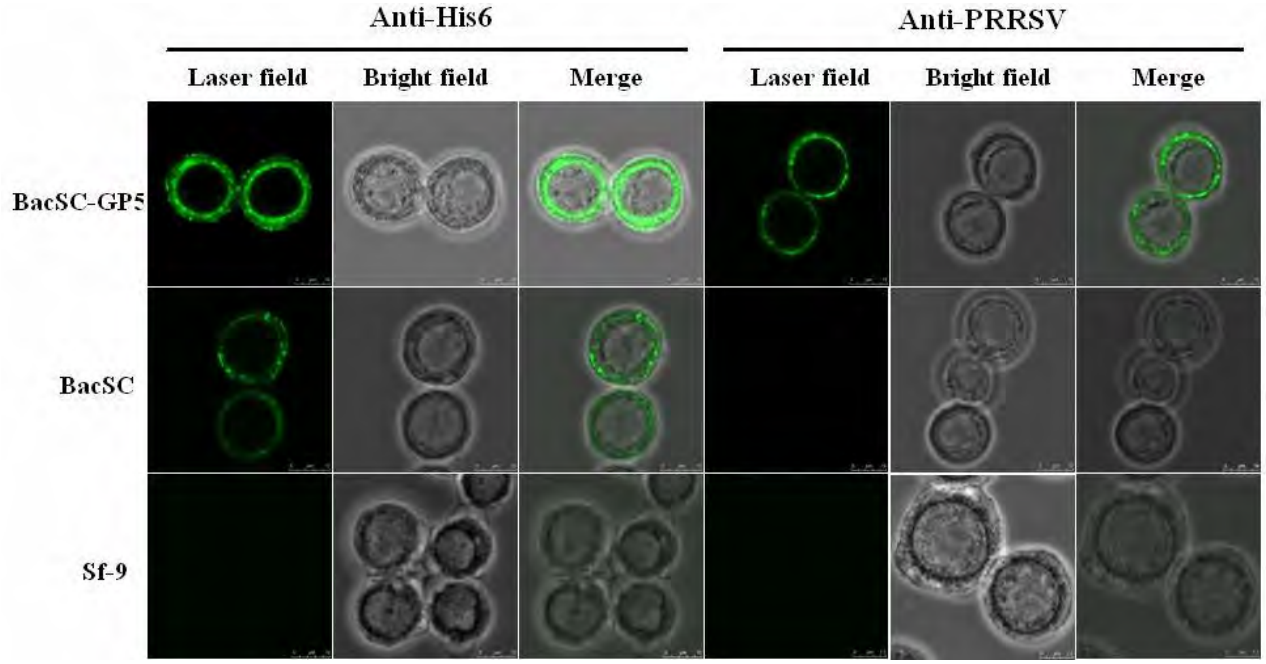

Figure 3. Confocal immunofluorescence microscopy of GP5 expression in insect cells. The cells were cultured on sterile cover slips, infected separately with BacSC-GP5 or BacSC at 10 MOI, and subjected to immunofluorescence examination by confocal microscopy 2 days after infection. The result showed that GP5 was translocated properly to the plasma membrane of the infected Sf-9 cells.

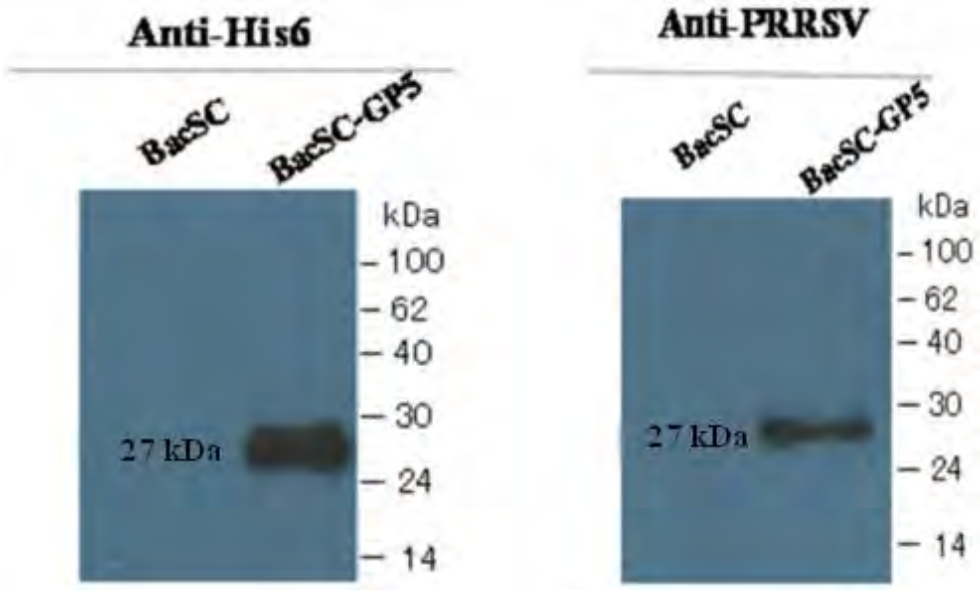

Figure 4. Western blot analysis of recombinant baculovirus. The budded viruses produced by infection of Sf-9 cells with recombinant viruses BacSC-GP5 or BacSC were purified by ultracentrifugation on sucrose gradient. The samples were subjected to Western blot assay and the GP5 fusion proteins (27 kDa) were detected by His-tag monoclonal antibody and PRRSV polyclonal antibody. No proteins were detectable in the negative control (BacSC).

immunized with BacSC-GP5 compared to those with BacSC or PBS ($P < 0.05$). These data indicate that the pseudotyped baculovirus BacSC-GP5 can induce humoral and cellular immune responses in the natural

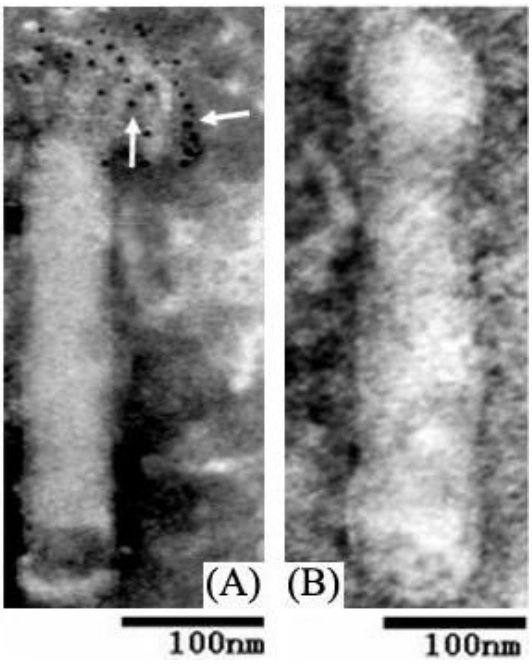

Figure 5. Incorporation of GP5 into the baculoviral envelope. Immunogold electron micrographs of purified baculovirus BacSC-GP5(A) or BacSC(B) detected by PRRSV polyclonal antibody as the primary antibody and anti-porcine IgG conjugated with 10-nm gold particles as the secondary antibody. No gold particles were displayed on the surface of baculovirus BacSC.

Table 1. Humoral and cellular immune responses of piglets to recombinant baculovirus.

6 weeks after primary immunization			
ELISA antibodies	**Neutralizing antibodies**	**Lymphocyte proliferative response (SI)**	
BacSC-GP5	83.2 ± 42.9	11.2 ± 4.38	1.77 ± 0.12[a]
BacSC	NR	NR	1.12 ± 0.09[b]
PBS	NR	NR	1.08 ± 0.13[b]

Data represent the mean ± standard deviation. Different letters (a, b, c) represent significant difference ($P < 0.05$) between groups; NR, no result

host.

Clinical signs and histological changes after PRRSV challenge

After PRRSV challenge, all piglets were maintained and observed for 21 days to evaluate clinical signs. Pigletsimmunized with BacSC or PBS appeared lethargic, moderate respiratory disease characterized by sneezing, coughing and markedly decreased appetite. And, the rectal temperature increased up to above 40°C at 4 dpc and fluctuated greatly during challenge. Meanwhile, piglets immunized with BacSC-GP5 did not show any obvious clinical signs and the rectal temperatures were not over 40°C and fluctuated steady during the experiment (Figure 6). At 21dpc, all piglets were dissected. No obvious macroscopic lesions of piglets immunized with BacSC-GP5 were found in the tonsils, spleens, kidneys and mesenteric lymph nodes except mild diffuse macular hemorrhage in the lungs. In contrast, severe hemorrhagic lungs and moderate hemorrhagic mesenteric lymph nodes were observed in piglets immunized with BacSC or PBS.

Histological changes were also evaluated in the collected lungs. Severe interstitial pneumonia was found in the lungs of piglets immunized with BacSC or PBS. In contrast, the piglets immunized with BacSC-GP5 only

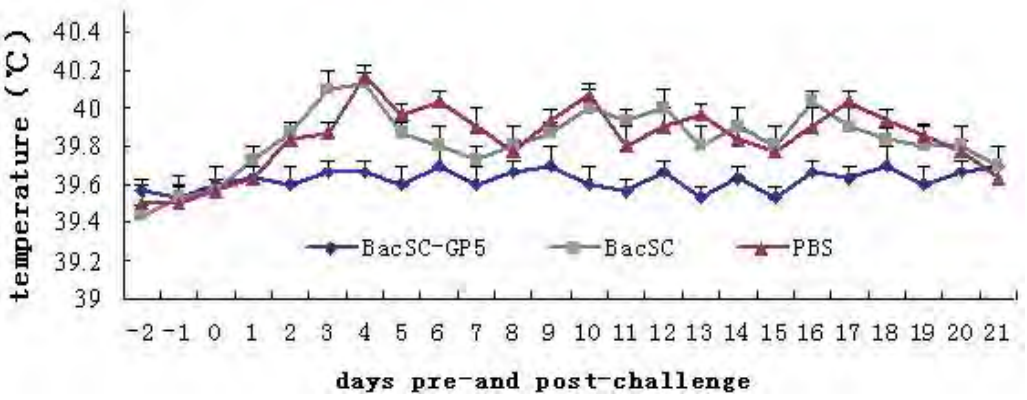

Figure 6. Mean rectal temperature of vaccinated piglets pre- and post- challenge with virulent PRRSV shaanxi strain.

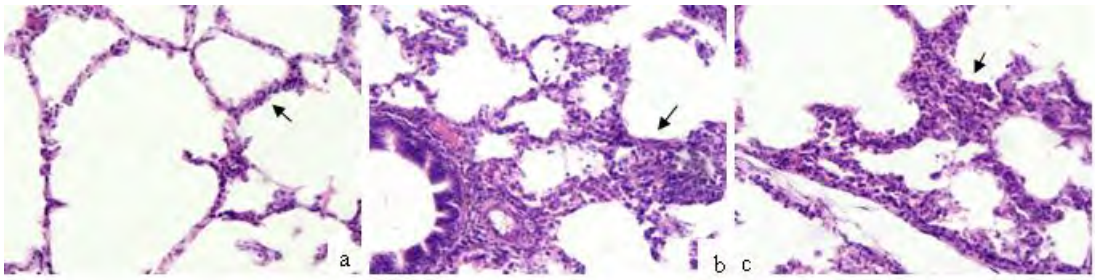

Figure 7. Histological changes in the lungs of piglets in BacSC-GP5-vaccinated group (a), BacSC group (b), and PBS group (c) at 21 dpc. a, Arrows indicate mild interstitial pneumonia with hyperemic small blood vessels; b, c Arrows indicate localized region of severe interstitial pneumonia with intensive accumulation of macrophages, and thickened alveolar septum; HE, staining. Magnification 400×.

exhibited mild interstitial pneumonia (Figure 7).

Real-time RT-PCR analysis of viral replication in serum

To determine the viral burdens in serum, the sera of five piglets in each group were collected at 7, 14 and 21 dpc for analysis. The viral was not detected in any of the serum samples on the day of challenge. The dynamic changes of PRRSV copies for all groups are shown in Figure 8. Viruses replicated at a high level in the serum of piglets immunized with BacSC or PBS. Compared with the viral burdens from the control group, significantly reduced ones were found in the serum of piglets immunized with BacSC-GP5 at 14 and 21 dpc ($P < 0.05$).

DISCUSSION

PRRSV infection is still a severe problem in the countries with intensive swine industries. Live attenuated and killed vaccines, the two types of commercial vaccine currently on the market, have been used wildly, and they play an extent effect in prevention and control of PRRSV. However, they cannot provide effective protection against PRRSV infection due to their inherent character (Opriessnig et al., 2002; Nilubol et al., 2004). Hence, new strategies should be developed for more efficacious vaccines against PRRSV infection. In the present study, we developed an experimental pseudotyped baculovirus vaccine and evaluated its protective efficacy. A baculovirus surface display system was firstly constructed to display the PRRSV GP5 protein as described in Figure 1. The gp64 SS would facilitate the translocation of the protein to the insect cell plasma membrane and then be cleaved, exposing the His6 tag to the outer surface, while the TM from baculovirus gp64 enabled the protein to anchor onto the plasma membrane. The CTD from baculovirus gp64 mediated the interaction with the budding nucleocapsid and the ensuing incorporation into the virus envelope (Oomens and Blissard, 1999). The results of confocal microscopy and immunogold electron microscopy indicated that the GP5 was displayed

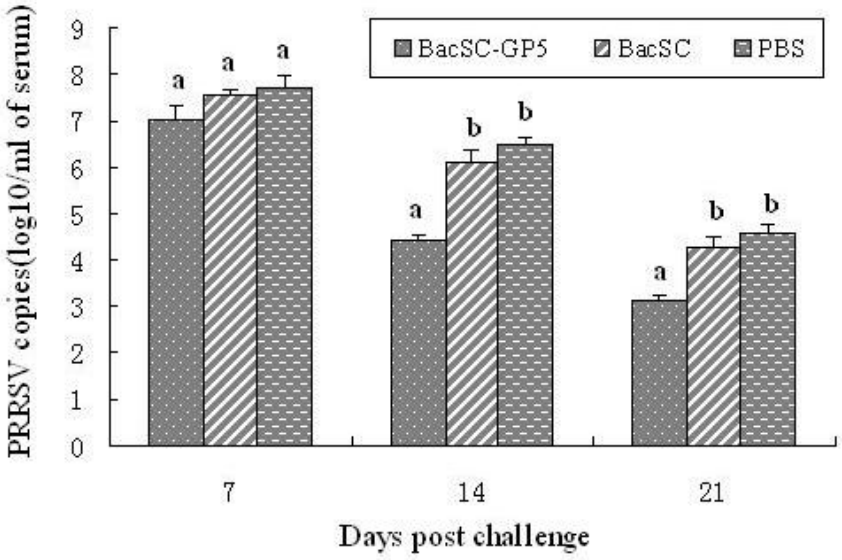

Figure 8. Real-time RT-PCR quantification of PRRSV viral copy numbers. The sera samples in each group were collected at 7, 14 and 21 dpc, and the viral RNA was extracted and then reverse-transcribed to cDNA. Plasmid encoding a 308 bp fragment of PRRSV ORF7 gene was used to obtain the standard curve for quantifying the PRRSV viral genomic copy numbers. Each column represents the mean of triplicate assay with standard deviation. Different letters (a, b, c) represent significant difference ($P < 0.05$) between samples.

successfully on the surface of the virus-infected cells as well as on the virions surface. The result of one-step growth curve indicated that the presence of the gp64 fusions did not alter the growth of the recombinant viruses and high titer stocks of these viruses could be achieved (Data not shown). Besides, the viral infectivity in cell cultures was also maintained.

It is now widely accepted that measures of humoral immunity can represent valuable parameters by which to evaluate the efficacy of vaccines against homologous viruses (McMurry et al., 2008). The cell-mediated immunity has also been suggested to have important implication for protective immunity against serologically distinct viruses (Doherty et al., 2006). Therefore, the simultaneous induction of strong humoral immunity and robust cellular immunity should be considered when developing a new generation of vaccines against PRRSV. In our study, we firstly evaluated the immunogenicity acquired with BacSC-GP5 in mouse model. The result showed that, significantly higher levels of GP5-specific ELISA antibody, neutralizing antibody, as well as lymphocyte proliferation responses could be induced in mice immunized with BacSC-GP5 than those received BacSC (Data not shown). Then, the immunogenicity was further evaluated in pigs. Similar results were acquired in piglets immunized with BacSC-GP5, indicating that the pseudotyped baculovirus is an alternative vector to express and present antigens of PRRSV. The strong immunogenicity of the recombinant baculovirus BacSC-GP5 may be attributable to the efficient antigen that exposes on the virions surface and increases the chance of being taken up by antigen-presenting cells (APCs). Meanwhile, protective efficacy was also evaluated by the challenge assay. The histological changes showed that piglets immunized with BacSC-GP5 exhibited mild interstitial pneumonia, while severe interstitial pneumonia was observed in those immunized with BacSC or PBS. The quantitative real-time RT-PCR also indicated that the PRRSV viral load was significantly reduced at 14 and 21 dpc from the serum in BacSC-GP5 group than that in the control group. These data indicate that the pseudotyped baculovirus BacSC-GP5 can confer partial protection against virulent PRRSV challenge.

In our previous study, we have demonstrated that the signal peptide and hydrophobic region affect the expression of proteins in different expression vectors including prokaryotic or eukaryotic system (Wang et al., 2010). Key et al. (2001) has also demonstrated that, GP5 protein without the signal peptide region would result in a better and higher expression maintaining the same immunogenicity. In the present study, we amplified the GP5 gene without the signal peptide of N-terminal 31 amino acid residues and demonstrated it could acquire a better immunogenicity.

It is known that a high degree of genetic and antigenic variability exists among different PRRSV isolates, which

makes it difficult to develop efficacious PRRS vaccine (Meng, 2000). To achieve more efficient cross-protective immunity against antigenic variant, several strategies have been developed recently. For example, to express a consensus immunogenic sequence derived from different PRRSV isolates or to co-express GP5 and other immunogenic proteins of PRRSV, such as M and/or GP3, has been demonstrated to elicit a broad and cross-protective immunity (Zheng et al., 2007; Jiang et al., 2008). The baculovirus vector has the advantage of being able to accommodate a large exogenous DNA genome (>30 kb) (Hu, 2005), which makes it suitable for co-expression of multiple heterologous antigens under independent promoters and allows for simultaneous induction of high-level humoral and cellular immunity. The unique advantage of the baculovirus provides the possibility to develop more immunogenic, broader-spectrum vaccines. Presently, these studies are ongoing in our laboratory.

In conclusion, the present study describes a recombinant baculovirus BacSC-GP5 displays the GP5 protein on baculovirus. Our data demonstrate that direct vaccination with recombinant baculovirus BacSC-GP5 can develop PRRSV specific humoral and cell-mediated immune responses in natural pig host, and can confer efficient protective immunity against PRRSV. In light of our results, the baculovirus-based vaccine may hold great promise as a novel platform to prevent the PRRS epidemic and can be envisaged as an alternative option in the priming-boosting vaccination scheme.

ACKNOWLEDGEMENTS

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Abbreviations: RT-PCR, Real-time polymerase chain reaction; **ELISA,** enzyme-linked immunosorbent assay; **TM,** transmembrane domain; **CTD,** cytoplasmic domain; **PRRSV,** porcine reproductive and respiratory syndrome virus; **PRRS,** porcine reproductive and respiratory syndrome; **VSV-G,** vesicular stomatitis virus; **RNA,** ribonucleic acid; **PVDF,** polyvinylidene fluoride; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **ECL,** electrochemiluminescence; **PBS,** phosphate-buffered saline; **SI,** stimulation index; **$TCID_{50}$,** tissue culture infection dose; **dpc,** days post-challenge; **CPE,** cytopathic effect; **DMEM,** Dulbecco's modified Eagle's medium

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Full Length Research Paper

Indigenous arbuscular mycorrhizae in areas with different successional stages at a tropical dry forest biome in Brazil

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Arbuscular mycorrhizal fungi mycelium creates multiple fungal links between roots of plants and could be an important component of plant succession in ecosystems. Our objective was to compare the spore number (SN), genus contribution and indigenous arbuscular mycorrhizal colonization (AMC) capacity of soils from a tropical dry forest (caatinga) ecosystem in Brazil at different levels of successional stages after culturing for 3 months in a greenhouse *Brachiaria decumbens* (= *Urochloa decumbens* Staph) as trap plants. Soil samples were collected from Parque Estadual Mata Seca (Manga, Minas Gerais State, Brazil) in four different areas: (a) Pasture (5 years without human activity, covered mainly by *Panicum maximum* Jacq.); and three different forest successional stages: (b) Initial (8 years in process of spontaneous recovery), (c) Intermediate (17 years without human activity) and (d) Late (no recorded human activity). At 90 DAP plants growing at intermediate disturbance soil had the lowest dry matter production, probably because of the lowest available soil P and Ca contents of this soil. The highest SN was found in soils from pasture while the lowest was in soils from the intermediate area. *Glomus* was, in general, the most abundant genus followed by *Acaulospora*, *Gigaspora* and *Scutellospora* (always present in intermediates rates). The contribution of *Archaeospora* was the lowest; however, in some cases it was absent. AMC was higher in plant roots growing in soils from pasture but the lowest AMC was in plant roots from the intermediate area. This could be associated with the soil acidity, Al and H contents of this area which could be toxic to AMF. Mycorrhiza formation and SN appeared to be more related to the physicochemical characteristics of the soil and the host plant development to the soil successional stage. Further studies are needed in order to identify appropriate management strategies for restoration of altered lands in order to contribute to the biodiversity conservation and microbial activity of ecosystems.

Key words: Arbuscular mycorrhizae, plant succession, soil factors.

INTRODUCTION

There is an emerging consensus that changes occurring aboveground (in the atmosphere or by consequences of land use) undoubtedly will affect plant, animal and belowground microbial biodiversity and that those compartments are intimately linked (van der Putten et al., 2009). Thus, recent studies are focused to understanding these relationships. Arbuscular mycorrhizal fungi (AMF) are belowground plant symbionts of roots that add another dimension to the plant-soil-microbe system because it can directly and indirectly influence other soil microbes and an adequate soil nutrient cycling. Mycorrhizal fungi are able to develop a symbiotic association with more than 90% of vascular or

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Abbreviations: AMC, Arbuscular mycorrhizal colonization; **AMF,** arbuscular mycorrhizal fungi; **Ar,** arbuscule content; **DAP,** days after planting; **SDM,** shoot dry matter; **SN,** spore number.

non-vascular terrestrial plants (van der Heijden et al., 1998; Pongrac et al., 2007; Feddermann et al., 2010). AMF are a diverse taxon, both systematically and functionally and mechanisms governing the formation and maintenance of the symbiosis have not been yet fully clarified. They belong to a monophyletic phylum, the Glomeromycota (Schüßler et al., 2001; Smith and Read, 2008), its taxonomic classification is constantly changing and a new list of families and genera has recently been published (Schüßler and Walker, 2010).

Mycorrhizal symbiosis is usually not host-specific, and displays abundant ecological differentiation and specialization to both their biotic and abiotic environments, including types that are highly specialized to unusual ecological niches (Fitter et al., 2004). AMF form a uniformly distributed mycelium in soil and hyphal proliferation occurs in response to several types of organic material deposition (Rillig and Mummey, 2006), connecting host plants and improving physical and chemical soil quality. The contribution of AMF on plant nutrition is widely documented, specially where available soil P limit plant growth of native plants as well agricultural crops (Menéndez et al., 2001; Covacevich et al., 2006, 2007; Siddiqui et al., 2008; Smith and Read, 2008; Guissou, 2009; Ndiaye et al., 2009). AMF provide other benefits to their host plants, stabilizing soil structure and increasing the tolerance to water stress, soil compaction, salinity and drought, root pathogens and heavy metals or others toxic substances present in the soil (Davies et al., 1993; Augé, 2001; Feng et al., 2002; Trotta et al., 2006; Hildebrandt et al., 2007; Siddiqui and Pichtel, 2008; Raviv, 2010; Miransari, 2011). The high amount of hyphae produced by AMF is correlated with significant increases in the aggregate stability of soils (Jastrow et al., 1998; Tisdall et al., 1997; Rillig, 2004; Treseder and Turner, 2007), modifying the soil's ability to mobilize nutrients, maintaining water content, facilitating roots penetration in soil and diminishing soil erosion potential.

The mycelium of AMF also interconnects the root systems of neighboring plants both of the same as different species (Allen et al., 2003). In this sense, mycorrhizal networks can create indefinitely large numbers of fungal linkages connecting together many plants in a community (Newman, 1988; Giovannetti et al., 2006). Changes in vegetation as a result of environmental changes may affect the spread of native AMF. This suggests that AMF formation could be an important element of plant succession in ecosystems (Bellgard and Williams, 2011). Consequently, AMF presents an increasingly interest for agriculture, agroforestry, altered lands restoration and natural ecosystems conservation. Observations on plant succession patterns in semiarid regions reported that AMF could play an important role in the composition and stability of plant communities. However, there are still no conclusive results on the relationship between mycorrhizal colonization and the spatial heterogeneity of ecosystems (Boerner et al., 1996).

Mycorrhizal fungi have a widespread presence in all environments and especially within the tropics (Fuchs and Haselwandter, 2004; Moreira et al., 2007; Makoi and Ndakidemi, 2009) although most of the knowledge regarding the effect of soil management on mycorrhizae comes from temperate regions (Raviv, 2010). In particular, little is known about the possible relationship between infectivity capacities of native AMF from a dry forest type called “Mata Seca” (in Portuguese), in the ***caatinga*** biome. The ***caatinga*** is of considerable importance because this type of tropical dry forest is the only biome of exclusively Brazilian distribution (Queiroz, 2006) and an important part of the world's biological richness is found in that ecosystem. The uniqueness and threat level of this vegetation type was reviewed by Espírito-Santo et al. (2009). The objective of this work was to evaluate, under controlled conditions in pots, the spread of indigenous mycorrhization, the contribution of genera and abundance of AMF spores on soils collected from Brazilian Mata Seca ecosystems with different successional stages.

MATERIALS AND METHODS

Study sites

Soil samples were collected from the Dry Forest State Park, in the north part of Minas Gerais State, Brazil (43° 97´02´´S -14° 64´ 09´´W and 44° 00´ 05 ´´ S -14° 53´ 08´´ W). The climate is tropical wet (Aw), the mean annual maximum temperature is 32°C, annual average 18 °C, annual rainfall is 660 mm (SIMGE, 2008). The region is characterized by a well marked dry season (May-October; rainfall less than 60 mm/month, temperature 24 to 26°C). Rains are irregular and concentrated in the summer (December-March).

Soil sampling at field sites and preparation of trap cultures for AMF

Soil samples (0-10 and 10-20 cm depth) were collected during the wet season (February, 2008) in a pasture area with 5 years without anthropogenic interference, covered mainly by ***Panicum maximum*** (= ***Urochloa maxima*** (Jacq.) Webster RD) grasses and in three areas with different stages of plant succession: (a) Initial (area with 8 years of spontaneous regeneration process), (b) Intermediate (area with 17 years without anthropogenic interference) and (c) Late (area without any sign of anthropogenic interference). For each area of study, plots of 1000 m^2 were delimited. For each area, soil samples (1500 g each) were collected with five replications. Any individual sample (from each area and depth) was composed by nine subsamples of (0-10 and 10-20 cm depth). Field soil was sieved through a 1 cm mesh, large root fragments were cut in pieces of 1 cm, they were returned to the soil and air dried in darkness at 10°C until use one month later.

The experiments were conducted at the Embrapa Agrobiologia, Seropédica-Universidade Federal Rural do Río de Janeiro, Seropédica, Rio de Janeiro, Brazil. Trap cultures of AMF in each area were made by using experimental units (n=60) which consisted of white plastic pots of 0.8 kg (used for plants growing until 45 days after planting-DAP) or 1 kg (used for plants growing until 90 DAP). All pots were filled with a substrate composed of not autoclaved homogenized soil from the field and twice autoclaved

Table 1. Soil characteristics at sites selected from the Dry Forest State Park. OC= organic carbon.

Site	pH	P	OC	Al	H
	(water)	(mg kg^{-1})	(g kg^{-1})	($cmol_c$ kg^{-1})	
Initial	7.02 a	0.61 b	22.03 a	< 0.00	0.86 b
Intermediate	5.06 b	0.20 b	18.01 b	0.58	3.61 b
Late	6.62 a	0.44 b	21.90 a	< 0.00	1.92 a
Pasture	6.42 a	1.87 a	14.42 b	< 0.00	1.89 b

Means within the same column followed by the same letter are not significantly different (LSD,P<0.05).

river sand in a proportion of 2:1. The experiment was set up with 5 replications, 4 areas (soil from 0-10 cm depth of Initial, Intermediate, Late and Pasture areas) and 2 harvest (45 DAP and 90 DAP). Additionally, 0.8 kg pots were filled with soil from the same areas at 10 to 20 cm depth with 5 replications and harvested at 45 DAP. To avoid preferences of mycorrhizal infection for a certain host plant, experiment was done using only ***Braquiaria*** grasses as host plant in all treatments. Seeds were sterilized with 4% sodium hypochlorite solution for approximately 5 min, rinsed with distilled-autoclaved water, pregerminated in sterile vermiculite and grown in a growth chamber during 5 days. Homogeneous-sized pre-germinated plants were planted in pots (4 plants pot^{-1} in 45 DAP pots, and 3 plants pot^{-1} in 90 DAP pots). Plants were grown in glasshouse under natural light conditions, daily watered with distilled water and the substrate was maintained at water holding capacity (65% w/s). Hoagland solution (P 30%) was supplied at 30 and 60 DAP.

Trap cultures harvest: Plant growth and mycorrhizal colonization assess

After 45 DAP and 90 DAP plants were harvested from 0.8 and 1 kg pots, respectively. Shoots were cut and plant material was oven dried at 60°C for shoot dry matter (SDM) estimation. As a measure of the infection potential of field samples, initial mycorrhizal root colonization was determined in the trap cultures (Oehl et al., 2004) after 45 and 90 DAP. Root material was washed out free of soil and collected on sieve (0.5 mm). Immediately, roots were cut into 1 cm pieces, thoroughly mixed, cleared with KOH (10%, 30 min, 90°C), acidified (HCl, 0.1 N), and stained with trypan blue (0.05% vol^{-1}, 5 min, 100°C) in lactoglycerol (1:1:1 lactic acid, glycerol, distilled water) according to the modified method described by Phillips and Hayman (1970). Presence of mycorrhizal infection was assessed by microscopic examination (40X) of the root system. A segment was considered infected if contained hyphae plus arbuscules, coils or vesicles. Mycorrhizal colonization was estimated as AMC (%) and percentage of arbuscules (Ar) of the root cortex, according to the Trouvelot et al. (1986) method.

AMF spore isolation and identification

AMF spores of the trap cultures were extracted by wet sieving and sucrose density gradient centrifugation (Daniels and Skipper, 1982). The procedure included passage of 50 cm^3 of harvested trap culture substrate through 500, 125, 53 and 37 µm sieves. The contents of the 125, 53 and 37 µm sieves were centrifuged at 800 × g for 3 min, the supernatant was discarded, and the pellet was layered onto a water-sucrose solution (80% [wt/vol]) gradient and centrifuged at 900 × g for 2 min. The resulting supernatant was passed through the 37 µm sieve, washed with tap water. Quantification of spore number (spores, spore clusters, and sporocarps) was carried out in 9 cm diameter Petri dishes with a gridline of 1 cm per side under a stereoscopic microscope at 50X (Lugo and Cabello, 2002). Ten divisions were counted and related to the total spores number (SN) (Schalamuk et al., 2006). Taxonomic identification was made at the Microbiology Laboratory-Estación Experimental Agropecuaria INTA, Balcarce (Argentina). For that, 80% of spores of each sample were mounted onto slides with the permanent mounting medium for fungi polivinil alcohol PVA (Omar et al., 1979) with or without Melzer's reagent to assist with the identification of AMF spores (Morton, 1988). About 70% of the spores fixed on slides could be identified at the genus level. Dead and decaying spores, lacking clear morphological features were not included in the number quantification or the genus identification. The spores' identification was based on current species descriptions and identification manuals (Schenck and Pérez, 1990; International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi http://invam.caf.wvu.edu/Myc_Info/Taxonomy/species.htm; Schüßler and Walker, 2010).

Statistical analysis

Data were analyzed by ANOVA (SAS 9.2 Institute Inc., 2002-2008). Means among treatments were compared with the least significant difference (LSD) test (P < 0.05). All presented data are means of untransformed values.

RESULTS AND DISCUSSION

Soil physicochemical attributes varied significantly among sites (Table 1) and the soil from the Intermediate area was the most different in the quantified characteristics in comparison to the other areas. In that sense, both the most acidic condition and highest Al and H contents were found in those samples. Furthermore, the lowest soil P content was determined at the Intermediate site whereas the highest soil P was found in the Pasture soil. Initial and Late soils showed the highest organic carbon contents.

The SDM, AMC, Ar and SN significantly varied among the different areas where the soil was collected (Table 2). SDM of ***Braquiaria*** was highest in plants growing in intermediate and pasture soils at 45 DAP (Figure 1). However, at 90 DAP plants growing in Intermediate soils showed lowest SDM production. This may be due in part to the lower available soil P and Ca content in Intermediate soils, which were two or three times lower than in the other areas, and also to the low pH, which

Table 2. Analysis of variance of successional areas (SA) and soil depth (SD) on shoot dry matter (SDM) production, arbuscular mycorrhizal colonization (AMC), percentage of arbuscules (Ar) of ***Braquiaria*** plants and spore numbers (SN) of soils at 45 days after planting.

Source of variation	Degree of freedom	SDM	AMC	Ar	SN
Replications	4	0.09	0.74	0.83	0.01
Successional area (SA)	3	0.05	0.01	0.10	<0.0001
Soil depth (SD)	1	<0.001	<0.0001	<0.0001	0.41
A×SD	3	<0.01	0.28	0.03	0.94

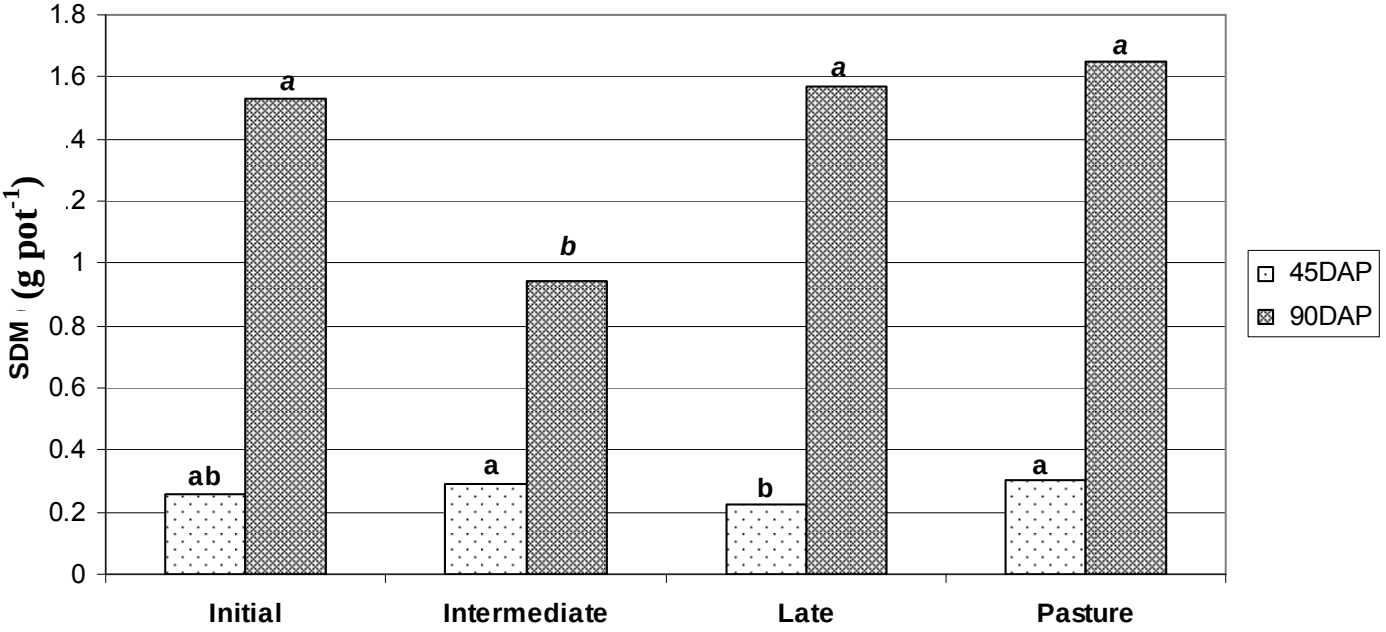

Figure 1. Shoot dry matter production (SDM) of ***Brachiaria*** at 45 and 90 days after planting (DAP) growing in soils from different successional ecosystems. For each sampling date (standard letters for 45 DAP, italics for 90 DAP), columns with the same letters show no significant differences in SDM among soils (LSD, P <0.05).

was about 1.5 times lower than in the other soils. With increasing soil depth, we found decreases in the percentage of roots colonized by AMF and in plant productivity. AMC and Ar of roots were significantly higher at 45 DAP in pots filled with the field soil of 0 to 10 cm depth comparing with 10 to 20 cm depth samples. This could indicate higher soil propagules content in the most superficial soil depth. Also Schwab and Reeves (1981), and Cooke et al. (1993) reported decreases in mycorhizal colonization through soil depth profiles. However, Covacevich et al. (2006) did not found differences in colonization between 0 to 10 and 10 to 20 cm of soil samples collected in agricultural field grasses of Argentina. On the other hand Neville et al. (2002) found that AM hyphae and total fungal colonization of ***Populus tremuloides*** Michx were significantly lower in the top 5 cm of the soil than in the 5 to 10 cm. Furthermore, at our experiment, the SN was not significantly different when comparing these 0 to 10 and 10 to 20 soil depths (Table 3). Ohel et al. (2005) found either differences or no differences in the SN between 0 to 10 and 10 to 20 cm of a Calcaric Regosol (pH 7, P 10.9 mg P kg^{-1}) and a Haplic Luvisol (pH 6.2, P 21.8 mg P kg^{-1}) soil samples collected in grassland fields from Upper Rhine Valley (Germany), respectively. It is an indicative that SN and AMF infective propagules are not always well correlated. Some incongruities among results indicate that AMF are differentially partitioned at different soil depths and it could be accounted by extreme differences in propagule richness related to soil characteristics and differences in time of germination of spores of different soil environments. Highest SN were found in soils from Pasture followed by Initial area while the lowest SN were found in pots with Intermediate area soil (Table 4). Silva Santos Santos (2010) working with field samples collected in the same areas as in this study also found that Pasture had highest SN (about 600 spores ×100 g $soil^{-1}$) followed by the Initial area (about 420 spores × 100 g $soil^{-1}$).

Table 3. Effect of soil depth on shoot dry matter (SDM) production, arbuscular mycorrhizal colonization (AMC), percentage of arbuscules (Ar) of ***Braquiaria*** roots and spore numbers (SN) at 45 days after planting.

Soil depth (cm)	SDM	AMC	Ar	SN (spores × 100 g soil^{-1})
0-10	0.308 a	44.7 a	30.9 a	87 a
10-20	0.232 b	25.1 b	15.2 b	80 a

Means within the same column followed by the same letter are not significantly different (LSD,P<0.05).

Table 4. Spore number (SN) and contribution of representative genera of arbuscular mycorrhizal fungi found in soils (0-10 cm) of different successional areas with ***Braquiaria*** plants grown until 45 and 90 days after planting (DAP).

Harvest	Site	SN (spores 100 g soil^{-1})	Genera (%)				
			Glomus	*Acaulospora*	*Archaeospora*	*Gigaspora*	*Scutellospora*
45 DAP	Initial	116 b	61	21	--	8	10
	Intermediate	10 c	26	32	2	10	30
	Late	51 c	48	36	--	9	7
	Pasture	172 a	42	33	5	9	11
90 DAP	Initial	133 b	52	29	1	9	9
	Intermediate	38 c	27	42	1	5	25
	Late	95 b	41	40	--	10	9
	Pasture	237 a	39	36	4	11	10

Means within the same DAP followed by the same letter are not significantly different (LSD,P<0.05).

However, the SN quantified at our study was about 3.5 to 6 folds lower than the amounts reported by Silva Santos Santos (2010). In the present study, the lowest SN of soil at the trap culture was found in soils collected at the Intermediate area. Silva Santos (2010) determined that the Late area had the lowest SN under field conditions (about 240 spores × 100 g soil^{-1}) and pointed that lowest SN could be an indicative of a highest environmental stability of soils. The most abundant genus was ***Glomus***, followed by ***Acaulospora*** that showed the highest frequency in Intermediate soils at 45 and 90 DAP and reached the same proportion of ***Glomus*** in Late soils at 90 DAP. Silva Santos (2010) also found that ***Acaulospora*** presented the highest frequencies at the Intermediate site under field conditions. The results obtained in the present study are agree with other studies that reports that ***Acaulospora*** could be found preferably under moderately acid soil-conditions at pH < 6.2 (Stürmer, 1999) and preferably within the range 3.5-5.8 (Trufem, 1995). Interestingly, no spores belonging to the genus ***Archaeospora*** were found in the pots filled with the Initial soils at 45 DAP nor in the Late soils during the two harvest of the experiment. When ***Archaeospora*** spores were found, their contribution to the total recorded genera was the lowest. Silva Santos (2010) did not found any spores belonging to the ***Archaeospora*** at the Late site under field conditions. On the other hand, ***Gigaspora*** and ***Scutellospora*** were always present at Intermediate rates from 5 to 25% (Table 4).

Silva Santos (2010) analyzed SN and mycorrhizal diversity of soil samples collected in the field. The present paper analyzed SN and most representative genera from soils collected at the same areas and at the same moment like Silva Santos (2010) in order to establish AMF-trap cultures using ***Braquiaria*** as host plants. Although the overall response patterns were similar between our results and those of Silva Santos (2010), some differences were found (for example in the overall amount of the SN). Differences between our results in pots and those found by Silva Santos (2010) under field conditions could be attributed to the fact that AMF sporulation varies with host and AMF species and also with growing conditions (affecting fungus directly and indirectly through host physiology). Many studies have demonstrated that not necessarily the same AMF species are found in a field site and in greenhouse cultures with soil from the same site and growing in “trap cultures” (Jansa et al., 2002; Oehl et al., 2003, 2004), although there is usually a considerable overlap between the same species groups. The period of time during the cultures are grown appears to be important. Sporulation often occurs within 3 to 4 weeks after onset of mycorrhizal colonization under almost any conditions except the high availability of soil P, which could depress or inhibits all fungal growth phases (http://invam.caf.wvu.edu/fungi/taxonomy/concepts/lifecycle.htm). Declerck et al. (2001) found that sporulation under controlled conditions increased from 4 to 5 weeks

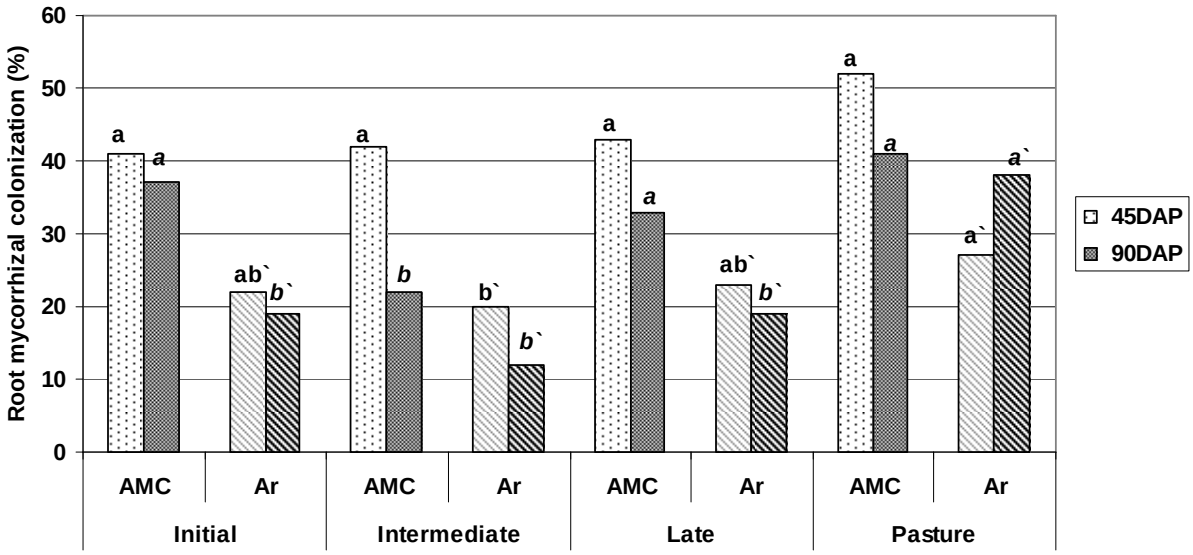

Figure 2. Arbuscular mycorrhizal colonization (AMC) and percentage of arbuscules (Ar) of ***Brachiaria*** roots at 45 and 90 days after planting (DAP) grown in soils from different successional ecosystems. For each sampling date (standard letters for 45 DAP, italics for 90 DAP) and value of root mycorrhizal colonization (without apostrophe for AMC, with apostrophe for Ar) columns with the same letters show no significant differences in mycorrhizal colonization among soils (LSD, P <0.05).

to approximately 10 weeks; at this time sporulation was stabilized. Oehl et al. (2004) found that there is a wide time range (4 to 20 months) in which AMF could sporuLate.

Mycorrhizal development of ***Braquiaria*** roots decreased from 45 to 90 DAP (Figure 2). It is likely that most of the photosynthates were allocated in the aerial part at 90 DAP and therefore less C was available in the roots to maintain the mycorrhizal symbiosis. AMC and Ar were highest in roots growing in pots filled with Pasture soil followed by the Initial and Late soils (Figure 2). The lowest formation of symbiosis was detected on plants grown in the Intermediate soil. This could be probably associated with a lower pH than in the other sites. Soil acidity is a factor that normally affects the development of mycorrhizal symbiosis (Sieverding, 1991; Bartolome-Esteban and Schenck, 1994; Fuchs and Haselwandter, 2004; Kittiworawat et al., 2010), mainly when the soil presents high Al^+ and H^+ contents, which are toxic to AMF (Porter et al., 1987) and sometimes interferes with roots colonization, because P adsorption makes it unavailable to the plant. It was mentioned that highest Al^+ and H^+ contents were found in Intermediate soils, which could explain the lowest presence of AMC in this site. However, Siqueira et al. (1990) mentioned that root colonization of ***Brachiaria*** was not greatly different between Brazilian soils of pH 4.51 to 6.0. But in a previous study, Siqueira et al. (1986) also showed that the ability to colonize roots grown on soil ranging in pH 4.5 to 6.3 was different among AMF species and that, in general, mycorrhiza formation of maize increased gradually as soil pH increased to pH 5, and decreases at the highest soil pH of 6.3. If in the present study the pH was not the cause of lower mycorrhiza formation at the Intermediate soil, it is likely that the lower amount of C in roots as a consequence of the symbiosis maintenance could result in a lower plant growth This is because AMF are obligate symbionts of plants that consume photosynthates from roots (Smith and Read, 2008).

Mycorrhizal root colonization and SN of soils followed, in general, the same pattern among sites with the lowest mycorrhizal development in soils from the Intermediate area. Changing environments can induce the predominance of Intermediate-sized spores, such as in ***Glomus*** species that in general moduLate the production of spores directly according to C availability, showing a direct reproduction investment as expected for r-strategists (Ijdo et al., 2010), as well as a reduction in AMF abundance and in richness species. However, Clapp et al. (1995) mentioned that AMC as well as SN could not necessarily reflect the actual AMF populations in the soils and Ahulu et al. (2006) showed that species with an abundant production of spores in the field or in the greenhouse do not always dominate the AMF community in the field roots. This could be explained because freshly formed spores can often not be readily distinguished from spores formed earlier in the season (Lee and Koske, 1994). Pianka (1970) reported that AMF that sporuLates early in trap cultures could potentially be representatives of the r strategy because they could dominate resource-rich uncolonized habitats in early successional stages of the fungal community. Thus, in the present study, ***Glomus*** also seems to have shown an r-strategy and could be found also in highest proportion in soils coming

from early and Late successional stages. Sýkorová et al. (2007) also found that some ***Glomus*** phylotypes showed an r-strategist behavior, rapidly colonizing ruderal habitats in early successional stages in French grasslands. They also mentioned that although AMF r-strategists typically invest heavily into their reproduction, it is possible that some of them are not prolific sporulators. The k-strategists would follow the opposite strategy showing slow growth rates under resource-limited conditions and occurrence in Late successional areas (Pianka, 1970). In that sense because ***Acaulospora*** increased in soils coming from Intermediate and Late sites (Table 4), it probably followed a k-strategy. However, it is difficult to draw direct conclusions about the life history strategy of the AMF detected by their spores.

It is known that belowground diversity of AMF is a major factor contributing to the maintenance of plant biodiversity and to ecosystem functioning; however, the increase/decrease of SN with plant succession is controversial. In Brazil, Carrenho et al. (2001) developed a plant restoration study along the river Moji-Guaçu, using a mixture of pioneer, secondary (early and Late) and climax plant species. They found that the rhizosphere of pioneer species (***Croton urucurana***) had the lowest number of spores, and this increased progressively until the occurrence of climax species such as ***Genipa americana***. They concluded that the number of AMF spores increases with the stage of succession. Contrarily, Zangaro et al. (2007, 2008) found that fast-growing native woody species in southern Brazil showed higher levels of AMC compared to slow growing ones. The last authors suggested that the occurrence of mycorrhiza and the density of AMF spores decreased along the succession, and it could be probably due to morphological root traits in fast-growing species that favored the contact with AMF propagules in the soil. Additionally, studies by Pagano et al. (2008, 2010) also in semiarid fields of Minas Gerais State, showed higher mycorrhizal sporulation in disturbed sites.

In this study the highest SN was found both at Initial like (Carrenho et al., 2001) and Pasture soils and it was in accordance to the highest mycorrhizal development of roots. Moreover, it was previously showed that ***Braquiaria*** and ***Panicum*** are mycorrhizal plants (Wang and Qiu, 2006) and that AMF enhance the growth and P uptake of these grasses (Siqueira et al., 1990; Roos and Allsopp, 1997).

Recently, Oehl et al. (2010) reported that soil type strongly affect AMF composition and the occurrence of many species. Our results also suggest that the physicochemical characteristics of soils influenced AMF formation more than the successional stages of the sites.

Regardless the origin and consequence of the disturbance (which sometimes can be a consequence of physicochemical differences among sites), ecosystems tend to recover naturally. It is suggested that this adaptation is mediated largely by soil microorganisms whose activity depends on the presence of indigenous AMF (Johansson et al., 2004; Artursson et al., 2006; Jin et al., 2010)

However, unexpected disruptions may occur in natural sites due to human interventions that can also accelerate natural processes of disturbance due to climatic change.

When a soil loses its natural condition (mainly by human action or when agricultural practices are installed) changes in the physical, chemical and biological soil properties undoubtedly occur. According to Lejon et al. (2005) these are the main factors controlling the transformation of organic matter and nutritional status in soils. In this sense, the monitoring of microbial populations (particularly AMF) (Pagano et al., 2011) and the physicochemical properties of soils can be useful tools to determine the state of disturbance in different sites. Studies suggest that establishment and maintenance of plant diversity in communities relies on the presence of a wide diversity of AMF (van der Heijden et al., 1998). Further, plant productivity is in general greater when a large array of AMF is associated with plants. In this regard, our results showed that Intermediate soils showed the lowest plant productivity and it was associated with the lowest mycorrhiza formation. Presumably, each AMF has a degree of specificity with hosts and it is also affected by the environment, resulting in a complex interaction whereby better combinations of fungal populations-plant-environment could contribute to plant growth and environmental sustainability. For a better understanding of the dynamics of AMF communities associated with different stages of environmental disturbance it will be necessary to classify AMF phylotypes according to their life history strategies and to monitor indigenous AMC. The present study provides the first steps in this direction. Our findings also emphasize that, in a short-term greenhouse experiment (3 month), only a certain subset of AMF genera, preferably comprising r-strategists, colonized roots of trap plants. Colonization and spore number appeared to be more reLated to soil physicochemical characteristics and host plant development than soil successional stage. However, mechanisms regulating and maintaining AMF species composition, spore density and fungus infectivity (as a measure of activity) among sites with different successional stages are not well understood. Therefore, more studies need to be aimed to identify involved mechanisms in order to ensure a successful management for the conservation and restoration of our diverse and threatened natural ecosystems.

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Full Length Research Paper

Prevalence of intestinal parasites at Ujjain, Madhya Pradesh, India: Five-year study

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This study was conducted to determine the prevalence of intestinal parasites in hospitals at Ujjain. A total of 5990 samples were collected in five years: 3580 from Ujjain Charitable Trust Hospital (Urban population) and 2410 from R. D. Gardi Medical College (Rural population). Overall prevalence rate of intestinal parasite was 21.4%. *Entamoeba histolytica* (10.5%) was the commonest protozoa followed by *Giardia lamblia* (3.9%). Among the helminths, *Ascaris lumbricoides* (2.8%) was the commonest. Multiple infections were seen in 70 samples. There was difference in prevalence between urban (20.2%) and rural (23.1%) population. Females (27.4%) were more affected than males (18.2%) and age group 0 to 10 years old had the highest rate of infection. The results indicate that there is a need to implement control measures in form of regular deworming, health education and provision of safe water supply.

Key words: Intestinal parasites, prevalence, rural, urban, Ujjain.

INTRODUCTION

Intestinal parasitic infestation represents a large and serious medical and public health problem in developing countries. Risk factors for this high prevalence being low levels of sanitation, lack of safe water supply, poor hygiene, low socio economic status and impoverished health services. Helminths such as ***Ascaris lumbricoides,*** hookworm***, Trichuris trichiuria, Enterobius vermicularis*** and protozoa ***Entamoeba histolytica*** and ***Giardia lamblia*** are some of the common intestinal parasites responsible for considerable morbidity in young and adult population (Koneman et al., 1997). In India, prevalence of intestinal parasites reported from different workers shows wide variations from 11.3 to 90% (Sethi et al., 2000; Rao et al., 2003; Chandrasedhar and Nagesha, 2003; Patel, 1986; Hedge and Patel, 1986) probably due to difference in time, place and method used. Prevalence has been reported in the past by: (a) Population study: giving us the prevalence of different types of parasitic infestation in given population (Kang, 1998) and (b) by analysis of reports of stool samples received at Microbiology laboratory: giving prevalence in symptomatic patients. In present study, stool samples received at Microbiology laboratories of R. D. Gardi Medical College (RDGMC) and Ujjain Charitable Trust Hospital (UCTH), both being part of one establishment, were included. RDGMC being situated at the outskirts of Ujjain city caters mainly rural population and UCTH situated in the heart of city receives urban population. This, thus, is a hospital based study the purpose of which was to obtain information as regards to the frequency of different types of helminthic and protozoal infestation in symptomatic patients. These studies are important as they provide basic data for the control of parasitic infection in future.

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MATERIALS AND METHODS

This study was conducted from January 2006 to December 2010 and includes stool samples of the patients admitted to the wards as well as those attending the outpatient department of the hospital. Stool samples were collected in screw capped, labeled plastic container, which were distributed to patients one day prior to the day of collection. Stool samples were subjected for complete examination – gross and direct microscopic examination

Table 1. Prevalence of intestinal parasites in 5-year period.

Intestinal parasite	2006	2007	2008	2009	2010	Total
I)Protozoa						
E. histolytica	135 (11.8)	123 (10.3)	131 (10.4)	119 (9.9)	126 (10.5)	634 (10.5)
G. lamblia	51 (4.4)	58 (4.8)	48 (3.8)	41 (3.4)	37 (3.0)	235 (3.9)
II) Helminths						
Ascaris	41 (3.5)	37 (3.1)	32 (2.5)	33 (2.7)	30 (2.5)	173 (2.8)
Hookworm	22 (1.9)	26 (2.1)	19 (1.5)	28 (2.3)	29 (2.4)	124 (2.0)
E. vermicularis	10 (0.8)	6 (0.5)	8 (0.6)	3 (0.2)	6 (0.5)	33 (0.5)
H. nana	12 (1.0)	11 (0.9)	10 (0.7)	11 (0.9)	13 (1.0)	57 (0.9)
Taenia spp.	4 (0.3)	3 (0.2)	5 (0.3)	2 (0.1)	3 (0.2)	17 (0.2)
T. trichiura	1 (0.08)	3 (0.2)	2 (0.1)	3 (0.2)	1 (0.08)	10 (0.1)
III) Multiple infection						
Two parasite	14 (1.2)	11 (0.9)	10(0.7)	9 (0.7)	16 (1.3)	60 (1.0)
Three parasite	3 (0.2)	0 (0.0)	1(0.07)	4 (0.3)	2 (0.1)	10 (0.1)
Total +ve/ Total sample examined = %	276/1141 = 24.1%	267/1192 = 22.3%	255/1256 =20.3%	240/1202 = 19.9%	245/1199 = 20.4%	1283/5990 = 21.4%

Figure in parenthesis indicates percentage.

(saline and iodine preparation) within 1 to 2 h of its collection. Negative samples were re-examined after formal ether concentration method. Protozoa and helminthes were identified according to morphological details (Garcia, 1999).

RESULTS

The distribution of eight different parasites identified among hospital patients is shown in Table 1. Out of 5,990 stool samples examined, 1283 (21.4%) were positive for one or more intestinal parasites. The overall prevalence of intestinal parasite showed gradual decline from 2006 (24.1%) to 2009 (19.9%) with slight increase in 2010 (20.4%). The protozoal infection was two times more common than helminthic infection. ***E. histolytica*** (10.5%), ***G. lamblia*** (3.9%) were the predominant parasite followed by ***A. lumbricoides*** (2.8%) and Hookworm (2.0%). Of all processed samples, 70 samples showed multiple infections. The prevalence of double infection being 1% and that of triple infection being 0.1%. Samples collected from UCTH (3580) were considered to represent urban population and those from RDGMC (2410) as to rural population as shown in Table 2. Higher prevalence was noted in rural population (23.1%) in comparison with urban population (20.2%).

Table 3 indicates that the rate of infection was higher in females (27.4%) than in males (18.2%). In age group distribution, most of the infected cases (24.0%) were in 1 to 10 year's group. No significant difference was noted in season wise distribution of intestinal parasite.

DISCUSSION

The prevalence of intestinal parasite in this study was 21.4% which is less when compared with the findings of Rao et al. (2003) (59.5%),

Table 2. Prevalence of intestinal parasites in urban and rural positives.

Intestinal parasites	Urban (n = 3580)	Rural (n = 2410)	Total
(i) Protozoa			
E. histolytica	357 (49.3)	297 (53.1)	634
G. lamblia	140 (19.3)	75 (13.4)	235
(ii) Helminths			
Ascaris	104 (14.3)	69 (12.3)	173
Hookworm	56 (7.7)	68 (12.1)	124
E. vermicularis	22 (3.0)	11 (1.9)	33
H. nana	29 (4.0)	28 (5.0)	57
Taenia spp.	10 (1.3)	7 (1.2)	17
T. trichiura	6 (0.8)	4 (0.7)	10
Total	724 (20.2)	559 (23.1)	1283

Figure in parenthesis indicates percentage.

Table 3. Prevalence of intestinal parasite by sex and age.

Parameter	Total no. of samples	Positive samples (%)
(i) Sex		
Male	3908	712 (18.2)
Female	2082	571 (27.4)
Total	5990	1283 (21.4)
(ii) Age group (years)		
1-10	2086	502 (24.0)
11-20	1008	195 (19.3)
21-30	940	207 (22.0)
31-40	995	189 (18.9)
> 40	961	190 (19.7)
Total	5990	1283 (21.4)

Figure in parenthesis indicates percentage

Chandrashekar and Nagesha (2003) (68%), Patel (1986) (75%), Hedge and Patel (1986) (90.6%) and Kang et al. (1998) (97.4%). But this finding is in agreement with reports of Vidyarthi (1969) (26.7%) and Chandrashekar et al. (2005) (21.3%).

The prevalence of protozoal infection was higher than that of helminthic parasites and ***E. histolytica*** was the commonest intestinal parasite isolated which is comparable to the study from Bombay (Patel, 1986). High prevalence has also been noted from Malaysia (21%) (Nor et al., 2003). Other studies (Chandrashekar et al., 2005; Nagaraj et al., 2004), however, have reported Giardia to be the commonest parasite, which appeared to occur in low percentage of patients (3.9%) here.

Among the helminthic parasite ***A. lumbricoides*** was the commonest; findings being in confirmation with the observations made by several other workers (Chandrasedhar and Nagesha, 2003; Patel, 1986; Hedge and Patel, 1986; Nagaraj et al., 2004). The prevalence of hookworm in this area seems to be much lower than that reported from Southern India where it is 61.5% (Kang, 1998). Prevalence of other helminths such as, ***Taenia*** spp., ***E. vermicularis, Hymenolepsis nana and Trichuris trichiura*** was quite low (<1%).

Prevalence rate was also higher in rural population, which is in concordance with studies from Karnataka (Chandrasedhar and Nagesha, 2003) and Western Nepal (Chandrashekar et al., 2005). This could be attributed to

poor environment and low socioeconomic condition prevailing in rural areas.

Our result showed high infection among females. However, the sex predominance for parasite infection is still not confirmed. Some report higher rate in males (Sethi et al., 2000; Rao et al., 2003) and some in females (Ali et al., 1999; Yong et al., 2000). The others reported similar rate in both sexes (Patel, 1986; Hedge and Patel, 1986). The infection may relate to the daily activity of the patients rather than sex. Concerning the relation of age group and parasite infection, our study revealed the high infection rate in age group of 1 to 10 years which might be due to high exposure of children to contaminated surrounding.

To conclude, parasitic diseases are still common and responsible for mild but chronic morbidity. To alleviate this prevailing health problem of the country, it requires multidisciplinary effort. Health facilities should be improved and provision made for adequate and safe water supply. Also, there is need for health programmes to be held regularly that will involve periodic deworming, health education concentrating on teaching the most elementary but important sanitary procedures. Regular surveys regarding the prevalence of intestinal parasites in hospitals and communities should be encouraged as these surveys not only give an estimate of prevalence of particular parasite, but also serve as an index of the communities' progress towards effective sanitation.

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Full Length Research Paper

Nematicidal activity of *Gymnoascus reesii* against *Meloidogyne incognita*

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In the present investigation, antagonistic effect of culture filtrates of *Gymnoascus reesii* at different concentrations were tested on eggs and juveniles of root knot nematode *(Meloidogyne incogint*a), *in vitro*. The percentage mortality and inhibition of hatching of root-knot nematode were directly proportional to the concentration of culture filtrates of *G. reesii*. Compared with the nematicides treatments of Cadusafos and Avermectins, the effects of higher concentration of culture filtrates of *G. reesii* were in some cases not statistically different. To our knowledge, this is the first report of culture filtrates of *G. reesii* observed to have nematicidal activity toward root-knot nematode and a nematicidal metabolite (3E,5E)-2,5-dihydroxy-2,7-dihydrooxepine-3-carboxylic anhydride was isolated based on bioassay-guided fractionation from the extracts of the fungus *G. reessii*.**

Key words: *Gymnoascus reesii*, nematicidal activity, *Meloidogyne incoginta.*

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) cause high levels of economic loss in a multitude of agricultural crops worldwide. They are capable of severely damaging a wide range of crops, in particular vegetables, causing dramatic yield losses mainly in tropical and sub-tropical agriculture (Sikora and Fernandez, 2005). For several decades the use of chemical nematicides is one of the primary means of control for root-knot nematodes. However, the potential negative impact on environment and ineffectiveness after prolonged use have led to a total ban or restricted use of most nematicides and an urgent need for safe and more effective options (Zuckerman and Esnard, 1994).

Biological control promises to be such an option. Application of microorganisms antagonistic to *Meloidogyne* spp. or compounds produced by these microbes could provide additional opportunity for managing the damage caused by root-knot nematodes. Research in this area has resulted in commercial biocontrol preparations reported to act against root-knot nematodes (Stirling, 1991; Fravel, 2000).

Over the last decades, research on root-knot nematode control was focused on proposing strategies for inhibition of egg hatch (Westcott and Kluepfel, 1993), degradation of hatching factor (Oostendrop and Sikora, 1989) or production of metabolites (Meadows et al., 1989). In particular, the search for nematode-antagonistic compounds from culture filtrates of fungi has greatly intensified in recent years because of the large number of toxins and potential new drugs among fungal metabolites. Number of fungi isolates from nematodes, soil and plants were proved to produce substances that inhibit nematode egg hatch or kill nematodes (Nitao et al., 1999). Some of these fungi produce toxic metabolites in culture filtrates (Khan and Saxena, 1997).

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For years, our group has been seeking nematode-antagonistic compounds from fungal metabolites. These studies have included screening the culture broths of 1,248 fungi isolated from soil for activity against *Meloidogyne hapla* (Liu et al., 2004). One fungal isolate that exhibited high nematicidal activity in these experiments was identified as *Gymnoascus reesii* Baran. Nematicidal potential of *G. reesii* Baran has remained unexplored except for few reports, which suggest that the culture filtrate of *G. reesii* displayed significant activity against the nematode *Haemonchus contortus*. However, there are no reports of nematicidal activity against *Meloidogyne* spp. in *G. reesii*.

The objective of this study was to evaluate the toxic activity of culture filtrate of our isolate of *G. reesii* against *M. incognita* eggs and second-stage juveniles (J2) *in vitro*.

MATERIALS AND METHODS

Fungal culture filtrates

The fungus was isolated using a soil dilution plate method directly from soil collected from the suburb of Beijing city, China and the isolate was identified to species as *G. reesii* Baran. A culture is maintained in the Institute of Microbiology, Chinese Academy of Sciences, China as isolate Za-130 (CGMCC No.2632). One-week-old *G. reesii* cultures on CMA plates (60 × 15 mm) were homogenized into Czapek-Dox broth medium (1 CMA plate/500 ml flask containing 125 ml Czapek-Dox broth) and incubated at 28°C on a shaker (180 rpm) for 5 days. After incubation, the culture broth was centrifuged at 13,700 g for 20 min, and the supernatant was sequentially passed through syringe filters designed for viscous samples containing particulates (GD/X series 1.0 µm GF/B and 0.45 µm GMF filters, Whatman, Clifton, NJ) and then sterile-filtered (GD/X sterile 0.2 µm PES filter, Whatman). The filtrates were used at concentrations of 1× solution (the original filtrate preparation) and diluted to -5× (1 part filtrate to 4 parts water), -10× (1 part filtrate to 9 parts water) by sterile water and condensed to +5× solution (a solution 5 times as concentrated as the 1x solution and +10× (a solution 10 times as concentrated as the 1 × solution) in vacuum at 40°C using a rotary evaporator.

Eggs and J2

Meloidogyne incognita was cultured in the greenhouse on tomato plants (New L-402, nematode susceptible) inoculated with a single nematode egg mass (Khan et al., 2005). After 55 days, egg masses were handpicked from galls of tomato roots and surface sterilized in 0.5% sodium hypochlorite for 3 min and washed with sterile water 3 times. J2 were hatched from the egg masses, collected daily and stored at 4°C. Eggs were extracted by the NaOCl technique (Coolen and D' Herde, 1972). Eggs were poured on a 75 µm-pore sieve and collected on 5 µm-pore sieve. The extracted eggs were gently washed with tap water to remove sodium hypochlorite (Oka and Yermiyahu, 2002; Nico et al., 2004).

Effect on egg mass hatch

Two sterilized healthy egg masses of nearly uniform size were transferred to a 6 cm-diameter autoclaved Petri dishes containing 3 ml filtrate of different dilutions. Egg masses placed in sterilized distilled water and 10 µl/ml Avermectins (1.8% avermectins emulsifiable manufactured by Hebei Veyong Bio-chemical Co. LTD, China) and 10 µg/ml Cadusafos (10% cadusafos granular manufactured by FMC Corporation, USA) served as three controls; three replicates of each treatment and control were included. Plate lids were sealed with parafilm and the plates were kept at 25°C. After 7 days hatched J2 were counted with the use of an inverted microscope and percentages of cumulative hatch and relative suppression of hatch rate were calculated.

Effect on individual egg hatch

One milliliter of egg suspension (containing about 250 eggs/ml) was combined with 2 ml of filtrate of different concentraions in 6 cm-diameter autoclaved Petri dishes. Eggs in sterilized distilled water and 10 µl/ml Avermectins and 10 µg/ml Cadusafos served as three controls. Treatments and controls were 3 replicates. Plate lids were sealed with Parafilm and the plates were kept at 25°C. After 7 days hatched J2 were counted with the use of an inverted microscope and percentages of cumulative hatch and relative suppression hatch rate were calculated.

Effect on J2

To determine the effects of culture filtrates on J2 of *M. incognita* 1 ml filtrates of different concentrations were transferred to wells of 24-well tissue culture plates to which about 100 J2 was added. Sterilized distilled water and 10 µl/ml Avermectins and 10 µg/ml Cadusafos again served as three controls. Three replicates were used per treatment. After 24 h at 25°C, the dead J2 were counted under an inverted microscope and the corrected mortality was calculated. Nematode mortality was confirmed after lack of movement to distilled water and observation after an additional 72 h (San Martin and Magnunacelaya, 2005). Mortality data was corrected for corrected mortality mortality following Abbot (1925). The experiment was repeated, and the results of the repeated trial were combined for analysis.

Extraction, isolation and identification of secondary metabolites of *G. reesii*

After incubation, the cultures were centrifuged, and the broth was filtered through a glass-filter funnel. The broth filtrate was extracted by mixing for 2 h with Amberlite XAD-16 resin equilibrated in H_2O (1 volume gel-6 volumes broth). In latter study, bioassay-guide was used throughout the isolation process. The resin was removed from the broth, washed with 3 bed volumes H_2O, and eluted with 3 bed volumes methanol (MeOH).

The MeOH eluate was evaporated under vacuum, and the residue was partitioned in 30% MeOH in H_2O and chloroform ($CHCl_3$).The chloroform fraction was evaporated to dryness and fractionated by Silica gel CC using chloroform-MeOH gradient elution to yield fraction A_1-A_5 according to thin layer chromatography (TLC) analysis. The active fraction A_2, obtained on elution with chloroform/ MeOH 90%, was further purified by recycling preparative high-performance liquid chromatography (HPLC) (Japan Analytical Industry Co., Ltd) eluted with MeOH/ H_2O (3:7,v/v) to furnish compound.

The structure of compound isolated from the cultures of *G. reesii* was determined by spectroscopic analysis. Infrared (IR) spectra was obtained in KBr pellets with a ATR, TENSOR27 spectrophotometer (ATR, Bruker, Karlsruhe, German). MS was performed on a Bruker APEX IV FTMS mass spectrometer (APEX, Bruker, Karlsruhe, German) spectrometers. The nuclear magnetic resonance (NMR) spectra were recorded on DRX-600 NMR

(Bruker, Karlsruhe, Germany) with TMS as an internal standard and coupling constants were represented in Hertz.

Statistical analyses

The experiment was repeated. A completely randomized design was used. Stat.10 for Windows (SPSS Inc. 2000: SPSS Base 10.1 User's Guide: SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Duncan's New multiple range test was employed to test for significant difference between treatments at P = 0.05 and means were compared with the Least Significant Differences (LSD) test (P =0.05).

RESULTS

Effect on egg mass hatch

The hatchability test (Table 1) indicated that culture filtrates of *G. reesii* strongly suppressed hatching of *M. incognita* egg masses. The percentage reduction ranged from 22.19 to 99.45%. The different concentrations of the culture filtrate exhibited significant differences compared to the water control; the culture filtrates at the original, +5× and +10× concentrations had no significant difference when compared to the Cadusafos and Avermectins controls (Table 1).

Effect on individual egg hatch

The culture filtrate of *G. reesii* also strongly decreased individual egg hatching of *M. incognita* (Table 1); hatching was suppressed by 8.02 to 97.18%. The difference between all filtrate concentrations except the -10× solution and the water control was significant; the relative suppression rate of the Cadusafos and Avemectins controls significantly differed from those of the filtrate treatments except at the +5× and +10× concentrations.

Effect on J2

All *G. reesii* filtrate concentrations exhibited nematicidal effects of various degrees on J2 of *M. incognita* (Table 1). The percentage mortality was directly proportional to the filtrate concentration. The corrected mortality of all treatments showed significance compared with the water control. The effect of culture filtrates at +10× concentration on percentage mortality significantly differed from the effects of the Cadusafos and Avermectins.

Extraction, isolation and identification of secondary metabolites of *G. reesii*

Based on bioassay-guided isolation, an active compound was obtained from the extracts of the fungus *G. reesii*. The EIMS of compound showed a molecular ion peak at m/z (100) 349.05248 $[M+Na]^+$, and the molecular formula was determined to be $C_{14}H_{14}O_9$ by MS and elemental analysis. The IR spectrum showed the absorption for carboxyl and hydroxyl (3360 cm^{-1}), unsaturation hydrocarbon (3124, 3096, 3067 cm^{-1}), saturation hydrocarbon (3956, 2910, 2868 cm^{-1}), carboxyl (1743 cm^{-1}), C=C double bond (1674, 1627 cm^{-1}), saturation hydrogen carbonide (1449, 1424, 1366 cm^{-1}). The NMR spectral data of the compound are presented in Tables 2 and the assignation was based in correlation experiments. Thus, the compound was identified as (3E,5E)-2,5-dihydroxy-2,7-dihydrooxepine-3-carboxylic anhydride.

DISCUSSION

Fungal natural products are very promising potential sources of new chemicals to manage plant-parasitic nematodes (Anke and Sterner, 1997). Culture filtrates of many fungi possess activity against nematodes, and the nematicidal action of these culture filtrates may involve the production of toxic metabolites by the fungi (Caroppo et al., 1990; Singh et al., 1991; Hallmann, 1996; Nitao et al., 1999; Miyako et al., 2000, 2003; Satoshi et al., 2004; Yoshinori et al., 2004; Dong et al., 2005, Heydari et al., 2006; Guohong et al., 2007; Asami et al., 2007; Ting et al., 2008; Lin et al., 2009).

Species of *Aspergillus, Penicillium, Trichoderma, Fusarium, Paecilomyces* and *Alternaria* are known to produce toxins and antibiotics like aflatoxin, penicillin virdin, fusaric acid, lilacin, and phyto-alternarin (Nafe-Roth, 1972; Arai et al., 1973; Wheeler, 1975; Ghewande et al., 1984). Adverse effect of the culture filtrates of several fungi on hatching and mortality of root-knot nematodes has been reported by others also (Mankau, 1969; Shukla and Swarup, 1971; Khan et al., 1984; Mani and Sethi, 1984; James et al., 1999, 2001; Meyer et al., 2004; Man-Hong et al., 2006; Sahebani, 2008).

In vitro results indicated that exposure to the culture filtrate of *G. reesii* reduced the ability of *M. incognita* eggs to hatch and induce mortality in J2 to a varying degree depending on the culture filtrate concentration. Our results indicated that nematicidal potential of the culture filtrate of *G. reesii* at higher concentrations than lower. The filtrate inhibited egg mass hatching greater than individual eggs; for example, the inhibition of egg masses and individual egg hatching of the 1× concentration were 97.74 and 73.94%, respectively. Therefore, this concentration could provide good control at the egg mass stage. Our results also indicated that the +5× and +10× concentrations showed great antagonistic activity towards J2 of *M. incognita* and that the relativity mortality rate of the +5× concentration (85.6%) and +10× concentration (98.2%) were both higher than those of the Cadusafos and Avermectins controls', indicating the possible implication of this biocontrol fungus as a new tool for an management program for the root-knot nematode.

Table 1. Effect of culture filtrate of *G. reessii* at different concentrations on hatching of eggs in egg masses, individual eggs and juveniles of of *M. incognita.*

Treatments	Mortality of juveniles (%)	Corrected mortality (%)	The number of hatched juveniles of individual eggs	Relative suppression rate (%)	The number of hatched juveniles of eggs in egg masses	Relative suppression rate (%)
-10×	38.6fF	35.6	65.3aA	8.02	182.3bB	22.19
-5×	60.4eE	58.5	47bB	33.80	154.7bB	33.97
Original filtrate	74.3dD	73.1	18.7cC	73.94	5.3cC	97.74
+5×	85.6bB	84.9	5dD	92.96	2.0cC	99.15
+10×	98.2aA	98.1	2dD	97.18	1.3cC	99.45
Water (ck1)	4.6gG	——	71aA	——	234.3aA	——
Cadusafos (ck2)	82.3bcBC	81.4	3.5dD	95.07	2.3cC	99.02
Avermectins (ck3)	77.4cdCD	76.3	3.3dD	95.35	2.7cC	98.85
LSD(p=0.05)	7.28		3.00		33.66	

Table 2. The ^{13}C, ^{1}H and HMBC spectrometry number of compound isolated from *G. reessii*.

Position	δ_C	δ_H	HMBC
1	59.06(s)	4.29,d,4.55,d	(5.96)
2	88.58(s)	5.88,d	4.29, 4.55, 5.96
3	108.58(d)	5.95,s	(4.29), 4.55
4	109.78(d)	5.96,s	(5.88)
5	146.45(d)		4.29, 4.55, 5.88, 5.96
6	152.08(d)		5.96
7	169.64(s)		5.95

Fungal antibiotics and other toxic compound present in metabolites might be responsible for the inhibition of egg hatch and J2 mortality. As *G. reesii* produces secondary metabolites such as Polyenylpyrroles and Polyenylfurans (Benjamin et al., 2006), aromatic butenolides (Ben et al., 2005) and Roquefortine E (Ben et al., 2005).

According to Benjamin et al. (2005, 2009), *G. reesii* displayed significant growth inhibitory activity against the bacterium *Bacillus subtilis*, the nematode *Haemoncs contortus*, the plant fungal pathogen *Septoria nodorum*, and a tumor cell line (murine NS-1). However, there is no report of nematicidal activity against the plant-parasitic nematode by *G. reesii.*

In summary, this is the first study of the nematicidal activity of culture filtrate of *G. reesii* on *M. incognita* and a compound was also isolated according to its nematicidal activity. We believe *G. reesii* is a new biological control factor that may be a potentially good source of a microbial nematicide that can be harnessed for successful nematode control.

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Full Length Research Paper

Determining the knowledge of food safety and purchasing behavior of the consumers living in Turkey and Kazakhstan

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Complete and balanced nutrition with reliable food consists of the basis of health and protective health services. Therefore, the current study was carried out to determine the knowledge of food safety level and purchasing behavior of 668 consumers living both in Turkey (n=348) and in Kazakhstan (n=320) and to compare the results. Volunteered consumers for the research were given a face to face interview between March and September 2010. It was found that the knowledge of purchasing behavior (14.43±2.56) of food safety (20.82±4.20) of the consumers living in Turkey was higher compared to those living in Kazakhstan (11.84±2.92, 14.74±3.86 respectively) and that the difference between the two countries was statistically significant (p<0.01). In addition, a positive correlation was found between knowledge of food safety and purchasing behavior (r=0.541, p< 0.01); age and purchasing behavior (r=0.325, p< 0.01) and knowledge score of food safety (r=0.148, p< 0.01).

Key words: Purchasing behavior, knowledge of food safety, consumer, Turkey, Kazakhstan.

INTRODUCTION

Food-borne diseases constitute a common public health at a global scale. Every year, millions of people worldwide die and many are hospitalized from foodborne diseases and illnesses as a result of consumption of contaminated food (Knight et al., 2003).

World governments concentrate their efforts on improving food safety, in order to promptly and properly respond to the increasing types and incidents of food-borne diseases. Food-borne infections are placed in the core of primary community health concerns, by both advanced and developing countries of the world (Ba¯, 2004; Eren, 2007). While it is hard to predict the actual number of incidents of food-borne diseases, it is a known fact that many lives were lost to diarrhea caused by food and water-borne microbiological agents, tolling around 1.8 million minors during 1998 and 2.1 million people, during 2000, in the developing world (except China). In industrial states of the world, on the other hand, it is stated that every one individual in a group of three is affected by food-borne diseases each year and almost 30% of the population in advanced countries are presented with food-borne diseases (Ba¯, 2004). In the US, approximately 76 million incidents of food-borne diseases are reported to take place in average during any year, where 325,000 people are institutionalized, and 5,000 ending up dead (Mead et al., 1999; WHO, 2002). There have been 29,901 cases of Salmonella paratyphii infection, 21,068 cases of dysentery and 8,824 cases of Hepatitis-A infections in 2004, Turkey, according to the data supplied by the Ministry of Health. Data available on food-borne diseases and food poisoning fail to reflect the actual situation, as there is not any statutory requirement in effect, for the reporting of food-borne or related diseases, in Turkey (Sanlier, 2009). Research made on recorded incidents of food poisoning among the consumer public in Kazakhstan, revealed no relevant data.

The economic outcomes of food contamination and food-borne diseases are presumed to be in a range of 3.3 to 12 million dollars for the US, as attributable to pathogens, generating some 6.5 to 35 billion dollars cost for the central government, on an annual basis, as a result of food-borne diseases, during 1995. The five

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major food-borne epidemics that occurred in England and Wales in 1996 were predicted to cost 300 to 700 million pounds sterling, including medical treatment costs and claims associated with deaths throughout these disasters. Predictions state that every 1 out of 10 persons in the UK or 1 out of 12 people in the US suffer from food-borne diseases each year, entailing to dramatic financial troubles (Redmond and Griffith, 2003). The predicted annual cost of 11,500-days of food poisoning cases for Australia has been calculated to be 2.6 million Australian Dollars (WHO, 2002). The customers represent the final link in the food safety chain. The purchasing power and level of awareness of the consumers is an important factor for ensuring food safety (Alpuğuz et al., 2009). The poor hygienic treatment of food during storage, processing and preparation may help creation of an environment suitable for bacterial growth, including the fast and easy spreader species such as Campylobacter, Salmonella and other infectious agents (Ba¯ et al., 2006). Many people are poisoned from day to day, for consuming food produced in non-hygienic environments, lacking sufficient knowledge or training on hygiene, using unclean water or due to inefficient storage conditions, lack of cleaning or mixing of chemicals with foodstuffs (Sanlier, 2009).

Food can be mishandled at many places during food preparation, handling and storage and several studies indicate that consumers have inadequate knowledge about procedures needed to prevent foodborne illnesses at home (Mederios et al., 2001; Meer and Misner, 2000; Redmond and Griffith, 2003; Woodburn and Raab, 1997). The prevention of foodborne illnesses requires educating food consumers on safe food handling practices (Jevsnik, Hlebec and Raspor, 2008). However, prior to education, it is important to assess food safety issues relevant to consumers. It has been demonstrated that level of education affects the level of knowledge or awareness in any casual consumer, in combination with age, sex and level of income (Angelillo et al., 2000; Redmond and Grifith, 2003; Bermudez-Millan et al., 2004; Mitkakis et al., 2004; Röhr et al., 2005; Sanlier, 2009; Sanlier, 2010). A majority of the consumers in Netherlands have been revealed to perceive the expiry dates marked on product labeling as the storage time for food, but having no idea of the fact that such dates become ineffective, once the product's package is actually opened. It has also been observed that respondents with kids of four or lower age were more careful and attentive on food product inserts than older consumers, who preferred to follow their experience patterns when storing food presenting no or little knowledge about the storage conditions of or the newly emerging products. There was a great gap in knowledge among respondents, on methods for storing food (Terpstra et al., 2005).

Varying demographies and life styles entail to situations that make life threatening, great epidemics out of food-borne diseases, in combination with the extraordinarily dangerous species of microorganisms and highly resistant bacteria (Haapala and Probart, 2004).

The increasing need for education on food safety has just recently been noticed in the US and EU, with the early sparkles of national initiatives aimed at effectively educating the young consumers and especially the potential food preparers of the future. Consequently, the need becomes eminent in this conjuncture, for education. There is benefit in expanding the outreach of consumer educations to cover wider communities through mass media, common public and formal education starting at early childhood. It is among the fundamental duties of the government to safeguard social wealth, improve and maintain high levels of health conditions, ensure full public access to healthier and high quality foodstuffs and retain comprehensive control of food from production stage to consumption by the end user, in order to ensure physically sound and mentally healthy newer generations (Anonymous, 2001).

Besides, there is not any public authority vested with the power and responsibility to carry out the controls regarding food safety, despite the lack of legislative arrangements to govern the issue, which is alarming, in both Turkey and Kazakhstan. Therefore, this study intends to demonstrate what attitudes are adopted by consumers living both in Turkey and Kazakhstan, from different cultural and educational backgrounds, in time of purchasing, as well as their levels of knowledge on and practical use of food safety.

MATERALS AND METHODS

This study was performed between March and September 2010 on a total of 668 individuals from Turkey (348) and Kazakhstan (320), consisting of 310 males and 358 females, who had shown full consent to attend it on a voluntary basis, to compare the purchasing behaviors and levels of knowledge on food safety, in both countries. The respondents were given a short brief on the subject and purpose of this study and general rules to follow, at the beginning. Survey forms prepared for the purpose were effectively used by the authors themselves through face-to-face dialogs. The average age for respondents from Turkey and Kazakhstan were 32.87 ± 9.60 years and 27.72 ± 10.96 years, respectively.

Instrumentation

There are 30 questions aimed at determining the level of knowledge in respondents on food safety and 20 expressions intended to identify their purchasing behaviors, on scale put up by the researchers, utilizing related articles (Haapala and Probart, 2003; Unusan, 2007; Sanlier, 2009). A pilot study has been performed on a group of 50 consumers, to check whether the questions on the scale were understood or not, and the forms were then reviewed and revised, with minor changes made in unclear questions. The answers given to questions relating to food safety and purchasing behaviors were evaluated as true and false. Scoring has been made so that a "True" answer would yield one point while a "False" one return "0" point. The information questions about food safety were evaluated in a score range of 0-30, while statements concerning purchasing behaviors covered a range of

Table 1. Demographic ınformatıon of participants.

Demographic characteristics		Turkey		Kazakhstan	
		n	%	n	%
Gender	Male	167	48.0	143	44.7
	Female	181	52.0	177	55.3
Marital status	Single	129	37.1	181	56.6
	Married	219	62.9	139	43.4
Education	Primary education	48	13.8	33	10.3
	High school	117	33.6	110	34.4
	Associate degree	43	12.4	106	33.1
	Undergraduate	99	28.4	71	22.2
	Postgraduate	41	11.8	-	-

0-20. Furthermore, the survey form was checked for reliability, as a result of which, the cronbach alpha values were found to be 0.73 on the purchasing behavior scale and 0.79 on knowledge on food safety scale.

Data analysis

The data thus obtained were evaluated using the SPSS 13.0 statistical calculations software bundle. For each answer provided to food safety knowledge and purchasing behavior inquiries, the responses given by the consumers are broken down in a table both in numbers and percentage, and comparisons made based on countries employed the x^2 test technique. The total scores were then calculated on both the food safety knowledge and purchasing behavior scales, which were subsequently subjected to comparisons between the two countries using the Student t test, with given arithmetical means ($\bar{}$) and standard deviation (SD) values. Also, the food safety knowledge scores, purchasing behavior scores and ages of consumers were correlated to study the relationships in between, while Pearson correlation factor (r) was used to determine the direction and level of the relations. The evaluations made took statistical significance level as 0.05 and 0.01.

RESULTS

Total of 348 Turkish respondents with the percentage of 48.0% male, %62.9 were married and 33.6% high school graduates while the Kazakh respondents were found to be 55.3% females, 56.6% singles and 34.4% high school graduates (Table 1).

Generally speaking, the true rates in answers provided by Turkish consumers to questions on purchasing behaviors were higher than those provided by their Kazakh peers ad there was a statistically significant difference between the true answering rates based on each country ($p<0.01$, $p<0.05$). An investigation of the true answering rates for statements on purchasing behavior immediately revealed that 98.3% of the Turkish consumers said "I check the product package for soundness", 98.0% said "I look at the expiry dates on labels when purchasing products", 97.7% said "I check the cleanliness of the store or sales point where it purchase my food", 96.0% said "I check the confirmation seal of a veterinary body when buying meat", 93.4% said "No additives in foodstuffs, that is what matters", 92.5% said "I totally reject and return a product which I later discover to be defective", 92.0% said "I check if the product I bought has any adverse affects on human health", 88.8% said "I check if the product package is made of materials which would not harm or damage the contained food product", 85.9% said "I strictly follow the instructions printed on the label when storing or cooking the product", 84.8% said "I read the label information provided on packages, before I buy foodstuffs", 83.9% said "I can comfortably consume any product regardless of where and how they were prepared and whether they are hygienic or not" 81.0 % said "I am ready to pay more for food products grown without the use of agricultural growth hormones", 80.2% said "Food should have good nutritional qualities, before good taste", and finally 76.1% said "I always take into account the nutritional value when I purchase food products". The above rates for Kazakh consumers have been 94.1, 88.8, 85.3, 83.8, 57.2, 78.4, 71.9, 75.6, 79.7, 73.1, 58.1, 50.0, 61.6 and 65.9%, respectively. There has been a statistically significant difference between the rates of accuracy of both country's in correctly identifying the true answer in statements on purchasing behavior ($p<0.01$, $p<0.05$), (Table 2).

However, the true answering rates of consumers of both countries for certain statements relating to purchasing behavior were found to be considerably low. The Turkish consumers performed low and returned less correct answers to the statements "Food sold in hypermarkets and big shopping malls are of high quality" by 43.4%, "Ads give all what we need to know about the product" by 40.8%, "Brands always contain high quality stuff" by 36.2%, "Food with higher nutritional qualities are

Table 2. The distribution of consumers' food purchasing behavior.

Statements	Turkey (n=348)		Kazakhstan (n=320)		P
	n	%	n	%	
I check the product package for soundness.	342	98.3	301	94.1	0.004**
I look at the expiry dates on labels when purchasing products.	341	98.0	284	88.8	0.000**
I check the cleanliness of the store or sales point where it purchase my food.	340	97.7	273	85.3	0.000**
I check the confirmation seal of a veterinary body when buying meat.	334	96.0	268	83.8	0.000**
No additives in foodstuffs, that is what matters.	325	93.4	183	57.2	0.000**
I totally reject and return a product which I later discover to be defective.	322	92.5	251	78.4	0.000**
I check if the product I bought has any adverse affects on human health.	320	92.0	230	71.9	0.000**
I check if the product package is made of materials which would not harm or damage the contained food product.	309	88.8	242	75.6	0.000**
I strictly follow the instructions printed on the label when storing or cooking the product.	299	85.9	255	79.7	0.032*
I read the label information provided on packages, before I buy foodstuffs.	295	84.8	234	73.1	0.000**
I can comfortably consume any product regardless of where and how they were prepared and whether they are hygienic or not.	292	83.9	186	58.1	0.000**
I am ready to pay more for food products grown without the use of agricultural growth hormones.	282	81.0	160	50.0	0.000**
Food should have good nutritional qualities, before good taste.	279	80.2	197	61.6	0.000**
I always take into account the nutritional value when I purchase food products.	265	76.1	211	65.9	0.004**
Food sold in hypermarkets and big shopping malls are of high quality.	151	43.4	112	35.0	0.027*
Ads give all what we need to know about the product.	142	40.8	84	26.3	0.000**
Brands always contain high quality stuff.	126	36.2	101	31.6	0.205
Food with higher nutritional qualities are always more expensive.	108	31.0	67	20.9	0.003**
The promotional stuff (gifts) given with foodstuffs influence my purchasing decisions.	105	30.2	90	28.1	0.561
The price is what drives my decision on which foodstuff to purchase.	45	12.9	60	18.8	0.039*

*p < 0.05; ** p < 0.01.

always more expensive" by 31.0%, "The promotional stuff (gifts) given with foodstuffs influence my purchasing decisions" by 30.2% and "The price is what drives my decision on which foodstuff to purchase" by 12.9%. The same situation is also true for Kazakh consumers. Their true answer ratings to the above statements were found to be 35.0, 26.3, 31.6, 20.9, 28.1 and 18.8%, respectively (Table 2).

Basing on the results obtained from Table 3, only 4 out of a total of 30 statements concerned with food safety have been found to have no significance in statistical terms, between the consumers of the two countries (p >0.05). A majority (95.4%) of Turkish respondents correctly affirmed the statement "Surfaces to be used for preparation of foodstuffs should be cleaned before operation", while only a few (30.7%) could have managed to give a true answer to the statement "Milk sold on streets may only be used after treatment with heat for half an hour". For the Kazakh side, a majority (78.4%) of the consumers correctly identified the statements "Peelable fruit and vegetables should be flushed with fresh running water" while only a few (12.8%) made the correct point about the statement "Leftovers should be put inside the fridge in no later than two hours of consumption".

The true answering rates of Turkish resident consumers to questions regarding food safety were found to be higher than Kazakh consumers. For instance, Turkish consumers correctly affirmed the statements "Surfaces to be used for preparation of foodstuffs should be cleaned before operation" (95.4%), "Peelable fruit and vegetables should be flushed with fresh running water" (93.7%), "Poultry like chicken, turkey and etc. should be washed before being cooked" (93.1%) "Hands are sources of contamination for food-borne diseases" (92.2%), "Hands contain the most intense populations of microorganisms in a body" (89.1%), "The bacteria passing to the food from the hands may create harmful toxins in the food" (86.5%), "Raw food and cooked food should be stored separately" (85.1%), "Thawed meat should not be frozen again" (83.6%), "Food containing cans with lumps and protrusions are inconvenient for use" (83.0%), and "Canned food may be stored in

Table 3. Distribution of correct answer on the food safety knowledge guestionnaire.

Statements concerning food safety	Turkey (n=348)		Kazakhstan (n=320)		P
	Number	%	Number	%	
Surfaces to be used for preparation of foodstuffs should be cleaned before operation.	332	95.4	238	74.4	0.000**
Peelable fruit and vegetables should be flushed with fresh running water.	326	93.7	251	78.4	0.000**
Poultry like chicken, turkey and etc. should be washed before being cooked.	324	93.1	246	76.9	0.000**
Hands are sources of contamination for food-borne diseases.	321	92.2	249	77.8	0.000**
Hands contain the most intense populations of microorganisms in a body.	310	89.1	206	64.4	0.000**
The bacteria passing to the food from the hands may create harmful toxins in the food.	301	86.5	184	57.5	0.000**
Raw food and cooked food should be stored separately.	296	85.1	231	72.2	0.000**
Thawed meat should not be frozen again.	291	83.6	138	43.1	0.000**
Food containing cans with lumps and protrusions are inconvenient for use.	289	83.0	220	68.8	0.000**
Canned food may be stored in shelves of their original warehouses	277	79.6	190	59.4	0.000**
Perishable foods in a short span of time should be put inside the fridge within two hours after the purchase	275	79.0	212	66.3	0.000**
Cooked foods should be left at room temperature until cool	273	78.4	229	71.6	0.004**
A food suspected of being corrupted can be used again after boiling.	265	76.1	145	45.3	0.000**
Raw chicken, fish and meat should not contact each other.	264	75.9	221	69.1	0.050
Leftovers should be put inside the fridge in no later than two hours of consumption.	259	74.4	41	12.8	0.000**
Food appearance is more important than hygiene.	258	74.1	75	23.4	0.000**
Food freezing process doesn't kill bacteria, it only stops their reproduction.	252	72.4	157	49.1	0.000**
Raw eggs or foods that contain raw eggs can be dangerous.	232	66.7	171	53.4	0.000**
There isn't an inconvenience to the use of cracked or broken eggs.	222	63.8	145	45.3	0.000**
Hard boiled egg can be kept at room temperature for more than two days.	218	62.6	169	52.8	0.011*
Pasteurized milk can be stored in the fridge temperature for a maximum of 3 days in its unopened box.	213	61.2	178	55.6	0.144
Saliva contaminates the air and food while blow cigarette smoke.	209	60.1	190	59.4	0.857
Internal temperature of chicken should be high for safe cooking.	206	59.2	56	17.5	0.000**
After touching raw food, you can not touch cooked food.	178	51.1	153	47.8	0.389
It should be taste milk rather than look at its expiry date to check if it is safe or not.	177	50.9	81	25.3	0.000**
Wiping the used surfaces of a meat cutting board right after use with a piece of paper towel would prevent bacterial growth before the board can be used for cutting any other food product.	152	43.7	75	23.4	0.000**
Food can be checked for taste to determine whether it is safe or not	151	43.4	60	18.8	0.000**
Frozen meat can be thawed over counter-central heating.	145	41.7	83	25.9	0.000**
A wiping cloth can be used as a cleaning material when preparing meals.	123	35.3	56	17.5	0.000**
Milk sold on streets may only be used after treatment with heat for half an hour.	107	30.7	68	21.3	0.005**

* $p < 0.05$ ** $p < 0.01$.

shelves of their original warehouses" (79.6%). The true answering rates for the above questions, of Kazakh consumers have been 74.4, 78.4, 76.9, 77.8, 64.4, 57.5, 72.2, 43.1, 68.8 and 59.4%, respectively. There has been a statistically significant difference between the rates of accuracy of both country's in correctly identifying the true answer to above statements on food safety ($p<0.01$), (Table 3).

Some of the statements on food safety were correctly answered by the consumers from both countries by less than 50%. While Turkish consumers correctly assessed the statements "Wiping the used surfaces of a meat cutting board right after use with a piece of paper towel would prevent bacterial growth before the board can be used for cutting any other food product" (43.7), "Food can be checked for taste to determine whether it is safe or

Table 4. Knowledge of food safety and purchasing behavior score according to countries.

Variables		Mean	Standard deviation	t test	P value
Purchasing behavior scores	Turkey (n=348)	14.43	2.56	12.197	0.000**
	Kazakhstan (n=320)	11.84	2.92		
Food safety knowledge scores	Turkey (n=348)	20.82	4.20	19.400	0.000**
	Kazakhstan (n=320)	14.74	3.86		

** p < 0.01.

Table 5. Correlation between age, participant scores of food safety knowledge and purchasing behavior (r).

Variables	Purchasing behavior scores	Food safety knowledge scores
Food safety knowledge scores	0.541**	
Age	0.325**	0.148**

** p < 0.01.

not" (43.4%), "Frozen meat can be thawed over counter-central heating" (41.7%), "A wiping cloth can be used as a cleaning material when preparing meals" (35.3%) and "Milk sold on streets may only be used after treatment with heat for half an hour" (30.7%) the Kazakh side's rate of accuracy in providing the right answers have been 23.4, 18.8, 25.9, 17.5 and 21.3%, respectively. There has been a statistically significant difference between the rates of accuracy of both country's in correctly identifying the true answer to above statements on food safety ($p<0.01$), (Table 3).

While the Turkish consumers scored 14.43 ± 2.56 for purchasing behavior and 20.82 ± 14.74 for food safety knowledge tests, their Kazakh peers performed 11.84 ± 2.92 and 14.74 ± 3.86, respectively. The difference between the two study groups were found to be statistically significant ($p<0.01$).

Finally, the purchasing behavior score of the consumers were analyzed as compared to their food safety knowledge scores and relations between their ages, and the resultant findings compiled into Table 5.

A positive and statistically significant correlation ($r=0.148$, $p<0.01$) was found to exist between the food safety knowledge and purchasing behavior scores ($r=0.541$, $p<0.01$), ages and purchasing behaviors ($r=0.325$, $p<0.01$) and food safety knowledge scores and ages of the consumers.

DISCUSSION

When consumers purchase foodstuffs, they guide the way in which the food safety system operates to the extent of the selectivity and rationalism reflected by their attitudes. In addition, they demand all standards-compliant, reliable, healthy and inexpensive food items and thereby ensure that food production plants and outlets operate in compliance with applicable laws on food, international norms and standards. Aware consumers also set the quality of food inspection ad controls conducted by the government to protect them. Consumers group after becoming aware individuals to form into non-governmental organizations to enforce and ensure the effective operation of the food safety system, while pressing the government to enact laws for the protection of consumer rights (Dağ and Merdol Kutluay, 1999).

Albayrak (2000) and Kucukkose (2002) found that consumers mostly check the product expiry dates, production dates and overall packing of foodstuffs, whether the packages are recyclable or not, type and quality of the material in which they are manufactured, their suitability for containing food and the state of soundness they present. Kolodinsky et al. (2008) observed that price is the topmost motivator of food purchasing behaviors and that the energy, nutritional elements and especially the amount of fat in the food as stated on product label have more or less influence on the choices of consumers. Alpoguz et al. (2009) have found in a study they performed on students that the students would never regard whether the expiry dates are overdue or packages are opened, when they buy foodstuffs, however, almost half of the youth never read information provided on product labels when purchasing packed food. Another study conducted in Italy showed that the relatively expensive sale prices of vegetables and fruit grown through organic farming methods influence the will to buy, in the consuming public, to purchase such products, due to low income levels (Boccaletti and Nardella, 2000). The contemporary changes in the areas of education, communication and technology also reflect on purchasing behaviors among

the consumer public, changing their nutritional habits and cultures as a result of changes in the social culture caused by globalization (Öztop and Babaoğul, 2004). The dazzling urbanization rates, vast diversification of products, ads communicated through mass media, rise in the per capita average income and women's integration into the business life affect the perspective and perception of food products in the consumer and therefore the purchasing behaviors. A consumer check of the food product in time of buying is essential for protecting the health of the consumer, while preventing him or her from being deceived economically. This study has revealed the need on the part of consumers living in both countries for having access to educational facilities to improve their inefficient purchasing behaviors in a more cautious manner, despite the fact that Turkish consumers appear to be more aware about the food purchasing behaviors (Table 2).

Lack of food safety entails to territorial and global problems. Food-borne diseases are frequently seen and reported in almost any country whether advanced or underdeveloped, although they differ more or less from one country to another, depending on social life styles and economic conditions (Unusan, 2007; Sanlier, 2009). It is crucial that conditions of hygiene are ensured in all processes through production to customer offering of foodstuffs, while keeping the consumer public well informed about the supply and use of safe food. Therefore, the accessibility of food should be handled as one and common concern with all its integrity, and the entire process from production to marketing through the distribution network should be brought under permanent control (Anonymous, 2001). The urgent need for protecting and preserving the health of consumer in terms of balanced and sufficient food consumption, which is a critical factor in people's gaining and retaining the ability to live, raise and age completely free of any immediate threats of diseases by consuming reliable (healthy) and quality food products and protection against all kinds of deceit when purchasing food highlight to the significance of the matter (Trepka et al., 2006).

Roseman and Kurzynske, in a study they performed recently (2006), found that age, sex, income and educational levels all influence the food safety knowledge and behaviors of the consumers. Other studies performed show that more information and higher perception is possessed in women then men (Bruhn and Schutz, 1999; Bryd-Bredbenner et al., 2008) and in adults than youth (Sanlier, 2009) in terms of food safety. Another study demonstrates that there is insufficient knowledge among the consumer public on food-borne diseases, hand-washing routines, purchasing food, separating raw and cooked food, thawing and cooling of frozen food and consumption of raw eggs and therefore, the obvious need for consumers to undertake education on food safety (Surujlal and Badrie, 2004). It has been reported in a study conducted with the aim to determine knowledge, attitudes and behaviors on food-borne diseases and food processing practices of Italian Mothers, that 36.0% of the moms studied knew or heard about pathogenic microorganisms. It was also observed that level of educatedness is an indicator of this knowledge and older and more educated women among the respondents have shown a positive attitude and approach to food-borne diseases at a high degree (Angelillo et al., 2001). In another study examining the food safety knowledge and attitudes of consumers, it was clearly shown that a majority of consumers were lacking any information about typhoid, gastro-intestinal inflammation and amebiasis, despite being knowledgeable in such food-borne diseases as cholera, food poisoning and jaundice (Sanlier et al., 2010). In a further study performed on US consumers, it was found tat consumers were especially clueless about microorganisms that cause food-borne diseases and foodstuffs being under threat of these microorganisms (Wilcock et al., 2004).

A recent study attempted to assess the level of knowledge in 904 consumers on food preparation and storage techniques both before and after a one week long education, using the survey method. The resultant findings revealed that knowledge of consumers were incomplete and faulty for the most part, while the rate of wrong information dropped after the education. For example, while only 31.7% of the respondents revealed knowledge of the fact that fridge temperature should be maintained in a range of 0 to 40°C, this rate grown to 78.4% after education. Besides, the numbers of people who had stored raw meat and cooked food in a wrong way in their refrigerators were declined to 63 and 65%, from a baseline of 144 and 133, before the education (Ghebrehewet and Stevenson, 2003). As this study clearly suggests education on food safety has a great influence on the consumer. Earlier studies also demonstrated the need in consumers for education on food safety (Bruhn and Schutz, 1999, Wilcock et al., 2004; Medeiros et al., 2004; Ba¯ et al., 2006; Unusan, 2007). Most of the consumers in Italy recognize ***Staphylococcus Aureus*** (92.9%) and ***Colostridium botulinum*** (87.5%) as food-borne pathogens. A 53% of the consumers believe that instant food would elevate he risk for food poisoning. The ratio of people knowing the requirement to separate raw food from cooked ones to those not knowing is 84.6 %. A 90.4% of the consumers know that thawed food should never be frozen again (Angelillo et al., 2001). In another study, knowledge of Turkish consumers about meat purchase, storage, preparation, cooking and serving in the domestic kitchen were investigated and it was found that many individuals failed to store meat at the correct temperature or did not defrost meat correctly. It was also reported that food handling practices differed according to socioeconomic group and the level of education of the consumers were noted (Karabudak, Bas and Kızıltan, 2008). In addition

to the survey studies concerning food safety, there have been also some observation based studies, where people are found to not follow many food safety rules when preparing meals. A 97% of the individuals volunteering the study has indicated that they would wash their hands with soap under running water, before preparing food. A 89% of the individuals who stated that meat cutting boards should be washed through with flushing water and soap, although only 60% were putting this practice in everyday life (Bermudez-Millan et al., 2004). A study conducted in the US showed that although 86% of the consumer public are aware of the fact that hand-washing practice prevent food poisoning, only 66% actually washed their hands and only after touching raw meat and poultry flesh (Wilcock et al., 2004).

At the end of this study, it was found that Turkish consumers had better levels of knowledge and information about food safety than their counterparts in Kazakhstan, but they still were below the sufficient levels (Tables 3 to 5). Although food safety lyes within the common authoritative and responsibility frames of the government, the food industry and the consumer, greater burden falls upon the government as the ultimate body responsible for setting and enforcing legal arrangements covering the food sector (Soydal, 1999). Governments have to establish an environment that, in addition to ensuring social, political and economic stability and justice, would bring peace and develop appropriate policies accordingly. With a global view, active cooperation seems a must between world governments inter alias and with UN institutions, financial institutions, intergovernmental organizations and non-governmental organizations to ensure food safety for all (Özel, 2003). The first measure to take and initial step toward performing a risks analysis in the field of food safety should be to educate consuming public on food safety. Savvy consumers present a motivating power for producers and industrialists in producing safe foodstuffs and for the government in establishing wide and effective control over food. Not only the food producers but also the food industrialists should assume offering safe food to consumer public as a social liability.

Misinformation of the public on food safety should be prevented. Professionals scientists and media should assume responsibility for this matter. The results obtained from the present study brings highlight to the importance of education once again, for which reason, there is a felt need to educate the consuming public on food safety. The data gathered from this study have revealed that there is an urgent need for food safety education in this target group. An effective food safety education program should cover information concerning temperature control of food, proper food preparation practices, prevention of cross contamination, suitable clean up procedures, causative foodborne illness agents, high risk groups, and other contributing factors to foodborne diseases and prevention strategies (Osaili et al., 2011). However, means should be provided to help seeding messages that any food safety education program would deliver in the minds of the consumers.

Following its completion, the education instructions should be repeated at regular intervals to ensure that knowledge learned throughout the classes entail to attitude and attitude results in behavior, with assurance of the continuity of education through surveillance controls. It is of common belief and opinion of the authors of this study that common research and studies to be performed through increasing cooperation between Turkey and Kazakhstan, two countries with a common past and culture would contribute much to raising public awareness. In the meanwhile, proper inclusion should be given to ensuring food safety in action plans, inter sectoral cooperation should be developed between the industries of both countries and efforts to be pursued in that context should gain effectiveness and speed in both states.

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Full Length Research Paper

Molecular characterization of norovirus from acute gastroenteritis patients in Malaysia

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Rotavirus and recently norovirus have been described as important and most common cause of acute gastroenteritis in children. The mode of transmission is fecal-oral. Diagnosis of both of these viruses can be made by rapid antigen detection of the viruses in stool specimens and strains can be further characterized by enzyme immunoassay or reverse transcriptase polymerase chain reaction. Stool specimens collected from various hospitals in Malaysia were examined for norovirus by both immunochromatography and reverse-transcription polymerase chain reaction techniques. Rotavirus antigen was screened by a commercially available latex agglutination test kit. Altogether, 168 stool samples were collected for both norovirus and rotavirus screening. Out of these, 77/168 (45.8%) were examined for rotavirus with 17/77 (22%) rotavirus antigen positive. Due to very small amount of fecal materials obtained in some cases, only 151/168 (89.8%) were sufficient for norovirus and 14/151 (9.3%) were positive for norovirus genogroup II (GII). This study highlights that rotavirus remains the main agent for acute gastroenteritis and identification for emerging norovirus among the children is becoming important for proper patient management.

Key words: Norovirus, rotavirus, immunochromatography, latex agglutination, RT-PCR, phylogenetic analysis.

INTRODUCTION

Noroviruses (NoV) are the worldwide major cause of viral gastroenteritis in human (Marshall et al., 2003). They belong to the ***Norovirus*** genus of the ***Caliciviridae*** family, which also includes the ***Sapovirus*** genus. NoV which is also called small round structured viruses (SRSVs) are non-enveloped, single-stranded positive sense RNA viruses (Frankhauser et al., 2002). The primary route of NoV transmission is through the fecal-oral route (Alice et al., 2004). Primary infection results from either the consumption of focal contaminated food or water (Rabanaeu et al., 2003). Currently, at least five NoV genogroups (GI, GII, GIII, GIV and GV) are being reported with GI, GII and GIII commonly found in humans and GII the most predominant genogroup causing worldwide acute gastroenteritis infecting persons of all age groups (Frankhauser et al., 2002; Alice et al., 2004; Kirkwood and Bishop, 2001). Outbreaks of gastroenteritis in USA showed that GII strains were the predominant type accounting to 73% in all outbreaks (Frankhauser et al., 2002), 94% of positive specimens in children hospitalized with acute gastroenteritis in Australia (Kirkwood and Bishop, 2001) and 64% in Thailand (Guntapong et al., 2004).

Rotavirus (RV) forms the genus Rotavirus of the ***Reoviridae*** family which are often associated with children under the age of 5 years, (Parashar et al., 2006), whilst NoV is common in the adults and older children (Kirkwood and Bishop, 2001). However, in Malaysia, the aetiology and frequency of viral gastroenteritis due to NoV is still poorly investigated and understood unlike the vast studies already reported on rotavirus (Zuridah et al., 2010; Yap et al., 1998; Rasool et al., 1993). The aim of our study was to detect for both RV and NoV and further characterization of NoV for epidemiological purposes.

MATERIALS AND METHODS

Stool samples were obtained from 8 different hospitals in Malaysia. These samples were tested for RV antigen using the Rotalex kit

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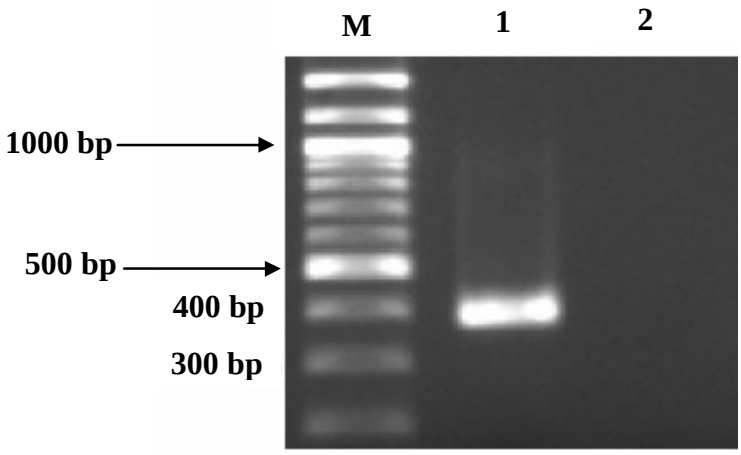

Figure 1. Detection of NoV GII gene from stool samples. M: marker (100 bp ladder); amplicons of NoV GII positive (Lane 1); negative control (Lane 2); PCR product for NoV GII: 379 bp.

(Orion Diagnostica, Finland). For NoV, antigens were screened by immunochromatography (IC) test (The RIDA® QUICK Norovirus, R-Biopharm, Germany) and ELISA (The RIDASCREEN® Norovirus, R-Biopharm, Germany). The IC test is based on the principle that it uses specific monoclonal antibodies directed against NoV capsid protein. Results were interpreted visually after 15 min.

The ELISA is based on monoclonal antibodies bound on the surface of the microwell strips to capture the antigen in stool specimens and samples were assayed accordingly (R-Biopharm, 2006).

For RNA extraction, NoV ssRNA was treated with Ultraspec^{TM}-II RNA isolation system (BioTecx/USA). The eluted ssRNA were then transferred to new clean sterile tubes and frozen until further use (BioTecx/USA). Next, the extracted ssRNA was used as the template for reverse transcription (RT) (Qiagen, Germany protocol). Two microlitre RNA template was added to a 20 µl reaction mixture containing a final concentration of 10X reverse transcription buffer, 2.0 µl of 5 mM dNTPs mixture, 2.0 µl of 10 µM random primers, 1 µl Sensiscript RT, 2 µl of RNase-free water and 10 units/µl RNase inhibitor (Fermentas) and incubated at 37°C for 60 min using the random primers (Qiagen, 2004).

Further amplification was performed using primers NV_OF2 and NV_OR (Bull et al., 2005). Five microlitre of cDNA was added to 20 µl of a PCR mixture containing 2.0 µl of 10X PCR buffer (Fermentas), 1.2 µl of 25 mM $MgCl_2$, 0.4 µl of 10 mM dNTPs mix (Promega), 0.24 µl of 50 µM each primer, and 0.4 µl of 2.5 U ***Taq*** polymerase (Fermentas). Pre-denaturation was carried out at 95°C for 5 min followed by 40 cycles of PCR (95°C for 30 s, 55°C for another 30 s, and 72°C for another final 60 s). Electrophoresis of the amplicons was carried out in 1% agarose gel with 1X TBE buffer with a 100 bp ladder (Fermentas) included.

For phylogenetic studies, all the 14 positive amplicons coded as C7, C4, C11, C12, C14, D1, D3, D6, D7, E1, E2, E8, and E9 were sent to an independent laboratory. The nucleotide sequences obtained were then analyzed using the public database (BLAST algorithm) and an alignment was made with the ClustalW algorithm.

RESULTS

Of the 168 specimens, 77 samples (45.8%) that were screened for RV antigen were from patients less than 5 years old by latex agglutination test. For NoV, not all the 168 samples were screened for NoV. Only 151 samples were screened, of which 14/151 (9.3%) samples were positive.

The amplicons with 379 bp size were confirmed to be NoV genogroup II (GII) (Figure 1). The sequences were compared against the GenBank database using the BLAST programme. Phylogenetic analysis showed that the NoV analyzed was grouped into 2 distinct genotypes GII/4 and GII/3 (Figure 2). GII/4 was the most predominant genotype, accounting for 71.4% (10 of 14), and followed by 29% (4/14) GII/3. All the samples were more than 95% similar to other strains found in Asian countries, especially in Japan. NoV GII gene of patients C7, C4, C11, C12, C14, D1, D3, D6, D7, E1 and E2 were clustered in the same branch with those in Japan (GII/4Nagano). Meanwhile, samples from patients E8 and E9 were clustered in the same branch with strains in Argentina (GII/3Arg320/95/AR).

DISCUSSION

This study describes the incidence and genetic diversity of NoV in diarrheal patients in Malaysia. The incidence found in this study (9.3%) which confirms that infection rates in our study population are comparable to Thailand, Taiwan and Vietnam (14, 8.2, and 5.4%, respectively) (Hansman et al., 2004b; Chen et al., 2007; Hansman et al., 2004a).

The predominant rotavirus infection (22%) was in concordance with previous investigations on the viral etiology of pediatric gastroenteritis (Zuridah et al., 2010; Chen et al., 2007; Zuridah et al., 2009; Nguyen et al., 2007). However, due to sampling volumes, no mixed infections were investigated in this study. This should be pursued in future because many studies have reported

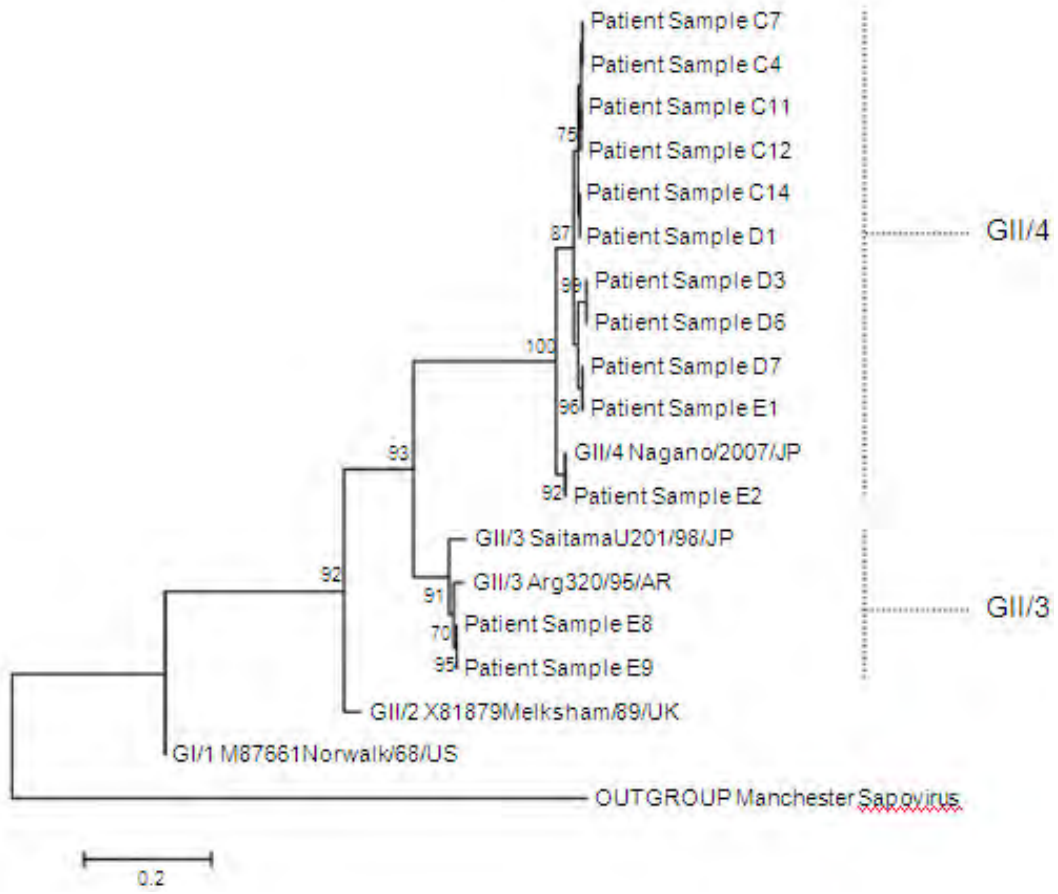

Figure 2. Phylogenetic analysis of NoV local strains (labeled as C7, C4, C11, C12, C14, D1, D3, D6, D7, E1, E2, E8 and E9) and other global strains GII-4 Nagano/2007/JP from Japan, GII-3Saitama from Japan, GII-3Arg from Argentina, GII-2X81879 Melksham from UK, GI/1M87661Norwalk from USA. OUTGROUP Manchester Sapovirus is used as an outgroup control.

that children are vulnerable to several viruses at the same time (Yap et al., 1998). Besides norovirus and rotavirus, adenovirus and astrovirus were also detected in small percentage (Tran et al., 2010).

To our knowledge, this is the first preliminary report on NoV infection in the hospitalized patients in Malaysia. Genetic diversity was observed, encompassing 2 genotypes, namely GII/3 and GII/4 with GII/4 the most prevalent in concordance to other reports worldwide (Nguyen et al., 2007; Bucardo et al., 2008).

However, in Japan, a change in distribution of predominant NoV genotypes, GII/4 to GII/3 in 2003 to 2004 and GII/3 to again GII/4 in 2004 to 2005 was observed in a surveillance conducted over a three year period (Phan et al., 2006a; Phan et al., 2006b). Therefore, continuous monitoring of NoV genetic property in Malaysian population is important for updating and comparing global trends of NoV infection. Great caution is needed in interpreting this result; moreover the prevalence strain cannot be concluded as final due to the small sample size analyzed thus this study warrants further investigation. In conclusion, this study concurs with other reports which indicate that NoV is another important enteric virus that can cause gastroenteritis.

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Full Length Research Paper

Diversity of yeasts involved in the fermentation of tchoukoutou, an opaque sorghum beer from Benin

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Opaque sorghum beers are traditional alcoholic beverages in several African countries. Known as *tchoukoutou* in Benin, the beer is often obtained from an uncontrolled fermentation. It is consumed in an actively fermenting state and has a sour taste. The present study characterized and identified the yeasts involved in the fermentation process of this type of beer using the phenotypical approach. Of 12 beers from 4 different locations, the mean values of the pH, titratable acidity, dry matter content and refractive index were respectively 3.67, 0.70 (% as lactic acid) 18.08% and 7.00. Lactic acid bacteria and yeasts were the predominant microorganisms involved in the fermentation of *tchoukoutou*. Their counts were respectively 9.1 log cfu/ml and 9.1 logcfu/g. Enterobacteriaceae were not detectable in the beer. Based on the phenotypic characters and the assimilation profiles of 40 isolated yeasts, four genera with seven species of yeasts were identified. The yeast species predominant in the Benin opaque sorghum beer tchoukoutou was *Saccharomyces cerevisae*.

Key words: Sorghum, beer, *tchoukoutou, Saccharomyces cerevisae*, yeast,

INTRODUCTION

Traditional alcoholic beverages are obtained in sub-Saharan Africa from carbohydrate-rich products (Sefa-Dedeh et al., 1999). Opaque sorghum beers are popular alcoholic beverages in Africa. The fermentation is often spontaneous and uncontrolled. The beverages often consumed in an actively fermenting state have short shelf-lives (Odunfa, 1985). In the West Africa region they are known as *tchoukoutou* in Benin, *dolo* in Burkina-Faso, *pito* in Ghana, and *burukutu* or *otika* in Nigeria (Odunfa, 1985; Glover et al., 2005; Kayodé et al., 2005). The beers have a sour taste, a relatively high dry matter content (5-13 g 100 ml^{-1}) and low alcohol content (2-3 ml 100 ml^{-1}), which make them suitable beverages for adults and teenagers (Agu and Palmer, 1998; Briggs et al., 2004). They are largely consumed by the poorest people and significantly contribute to the diet of millions of consumers. The beers are mostly prepared with Guinea corn (*Sorghum bicolor*) but other cereals such as millet or maize can be used as adjunct or as substitutes (Kayode et al., 2005). The manufacturing process consists of malting (soaking, germination, sun drying), brewing (mashing, boiling, filtration) and fermentation (Haggblade and Holzapfel, 1989). Depending on the

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geographic location, variations may occur in the process (Odunfa, 1985; Haggblade and Holzapfel, 1989).

Lactic acid bacteria and yeasts were identified during the fermentation of several cereal products in Africa (Jespersen et al., 1994; Muyanja et al., 2002; Mugula et al., 2003; Vieira-Dalodé et al., 2007; Muyanja et al., 2010). The yeast species most often reported in African traditional food and beverages belong to the species *Saccharomyces cerevisiae.* (Jespersen, 2002). Few studies investigated the micro-organisms involved in African opaque beers. The microorganisms isolated from the beer consist essentially of yeasts and lactic acid bacteria (LAB) (Van der Aa Kühle et al., 2001; Demuyakor and Ohta, 1991; Sefa-Deheh et al., 1999; Sanni and Lönner, 1993; Faparusi et al., 1973). However, the species isolated and their frequency distributions in the product vary according to the regional location. *Saccharomyces cerevisiae* (33%), *Kluyeromyces* spp, *Candida* spp., and *Torulaspora delbrueckii* are predominant yeasts in Ghanaian opaque beer (Demuyakor and Ohta, 1991; Sefa-Deheh et al., 1999). In Burukutu Nigeria, Sanni and Lönner (1993) found the predominance of Candida spp., *Geotrichum candidum*, *S. cerevisiae, Kloeckera apiculata* or *Torulaspora delbrueckii*. Van der Aa Kühle et al. (2001) demonstrated the predominance of *Saccharomyces* spp (of which 45% were identified as *Saccharomyces cerevisiae*) in the fermentation of sorghum beer from Ghana and Burkina-Faso. Very limited information exists on the micro-biological attributes of the Beninese opaque beers. The objectives of this study are two-fold. Firstly, to characterise the Benin opaque sorghum beer in terms of its physical and microbiological attributes. Secondly, to characterise and identify the yeasts involved in the fermentation of the beer.

MATERIALS AND METHODS

Sampling

Samples of actively fermenting (about 12 h of fermentation) opaque sorghum beer were collected from twelve commercial processing sites in northern Benin. The processors (one per site) were selected on the basis of their rich beer brewing tradition. The samples were collected in screw-capped bottles, packed in an insulated icebox, transported to the laboratory and analysed immediately for microbiological analysis (Hounhouigan et al., 1993).

Physico-chemical analysis

Dry matter was determined according to American Association of Cereal Chemistry (AACC) methods (AACC, 1984). Titratable acidity pH and were determined as described by Nout et al. (1989). The refractive index was measured using a refracto meter (Sopelem 9596, France).

Enumeration of micro organisms

Duplicate aliquots of *tchoukoutou* (10 ml) were diluted in 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, and 1000 ml distilled water, pH = 7.0) and homogenised with a Stomacher lab-blender (type 400, London, UK). Decimal dilutions were plated. Total counts of aerobic mesophilic bacteria (TC), lactic acid bacteria (LAB), yeasts and Enterobacteriaceae were enumerated as described by Hounhouigan et al. (1993).

Identification of yeast

For identification of yeasts, isolates from three representative production sites were purified by successive sub-culturing on malt extract agar (MEA, CM 59, Oxoid). Preliminary confirmation was based on microscopic observation. The isolates were tested for the fermentation of sucrose, lactose, glucose and raffinose, as well as the assimilation of selected nitrogen sources i.e. nitrate, ethylamine, L-lysine, cadaverine, and creatine. The assimilation of carbon sources was performed using API 20 C AUXstrips (BioMérieux, Lyon, France) according to the manufacturer's instructions. The Diazonium Blue B reaction, a test to differentiate between ascomycetous and basidiomycetous yeasts, was performed as described by Kurtzman et al. (2003).

Data analysis

Mean values and standard deviation are reported. The data were analysed using the statistical program SPSS 11.0. The on-line available software (http:www.cbs.knaw.nl) of Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands was used for identification of yeasts.

RESULTS AND DISCUSSION

Physico-chemical characteristics of the Benin opaque beer

The mean value of the pH was 3.67 and the titratable acidity of the beer averaged to 0.7 (% as lactic acid). The dry mater content of the beer is high but variable (15-20%) and averaged to 18.1%. The refractive index averaged to 7.0 (Table 1). It has been demonstrated that at pH < 4.0 in food products, the growth of diarrhea causing pathogens is inhibited (Motarjemi and Nout, 1996). Thus it can be expected that the *tchoukoutou* beer is inherently safe from a microbiological point of view. The Benin opaque beer resembles *pito,* the Ghanaian opaque beer, in terms of acidity content (Sefa-Dedeh et al., 1999) but its refractive index is higher.

Microbial content

The mean counts of lactic acid bacteria and yeasts were respectively 9.1 and 9.1 log cfu/ml. The Enterobacteriaceae were not detectable in the beer (log cfu/ml< 1) (Table 2). The yeast concentration in *tchoukoutou* is relatively higher than reported in *pito* beer (Glover et al., 2005) but close to values reported for *burukutu*, another opaque sorghum beer from Nigeria

Table 1. Physico-chemical characteristics of the Benin opaque sorghum beer *tchoukoutou.*

Sample origin	pH	Titratable acidity (%lactic acid)	Dry matter (%)	Refractive index
Tchaourou (n=3)	3.5±0.19a	0.8±0.3a	20.2±2.03a	5.0±2a
Parakou (n=3)	3.7±0.26a	0.6±0.3ab	18.1±2.37a	10.0±2b
Perere (n=3)	3.6±0.12a	0.8±0.2a	15.4±1.95ab	5.0±1a
N'dali (n=3)	3.8±0.22a	0.6±0.03ab	18.6±4.39a	8.0±1ab
Mean	3.7	0.7	18.1	7.0
CV (%)	3.5	16.5	11.1	35.0

Table 2. Microbiological characteristics of the Benin traditional opaque sorghum beer *tchoukoutou.*

Sample origin (Northern Est of Benin)	Lactic acid bacteria (log cfu/g)	Yeasts (log cfu/g)	Total mesophilic aerobic bacteria (log cfu/g)	Enterobacteriaceae (log cfu/g)
Tchaourou (n=3)	9.5	9.3	9.9	< 1
Parakou (n=3)	8.6	8.9	9.0	< 1
Perere (n=3)	9.6	9.1	9.7	< 1
N'dali (n=3)	8.6	8.9	9.6	< 1
Mean	9.1	9.1	9.67	-
CV(%)	6.2	2.2	4.3	-

(Faparusi et al., 1973). Indeed, lactic acid bacteria and yeasts are the predominant microorganisms involved in most African fermented beverages and food (Odunfa, 1985; Mugula et al., 2003; Muyanja et al., 2002).

Phenotypic characters of yeast isolates

After preliminary microscopic confirmation all 40 yeasts were subjected to morphology, fermentation and assimilation tests. None of the isolates could ferment lactose whereas the majority fermented glucose (100%), sucrose (95%) and raffinose (90%). Only 5% of the isolates assimilated nitrate, ethylamine or creatine, whereas 15% assimilated L-lysine. All isolates were ascomycetous yeasts as revealed by Diazonium Blue B test (Table 3). On the basis of their fermentation profile and their nitrogen assimilation pattern the 40 yeasts could be grouped into 5 distinct clusters with the majority (63%) present in one group which showed a metabolism profile typical of *Saccharomyces* spp.

Assimilation profile and identification of yeasts isolates

Based on their assimilation of carbon compounds, sixteen assimilation profiles were distinguished. All yeasts assimilated glucose and maltose (100%), 42.5% assimilated sucrose, 23% assimilated raffinose and only 2.5% could assimilate trehalose. None of them assimilated arabinose, sorbitol and methyl- D-glucopyranoside (Table 4). On the basis of their phenotypic characteristics, the 40 yeasts were found to belong to four genera and seven species of yeast (Figure 1). Clearly, *Saccharomyces cerevisiae* (68%) predominates in the Benin opaque sorghum beer. Our result ressembles findings by Konlani et al. (1996) who reported a prevalence of 55-90% for *S. cerevisiae* in sorghum beer from Togo and Burkina-Faso, two regions close to our study area. Van der Aa Kühle et al., 2001 also identified a large number (45%) of the yeasts involved in the fermentation of opaque beers from Burkina-Faso and Ghana as *S. cerevisiae.* From a study carried out on *pito* in Northern Ghana, Glover et al. (2005) identified 72% of 247 isolates as *S. cerevisiae* based on their assimilation profiles. Twenty seven percent of isolates had narrow assimilation profiles atypical of the specie and could not be clearly identified.

For an isolate to be accepted as *S. cerevisiae* it must be able to assimilate glucose, sucrose, maltose, trehalose, raffinose and ethanol (Vaughan-Martini and Martini, 1998). In the present study, even though many isolates could not assimilate all of these sugars, they were identified as *S. cerevisiae.* Van der Aa Kühle et al. (2001) and Demuyakor and Ohta (1991) also identified many isolates from Ghanaian and Burkina-Faso sorghum beers as *S. cerevisiae,* even though these microorganisms showed carbon assimilation profiles different from the taxonomical key proposed by Vaughan-Martini and Martini (1998). Like in our result, many of the isolates analysed by *these* authors were not able to assimilate sucrose, raffinose and trehalose.

Table 3. Phenotypic characters of yeasts isolated from *tchoukoutou.*

Cluster	Isolates Nr	Fermentation				Assimilation of nitrogen source					DBB test[2]
		Glu[1]	Lac	Suc	Raf	Nit	Eth	Lys	Cad	Crt	
I	26, 28, 11, 12, 22, 37, 24, 34, 8, 15, 16, 19, 17, 20, 35, 2, 9, 38, 39, 7, 10, 21, 40, 31, 3	+	-	+	-	-	-	-	-	-	-
II	4, 13, 32, 27,33, 29, 14	+	-	+	+	-	-	-	-	-	-
III	25, 30, 5, 6	+	-	+	-	-	-	+	-	-	-
IV	1, 18	+	-	-	+	-	-	-	-	-	-
V	23, 36	+	-	+	+	+	+	+	-	+	-
Frequency (%)		100	0	95	90	5	5	15	0	5	0

[1] Glu = glucose, Lac = lactose, Suc = sucrose, Raf = rafinose, Nit = nitrate, Eth = ethylamine, Lys = L-lysine, Cad = cadaverine, Crt = creatine [2] DBB= diazonium Blue B.

Table 4. Assimilation profiles of yeasts isolated from *tchoukoutou.*

	a*	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	Total (%)
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Glycerol	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	5
Calcium 2-keto-Gluconate	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	2.5
L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
D-xylose	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	10
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	2.5
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
D-galactose	+	+	-	+	-	+	+	+	-	-	+	+	+	+	+	+	67.5
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	2
D-sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Methyl- D-Glucopyranoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
N-acetyl-glucosamine	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	2.5
D-cellobiose	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	2.5
D-lactose	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	2.5
D-maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
Sucrose	+	+	-	+	-	-	-	+	+	+	-	+	+	-	+	+	57.5
D-trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	2.5
D-melezitose	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	5
D-raffinose	-	+	-	-	-	-	-	+	-	-	-	+	+	-	-	+	22.5
Nb of isolate (%)	15	12.5	12.5	12.5	7.5	5	5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	

*a = isolates 11, 12, 22, 26, 28, 37, b = isolates 8, 24, 27, 33, 34, c = isolates 10, 15, 16, 18, 19, d = isolates 17, 20,25, 30, 35,e = isolates 4, 13, 32, f = isolates 2, 9, g = isolates 31, 36, h = isolate 23, i = isolate 1, j = isolate 14, k = isolate 5, l = isolate 7, m = isolate 6, n = isolate 21, o = isolate 40, p = isolate 3.

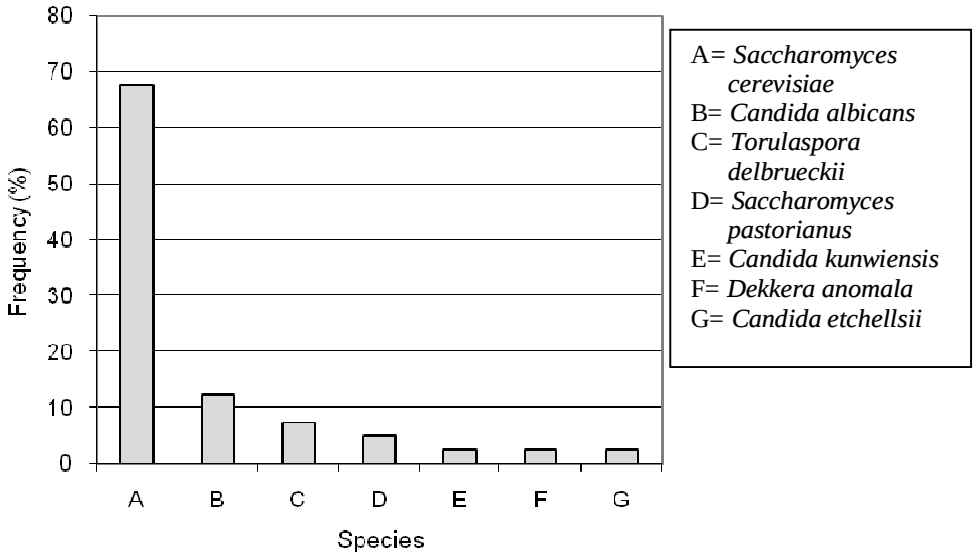

Figure 1. Frequency distribution of yeast species involved in *tchoukoutou* fermentation.

Conclusion

Based on the phenotypic characterisation, *S. cerevisiae* was found to be the predominant yeast species in the fermentation of Benin opaque sorghum beer. There is a need of genotyping the isolates for a best characterisation of the yeasts involved. In view of controlling the quality of *tchoukoutou*, the predominant species identified can be selected and used as single or mixed starter cultures for a more predictable fermentation outcome.

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Full Length Research Paper

Structural characterization and antioxidant activity of purified polysaccharide from cultured *Cordyceps militaris*

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The polysaccharide fraction, CM-hs-CPS2, was isolated from fruiting bodies of cultured *Cordyceps militaris* grown on solid rice medium by hot water extraction and ethanol precipitation, and purified by ion exchange column (DEAE-cellulose-52) and gel filtration column (Sephadex G-100) chromatography. Its structural characterizations were analyzed by gas chromatograph (GC) and fourier transform infrared spectroscopy (FT-IR). Results indicated that CM-hs-CPS2 was a kind of sulfating acidic polysaccharide containing acetamido group. The monosaccharide composition analysis showed that CM-hs-CPS2 was composed mainly of mannose, glucose and galactose in a molar ration of 1.35: 8.34: 1.00, and linked by α-glycosidic linkage. The studies on antioxidant activities of CM-hs-CPS2 were evaluated by various methods *in vitro*. Results showed that DPPH· scavenging activity, reducing power and ferrous ion chelating activity of CM-hs-CPS2 (8 mg/ml) were 89%, 1.188 and 85%, respectively.

Key words: *Cordyceps militaris*, polysaccharide, structure, bioactivity.

INTRODUCTION

Cordyceps sinensis Berk. Sacc, as a tonic herb in Chinese traditional medicine, is one of entomogenous fungi that form a fruiting body mainly on an insect larva. It has been widely used as a general tonic for protecting and improving lung and kidney functions (Leung et al., 2009), anti-cancer (Bok et al., 1999), immunomodulation (Kuo et al., 2001) and hypotensive effect (Chiou et al., 2000).

However, naturally occurring *C. militaris* is not easily available for food or medicine in large amounts because of its rare and expensive. Therefore, much effort has been invested to look for alternative species. *C. militaris*, a species of *Cordyceps* spp., has also been used as a folk medicine in China for hundred years. Studies have proved that it possesses similar constituents and pharmacological efficacy to those of *C. sinensis* (Wei et al., 2004; Zheng et al., 2004). As a result, *C. militaris* is considered to be the suitable alternative of *C. sinensis* (Gui et al., 2008).

Polysaccharide is one of major bioactive constituents of *Cordyceps* and many other medicinal fungi, which has proven anti-cancer, immunomodulation activities (Wasser, 2002), and the notable anti-oxidation effect (Liu et al., 1997). The anti-oxidant properties of herbs are most relevant to their health protecting functions.

The anti-oxidant function of polysaccharides from various natural products has been extensively studied. However, studies on the anti-oxidant properties of *C. militaris* polysaccharides are seldom reported. The aim of this study was to better understand and characterize the structural characteristics of the polysaccharide, CM-hs-CPS2, which was isolated and purified from the strain CM-hs of cultured *C. militaris* in our lab. Furthermore, we investigated antioxidant activity of CM-hs-CPS2 *in vitro*. The results will be helpful for further revealing the

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relationship between structure and bioactivities of *C. militaris* polysaccharides.

MATERIALS AND METHODS

Fungal strains and Materials

The *Cordyceps militaris* strain, CM-hs, was conserved in in the lab of biological resource function, Jiangsu University of Science and Technology and used for this study. Fresh fruiting bodies grown on solid rice medium were obtained. Ascorbic acid (Vitamin C), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, potassium ferricyanide, ferrous chloride and ferric chloride were purchased from Bio Basic Inc of China. Other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd. All reagents were of analytical grade.

Extraction of polysaccharides

The dried powder of cultured *C. militaris* (100 g) was defatted with ethanol at 70°C for 10 h at twice, and exhaustively extracted with 20 volumes of water at 80°C for 10 h at twice. The extract was concentrated under reduced pressure to a volume of 100 ml, deproteinated by Sevag method (Liu et al., 2002) and dialyzed against distilled water for 4 days to remove low molecular weight compounds. The crude polysaccharides were obtained through precipitation with ethanol to the final concentration of 90%, and overnight at 4°C. The precipitate was collected as the crude CM-hs-CPS and lyophilized.

Isolation and purification of polysaccharides

The crude polysaccharide, CM-hs-CPS (100 mg), was dissolved in 10 ml distilled water, centrifuged at 8000 rpm for 10 min, and loaded onto a DEAE-cellulose-52 column (2.6 × 30 cm). After loading with sample, the column was eluted with 80 ml in order of distilled water, 0.1 mol/L NaCl, 0.2 mol/L NaCl and 0.3 mol/L NaCl, respectively, at flow velocity of 1.0 ml/min. The elute was collected at 4 ml/tube. This process was monitored by the phenol-sulfuric acid method (Xu et al., 2005). The resulting fraction (5 mg) was loaded onto a Sephadex G-100 column (2.6 × 30 cm) for purification, and then eluted with distilled water. Consequently, the homogeneous fraction CM-hs-CPS2 was obtained and lyophilized.

Polysaccharides ultraviolet spectroscopy

The purified CM-hs-CPS2 was dissolved in distilled water to the concentration of 0.1 mg/ml, and scanned with UV spectrophotometer (UV-2450, SHIMADZU) in wavelengths from 800 to 200 nm.

GC analysis

Derivatives of acid hydrolytic products from CM-hs-CPS2 were analyzed by gas chromatography (GC) to identify the monosaccharide components. The CM-hs-CPS2 fraction (10 mg) was placed in an ampoule, hydrolyzed with 3 ml of 4 mol/l trifluoroacetic acid at 115°C for 12 h. The ampoule was filled with nitrogen and sealed. The acidolysis solution was dried with a stream of N_2 at 65°C in a water bath. The solid residual was re-dissolved in methanol, and then distilled at 65°C with a stream of N_2. This process was repeated three times to remove the acid. The solid hydrolysate was mixed with hydroxylamine hydrochloride and inositol as an internal standard. Then pyridine added to dissolved with shaking at 95°C for 30 min. After that, the sample was cooled down to room temperature, and then added acetic anhydride to carry on acetylation reaction at 95°C for 30 min. Following, the solution was mixed with methanol, and dried with a stream of N_2. The derivatives were dissolved in chloroform and analyzed by GC. The derivation process of mixed standard monosaccharide (rhamnose, arabinose, xylose, mannose, glucose, galactose) was operated with the same method mentioned above. The GC was performed on Agilent 6820 GC (Agilent Corporation) equipped with a flame ionization detector (FID), through a fused-silica capillary column (0.23 mm × 30 m). N_2 was used as carrier gas, and H_2 as burning gas. Sample injection volume was 1 ¯l on condition of N_2 flow of 50 ml/min at split ratio of 50:1. Temperatures of injection and FID detector were controlled at 230°C. The column temperature was first fixed at 130°C for 20 min, increased to 190°C at 5°C/min, and maintained for 20 min, then increased to 230°C with the ratio of 5°C/min for 10 min.

IR analysis

Infrared (IR) spectrometry of CM-hs-CPS2 was performed at 4000 - 400 cm^{-1} wave range. The dried CM-hs-CPS2 was pressed into KBr disks, and then scanned with a Fourier transform infrared (FT-IR) spectrometer (Tensor 27, BRUKER).

In vitro antioxidant assay

DPPH radical scavenging assay

The DPPH radical scavenging capacity was assayed according to the method of Luo et al. (2009) with slightly modification. Each sample at different concentrations (2, 4, 6 and 8 mg/ml) was mixed with 2 ml of 0.04 mg/ml DPPH· in ethanol. The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then, it was centrifuged at 5000 rpm for 10 min. After that, the absorbance of the supernatant was measured at 517 nm. Vitamin C (Vc) was used as the positive control. The DPPH radical scavenging capacity was calculated by the following formula:

$$\text{Scavenging capacity (\%)} = \frac{1-(A_1-A_2)}{A_0}\times 100$$

Where A_0 is the absorbance of the control (ethanol sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the blank.

Reducing power assay

The reducing power was determined by the method of Tsais (2006). Briefly, 1 ml sample of different concentration (2, 4, 6 and 8 mg/ml) in phosphate buffer (0.2 mol/l, pH 6.6) was mixed with 2 ml potassium ferricyanide (1%, w/v), and incubated at 50°C for 20 min. After that, 2 ml of trichloroacetic acid (10%, w/v) was added to mixture and stop the reaction. After centrifugation at 5000 rpm for 10 min, the supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml ferric chloride (0.1%, w/v). Ten minutes later, the absorbance was measured at 700 nm. Vitamin C (Vc) was used as the positive control.

Ferrous ion chelating capacity assay

The ferrous ion chelating capacity was determined follow by the

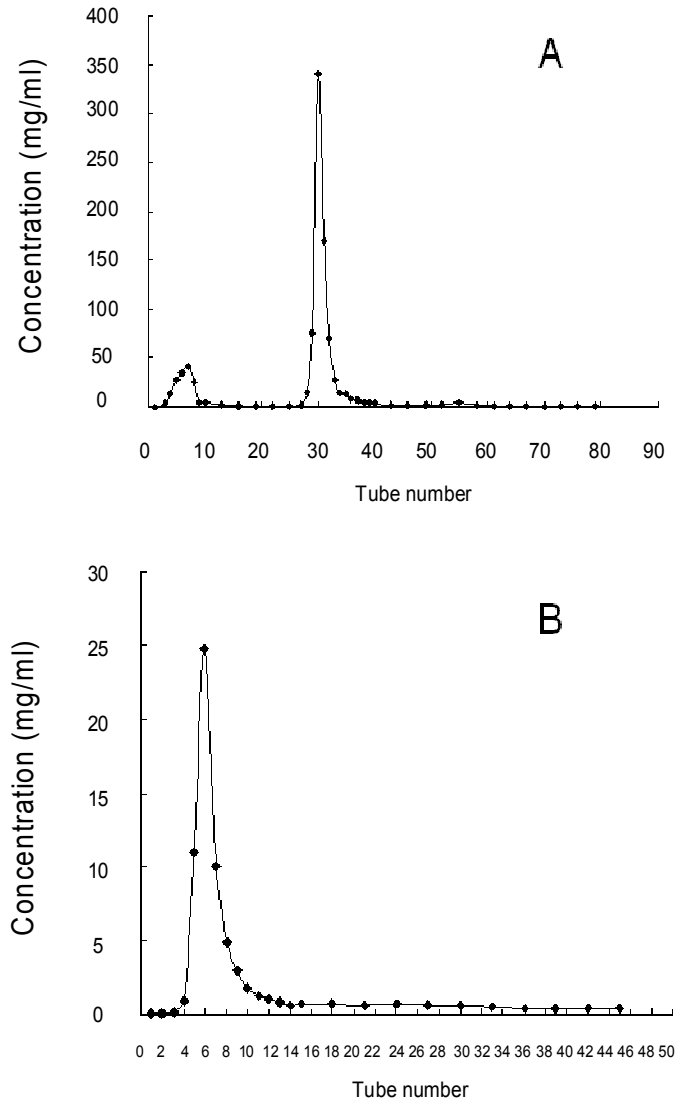

Figure 1. Elution profiles of polysaccharides extracted from cultured C. militaris by column chromatography. (A) Ion exchange chromatogram of the crude polysaccharides, CM-hs-CPS, on a DEAE-cellulose-52 column. (B) Gel filtration chromatogram of the acidic polysaccharide fraction, CM-hs-CPS2, on a Sephadex G-100 column.

method of Decker (1990) with slightly modification. The reaction mixture, containing 3 ml of sample with different concentrations (2, 4, 6 and 8 mg/ml), 0.05 ml of 2 mmol/L ferrous chloride ($FeCl_2$) solution and 0.2 ml of 5 mmol/L ferrozine solution, was shaken vigorously and incubated at room temperature for 10 min. The absorbance of the mixture was then measured at 562 nm. Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as the positive control. The ferrous ion chelating capacity of sample was calculated as follows:

$$\text{Chelating capacity (\%)} = \frac{A_0-(A_1-A_2)}{A_0}\times 100$$

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the blank.

Statistical analysis

All treatments and assays were carried out in triplicates for three separate experiments. Values are represented as mean±SD (standard deviation).

RESULTS

Isolation and purification of crude CM-hs-CPS

The crude polysaccharide, CM-hs-CPS, was obtained from fruiting bodies of cultured *C. militaris* by hot water extraction and ethanol precipitation. Figure 1 shows the elution profile of the deproteinized CM-hs-CPS on the DEAE-cellulose-52 ion exchange column. The first peak eluted with the distilled water is ascribed to a neutral polysaccharide fraction. The main peak eluted with NaCl solution is an acidic polysaccharide fraction, termed CM-hs-CPS2. This fraction was collected and applied to the Sephadex G-100 gel filtration column, resulting in the elution profile in Figure 1. CM-hs-CPS2 was eluted as a single peak. The UV absorption spectra (Figure 2) of CM-hs-CPS2 showed no absorption at 260 and 280 nm, indicated that nucleic acid and protein were absent in this polysaccharide.

Structural composition of CM-hs-CPS2

GC analysis of CM-hs-CPS2

The alditol acetates derived from the acetylation of CM-hs-CPS2 hydrolysate and standard monosaccharide were measured by gas chromatography (GC). As shown in Figure 3, mixture of monosaccharide and inositol were separated completely. The peaks emerged in the order of rhamnose, arabinose, xylose, mannose, glucose, galactose and inositol. Three monosaccharides, termed mannose, glucose and galactose were identified in the hydrolysate of CM-hs-CPS2 on the basis of the standard monosaccharide retention time and correction factor (Figure 3). According to the peak area, the mole ratio of mannose:glucose:galactose in CM-hs-CPS2 was 1.35: 8.34:1.00.

IR analysis of CM-hs-CPS2

Infrared (IR) spectrometry of CM-hs-CPS2 showed that the largest absorption peak at 3396 cm^{-1} is ascribed to the stretching of the hydroxyl (OH) group and the hydrogen bond within or between the molecules (Figure 4). The peak at 2929 cm^{-1} is ascribed to the C-H stretching band of the saccharide. And the weak peaks

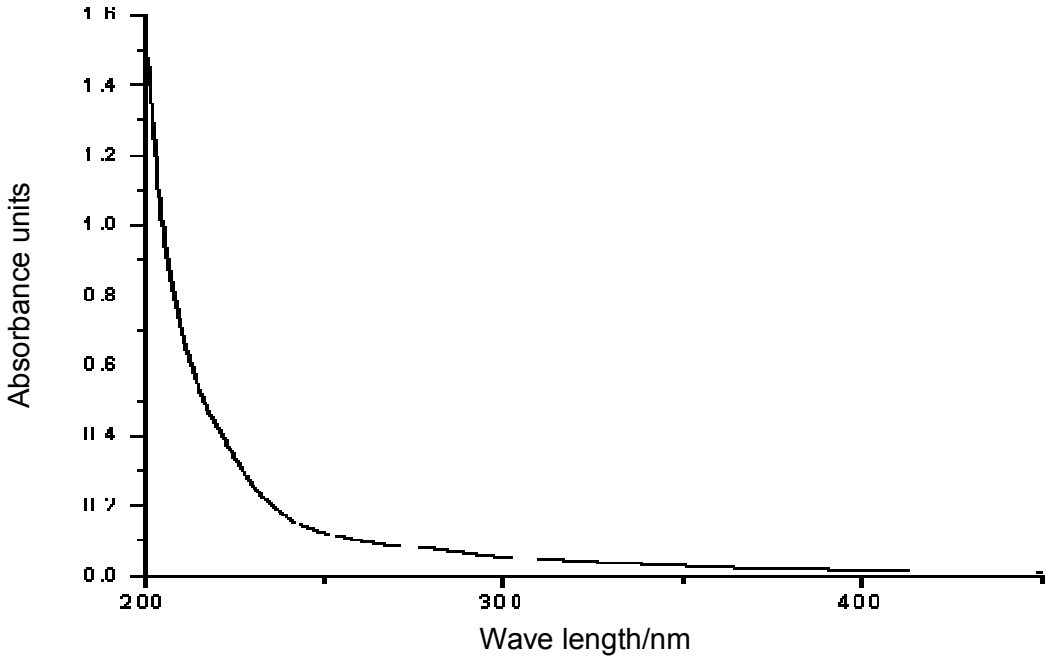

Figure 2. Ultraviolet absorption curve of CM-hs-CPS2.

between 1400 and 1200 cm^{-1} are ascribed to C-H bending vibration. The bands at 1651 and 1541 cm^{-1} are assigned to stretching vibration of the C=O bond and the bending vibration of the N-H bond, which suggests the presence of acetamido group. The two absorption bands near 1240 cm^{-1} and 850 cm^{-1} resemble the stretching band of S=O and C-O-S, respectively, which indicates the existence of $–O-SO_3$. There is a group of strong absorption peaks from 1200 to 950 cm^{-1}, which can be attributed to the ether linkage (C-O-C) and the hydroxyl presented in the ring of pyranose. And the peak at 761 cm^{-1} was the symmetric ring stretching of pyranose, impling the monosaccharide composing CM-hs-CPS2 was pyranose. The presence of the α-glycosidic linkage was proven by the C-H bending vibration at 848 cm^{-1}. And the absorption peaks at 931 cm^{-1} and 761 cm^{-1} imply the existence of α-D-glucopyranose (α-D-Glcp).

In vitro antioxidant activities of CM-hs-CPS2

DPPH radical scavenging activity of CM-hs-CPS2

As depicted in Figure 5, CM-hs-CPS2 exerted concentration-dependent DPPH· scavenging activity. At a concentration of 8 mg/ml, the DPPH· scavenging activity of CM-hs-CPS2 reached to 89%. Compared with positive control (Vc), the scavenging activity of CM-hs-CPS2 was a little lower than Vc at each concentration. These results indicated that CM-hs-CPS2 had strong DPPH· scavenging activities.

Reducing power of CM-hs-CPS2

The reducing power is an important measure of the antioxidant capability of antioxidant. It was determined by absorption spectrometry to monitor the transformation of Fe^{3+} to Fe^{2+} in the presence of the antioxidant. The increasing absorbance is correlated with the increasing reducing power. As shown in Figure 6, the sample showed a dose-dependent reducing capacity. The reducing power of CM-hs-CPS2 was 1.188 at the concentration of 8 mg/ml, compared with the Vc at 2.108. It was obvious that the reducing activity of CM-hs-CPS2 was much lower than that of Vc, suggesting CM-hs-CPS2 had a moderate reducing power.

Ferrous ion chelating activity of CM-hs-CPS2

The ferrous ion chelating capacity is also one of the important antioxidant properties. Transition metals such as Fe^{2+} are known to play key roles in lipid peroxidation by generating hydroxyl radicals through Fenton reaction. In the assay, the ferrous ion chelating capacity of the antioxidants was detected by inhibiting the formation of red-colored ferrozine- Fe^{2+} complex.

The results in Figure 7 showed that the ferrous ion chelating activity of CM-hs-CPS2 was 85% at a concentration of 8 mg/ml. It is notable that the positive control of EDTA-2Na has excellent ferrous ion chelating capacities with 100% at each concentration. As a result, CM-hs-CPS2 exhibited strong Fe^{2+} chelating activities at

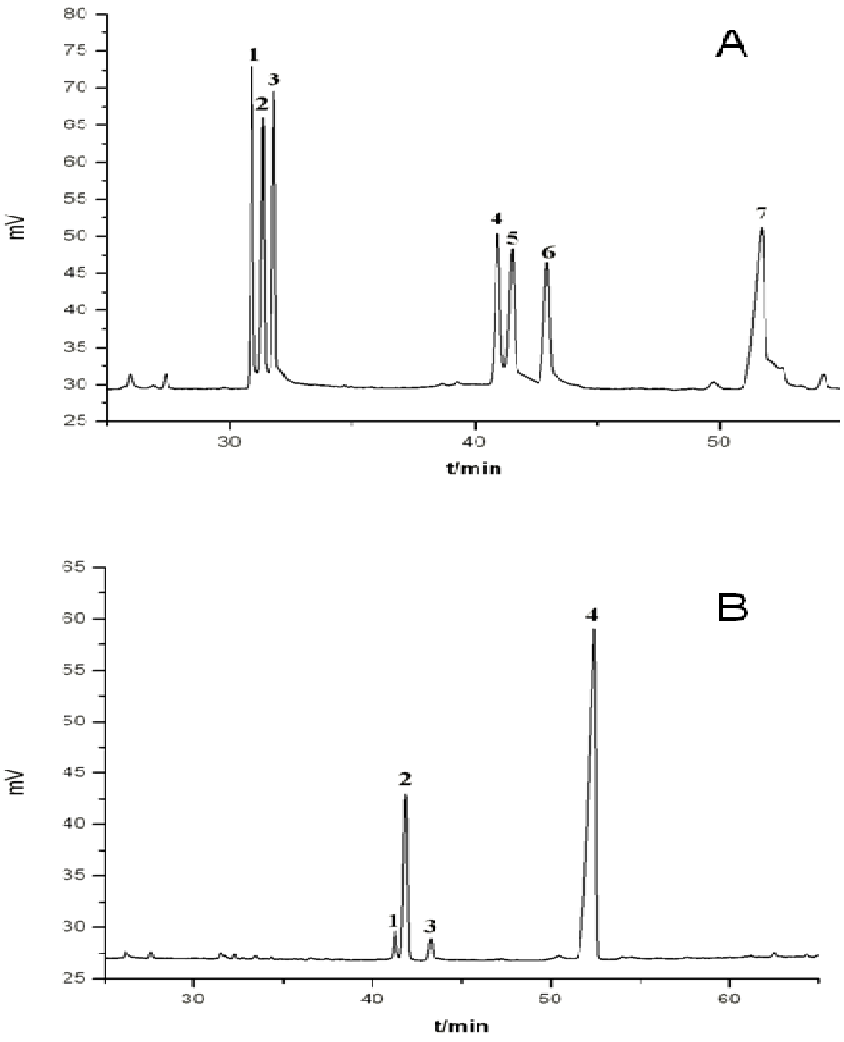

Figure 3. GC profile of standard monosaccharide (A) and CM-hs-CPS2 (B). (A) Peak identity: 1, Rhamnose (rt: 30.897); 2, Arabinose (rt: 31.336); 3, Xylose (rt: 31.803); 4, Mannose (rt: 40.963); 5, Glucose (rt: 41.530); 6, Galactose (rt: 42.978); 7, Inositol as an internal standard. (B) Peak identity: 1, Mannose (rt: 41.249); 2, Glucose (rt: 41.862); 3, Galactose (rt: 43.272); 4, Inositol as an internal standard.

the concentration of 4, 6 and 8 mg/ml.

DISCUSSION

Currently, cultivated fruiting bodies of *C. militaris* are commercially available as medicine materials and health food products in China, Korea, and South East Asia (Li et al., 2006). Biologically active polysaccharides are widespread among mushrooms, and most have unique structures in different species. As a result of this phenomenon, several studies have been conducted to determine accurately the structures of these different polysaccharides.

In this study, CM-hs-CPS2 was extracted from fruiting bodies of *C. militaris* which were cultivated on solid rice medium. The results of its structure composition exhibited that CM-hs-CPS2 was composed by mannose, glucose and galactose in the mole ratio of 1.35:8.34:1.00. The IR spectrum results revealed that the CM-hs-CPS2, composed by pyranose and linked by α-glycosidic linkage, was a kind of sulfating acidic polysaccharide containing acetamido-group. In the last few years, the structural characterizations of several polysaccarides obtained from cultured *C. militaris* were reported. Two polysaccharides (CPS-2 and CPS-3) were isolated from cultured *C. militaris* by Yu and his group. CPS-2 was made mainly of rhamnose, glucose and galactose in a mole ratio of 1:4.46:2.43. CPS-3 was a homogeneous polysaccharide, which contained glucose with a major linkage form of α-D-glucose (Yu et al., 2004). After that, polysaccharide P70-1 was also obtained from the fruiting

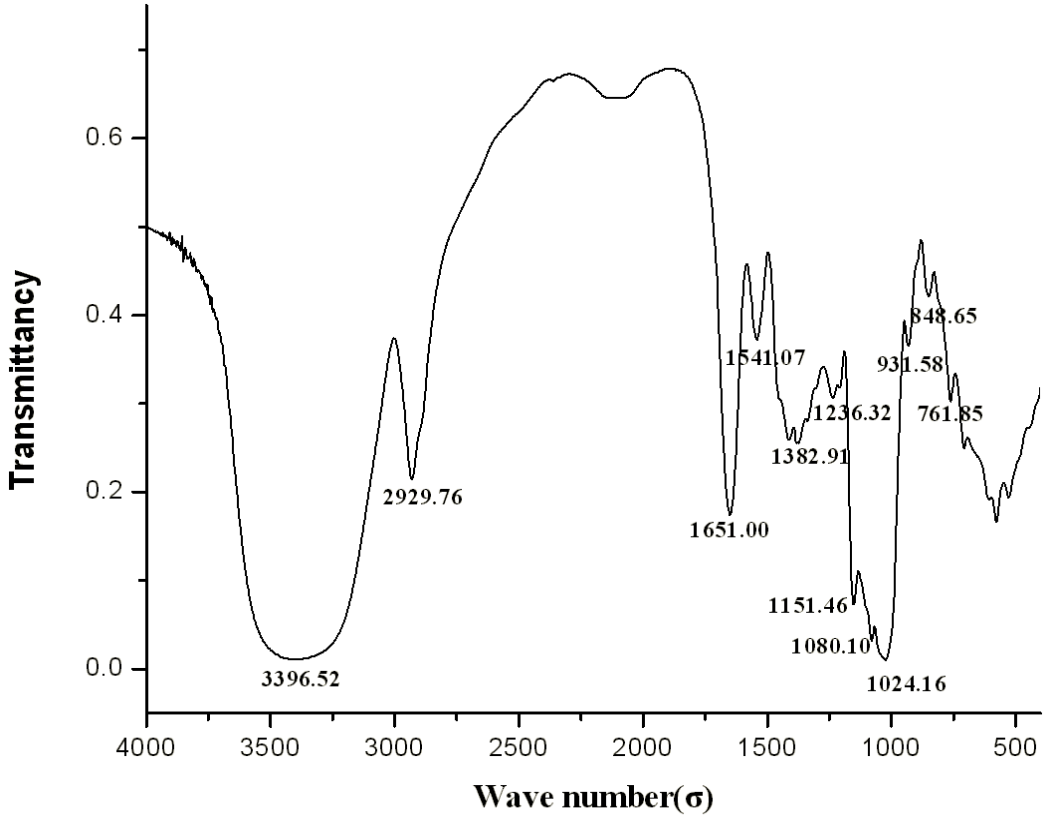

Figure 4. IR spectrum of CM-hs-CPS2.

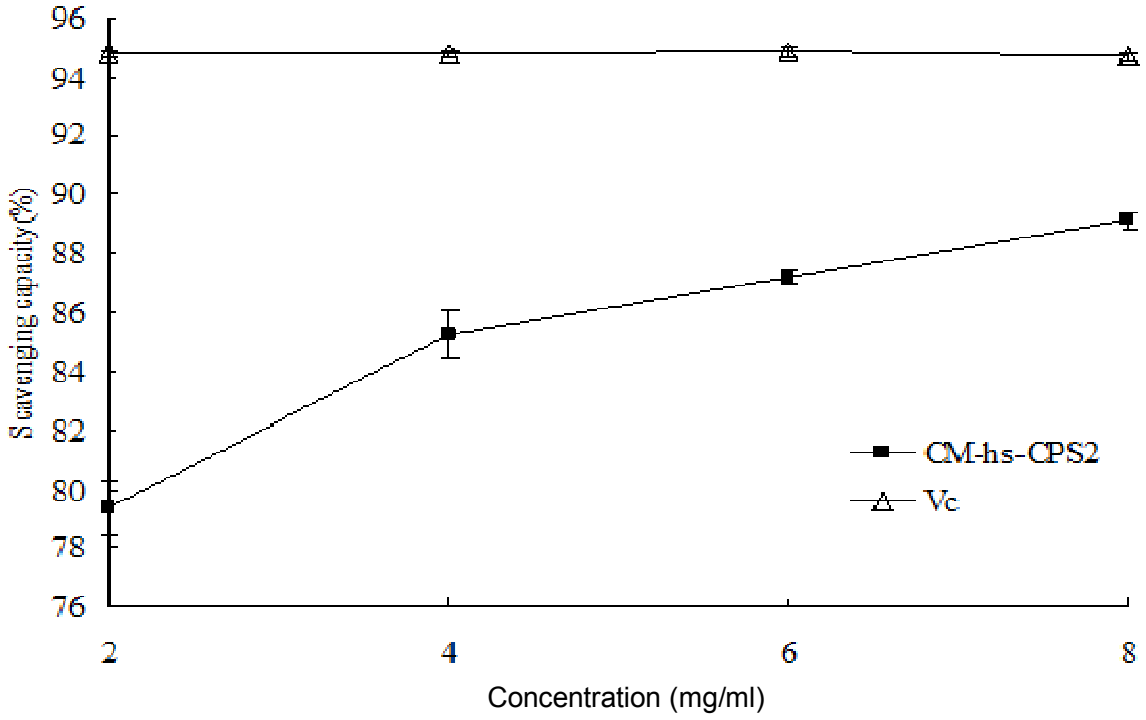

Figure 5. The DPPH radical scavenging activities of CM-hs-CPS2, with Vc as the positive control. Values are represented as mean±SD (n=3).

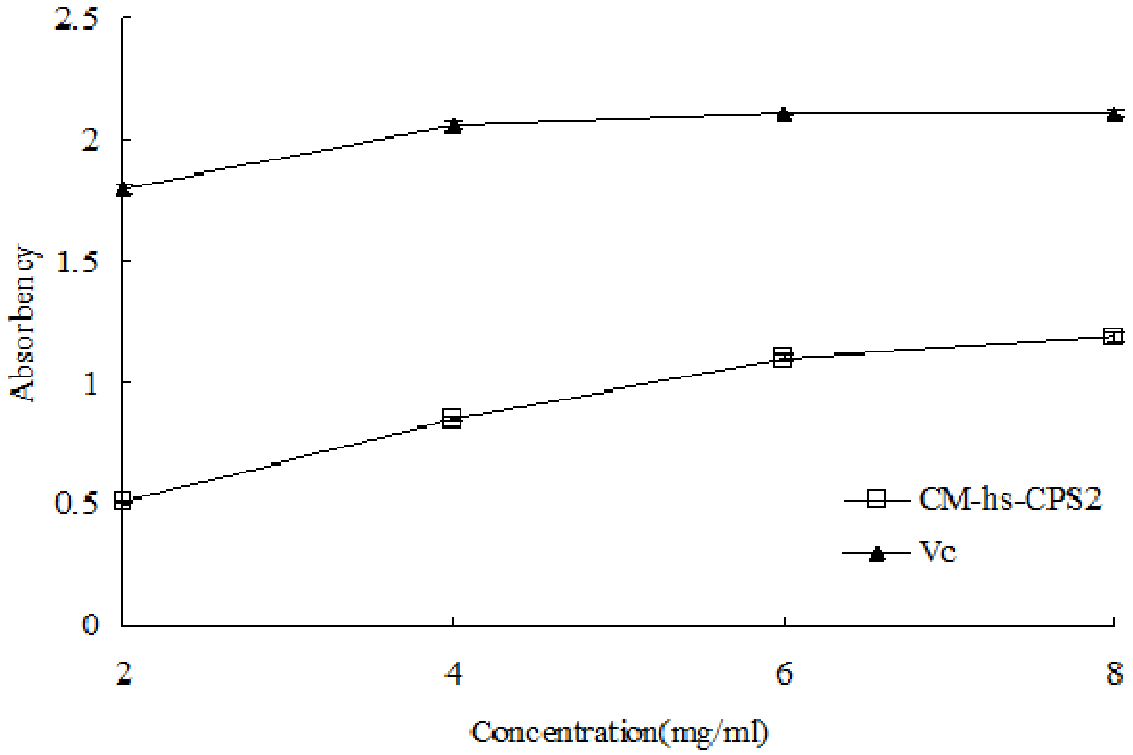

Figure 6. The reducing power assay of CM-hs-CPS2, with Vc as the positive control. Values are represented as mean±SD (n=3).

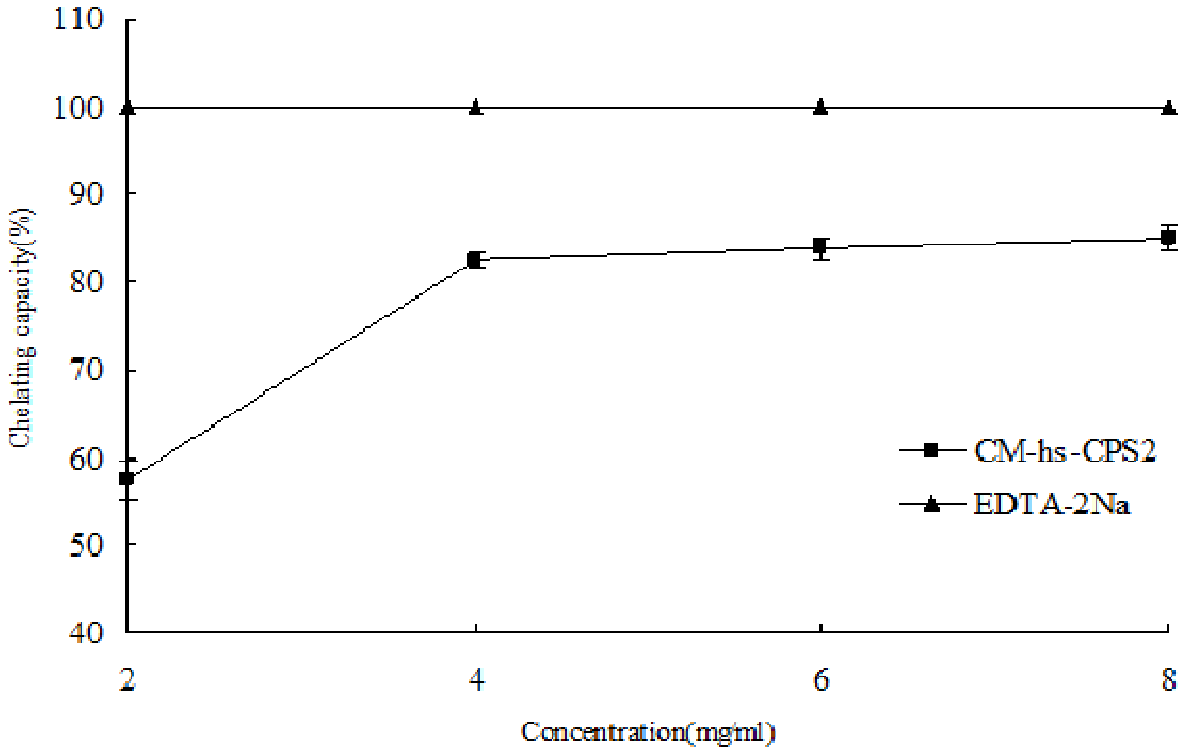

Figure 7. The ferrous ion chelating capacity of CM-hs-CPS2, with EDTA-2Na as the positive control. Values are represented as mean±SD (n=3).

bodies of cultured *C. militaris* (Yu et al., 2007). An acidic polysaccharide isolated from *C. militaris* grown on germinated soybeans was composed of galactose, arabinose, xylose, rhamnose and galacturonic acid (Ohta et al., 2007). Lee and his colleagues have reported the structural properties of CPMN Fr II and CPMN Fr III which were obtained from cultured mycelia of *C. militaris*. CPMN Fr II was a 1, 6-branched-glucogalactomannan with the β-linkage and random coil conformation (Lee et al., 2010a). CPMN Fr III was a β-1, 4-branched-β-1, 6-galactoglucomannan (Lee et al., 2010b). The interpretation of polysaccharide structural differences from above results may relative to the fact that they were different *C. militaris* strains. Furthermore, different culture medium (silkworm pupa, solid rice medium and broth) may contribute to different structure of the polysaccharide.

The antioxidant properties of natural products are most relevant to their health protecting and anti-cancer functions. Firstly, CM-hs-CPS2 was able to scavenge the stable free radical DPPH efficiently. This may attributed to their electron transfer or hydrogen donating ability. It has been suggested the existence of hydroxyl group in polysaccharides could donate electrons to reduce the radicals to a more stable form or reacts with the free radicals to terminate the radical chain reaction (Leung et al., 2009). There was a direct correlation between antioxidant activities and reducing power (Duh et al., 1999). Secondly, the presence of reductant associated with the reducing power. Reductant has been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Zhang et al., 2010). As a result, CM-hs-CPS2 showed a moderate reducing power, which may due to the presence of -OH in its structure. Thirdly, the results of the ferrous ion chelating capacity assay showed CM-hs-CPS2 exhibited strong Fe^{2+} chelating activities. It has been demonstrated that compounds with metal chelating activities usually contain two or more of the following functional groups: -OH, -SH, -COOH, $-PO_3H_2$, -C=O, $-NR_2$, -S- and -O- (Yuan et al. 2005). Accordingly, the ferrous ion chelating capacities of CM-hs-CPS2 was partially accounted for the presences of –OH, C=O, -S- and -O- groups in its structure. Moreover, it has reported that the antioxidant capacity of polysaccharides also depends strongly on the type and organization of the monosaccharide, the linkage pattern of the main chain (alpha or beta) and the branching configuration (Liu et al., 2007).

The fundamental findings in this study are beneficial to the interpretation in the connection of the polysaccharide structures and its biological activities. Due to the complex structures of bioactive polysaccharides, it is difficult to elucidate their chemical properties and relationships between their structure and activity. Therefore, great efforts should be devoted to reveal the structure-activity relationship of polysaccharides from *C. militaris* in further study.

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Full Length Research Paper

The effects of four weeks training on leptin levels in junior female judokas

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The purpose of this study is to investigate the effects of four weeks judo training on plasma leptin levels. Twenty-five female national team athletes with a mean age of 18.12±1.12 years; body weight of 62.72±22.72 kg; body height of 164.28±7.28 cm, BMI of 23.17±6.28 kg/m^2; training experience of 6.56±5.44 years participated in this study. The data were collected from junior female judokas who trained six times in a week for two hours daily. Physical measurements and blood samples were taken before and after four weeks of training including technical, tactical and physical preparations. Body fat percentages were measured by using the Tanita Bioimpedance BC-418 (Tokio, Japan). Statistical Package for Social Science 15.0 (SPSS) was used to analyse the obtained results. Before and after the training, paired samples t-test was used to evaluate the differences between serum leptine and body fat percentages. Results of this study showed that pre-training serum leptin levels were significantly lower than post-training levels (p<0.01). There was also significant decrease in body fat percentages after four week training (p<0.01). Four weeks judo training results in a decrease in total body fat and serum leptin level in junior female judokas. It may be considered that lowered serum leptin levels were due to the decrease of the body fat percentage after four weeks training.

Key words: Plasma leptin, body fat, female judokas.

INTRODUCTION

Sport of judo needs dynamic and intermittent workloads with a high-intensity anaerobic activity (Degoutte et al., 2000). Judokas spend significantly high amount of energy during the training and competitions which affects energy balance, hormonal profile and metabolic hemostasis (Filaire et al., 2001). There was a limited number of studies related females judokas in the literature (Prouteau et al., 2007).

Leptin hormone has been a topic in several researches as it is associated with food intake and energy use directly. Regulation of the body weight and energy balance for human organism is one of the basic functions controlled by endocrine and central nevrous system.

Leptin has been recently defined as an adipocyte derived hormone and regarded as a regulator of the body weight and energy balance (Simsch et al., 2002). There are several studies showing the effects of exercise on leptin levels. Many of these studies are related to why leptin responds to exercise and shows adaptation to exercise (Robert et al., 2002). Leptin is efficient in controlling the body weight in professional athletes and exercise density and the amount of the energy spent are important in alternation in serum leptin level (Gomez-Merino et al., 2002). Exercise results in a decrease in fat mass and thereby effecting leptin levels. However, the exercise types requiring more calory and energy expenditure may result in more alterations in leptin levels (Perusse et al., 1997; Loucks et al., 1998). Decrease in plasma leptin levels associated with exercise may be balanced by nurition (Koistinen et al., 1998). Low leptin levels in athletes occur together with the energy deficit caused by the exercise and associated decreased fat percentage (Casimiro-Lopes et al., 2009). But, low fat proportion may not always indicate low leptin level. This result can be seen in some studies (Koury et al., 2007). They showed that unaltered fat proportion values occurred in spite of low leptin levels after the training (Koury et al., 2007). It is thought that exercise makes its effects on leptin through sympathetic adrenergic system (Torjman, 2001). It was suggested that alterations occuring in the levels of growth hormone, cortisol, insülin, testosteron, epinefrin and norepinefrin during the

Table 1. Plasma leptin levels pre-training and post-training.

Plasma leptin levels		N	X	S	t	Sig
Leptin	Pre-training	25	4.70	3.48	5.307	0.000**
	Post-training	25	2.96	1.96		

**There was significant difference at 0.01 level.

Table 2. Body fat levels pre-training and post-training.

Body fat levels		N	X	S	t	Sig
Body fat (%)	Pre-training	25	17.31	1.52	11.290	0.000**
	Post-training	25	15.48	1.60		

**There was significant difference at 0.01 level.

exercise result in delayed decrease in leptin (Essing et al., 2000). In this respect, there are also studies in the literature showing that short-term exercise (<60 min.) has no effect on serum leptin levels (Weltman et al., 2000; Essing et al., 2000; Houmard et al., 2000; Olive and Millller, 2001; Sütken et al., 2006; Yamaner et al, 2010).

The aim of this study is to investigate the effects of four weeks training on plasma leptin levels and body fat percentages in junior female judokas.

MATERIALS AND METHODS

Subjects

The mean values of the subjects were 18.12±1.12 years for age, 62.72±22.72 kg for body weight, 164.28±7.28 cm for body height, 23.17±6.28 kg/m^2 for body mass index, and 6.56±5.44 years for training experience, respectively. Body mass index was calculated using following formula, BMI=body weight (kg)/body height^2 (m) (Fahey et al., 2007).

Sample collections

The data were collected from junior female judokas who trained six times in a week for two hours daily for world championship. Physical measurements and blood samples were taken before and after four week training including technical, tactical and physical preparations. Body fat percentages were measured by the Tanita Bioimpedance BC-418 (Tokio Japan).

Statistical analysis

Statistical Package for Social Science 15.0 (SPSS) was used to analyse the obtained results. Before and after four weeks training, paired samples t-test was used to evaluate the differences between serum leptine and body fat percentages.

RESULTS

Serum leptin levels obtained after the training was statistically sigificantly lower than plasma leptin levels before the training (p=0.000) (Table 1).

Body fat levels obtained after the training was statistically significantly lower than body fat levels before the training (p=0.000) (Table 2).

DISCUSSION

Results of this study showed that plasma leptin and body fat percentages of judokas after judo training were significantly decreased. There are similarities with other studies reported in the literature (Landt et al., 1997; Leal-Cerro et al., 1998; Essig et al., 2000; Olive and Millller, 2001; Zaccaria et al., 2002; Yamaner et al., 2010). Burning the fat tissues through training and energy deficit associated with this case result in the same percentage of decrease in serum leptin levels (Witek et al., 2003). It was reported that long-term medium level exercises supress the plasma leptin levels related to the decrease of body fat percentage presented by BMI. But, it was noted that serum leptin levels did not alter in short-term exercise (Sütken et al., 2006).

Some researchers reported that these results were also in disagreement with the some studies (Essing et al., 2000; Olive and Millller, 2001). They found that exercise had no acute effect on leptin levels and they also reported that serum leptin levels were decreased 30 to 40% after the exercise (Essing et al., 2000; Olive and Millller, 2001). In another study on acute effects of training, there was a decrease in fat mass, while there was not any alteration in serum leptin levels after the training (Perusse et al., 1997).

In a study made by Türkmen, judo and cycling exercises were found to have no influence on leptin, LDL, triglyceride, total cholesterol level (Türkmen, 2011). In a similar study, Kishali found out that 8 weeks of exercise does not change serum leptin levels and body fat percentages of male university students (Kishali, 2011).

Some studies were carried out on animals in order to

determine the effects of exercise on leptin levels (Pagano et al., 1999). They reported that no alteration was found in leptin levels of obese animals after the exercise while leptin levels of animals with lower body weight showed decrease (Pagano et al., 1999). Conversely there were also other studies in which serum leptin levels of different subjects having different fat mass show difference in terms of their responses to exercise (Hickey et al., 1996; Moller et al., 1998).

Conclusions

It was found that judo training had a short term effect on plasma leptin level and body fat percentages. Following the four weeks training, there was a decrease in serum leptin levels and body fat percentages. The decrease in plasma leptin levels after training programme was associated with the decrease in body fat percentage. In other words plasma leptin levels may be altered depending on the body fat percentage.

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Full Length Research Paper

Effects of the electromagnetic radiation on oocysts of *Eimeria papillata* infecting mice

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Electromagnetic radiation (EMR) produced by many telecommunication systems, has short and long term biological effects on living cells. The aim of this study was to investigate the influence of EMR on the outcome of coccidiosis induced by *Eimeria papillata*. Oocysts from *E. papillata* infected mice were exposed to the EMR in the form of gamma rays, ultraviolet rays and radiations emitted from the mobile phone. Sporulation rate, oocysts shedding as well as the histological alterations in jejunum of mice irradiated with oocysts exposed to EMR were determined. Oocyst output was reduced in mice exposed to EMR. The jejunum histopathology was improved after inoculation of mice with irradiated oocysts. We suggest that EMR has anticoccidial activities and its application could serve as an alternative to the anticoccidial drugs currently used in poultry production.

Key words: Electromagnetic radiation, *Eimeria papillata*, mice.

INTRODUCTION

Coccidiosis is a common infectious disease in poultry, causing major economic losses. The protozoan parasite of the genus *Eimeria* multiplies in the intestinal tract of poultry and produces tissue damage, resulting in reduced growth and increased susceptibility to pathogens (McDougald, 2003). The Eimerian parasites are characterized by fast reproduction and by infecting especially young animals (Gres et al., 2003; Pakandl, 2005). Infection begins with oral uptake of Eimerian oocysts, which release infectious sporozoites in the intestine. These, in turn, invade mainly epithelial cells of the intestine, in which they asexually multiply before oocysts are finally discharged with the feces.

It is known that electromagnetic radiation (EMR) produced by many telecommunication systems, has short and long term biological effects on living cells (Galeev, 2000; Gos et al., 2000). Related to this subject, numerous *in vivo* and *in vitro* researches are carried out either on humans and animals or microorganisms (Juutilinen and Seze, 1998; Gos et al., 2000). When researches on the effects of EMF are investigated, it is seen that the number of studies on the influence of electromagnetic radiation on single cell protozoa is very limited (Berk, 1997). EMR have demonstrated the production of cytokines, increased immune parameters and stress effects and concluded that EMR causes stress at the cellular level and that this leads to production of cytokines and consequently biological response, including immune response (Markov et al., 2006).

Exposure of *E. tenella* oocysts to EMR decreased infectivity (Bajwa and Gill, 1977). The current study aimed to investigate the possible anticoccidial activity of the electromagnetic field.

MATERIALS AND METHODS

Animals

Male Swiss albino mice were bred under specified pathogen-free conditions and fed a standard diet and water *ad libitum*. The experiments were performed only with male mice at an age of 8–9 weeks and were approved by state authorities and followed Saudi Arabian rules for animal protection.

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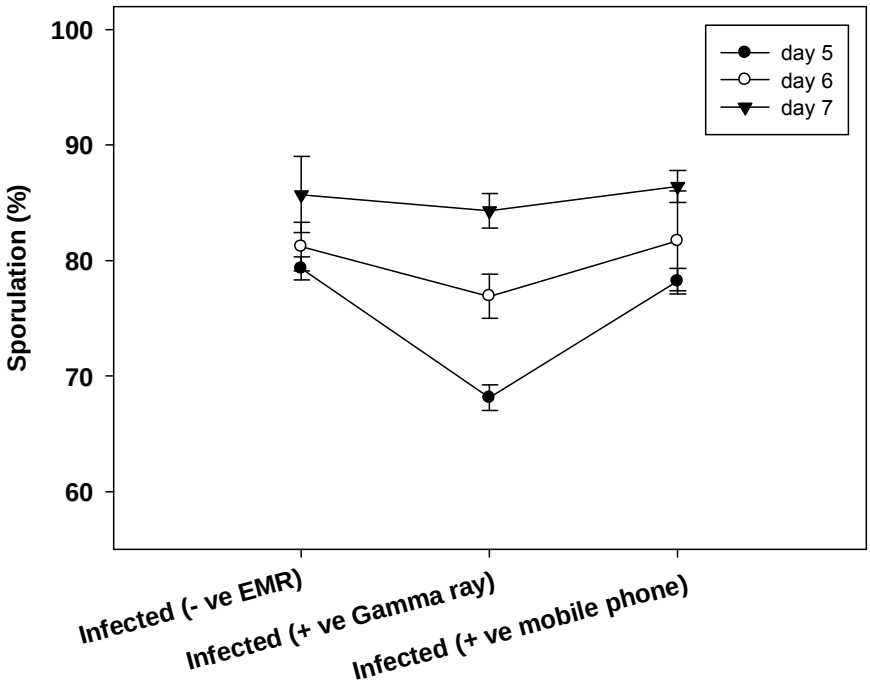

Figure 1. Changes in sporulation of *E. papillata* oocysts due to EMR. Values are means ± SD.

Irradiation of *E. papillata* unsporulated oocysts

E. papillata, kindly provided by Prof. Mehlhorn (University of Duesseldorf, Germany), was previously characterized (Hnida and Duszynski, 1999; Zhao and Duszynski, 2001). *E. papillata* oocysts were obtained from infected mice at day 4 postinfection by both cecal harvest and fecal collection using standard procedures (Schmnatz et al., 1984). Unsporulated oocysts were divided into 4 groups and placed in 4 Petri dishes; each contained at least 5000 oocysts in 10 ml distilled water. The first group was unexposed to the electromagnetic radiation (-ve EMR). The second group was exposed to a dose of 500 Gy gamma-radiation using Gamma Cell 200 Irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) utilizing a ^{60}Co source located at the Research Center of College of Science, King Saud University, Saudi Arabia. The third group was exposed to a dose of ultravilot radiation from Molecular Imager Gel Doc XR System (Hercules, California, USA) in a dose of 302 nm for 30 min. The fourth group was exposed to an electromagnetic radiation from a mobile phone (Nokia 6120) receiving a call for 1 h. Directly after exposure of all groups to the EMR, oocysts were allowed to sporulate as described by Schito et al. (1996). Sporulation rate of each group was calculated on day 5, 6 and 7.

Effect of irradiation on oocyst production

Oral gavage of mice from all groups was done with 1000 sporulated oocysts of *E. papillata* suspended in 100 ¯l sterile saline. Once a day, fresh fecal pellets were collected and weighed for each mouse, and the bedding in the cages was changed to eliminate re-infection. Faecal pellets were diluted in saturated NaCl, causing the oocysts to float. The latter were counted in a McMaster chamber and expressed as number of oocysts per gram of wet feces (Schito et al., 1996). The oocyst output was calculated in day 4 p.i.

Histological analysis

Pieces of jejunum were freshly prepared, fixed in 10% neutral buffered formalin, and then embedded in paraffin. Sections were cut and then stained with hematoxylin and eosin according to Drury and Wallington (1980). According to Dommels et al. (2007), tissue sections were scored for inflammatory lesions (infiltrations by mononuclear cells, neutrophils, eosinophils, and plasmacytes, for fibrin exsudation and lymphangiectasis, for tissue destruction (enterocyte loss, ballooning degeneration, edema, and mucosal atrophy), and for tissue repair (hyperplasia, angiogenesis, granulomas, and fibrosis). A rating score between 0 (no change from normal tissue) and 3 (lesions involved most areas and all the layers of the intestinal section including mucosa, muscle, and omental fat) was given for each aspect of inflammatory lesion, tissue destruction, and tissue repair. The sum of inflammatory lesions, tissue destruction, and tissue repair scores was used to represent the total histological injury score (HIS) for each intestinal section. The sum of the inflammatory lesions was multiplied by 2 to give more weight to this value since the tissue changes were mainly characterized by inflammatory lesions.

Statistical analysis

Statistical analyses were performed using Student's *t*-test at $p < 0.01$.

RESULTS

Electromagnetic radiation could induce a change in sporulation rate as indicated in Figure 1. The percentage of sporulation of the irradiated oocysts with 500 Gy

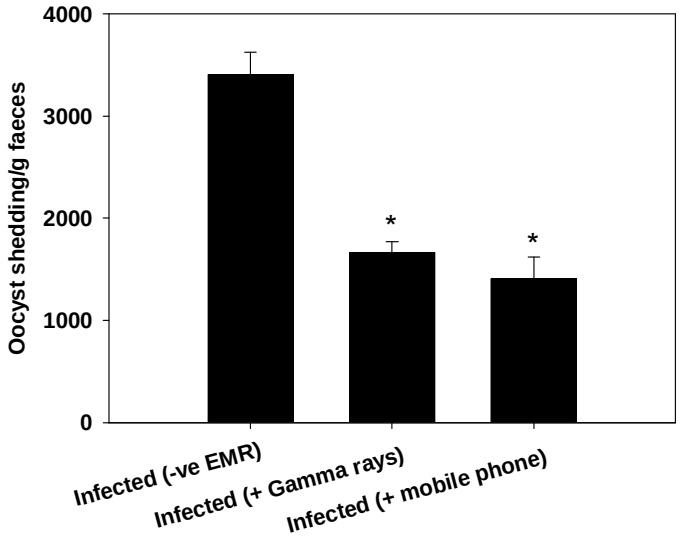

Figure 2. Oocyst output in mice infected with irradiated *E. papillata* oocysts. Values are means ± SD. *, significant difference at (p<0.01) compared to infected –ve EMR group.

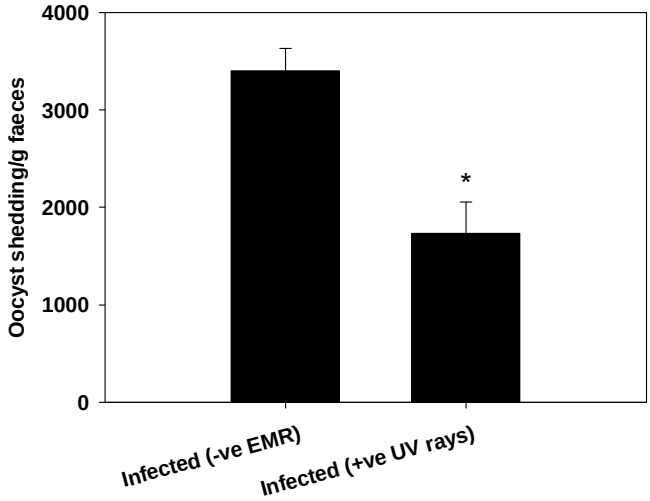

Figure 3. Oocyst output in mice infected with irradiated *E. papillata* sporulated oocysts. Values are means ± SD. *, significant difference at (p<0.01) compared to infected –ve EMR group.

gamma rays was lower in days 5, 6 and 7 than that of the control group. EMR emitted from the mobile phone could also alter the sporulation rate in day 5 post sporulation in dichromate. Surprisingly, unsporulated oocysts exposed to UV for 30 min could inhibit sporulation process.

Oocyst output in faeces of mice at day 4 p.i. were significantly decreased to more than 50% in mice inoculated with oocysts irradiated with gamma rays (Figure 2). EMR emitted from mobile phone could also significantly reduce the shedded oocysts from mice infected with *E. papillata* (Figure 2).

Sporulated oocysts of *E. papillata* post-irradiated with UV for 30 min could significantly ($p < 0.01$) decrease the oocyst out put in faeces of mice (Figure 3).

Histological analysis revealed that mice infected with sporulated oocysts of *E. papillata* suffered a moderate inflammatory injury in jejunum (Figures 4 and 5). This injury was reduced when mice were exposed to EMR (Figure 5). The histological injury score reached approximately 16, 11, 10 and 12 in infected (-ve EMR), infected (+ gamma rays), infected (+ UV rays) and infected (+ mobile phone) groups, respectively (Figure 5).

DISCUSSION

Oocysts of *E. papillata* spend a part of their life cycle outside the host when reproductive stages are released from the host into the external environment, where they wait to be transmitted to a new host. Hence, the fitness of the parasite depends, at least in part, on its ability to resist adverse external conditions before transmission. Our results clearly show that exposure of unsporulated oocysts to the EMR significantly reduced the oocysts output in faeces of mice. This reduction occurrence may be due to the reduction in the viability of the oocysts (Martinaud et al., 2009).

Some of the researchers investigating the biological effects of EMR, used mobile phones (Irmak et al., 2002) for their experiments. We preferred to produce EMR by means of a GSM mobile phone turned to speech position for 1 h. Also, we used UV rays and gamma which produce electromagnetic waves. EMR from these sources affected the sporulation process of *E. papillata* as well as the oocysts output after inoculation into mice.

EMR mediated a reduction of the severity of lesion in the jejunum of the infected mice. These results generally support the observations of Gilbert et al. (1998) who found that irradiation of sporulated oocysts of *Eimeria* reduced the pathogenic effects when oocysts were inoculated into animals and reduced the ability of sporozoites to develop in cultured cells.

Remarkably, the jejunum of mice infected with *E. papillata* is characterized by low inflammation (Al-Quraishy et al., 2011). Exposure to EMR may have anti-inflammatory effect (Vallbona and Richard, 1999; Jasti et al., 2001), and electromagnetic signals have been reported to stimulate the production of cytokines, mediating an enhanced immune response (Blank et al., 1992; Mevissen et al., 1998; Simko and Mattsson, 2004).

The novel observation was that exposure of the unsporulated oocysts to EMR antagonized the effect of infection with *E. papillata*. Further work on the effect of EMR exposure on coccidiosis infection is needed. Perhaps EMR could serve as an alternative to the anticoccidial drugs currently in use.

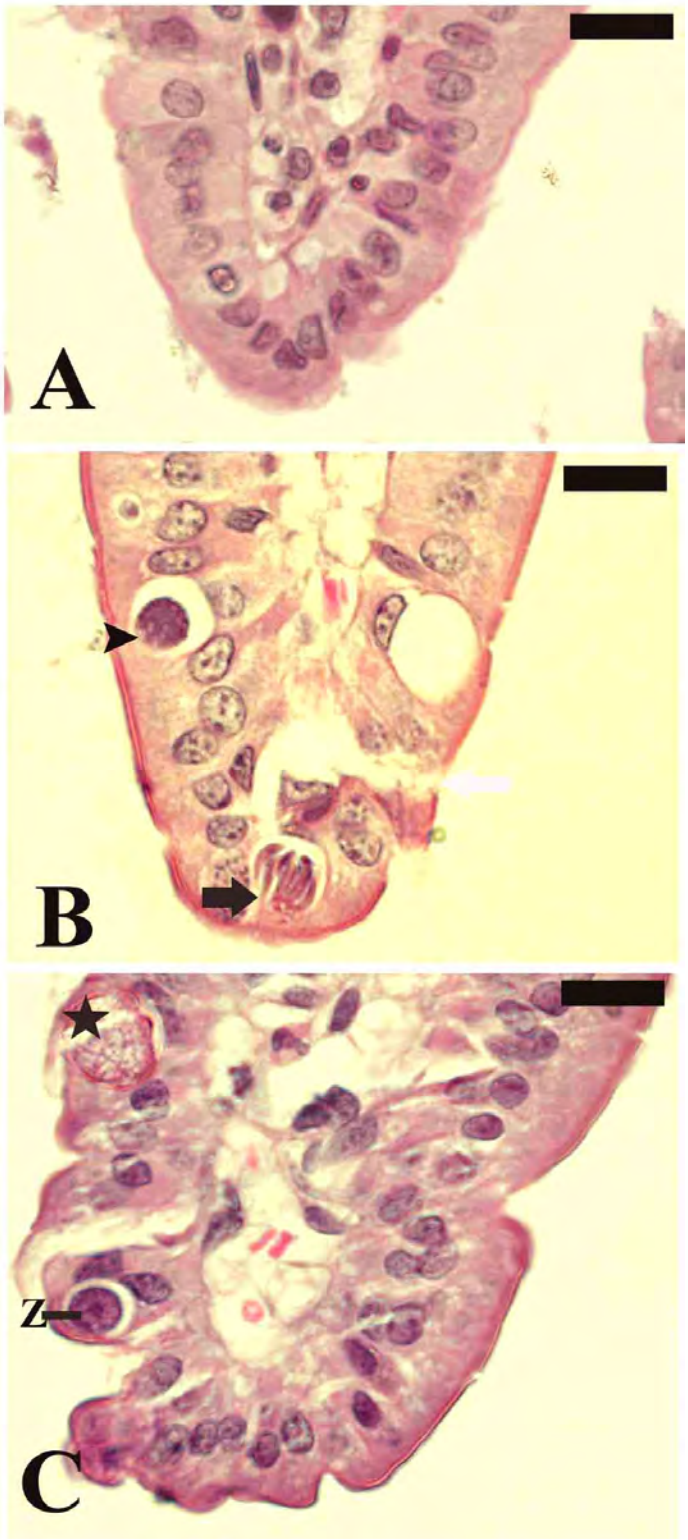

Figure 4. Sections of mouse jejunum infected with *E. papillata* on day 4 p.i. A, Non-infected jejunum with normal architecture. B and C, Infected jejunum with some pathological changes and developmental stages appearing in the inner epithelium. Destructive wall (white arrow). Arrow heads indicate Macrogamete, black arrow indicates Merozoites. Zygote (Z) and oocyst (star) appeared in C section. Sections are stained with hematoxylin and eosin. Bar=25 ¯m.

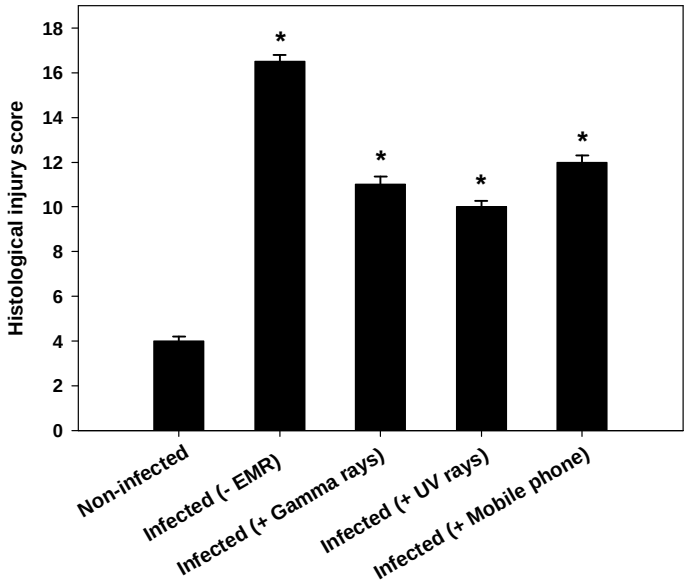

Figure 5. Total HISs in jejunum of mice infected with *E. papillata* on day 4 p.i.. Values are means±SD. Asterisk significant difference vs. the control ($p<0.01$) and section sign significant difference between infected and infected + EMR exposed mice ($p<0.01$).

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Full Length Research Paper

Influence of bispyribac sodium on nitrogenase activity and growth of cyanobacteria isolated from paddy fields

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The goal of this study was to determine the effect of bispyribac-sodium on the nitrogenase activities and growth of cyanobacteria isolated from paddy fields. Ten cyanobacterial species were used in this study. Cyanobacterial species were isolated from soil and water samples obtained from rice fields in Corum, Turkey. Among all *Anabaena* strains, the maximum activity was determined in *Anabaena* sp. O-22 (2.26 µl ethylene/mg.h) whereas; the lowest activity was shown in *Gloeothece* sp. O-Y (0.04 µl ethylene/mg.h). The maximum inhibition was seen in *Anabaena* sp. O-22, *Synechocystis* sp. O-X and *Anabaena* sp. O-16 in 100 µg/ml bispyribac-sodium concentration. Although low bispyribac-sodium concentrations somewhat stimulated growths of *Anabaena* sp. O-X2, O-Ç, O-4, O-16, O-8 and *Synechocystis sp.* O-X, the biomass of all cultures were severely inhibited at higher concentrations. The growths of *Anabaena* sp. O-22 and *Synechocystis* sp. O-X completely repressed at 100 µg/mL and at higher bispyribac-sodium concentrations, whereas, *Anabaena* O-X2, O-6, O-4 and O-16 completely suppressed at 500 µg/mL bispyribac-sodium concentration. The end of the study *Anabaena* sp. O-22 has been proposed as biofertilizer. The results obtained may be useful for the production of rice.

Key words: Cyanobacteria, nitrogenase activity, herbicide.

INTRODUCTION

The utilization of nitrogen gas (N_2) as a source of nitrogen is called nitrogen fixation and it is a property of only certain prokaryotes (Manahan, 1997; Madigan et al., 1997). Nitrogen fixing cyanobacteria are important photosynthetic microorganisms because they contribute to soil fertility by fixing the atmospheric nitrogen.

Nitrogen fixing cyanobacteria are found in many different ecosystems. Certain photosynthetic bacteria fix N_2, but only under anaerobic conditions. Nitrate, phosphate, light intensity, metal, osmotic and herbicide stresses are important environmental conditions affecting algal growth and nitrogenase activity (Meeks et al., 1983; Castenholz, 1988; Lehtimaki et al., 1997; Liengen, 1999; Banerjee et al., 2004; Okmen and Donmez, 2007a; Okmen et al., 2007b; Okmen et al., 2007c). These effects depend on the type and nature of environmental conditions, the organisms present as well as the experimental conditions (Tozum and Sivaci, 1993).

The herbicide Nominee commonly know as bispyribac-sodium. Sangakkara et al. (2004) is reported to increase the rice yield by selectively eliminating weeds from paddy fields. Although, the use of the herbicide is aimed at eliminating weeds, a major portion is deposited on the surface of the soil and might adversely affect the nontarget soil microflora.

Bispyribac-sodium, sodium 2.6- bis [(4, 6- dimethoxy-2- pyrimidinyl) oxy] benzoate, which was first developed by Japan Kumiai Chemical, belongs to the pyrimidinyl oxybenzoic acid group (Wu and Mei, 2011). Bispyribac-sodium has been applied post emergence to control many weeds. Rice-field herbicides while protecting rice-seedlings selectively, destroy the weeds and indirectly bring an increase in grain yield (Still and Kuziriam, 1967; Park and Park, 1971; Fischer et al., 2000). This class of herbicides (penoxsulam, imazamox and bispyribac-sodium) act through inhibition of acetolactate synthase (specific to plants and microorganisms) and thereby block the biosynthesis of the branched-chain aminoacids which lead to decreased protein synthesis and cessation of growth (Osuna et al., 2002). However, bispyribac-sodium inhibits the synthesis of key aminoacids causing susceptible plants to stop growing and die within about two to three weeks (Slade et al., 2006).

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Many reports available indicate interaction between cyanobacteria and herbicides, including effects of herbicides on algal growth, photosynthesis, nitrogen fixation, biochemical composition and metabolic activities as well as degradation and removal of herbicides by algae and cyanobacteria (Lundqvist, 1970; Ibrahim, 1972; Singh, 1974; Dasilva et al., 1975; Tiwari et al., 1981; Maule and Wright, 1983; Stratton, 1984; Mattoo et al., 1984; El-Sawy et al., 1984; Singh et al., 1986; Goyal, 1986; Tandon and Lal, 1988; Singh and Tiwari, 1988; Mishra and Pandey, 1989a; Bhuniaa et al., 1991; Leganés and Fernández-Valiente, 1992; El Sheekh et al., 1994; Caux et al., 1996; Jeong-Dong and Choul-Gyun, 2006; Okmen, 2007c;).

Until now, very little work has been done on the effects of rice field herbicides on nitrogen fixation and the studies carried out provide a preliminary idea about the inhibitory or stimulatory effect of the herbicides on diazotrophic growth in cyanobacteria.

Previous studies have investigated the influence of selective pesticides on the growth of cyanobacteria (Pandey, 1985; Singh and Tiwari, 1988; Mishra et al., 1989b; Prosperi et al., 1993; Jin et al., 1996; Nystrom et al., 1999; Jianyi et al., 2002; Okmen et al., 2007c). Most reports demonstrated that the sensivity of cyanobacteria toward herbicides and their growth and nitrogen fixation behavior changed in the presence of herbicides. There are no reports on the effects of bispyribac-sodium on nitrogenase activity of cyanobacteria. In this paper, we report the experimental findings obtained on the effect of a rice herbicide bispyribac-sodium, on the nitrogenase activity, growth of ten diazotrophic cyanobacterial strains, namely ***Anabaena***, ***Synechocystis*** and ***Gloeothece*** sp.

MATERIALS AND METHODS

Test organisms

Samples were collected from paddy fields in Osmancik, Corum-Turkey. The unicellular and filamentous, heterocystous cyanobacteria used in this study (***Anabaena***, ***Synechocystis*** and ***Gloeothece*** sp.) were isolated from soil and water samples obtained from rice fields in Osmancik, Corum, Turkey. Nitrogen-free BG-11 medium was used for isolation of nitrogen fixing cyanobacteria. Isolation and purification were performed by dilution and plating of soil and water samples. Stock cultures were grown in the N-free BG-11 medium as previously described (Castenholz, 1988). Temperature was maintained at 20°C and cultures were grown under a cool white light. Cells in the logarithmic phase of growth were collected from cultures and used as inocula for experiments. Experiments were conducted in batch cultures by using 10 ml of inoculated medium flasks in 25 ml. Culture media were adjusted accordingly pH 8 with 1 N NaOH and 1 N HCl. Illumination was supplied with 11 µmol/m^2 cool white light (Fogg et al., 1973; Rippka, 1988).

Determination of nitrogenase activity

Nitrogenase activity was measured by acetylene reduction technique (Burlage et al., 1998). Cultures (10 ml) were grown under the different concentrations of bispyribac-sodium and were enclosed by parafilm plastic. Then 1 ml of acetylene gase was injected into the serum bottles and cultures were incubated for 12 h under the experiment conditions. After the incubation periods, samples (1 ml) were taken from serum bottles with gas-tight syringes, injected into the gas chromatograph, and ethylene concentrations were determined using Agilent 6890 GC-FID.

Determination of dry weight

The pellets of centrifuged cultures were washed with distilled water three times, then dried to a constant weight at 70°C for 12 h and dry weights were measured (Castenholz, 1988; Cappuccino and Sherman, 2001).

Influence of bispyribac – sodium on nitrogenase activity and growth

The influence of different concentrations of bispyribac– sodium (5-500 µg/mL) on the nitrogenase activity were also tested on ***Anabaena***, ***Synechocystis*** and ***Gloeothece*** sp. The experimental cultures were grown in 25 ml flasks containing 10 ml N-free BG-11 medium under the same conditions as described below. According to Rippka (1988), the cultures were grown in a liquid sterilized medium at 20 ± 2°C under cool white light for 35 days. At the end of 35 days, nitrogenase activity of the cultures were determined using the acetylene reduction technique.

Appropriate control systems containing no solvent and pesticide were included in each experiment. Control and treated cultures were grown under the same temperature and light intensity as mentioned above. All experiments were performed in triplicate and the average values were presented.

RESULTS

Cyanobacterial species were isolated from soil and water samples obtained from rice fields in Corum, Turkey. In this study, 10 cyanobacteria had studied and determined the effets of different concentrations of bispyribac-sodium on nitrogenase activities of cyanobacteria. These included 8 ***Anabaena*** sp., 1 ***Synechocystis*** sp. and 1 ***Gloeothece*** sp. strains.

When ***Anabaena***, ***Synechocystis*** and ***Gloeothece*** sp. were cultured in the presence of various bispyribac-sodium concentrations, distinct effects were seen on nitrogenase activities and growths. The growths and nitrogenase activities of cyanobacteria treated with different concentrations of bispyribac-sodium under 11 µmol/m^2 light intensity are listed in Tables 1 and 2.

Among all ***Anabaena*** strains, the maximum activity was determined in ***Anabaena*** sp. O-22 (2.26 µl ethylene/mg.h) whereas, the lowest activity was shown in ***Gloeothece*** sp. O-Y (0.04 µl ethylene/ mg.h). The nitrogenase activities of ***Anabaena*** sp. O-X2, O-4 and O-8 were stimulated in initial period but, increasing concentrations repressed the nitrogenase activity. Bispyribac-sodium experiments have shown that the initial nitrogenase activity of ***Gloeothece*** sp. O-Y at low concentrations of bispyribac-sodium (5 to 10 µg/mL) did not change but, the activity repressed with increasing bispyribac-sodium concentrations in ***Gloeothece*** sp.O-Y (Table 1). The maximum inhibition was

Table 1. Effects of bispyribac- sodium on nitrogenase activity of cyanobacteria.

Microorganisms	Ethylene amount (µL/ mg.h)						
	Concentrations (µg/mL)						
	Control	5	10	25	50	100	500
Anabaena sp. O-X2	0.58±0.002	0.65±0.01	0.53±0	0.44±0	0.29±0	0.23±0.01	-
Anabaena sp. O-Ç	1.50±0.001	0.22±0.01	0.33±0.014	0.31±0.05	0.18±0	0.15±0.02	-
Anabaena sp. O-6	1.2±0.003	0.35±0.001	0.26±0.014	0.23±0.001	0.28±0	0.05±0	-
Anabaena sp. O-K	1.28±0.001	0.9±0.009	0.31±0.035	0.01±0.05	0.04±0.02	0.05±0.02	0.05±0.01
Anabaena sp. O-4	0.3±0.001	0.77±0.002	0.6±0.002	0.59±0.09	0.6±0.001	0.6±0.05	-
Anabaena sp. O-22	4.45±0.005	2.26±0.2	0.03±0.2	0.009±0	0.008±0.5	-	-
Anabaena sp. O-16	4.8±0.005	0.95±0.006	0.96±0.05	0.34±0.005	0.14±0.4	-	-
Anabaena sp. O-8	0.86±0.001	1.04±0.001	2.1±0.08	1.38±0.16	0.84±0.001	0.82±0.08	-
Synechocystis sp. O-X	2.62±0.002	1.18±0.008	1.40±0.001	1.45±0.003	0.9±0.04	-	-
Gloeothece sp. O-Y	0.30±0.004	0.25±0.001	0.27±0	0.20±0.007	0.08±0.02	0.04±0.07	0.04±0.01

(-): No effect Values are mean ± Standard deviation.

Table 2. Effects of bispyribac- sodium on growth of cyanobacteria.

Microorganisms	Dry weight (mg/mL)						
	Concentrations (µg/mL)						
	Control	5	10	25	50	100	500
Anabaena sp. O-X2	1.2±5	1.8±15	1.6±10	1.5±10	1.5±0	0.7±10	-
Anabaena sp. O-Ç	0.4±8	0.5±20	0.5±0	0.7±10	0.4±14.5	0.4±20	0.1±12
Anabaena sp. O-6	5±10	0.9±10	1.1±14	1.1±4	1.4±18	0.4±20	-
Anabaena sp. O-K	1.4±9	0.8±5	0.6±16	0.4±6.3	0.2±17	0.01±25	0.01±20
Anabaena sp. O-4	0.3±10	1.2±7	1.5±0	0.8±5	0.9±15	1±1.8	-
Anabaena sp. O-22	0.1±15	0.1±7	0.004±10	0.004±20	0.004±20	-	-
Anabaena sp. O-16	0.3±2	0.8±10	0.3±10.5	0.3±24	0.1±16	0.1±20	-
Anabaena sp. O-8	0.7±2.5	1.1±0	1.1±0	0.9±10	0.8±20	0.7±15	0.5±20
Synechocystis sp. O-X	0.2±0	0.3±10	0.4±7	0.5±14	0.2±20	-	-
Gloeothece sp. O-Y	0.7±0	0.6±7.8	0.7±5	0.4±18	0.3±0	0.4±10	0.4±18

(-): No effect Values are mean ± Standard deviation.

seen in ***Anabaena*** sp. O-22, ***Synechocystis*** sp. O-X and ***Anabaena*** sp. O-16 in 100 µg/ml bispyribac-sodium concentration. With the exception of ***Anabaena*** sp. O-K and ***Anabaena*** sp. O-Y, the nitrogenase activities of all other strains were inhibited at 500 µg/mL bispyribac-sodium concentration.

The growths of ***Anabaena*** sp. O-X2, O-Ç, O-4, O-16, O-8 and ***Synechocystis*** sp. O-X were stimulated in low bispyribac-sodium concentrations, but the biomass of all cultures were severely inhibited in higher concentrations. Bispyribac-sodium experiments have shown that the initial biomass of ***Anabaena*** sp. O-22 at low concentrations of bispyribac-sodium (5 µg/ ml) did not change but, the growth repressed with increasing bispyribac-sodium concentrations. The negative impacts of high bispyribac-sodium on the biomass of ***Anabaena*** sp. O-6 and O-K cultures were also demonstrated. The growths of ***Anabaena*** sp. O-22 and ***Synechocystis*** sp***.*** O-X completely repressed at 100 µg/ mL and at higher bispyribac-sodium concentrations, whereas, ***Anabaena*** O-X2, O-6, O-4 and O-16 completely suppressed at 500 µg/mL bispyribac-sodium concentration (Table 2).

DISCUSSION

Variation in growth conditions influenced the growths and nitrogenase activities of all genera. Although, the use of the herbicide is aimed at eliminating weeds, a major portion is deposited on the surface of the soil and might adversely affect the nontarget soil microflora. The nitrogen- fixing cyanobacteria are known to dominate the water-logged paddy fields and help in the nitrogen economy of rice agriculture (Singh, 1961; Stewart, 1967;

Henriksson et al., 1975). Information on resistance to herbicides, and for bispyribac in particular, is lacking. In Turkey, bispyribac-sodium is mostly used for eliminating weeds in paddy fields in the Corum- Osmancik region (THOA, 2002). For this reason, the herbicide was chosen for this study.

In this study, bispyribac–sodium stimulated nitrogenase activity of ***Anabaena*** sp.O-X2, O-4 and O-8 at 5 µg/ml but not in higher concentrations. It was demonstrated that ***Anabaena*** sp. was capable of growing both photoautotrophically and photoheterotrophically like bacteria to a great extent (Jin et al., 1996; Yan et al., 1997).

In the other cyanobacteria tested the nitrogenase activities and growths were inhibited during the initial concentration (5 µg/ml) (Table 1). Gonzalez-Barreiro et al. (2006) showed that the serious effects on growth for microalgae by herbicide added to culture medium. The main characteristic of cell death or decrease of cell viability, whether from senescence, acute stress, or aging, seems to be the loss of the ability of cells to maintain homeostasis (Gahan, 1984). Most reports have demonstrated that the inhibitory effect of herbicide became greater with an increase in herbicide concentration and suggested that the reduction in the dry matter of algae may be due to a decrease in algal photosynthesis caused by the inhibition of synthesis of chlorophyll, which is the most important pigment in algal cells for collecting solar energy for photosynthesis (Caux et al., 1996; Prosperi et al., 1993).

The nitrogenase activities of ***Anabaena*** sp. O-K, O-6 and O-16 were more inhibited than growths. The nitrogenase activity of ***Anabaena*** sp. O-22 was repressed about 50% by 5 µg/ml bispyribac-sodium but growth was unaffected. In the case of ***Synechocystis*** sp. O-X, the herbicide also inhibited nitrogenase activity by about 50% but the dry matter increased. Powell et al. (1991) reported that nitrogenase activity was more sensitive to the isopropylamine salt of glyphosate than was photosynthetic O_2 evolution.

In ***Anabaena*** sp. strains O-X2, O-4 and O-8 it were found that very low concentrations of bispyribac-sodium (5 µg/ml) stimulated both nitrogenase activity and growth but increased concentrations repressed both nitrogenase activity and growth. The observed effects of bispyribac-sodium on cyanobacterial growth in this study are similar to those reported by Shen et al. (2005) for butachlor and acetochlor on several ***Anabaena*** species. Butachlor and acetochlor stimulated growth of these cyanobacteria under low concentrations (1 to 8 mgL^{-1}), but showed high toxicity at concentrations above 16 mgL^{-1}. Hammouda (1999), on the other hand, demonstrated the utilization of carbofuran at low concentrations by a nitrogen-fixing cyanobacterium, ***Anabaena doliolum***. The 2.4D, a synthetic growth hormone analog, is reported to stimulate growth and heterocyst formation in the cyanobacterium at lower concentrations (Mishra and Tiwari, 1986). Growth studies showed that the strains used in the present study were capable of growing both photoautotrophically and photoheterotrophically (Yan et al., 1997; Guoan et al., 1997).

The data obtained in this study provide information about the inhibitory effect of the bispyribac- sodium on growths and nitrogenase activities of cyanobacteria, which exhibits different sensitivity to the herbicide. These findings suggest a limit or avoidance of the use of bispyribac- sodium in paddy fields, due to its inhibitory effect on biological nitrogen fixation and hence a possible reduction in rice crop yields.

In this study, we have shown a clear physiologic distinction betweeen ***Anabaena*** sp O-22 sp. and the other strains. Generally ***Anabaena*** sp O-22 had the best optimal performance of nitrogenase activity in all environmental conditions, so it is thought that it is a suitable genus for biofertilizer. A better understanding of the mechanism of action of the herbicide on biological nitrogen fixation requires further study of the biochemical targets of the herbicide in cyanobacteria.

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Full Length Research Paper

Antimicrobial activity of crude venom extracts in honeybees (*Apis cerana, Apis dorsata, Apis florea)* tested against selected pathogens

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Antimicrobial activity of crude venom extracts from different honeybee species was tested against selected pathogens. Toxicity of bee venom is known to man since ages, which varies from mild inflammations to death. Bee venom is synthesized in the venom glands of worker bees and queen and is stored in the venom sac. In the present study the toxic potentialities of honeybee venom pertaining to different honeybee species *Apis cerana, Apis dorsata* and *Apis florea* was carried out *in vitro* on selected species of bacteria and fungi. The selected bacteria and fungal species were *Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Xanthomonas subtilis, Proteus vulgaris, Salmonella typhimurium* and *Candida albicans*. They were collected from St. John Medical College, Bangalore. The antimicrobial activity of different species of *Apis* bee venom (ABV) was studied by Disc Diffusion Assay. Minimal inhibitory concentration (MIC) was determined using Broth dilution method at lowest dilution (0.5 mg/ml). Bacterial growth was assessed by the measurement of inhibitory zone. The order of susceptibility of the pathogens against the ABV recorded was: *A. cerana* > *Apis dorsata*> *Apis florea.* The results showed that, ABV has significant antimicrobial effects and could be a potential alternative antibiotic.

Key words: Bee venom, *Apis* species, broth dilution, inhibitory zone, bacteria, fungi.

INTRODUCTION

Honeybees are the earliest known social insects to man. They have survived alongside their ever-changing environment for 120 million years. They are recognized and appreciated as the single most important insect pollinators and thus, increase the productivity of food plants on earth (Free, 1993; Jyothi, 1994; Chaudary et al., 2001; Sharma and Gupta, 2001). Besides pollination, honeybees provide honey, bee wax, royal jelly, pollen, venom and propolis. The venom gland of worker bee is located in posterior portion of the abdomen, between the worker's rectum and ovaries (Owen and Bridges, 1984).

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The two glands (Dufours and Venom gland) associated with sting apparatus of the worker produce venom. The venom gland is a thin long, distally bifurcated integumentary gland with cuticular lining. It consists of a secretary filamentous region, connected to a reservoir at its proximal portion, in which the venom is stored (Kerr and Lello, 1962). The small flat cells also bearing canaliculi form the distal region of the reservoir where their products contributes to venom composition (Lello, 1971). The workers sting only once, which leads to their death.

Venom contains 88% water. At least 18 pharmacologically active components have been described so far; including various enzymes, peptides and amines (Dotimas and Hider, 1987). The glucose, fructose and

phospholipids contents of venom are similar to those in bee's blood. Venom from *Apis* species is similar, but even the venoms from various races within each species are slightly different from each other. Bee venom is haemorrhagic and contains apamine, melittin, phospholipase, hyaluronidase. These oppose the inhibiting action of the nervous system and also stimulate the heart and adrenal glands. Sulphur is the main element in inducing the release of cortisol from the adrenal glands which protects the body against infections. The venom also contains mineral substances, volatile-organic acids, formic acid and some antibiotics. Venom is one of the products of honeybee, which is an important component in the pharmaceutical industry. Use of naturally available substances as medicines, in Asia represents a long history of human interactions with the environment. The medicinal value of these substances lies in some chemicals that produce a definite physiological action on the human body. The venom production is usually complete within two weeks and then glands start to degenerate in the adults. Not only has the age affected the venom composition but also seasonal factors like availability of food sources etc. A newly emerged bee has very little venom content, but the amount gradually accumulates with age, to about 0.3 mg in a 15 day old *A. mellifera* worker bee (Owen and Bridges, 1976), after the age of 18 days no additional venom is produced. Subsequently, the weight of the venom in the venom sac remains unchanged (Cruz- landim et al., 1966, 1967).

The study of social Hymenoptera (bees, wasps, and ants) venom proteins is of great interest, since these venoms can trigger serious allergenic reactions in humans. The allergenic reactions of Hymenoptera venoms are caused mostly by low molecular weight compounds, which can result in pain, local inflammation (Hider, 1981, 1988; Hoffman, 1977), itching, and irritation as immediate responses that after some hours are attenuated (Golden et al., 1989). Melittin is the main compound responsible for most of these reactions, and it is present in several bee venoms.

Bee venom has interesting pharmacological properties (Dong. et al., 2007) and is used in the treatment of various health conditions such as arthritis, rheumatism (Putz et al., 2006), pain (Kim et al., 2003), cancerous tumors (Russell et al., 2004) and skin diseases.

MATERIALS AND METHODS

Collection of bee venom

Venom, from forager honeybees of three *Apis* species descended from naturally mated queens were used to study its antimicrobial activity. These bees, derived from a pool of 3 colonies were collected from the Apiary of the Centre for Apiculture Studies, Department of Zoology, Bangalore University, Jnana Bharathi Campus, Bangalore. They were immobilized by quick freezing at -20°C. The venom reservoirs were extracted at 4°C by dissecting the stinging apparatus and stored at -20°C until required. Venom sacs were re-suspended in deionized water (MilliQ) and extracts of whole bee venom (WBV) were made by reservoir disruption under rapid defrosting and light pressure by a glass rod. These samples were centrifuged at 10,000 g at 4°C for 5 min, and the supernatants were used as protein and enzyme sources and then lyophilized (Biotran, Speed vaccum concentrator, Model: Ecospin 3180C). Lyophilized Bee venom was dissolved in MilliQ water and filtered using 0.22 mμ syringe filter to further estimations.

Collection of bacterial isolates

The test clinical control isolates used in the present study were collected from Research Laboratories of St. John's Medical College and Hospital, Bangalore. These clinical isolates were identified based on the standard microbiological techniques (Chees brough, 1998) and drug susceptibility test for each clinical isolate was done following the standard agar Disc Diffusion method (Bauer et al., 1966). The microbial strains, *P. aeruginosa, C. albicans, E. coli, K. pneumoniae, X. subtilis, P. vulgaris and S. typhimurium* were used to determine the antimicrobial activity of venom from different *Apis* species.

Pure culturing of microbial strains

The collected clinical control microbial strains were maintained in the laboratory on Nutrient Agar (Hi-Media) by Slant–Streak technique for further pure cultures (Mackie Mc Cartney, 1999).

The nutrient Agar Hi-Medium composed of 5 g peptone, 2 g Beef extract, 5 g Sodium chloride and 20 g Agar-Agar was dissolved in one liter of double distilled water and pH was maintained at 7.0 ± 0.2. The mixture of contents were later transferred into a sterile conical flask and plugged with cotton for air tightening. The conical flask with contents was autoclaved and the flasks were cooled and stored at 5 to 10°C.

Under sterile conditions, the contents when needed were dissolved on heating mantle and 10 ml of medium was poured into sterile test tubes and cooled in Laminar Air Flow by placing in slanting position.

The solidified medium was streaked with specific bacterial strains using sterile inoculation loop. The slants with strains were incubated in Bacterial incubator at 35 to 37°C for a period of 24 to 48 h. The slants with strains were stored at 4°C.

Maintenance of pure bacterial culture suspension in Nutrient Broth

Under aseptic conditions, pure colonies of Bacterial isolates from slants were picked with an inoculating loop and suspended in 3 to 4 ml of nutrient broth in sterile test tubes and incubated for 24 h at 37°C. The contents were transferred into sterile conical flask and plugged with cotton (Andargrchewmulu et al., 2004).

Investigation of antibacterial potency of venom

The nutrient agar Disc Diffusion method (Molan, 1992; Sommeijer and Francke, 1995; Perumalsamy et al., 2006) was employed to test the antibacterial activity of venom of different species. The sterile control bacterial nutrient broth was further used to prepare the culture suspension in order to set an inoculum's density of 100 µl: $0.1A_{600}$ culture containing 3.2×10^8 colony forming unit (cfu /ml). Further, 20 µl was spread on to 20 ml of sterile agar plates by using a sterile cotton swab. The surface of the medium was allowed to

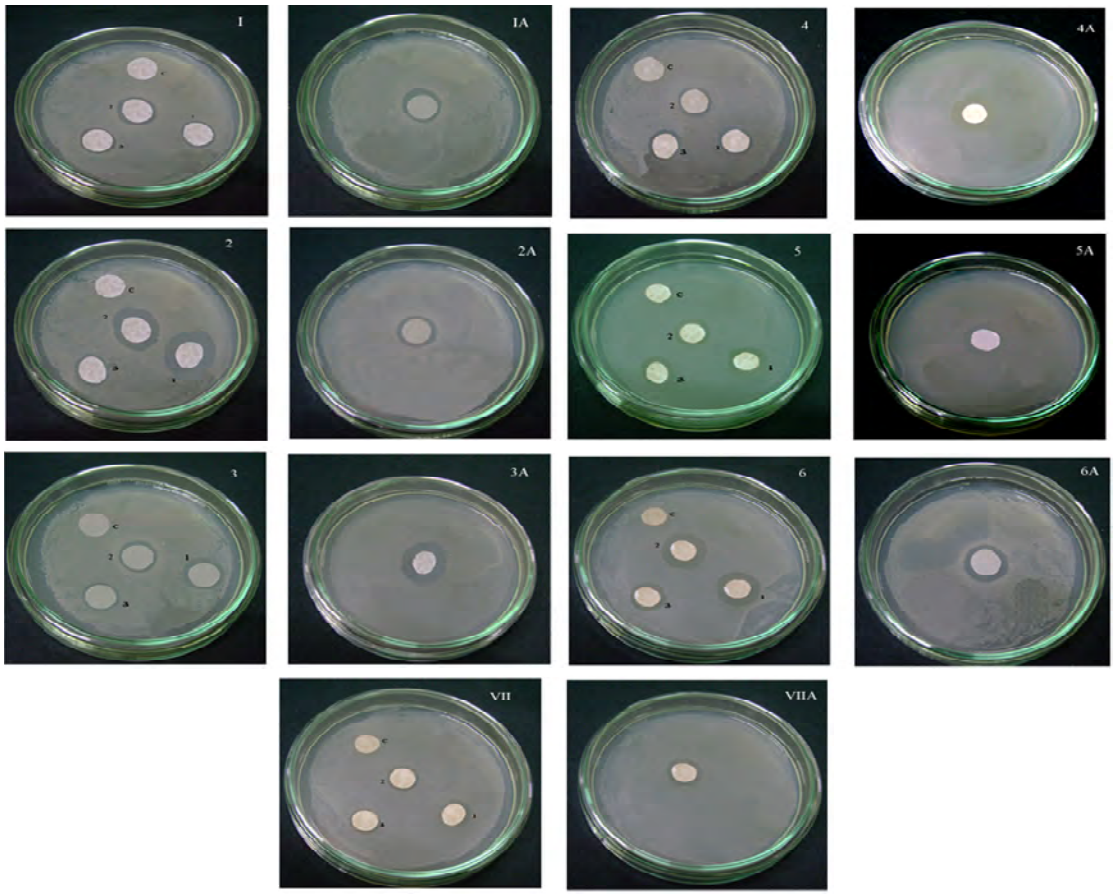

Figure 1. Inhibition zones of venom from different *Apis* species (1-3) with Control (C) against selected microbial strains (I-VII) along with their Ampicillin Standards (IA-VIIA). C: Control, 1: *Apis cerana*, 2: *Apis dorsata*, 3: *Apis florea*.
I and IA: *Escherichia coli*; II and IIA: *Pseudomonas aeruginosa*; III and IIIA: *Klebsiella pneumonia*; IV and IVA: *Proteas vulgaris*; V and VA: *Xanthomonas camperstris*; VI and VIA: *Solmonella typhimurium*; VII and VIIA: *Candida albicans.*

dry for about 3 min and sterile paper discs (10 mm in diameter) were placed on them. 20 µl of various concentration of proteins that is, 10, 20, 30 and 25 µg of venom sample was added per disc. Disc containing 20 µl of MilliQ water served as control. The bacterial plates were incubated at 37°C for 24 h and fungal plate at 37°C for 48 h. The diameters of inhibition zones were measured. The results were compared with standard Ampicillin. Inhibition effects were measured in terms of zone of inhibition around the paper disc with scale. The zone of inhibition was measured from the centre of the disc till the edge of the inhibition zone. This was repeated at four directions in the inhibition zone and the mean radius was calculated. The area of zone of inhibition was calculated using the formula:

$A_{in} = \pi R^2 - \pi r^2$

Where A_{in} = inhibition zone
π = 22/7, R = radius of the inhibition zone and r = radius of the paper disc.
πr^2 in this case was the area of the paper disc that was deducted from the area of the zone of inhibition to obtain the mean area of inhibition produced due to the action of bee venom against respective pathogens. Various clear zones, more than 10 mm in diameter, that were observed at different concentrations of venom for varied bacterial strains were measured in millimeter and the average of the inhibition zones were recorded. In this experiment as a positive control, we used standard antibiotic Ampicillin disc (10 µg/ml each).

Protein estimation

The protein content in the honeybee venom samples was estimated by using Bovin serum albumin as standard at 610 nm (Lowrys et al., 1951).

Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE) was performed using 12% polyacrylamide at 120 V and 20 mA. Venom samples were dissolved in 20 µl of doubled distilled water, 5 µl of sample buffer (0.001% mercaptoethanol, 75% of 0.313 M Tris- HCl and 10% glycerol) and 0.001% bromophenol blue (pH 6.8). The samples were boiled for two minutes, shaken in vortex for 30 s and loaded onto the gel. The gels were stained in 0.25% Coomassie Brilliant Blue R-250 solution and destained with 30% methanol and 10% acetic acid to reveal proteins.

Table 1. Antimicrobial activity of crude honeybee venom extract from different species of honeybee workers tested against the selected microorganisms showing zone of inhibition in diameter (mm^2) N=5.

Concentration (µg)	*Escherichia coli* (mm^2)	*Pseudomonas aeruginosa* (mm^2)	*Klebsiella pneumoniae* (mm^2)	*Proteus vulgaris* (mm^2)	*Xanthomonas subtilis* (mm^2)	*Salmonella typhimurium* (mm^2)	*Candida albicans* (mm^2)
			Apis cerana				
10	10.50 ± 0.25	12.50 ± 0.25	19.00 ± 0.25	12.00 ± 0.50	14.00 ± 0.25	18.00 ± 0.25	13.00 ± 0.25
20	12.00 ± 0.20	13.00 ± 0.20	20.00 ± 0.14	13.50 ± 0.10	16.00 ± 0.32	19.65 ± 0.25	14.00 ± 0.20
25	12.50 ± 0.20	14.00 ± 0.29	21.00 ± .20	12.75 ± 0.20	17.50 ± 0.12	20.25 ± 0.10	15.50 ± 0.20
30	13.50 ± 0.12	15.00 ± 0.16	22.50 ± 0.18	15.00 ± 0.12	18.50 ± 0.12	21.30 ± 0.10	17.00 ± 0.31
			Apis dorsata				
10	11.50 ± 0.21	10.50 ± 0.20	16.00 ± 0.18	10.75 ± 0.16	13.00 ± 0.29	17.00 ± 0.20	10.50 ± 0.20
20	12.00 ± 0.33	11.00 ± 0.25	17.00 ± 0.43	11.50 ± 0.15	14.00 ± 0.25	18.50 ± 0.25	11.50 ± 0.13
25	13.00 ± 0.25	12.00 ± 0.02	18.00 ± 0.15	12.50 ± 0.25	14.50 ± 0.15	19.5 ± 0.25	13.50 ± 0.25
30	14.00 ± 0.25	13.50 ± 0.25	19.00 ± 0.25	13.50 ± 0.25	16.50 ± 0.15	21.50 ± 0.25	14.00 ± 0.14
			Apis florae				
10	11.00 ± 0.25	0.00 ± 0.00	13.50 ± 0.15	0.00 ± 0.00	11.00 ± 0.05	14.25 ± 0.25	0.00 ± 0.00
20	11.50 ± 0.13	10.50 ± 0.14	14.00 ± 0.25	13.50 ± 0.15	13.00 ± 0.25	16.00 ± 0.25	11.00 ± 0.07
25	12.00 ± 0.23	11.00 ± 0.03	15.00 ± 0.12	14.25 ± 0.00	14.00 ± 0.25	15.25 ± 0.10	11.50 ± 0.16
30	13.00 ± 0.25	12.00 ± 0.18	16.00 ± 0.25	15.00 ± 0.25	16.00 ± 0.25	17.75 ± 0.25	12.00 ± 0.25

Two molecular weights were estimated using standard molecular. Weight marker (1KDa) was obtained from Genei pvt Limited, Bangalore, India.

RESULTS AND DISCUSSION

In the present investigation six Gram-negative bacteria and fungal strains were used to investigate the antimicrobial properties of venom from different species of honeybee by using Disc Diffusion Assay. The sensitivity of seven microbial strains was tested against the venom of three *Apis* species (*A. cerana, Apis dorsata, Apis florea*). Venom of all three species showed antimicrobial activity against selected microbial strains (Figure 1). The antimicrobial spectrum of venom of three *Apis* species in terms of inhibition zone (IZ) is shown in Table 1. The deionised water (MilliQ) had no inhibitory effect on tested microbial strains.

The venom from *A. cerana* showed high antimicrobial activity and exhibited larger zone of inhibition among three species (Table 1, Figure 2). The venom concentration of 30 µg, showed highest inhibition zone against *K. pneumoniae* (22.50 ± 0.18) followed by *S. typhimurium* (21.30 ± 0.10), *X. subtilis* (18.50 ± 0.12), *C. albicans* (17.00 ± 0.31), *P. aeruginosa* (15.00 ± 0.16), *P. vulgaris* (15.00 ± 0.12) and *E. coli* (13.50 ± 0.12).

The venom of *A. dorsata* showed at the 30 µg concentration of venom showed highest inhibition zone against *S. typhimurium* (21.50 ± 0.25) followed by *K. pneumoniae* (19.00 ± 0.25), *X. subtilis* (16.50 ± 0.15), *E. coli* (14.00 ± 0.25), *C. albicans* (14.00 ± 0.14), *P. aeruginosa* (13.50 ± 0.25) and *P. vulgaris* (13.50 ± 0.25). *P. aeruginosa*, *P. vulgaris, C. albicans* and *E.*

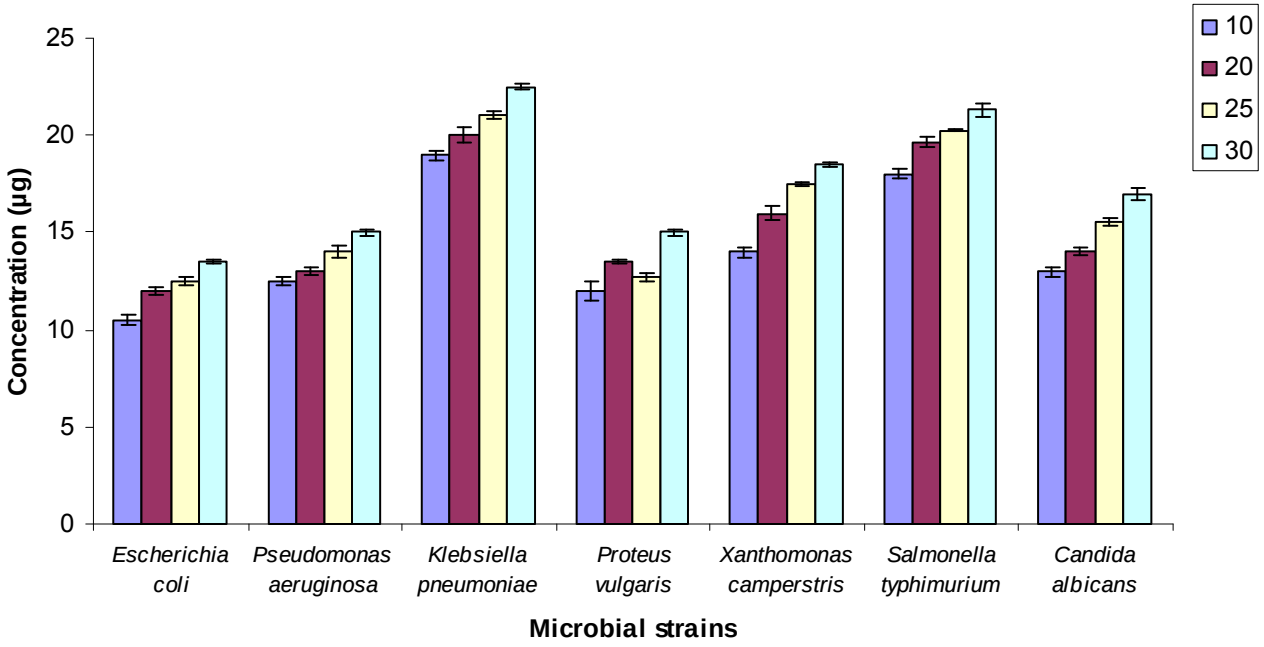

Figure 2. Inhibitory zones at different venom concentration of *Apis cerana* tested against the selected microbial strains.

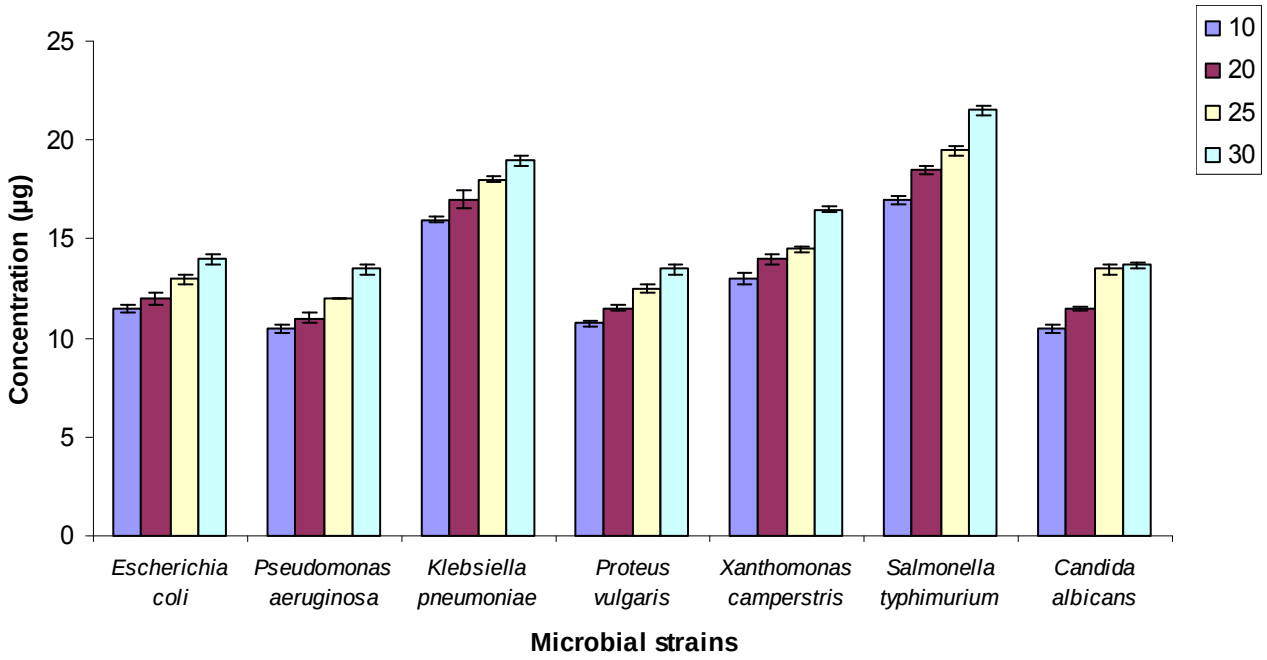

Figure 3. Inhibitory zones at different venom concentration of *Apis dorsata* tested against selected microbial strains.

coli showed almost similar effects at the same concentration (Table 1, Figure 3).

A. florea showed minimum antimicrobial activity when compared to *A. cerana* and *Apis dorsata*. The concentration of 30 µg showed highest inhibition zone against *S. typhimurium* (17.75 ± 0.25) followed by *X. subtilis* (16.00 ± 0.25), *K. pneumoniae* (16.00 ± 0.25), *E coli* (13.00 ± 0.25), *P. aeruginosa* (12.00 ± 0.18) and *C. albicans* (12.00 ± 0.25). *C. albicans, P. aeruginosa, K. pneumoniae,* and *X. subtilis* showed similar effects at 30 µg venom concentration (Table 1, Figure 4).

The present examination also showed that the

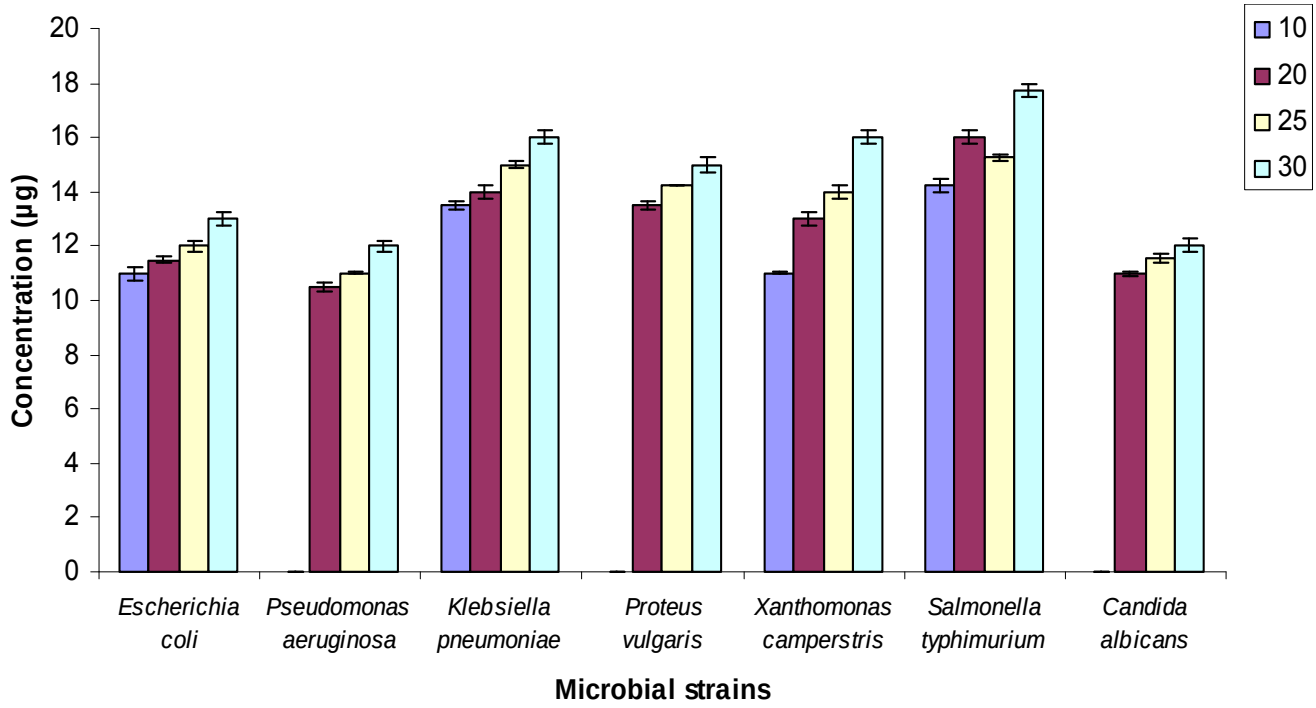

Figure 4. Inhibitory zones at different venom concentration of *Apis florea* tested against selected microbial strains.

Table 2. The zones of inhibition diameter (mm^2) of Ampicillin standard (10 µg/ml).

Names of microbial strains	Zone of inhibition (mm^2)
Pseudomonas aeruginosa	15.00
Klebsiella pneumoniae	22.00
Escherichia Coli	13.00
Xanthomonas subtilis	18.00
Proteus vulgaris	17.00
Salmonella typhimurium	19.00
Candida albicans	16.00

antimicrobial activity of venom of three *Apis* species at the concentration of 25µg was almost equal to the effect of Ampicillin at 10µg (Table 2, Figure 5). The SDS PAGE gel also confirmed the constituent proteins from the lyophilized crude venom such as phospholipase A2, Melittin and some of the small peptides with molecular weight ranges of 35, 34, 30, 27, 31, 16, 15, 11, 9, 8, 7, 6, 5 and 4 KDa were common in all the three *Apis* species (Figure 6).

The venom of *A. cerana* inhibits the growth of fungi; *C. albicans* as compared to venom of *A. dorsata* and *Apis florea*. Similarly, the venom of *A. cerana* inhibited the growth of bacteria such as *E coli, P. vulgaris, P. aeruginosa, X. subtilis, K. pneumoniae* and *S. typhimurium* to a maximum extent as compared to the venom of *A. dorsata* and *Apis florea.*

Since, reliable research information on the antimicrobial properties of Indian honeybee venom is not available; such a study has been carried out for the first time to know the functional status of bee venom especially on growth inhibition of fungi and bacteria. Benton et al. (1963) determined the inhibition rate on the spores of *Bacillus subtilis* from the venom of *A. mellifera*. Similarly Hancock et al. (1995) reported that Melittin in the venom of *A. mellifera* had wide spectrum of biological effects including antimicrobial activity. The toxicity of *A. cerana* venom has been reported to be twice as high has that of *Apis mellifera* (Benton and Morse, 1968). The non selective cytotoxic venom protein Melittin, was the most abundant polypeptide in the venom of *A. mellifera* (Habermann et al., 1967). This antimicrobial activity and protein profile of honeybee venoms reported herein will

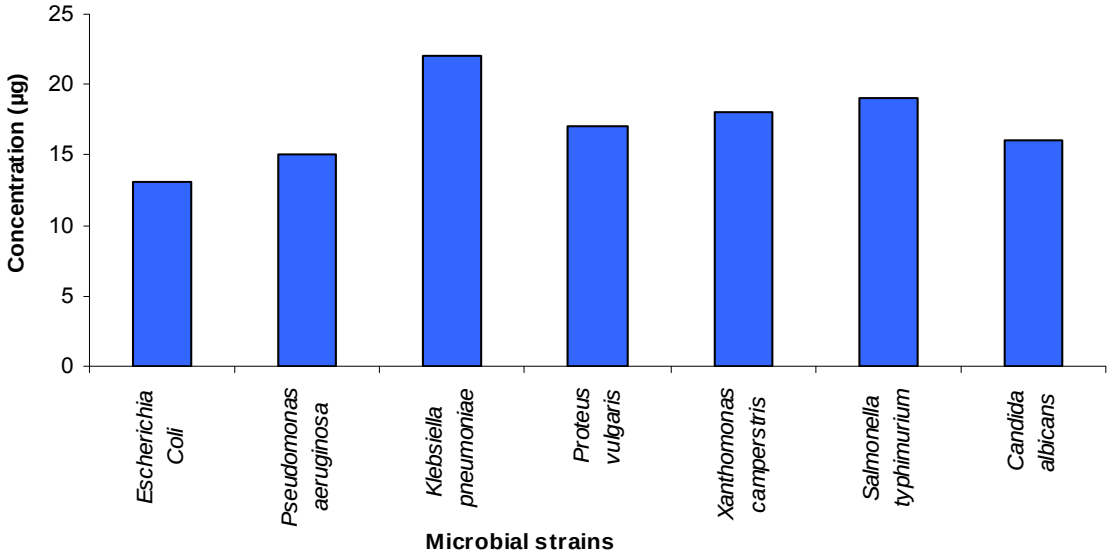

Figure 5. Zone of inhibition (diameter, mm^2) showed by Ampicillin standard against the selected microbial strains.

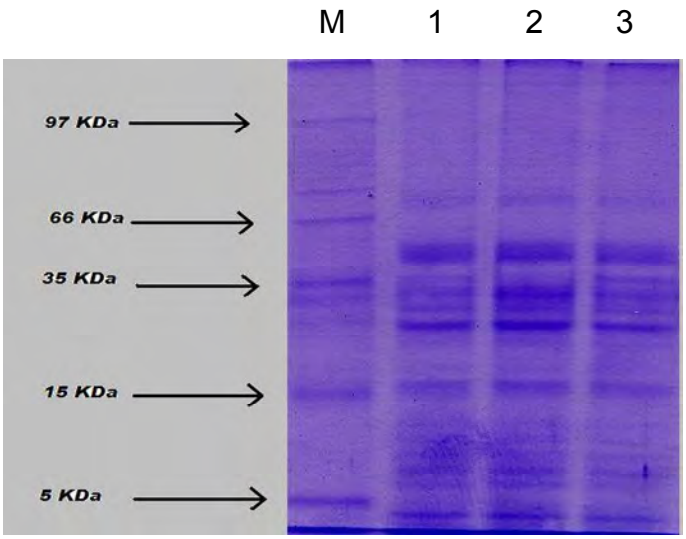

Figure 6. SDS-PAGE (12%) was performed with molecular marker to know the presence of different proteins and their molecular weight. M – 1 KDa standard marker, 1 - *Apis cerana*, 2 - *Apis dorsata*, 3 - *Apis florae.*

be useful in the search for potential antimicrobial agents against drug resistant microorganisms.

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Full Length Research Paper

The bactericidal and fungicidal effects of salicid on pathogenic organisms involved in hospital infections

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Accepted 15 July, 2011

The study was designed to investigate bactericidal and fungicidal actions of salicid (pH: 6.7, ORP: 760 mV, residual chlorine of 2 to 5 ppm) on hospital infections. Four of the most common opportunistic pathogens (*Klebsiella pneumoniae*, *Staphylococcus aureus*, *Rhizopus oryzae*, and *Aspergillus fumigatus*) were used for this study. Cultures were inoculated in 9 mL of salicid and incubated for 0.5, 1, 3, 5, 7 and 10 min at room temperature (23 ± 2°C). A dipping method was followed for this study. Untreated pathogens were treated as control. Compared to the untreated control, a reduction of 1.10 to 6.08 $\log_{10}$ CFU/mL for aforementioned pathogens were recorded as the result of treatment with 2 and 5 ppm salicid, respectively. The highest bactericidal effect was found with *S. aureus* for 5 ppm salicid with 0.5 min immerse time. Salicid treatment with 0.5 min dipping and 5 ppm residual chlorine also reduced *K. pneumoniae*, *R. oryzae*, and *A. fumigatus* by 5.00, 3.50 and 2.63 $\log_{10}$ CFU/mL, respectively. Our findings showed that in each pathogen, efficacy of salicid decreased significantly ($p < 0.05$) with increased dipping time, from 0.5 to 10 min and there was significant difference ($p < 0.05$) observed between 2 and 5 ppm salicid treatment in reducing pathogens. The results indicate that salicid may be a useful disinfectant for hospital infections, but its clinical application has still to be evaluated.

Key words: Salicid, bactericidal and fungicidal effect, hospital infections.

INTRODUCTION

Hospital infections are a serious medical, social, and economic problem for public health services all over the world (Vorobjeva et al., 2004). *K. pneumoniae*, *S. aureus*, *R. oryzae*, and *A. fumigatus* are the most opportunistic human pathogens involved in hospital infections. *K. pneumoniae* is a major cause of nosocomial infections (DeChamps et al., 1991; Johnson et al., 1992). As a general rule, Klebsiella infections tend to occur in people with a weakened immune system. Many of these infections are obtained when a person is in the hospital for some other reason. The most common infection caused by *Klebsiella* bacteria outside the hospital is pneumonia. *S. aureus* is the most common organism responsible for postoperative wound infections (Wenzel and Perl, 1995) and a leading cause of septicaemia, intravenous catheter-related infections and skin and soft-tissue infections. Auto-infection of surgical wounds by *S. aureus* is common, and is associated with considerable morbidity and represents an important medical and economic problem. *R. oryzae* is the most common cause of zygomycosis, a life-threatening infection that usually occurs in immunocompromised patients (Ibrahim et al., 2005). Infections caused by *Aspergillus* species have grown in importance in recent years (Pasqualotto, 2009). As most of the *Aspergillus* infections are caused by *A. fumigatus*, the majority of studies have focused on this species. Such pathogens are typically characterized by a wide variety of sources, ways, and factors of transmission, appearing in different types of clinics and preventive hospitals. In this context, the methods of asepsis and active chemical antisepsis are currently becoming increasingly important in terms of the prevention of hospital infections.

Salicid is the first potable product treating infectious disease on human mucosa and skin using its

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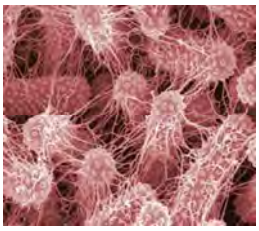
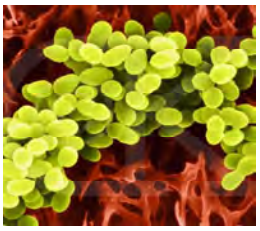
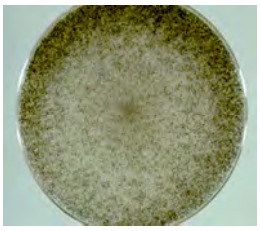

Klebsiella pneumoniae


Staphylococcus aureus


Rhizopus oryzae

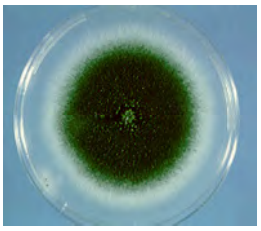

A. fumigatus

Figure 1. Challenge microorganism.

antimicrobial efficacy for bacteria, fungi and viruses. When electrolyzing brine with salicid, small sized chlorine bubbles are generated and this chlorine gas dissolves in the solution, producing the free chlorine. Salicid hardly change the pH value of used water, so it is the product available for medical use which can use hypochlorous acid which is a predominant species of free chlorine at the pH range of weak acidic and neutral. The free chlorine solution generated by salicid is below 5 ppm which is allowed by WHO for drinking water and swimming pool water and also is safe to human mucosa. Accordingly, salicid is the first antiviral product which can be applied for human mucosa and is the first product which has antimicrobial efficacy for bacteria and fungi, simultaneously.

The objective of this study was to evaluate the bactericidal and fungicidal effect of salicid obtained in the salicid electrolysis device on common hospital pathogens under *in vitro* conditions.

MATERIALS AND METHODS

Challenge microorganisms

K. pneumoniae- ATCC 8724, *S. aureus*- ATCC 12488, *R. oryzae*- ATCC 24794, *A. fumigatus*- ATCC 26430 (Figure 1).

Media and reagents

Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB), Potato Dextrose Agar (PDA), 0.85% saline solution (ss), Neutralizer used: 0.85% Nacl containing 0.5% $Na_2S_2O_3$

Laboratory equipments

Sterile test tubes, Sterile flasks, Sterile Beaker, Sterile pipettes, Petridishes, Stopwatch, Incubator capable of maintaining 35°C, Incubator capable of maintaining 25°C, Pincer, Cheese gauge cloth, Haemocytometer, Microscope, Glass rod, Calibrated thermometers

Inocula preparation

K. pneumoniae and *S. aureus* stock cultures were transferred into tryptic soy broth (TSB) and incubated for 24 h at 35°C. Following incubation, 10 ml of each culture was sedimented by centrifugation (3000 x g for 10 min), washed and resuspended in 10 ml of 0.1% peptone water (pH 7.1) to obtain a final cell concentration of 10^9 CFU/mL. The bacterial population in each culture was confirmed by plating 0.1 ml portions of appropriately diluted culture on tryptic soy agar (TSA) (Difco Laboratories) plates and incubating the plates at 35°C for 24 h. 1 ml of the suspension was used as the inoculum (10^9 CFU).

The two fungal strains *A. fumigatus* and *R. oryzae* were inoculated from the stock culture onto PDA and incubated at 25°C for 10-15 days or until sporulation occurred. When the cultures appeared to be mature, the mycelial mats was removed from the surface of at least five plates and macerated with ss with a sterile glass rod. The suspension was filtered through sterile cheese gauge cloth to remove the hyphae. In order to remove any organic matter, each of the prepared fungi suspensions was washed in the same manner as described for the bacteria.

Test agent preparation

For each generation of the test material, one salicid packet was added to the test device containing 35 mL of sterile tap water (pH 7.0). The device was shaken 10 times. The generation button was activated by pressing the button and let it electrolysis for 20 s. After 20 s the activation button was stopped manually, then the device was shaken 2-3 times and the product was immediately dispensed for testing. After 20 to 21 s electrolysis we got 2 ppm salicid and in the same way after 44-45 s electrolysis we got 5 ppm salicid (pH: 6.5-7.0, ORP: 760-775 mV).

Test devices and material

Salicid (device), serial no. 001170, Salicid (device), serial no. 001171, Salicid salt packet, (Executive Summary former salt 315 mg/ saline 35 ml), DPD Free Chlorine Reagent (Figure 2).

Test

For each replicate, a 1 mL aliquot of prepared inoculum was added to a sterile test tube. Once the test material is generated, 9 mL of the prepared test agent was added to the tube containing 1 mL of the prepared inoculum; a timer started and the tube was mixed immediately. After each contact times, a 1 mL sample was transferred to a tube containing 9 mL of neutralizer. Serial ten-fold dilutions were performed in 0.85% ss dilution blanks. There were three replicates performed for each microorganism.

Contact times

30 s, 1 , 3, 5, 7 and 10 min.

Salicid device | Dry cell | Salicid salt packet

Figure 2. Test devices.

Temperature

Ambient room temperature

Incubation and enumeration

Upon completion of the test, all plates were inverted and incubated for the appropriate time and temperature as follows: *k. pneumoniae* and *s. aureus* were incubated for 18 to 24 h at 35°C. *A. fumigatus* and *R. oryzae* were incubated for 3-5 days at 25°C. Following incubation, all plates were removed from the incubator, the colonies were counted and the CFU/mL at each contact time was determined.

Statistical analysis

For each treatment, the data from the independent replicate trials were pooled and the mean value and standard deviation were determined.

RESULTS AND DISCUSSION

The mean counts, percent reduction and $\log_{10}$ reduction per test material, contact time and organism are presented in Tables 1 to 4. $\log_{10}$ reduction and percent reduction were calculated using the following equations:

Enumeration is expressed as colony-forming units (CFU/mL)

$$\frac{\text{Average Initial Counts Control} - \text{Test Results}}{\text{Average Initial Counts Control}} \times 100 = \text{Percent Reduction}$$

$\log_{10}$ (Average Initial Counts Control) – $\log_{10}$ (Test Results) = $\log_{10}$ Reduction

Cell suspensions of *K. pneumoniae*, *S. aureus*, *R. oryzae* and *A. fumigatus* were treated at RT (23 ± 2°C) for 0.5, 1, 3, 5, 7 and 10 min with Salicid. Salicid had major antibacterial activity compared to yeast and mould on different challenge microorganisms. Our results revealed that salicid containing 5 ppm of residual chlorine was more effective ($p < 0.05$) than that of 2 ppm salicid in reducing populations of bacterial and fungal strains regardless of dipping time. Reduction of bacterial count was ranged from 3.15 to 6.08 $\log_{10}$ CFU/mL and 1.10 to 3.50 $\log_{10}$ CFU/mL reduction was gained in yeast and mould, respectively. With the increased dipping time rate of log reduction was decreased. The available chlorine concentration (ACC) reduced with an increase in dipping time which could have resulted in lower reductions at increased dipping times. Sensitivity to sanitizers depends on pathogens' characteristics and properties of sanitizers. From our tested pathogens bacteria were more sensitive to salicid compared to fungi and *A. fumigatus* was more resistant to salicid treatment than other three pathogens. Chemical compounds such as formaldehyde, phenol, glutaraldehyde, tricresol and chloramines have shown bactericidal and fungicidal effects (Hegna and Clausen, 1988). However, most of these disinfectants are made from the dilution of condensed solutions, which in handling involves some risk and is troublesome. A disinfectant that is not produced from the dilution of a hazardous condensed solution is required for practical use.

In recent years, electrolyzed oxidizing water (EOW) has gained interest as a disinfectant used in agriculture, dentistry, medicine and food industry (Huang et al., 2008). An advantage of EOW is that it can be produced with tap water, with no added chemicals other than sodium chloride. EOW has been attracting as a disinfectant because of its strong microbicidal activities on a broad variety of bacterial pathogens (Venkitanarayanan et al., 1999). Moreover, EOW exerts fungicidal activity (Suzuki et al., 2002a, b). Xiong et al. (2010) reported that the OH radical ($^{\cdot}OH$) that exists in neutralized electrolyzed oxidizing water (NEW) and acidic electrolyzed oxidizing water (AcEW) was found to have an important fungicidal factor. However, the potential application of EOW is limited because of its low pH values (≤ 2.7) and it corrosive characteristics. At this low pH, dissolved Cl_2

Table 1. *In vitro* inactivation of *Klebsiella pneumoniae* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
1.0×10^8	0.5	$1.5 \times 10^4 \pm 0.10$	$1.0 \times 10^3 \pm 0.06$	99.9850	99.9990	3.82^a	5.00^b
	1	$1.6 \times 10^4 \pm 0.09$	$1.0 \times 10^3 \pm 0.09$	99.9840	99.9990	3.80^a	5.00^b
	3	$1.9 \times 10^4 \pm 0.10$	$1.5 \times 10^3 \pm 0.20$	99.9810	99.9985	3.72^a	4.82^b
	5	$2.8 \times 10^4 \pm 0.18$	$2.5 \times 10^3 \pm 0.09$	99.9720	99.9975	3.55^a	4.60^b
	7	$5.5 \times 10^4 \pm 0.10$	$6.5 \times 10^3 \pm 0.06$	99.9450	99.9935	3.26^a	4.19^b
	10	$7.0 \times 10^4 \pm 0.08$	$1.1 \times 10^4 \pm 0.13$	99.9300	99.9890	3.15^a	3.96^b

Values with different letters within the same column differ significantly at $p < 0.05$.

Table 2. *In vitro* inactivation of *Staphylococcus aureus* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
1.2×10^8	0.5	$3.5 \times 10^2 \pm 0.10$	$1.0 \times 10^2 \pm 0.1$	99.9997	99.9999	5.54^a	6.08^b
	1	$4.0 \times 10^2 \pm 0.10$	$1.0 \times 10^2 \pm 0.26$	99.9997	99.9999	5.48^a	6.08^b
	3	$6.0 \times 10^2 \pm 0.18$	$1.5 \times 10^2 \pm 0.17$	99.9995	99.9999	5.30^a	5.90^b
	5	$1.1 \times 10^3 \pm 0.21$	$2.0 \times 10^2 \pm 0.18$	99.9991	99.9998	5.04^a	5.78^b
	7	$1.5 \times 10^3 \pm 0.20$	$3.0 \times 10^2 \pm 0.25$	99.9988	99.9998	4.90^a	5.60^b
	10	$2.0 \times 10^3 \pm 0.18$	$4.5 \times 10^2 \pm 0.22$	99.9983	99.9996	4.78^a	5.43^b

Values with different letters within the same column differ significantly at $p < 0.05$.

gas can be rapidly lost due to volatilization, adversely affecting human health and the environment. Moreover, the high acidity of EOW may cause the corrosion of equipment and consequently limit its practical application (Abadias et al., 2008; Guentzel et al., 2008). So, as an alternative to EOW we used salicid in our study. Salicid with a pH value of 6.5–7.0, also known as slightly acidic low concentration electrolyzed water (SlALcEW), is commonly produced by electrolyzing a dilute salt solution (0.9% NaCl) in a non-membrane electrolytic cell (Rahman et al., 2010). At a near-neutral pH, the predominant chemical species is the highly biocidal hypochlorous acid species (HOCl, approximately 95%). The advantage of salicid are numerous: non-corrosive due to near-neutral pH, low current and minimum time required to produce it, it does not leave residuals due to low content of ACC (2 - 5 mg/L), comparatively inexpensive, and a less potential health hazard to the worker due to the lack of Cl_2 off-gassing. To produce salicid, an apparatus is required that utilizes common salt and an electric source. Salicid can be produced at site, as the size of the machine is quite small. Therefore, the widely used EOW might be replaced by salicid as an effective and environmentally friendly sanitizer in medical use.

Based on our study this salicid can be recommended for use as a strong disinfectant for the equipment, and diagnostic and medical devices in hospitals. However, future clinical research has to be done under an *in vivo* system, in order to

Table 3. *In vitro* inactivation of *Rhizopus oryzae* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation Units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
7.5×10^6	0.5	$7.4 \times 10^3 \pm 0.13$	$2.4 \times 10^3 \pm 0.09$	99.9013	99.9680	3.01^a	3.50^b
	1	$8.0 \times 10^3 \pm 0.11$	$2.5 \times 10^3 \pm 0.01$	99.8933	99.9667	2.98^a	3.48^b
	3	$1.1 \times 10^4 \pm 0.09$	$3.6 \times 10^3 \pm 0.08$	99.8533	99.9520	2.84^a	3.33^b
	5	$1.5 \times 10^4 \pm 0.08$	$5.3 \times 10^3 \pm 0.04$	99.8000	99.9293	2.70^a	3.16^b
	7	$2.7 \times 10^4 \pm 0.11$	$1.2 \times 10^4 \pm 0.09$	99.6400	99.8400	2.45^a	2.78^b
	10	$4.8 \times 10^4 \pm 0.08$	$3.0 \times 10^4 \pm 0.29$	99.3600	99.6000	2.20^a	2.41^b

Values with different letters within the same column differ significantly at $p < 0.05$.

Table 4. *In vitro* inactivation of *Aspergillus fumigatus* using salicid.

Initial count CFU/mL recovered	Dipping time (min)	Calculation Units					
		CFU/mL recovered		Percent reduction		Log_{10} reduction	
		2 ppm	5 ppm	2 ppm	5 ppm	2 ppm	5 ppm
2.4×10^7	0.5	$2.6 \times 10^5 \pm 0.05$	$5.6 \times 10^4 \pm 0.06$	98.9167	99.7667	1.97^a	2.63^b
	1	$3.0 \times 10^5 \pm 0.03$	$6.0 \times 10^4 \pm 0.03$	98.7500	99.7500	1.90^a	2.60^b
	3	$4.0 \times 10^5 \pm 0.26$	$9.0 \times 10^4 \pm 0.05$	98.3333	99.6250	1.78^a	2.43^b
	5	$6.0 \times 10^5 \pm 0.20$	$1.4 \times 10^5 \pm 0.11$	97.5000	99.4167	1.58^a	2.23^b
	7	$1.0 \times 10^6 \pm 0.23$	$1.8 \times 10^5 \pm 0.08$	95.8333	99.2500	1.38^a	2.13^b
	10	$1.9 \times 10^6 \pm 0.10$	$2.5 \times 10^5 \pm 0.04$	92.0833	98.9583	1.10^a	1.98^b

Values with different letters within the same column differ significantly at $p < 0.05$.

evaluate the stability of salicid under operating conditions and its safety for medical personnel and patients.

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Full Length Research Paper

Isolation of *Legionella pneumophila* from surface and ground waters in Osogbo, Nigeria

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Legionella **is a ubiquitous water environmental organism. Isolation/detection of Legionellae has been reported worldwide. However, there is no reported isolation of Legionellae in countries in Africa with the exception of South Africa. The aim of this study was to survey the surface, ground waters and air conditioner water systems (ACWs) in Osogbo, Nigeria for the presence of *Legionella pneumophila* by cultural isolation method. A total of 313 water samples from the streams (surface water), wells (groundwater) and ACWS were cultured on buffered charcoal yeast extract (BCYE) medium. The isolates were identified by Gram staining techniques, biochemical tests and demonstration of pore forming activity. Polymerase chain reaction for amplification of 0.52 kb fragment of the part of dot/icm region of *L. pneumophila* was used for the final identification. The results obtained showed that the prevalence of *L. pneumophila* from streams (8%) was statistically significant when compared to wells (1%) (Chi square, P < 0.05). *L. pneumophila* was not isolated from any of the water samples taken from air-conditioner water systems. The study concludes that *L. pneumophila* is present in natural aquatic environment in Nigeria.**

Key words: Cultural isolation, ***Legionella pneumophila***, Nigeria.

INTRODUCTION

Legionella sp is an environmental organism that has gained recognition as a public health organism over the years as a result of changes in our life style. It is a Gram negative, intracellular pathogen that has the ability to survive within the amoebae and macrophages (Abu Kwaik et al., 1998; Adeleke et al., 1996; Alli et al., 2002). The organism was first brought to a limelight in 1976 as a result of Legionnaire convention in Philadelphia, United States of America (Fraser et al., 1977). From its natural environment - water, legionellae can be transmitted to humans by inhalation of infectious droplets. There are at least 48 species of ***Legionella*** that have been identified of which five species have been designated ***Legionella like*** amoebic pathogens (LLAPs) (Adeleke et al., 1996; Lo Presti et al., 2001) and there is still room for discovery of new species as a result of more surveillance of water bodies. Within the species of ***Legionella*** are 70 serogroups with more than half been implicated in diseases (Benson and Fields, 1998; Fields et al., 2002). ***Legionella pneumophila*** is responsible for most cases of Legionnaires' disease in the world, with ***L. micdadei*** coming distantly second (Benin et al., 2002; Joshi and Swanson, 1999). ***L. longbeachae*** and ***L. dumoffii*** rank third and fourth, respectively (Benin et al., 2002).

Legionellae multiply within its host cell (human macrophages and amoebae) but can also grow outside its host cell and can be cultured by routine methodologies that involve the use of selective medium (Feeley et al., 1978; 1979). In aquatic environments, ***L. pneumophila*** replicates within protozoan hosts. At least 13 species of amoebae and 2 species of ciliated protozoa support the intracellular growth of ***L. pneumophila*** (Fields, 1996). The dot/icm loci have been shown to play important roles in the pathogenicity of Legionellae (Berger and Isberg, 1993; Marra et al., 1992; Molmeret et al., 2002a; 2002b). In fact, the loci have been demonstrated to help in the

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intracellular multiplication in both protozoan and macrophages (Brand et al., 1994; Segal and Shuman, 1999).

After intracellular multipli-cation and exhaustion of nutrients in the hosts, in which the host cells are filled with the bacteria, Legionellae use the dot/icm to egress from the host cells as a result of pore forming activity of the bacteria that help in escaping the cell so as to infect fresh host cells (Alli et al., 2002; Molmeret et al., 2002a; 2002b). Inside the macrophage, the phagosome is prevented from fusing with lysosome, hence the bacteria live happily inside the cells (Horwitz and Maxfield, 1984). Apoptosis and necrosis have been shown to contribute to cell death of the host cells (Gao and Abu Kwaik, 1999; 2000). The dot/icm loci have been demonstrated in all species of Legionellae to date by hybridisation (Alli et al., 2003), however, the functionality of these loci are in doubt.

Epidemiologically, the first outbreak of legionellosis was reported in 1976 in Philadephia in USA, an outbreak that claimed the lives of many attendees of Legionnaires' convention (Fraser et al., 1977). Since then, outbreaks of legionellosis had been reported all over the world. In USA where surveillance for legionellosis is put in place, between 1980 and 1998, an average of 356 cases were reported to CDC each year with no particular trend (Fields et al., 2002). This is considered to be a fraction of 8,000 to 18,000 cases estimated to occur each year (Marston et al., 1997). Majority of the cases reported in the USA are sporadic. Outbreaks in the USA have been frequently associated with cooling towers in summer and fall (Bentham and Broadbent, 1993). In England and Wales, the Legionellae surveillance carried out showed a similar trend to what had been reported in the USA, reaching its peak in 1988 (Joseph et al., 1995; 1997).

In Holland, the 1999 outbreak of ***Legionella*** infection among attendees at flower show resulted in 133 confirmed and 55 probable cases of Legionellae (Den Boer et al., 2002). April 2000 outbreak of legionellosis in Australia among people visiting newly constructed aquarium in Melbourne claimed 4 lives with total number of people infected put at 119 (Anonymous, 2000). In Africa, the only country that reported outbreak of ***Legionella*** in a hospital is South Africa (Strebel et al., 1988).

Apart from this, many researchers all over the world including South Africa have reported the detection and isolation of Legionellae in their aquatic environments (Bartie et al., 2003; Erdogan and Arslan, 2007; Kuroki et al., 2009). It is inconceivable to think that ***L. pneumophila*** is not present in the authors' aquatic environment. The only information we have on the possible presence of Legionellae can be derived from seroprevalence study conducted about two decades where they reported 9% seroprevalence to ***Legionella*** (Sixl et al., 1987), suggesting possible exposure to Legionellae. Since the seroprevalence study, no researcher has reported the detection/isolation of Legionellae in water bodies in Nigeria or West Africa. The information on this is needed to determine the risk of acquiring Legionellae from the environmental irrespective of whether it is man-made or natural. It was in view of this that this study was instituted. The aim of this study was to survey aquatic environments for the presence of ***L. pneumophila*** in order to determine the prevalence of this species of ***Legionella*** in water bodies and air conditioner water system in Osogbo, Nigeria.

MATERIALS AND METHODS

Sample collection

Three hundred and thirteen (313) water samples from three different sources (air conditioner water systems, wells and streams) were examined for the presence of ***Legionella*** spp, of which 165 samples were collected from air conditioners while 98 and 50 samples were collected from wells and streams, respectively. Samples for investigation were collected from different locations within Osogbo, Osun State, Nigeria. One hundred millilitre volumes were collected from wells and streams into a sterile wide mouth screw capped specimen bottles, respectively. Swab sticks were used to scrape the mouth of air conditioner pipes after which 100 ml of water draining from the pipe was collected. All samples were stored in the laboratory at room temperature before investigation commenced.

The sample was processed for isolation of ***Legionella*** spp. as described in standard microbiology manual (Collins et al., 2004). A volume of 100 ml of each sample was placed in centrifuge tube and centrifuged at 5000 x g for 10 min, after which the supernatant was discarded carefully leaving about 2 ml of water with the sediment. The sediment was vortex mixed for one minute. Thereafter, 1 ml of sample concentrate was incubated at 50°C in a water bath for 30 min; in order to reduce the number of non-***Legionella*** bacteria from water samples before culture, and the remaining samples were stored at room temperature for possible use later. The heated samples were placed on bench for 10 min to attain room temperature.

Cultural isolation and Identification

About 100 µl of the treated sample suspension above was inoculated onto buffered charcoal yeast extract (BCYE) medium. After inoculation, the plate was incubated at 37°C in a humidified 5% CO_2 incubator. All cultures were examined after 48 h for possible bacteria growth and plates which showed no growth was re-incubated for another 24 h. Macroscopic examination of culture was thereafter done to identify typical Legionellae colonies. Each suspected colony was aseptically sub-cultured on BCYE agar containing supplement (cysteine and ferric chloride) and BCYE agar with no supplement. Isolates that were able to grow on BCYE agar with supplement and not able to grow on BCYE without supplement were taken for further identification as described below. Only isolates that were Gram negative rods were selected for biochemical test. Catalase, oxidase, hippurate hydrolysis and gelatine liquefaction tests were carried out as described in a standard medical microbiology laboratory manual (Cheesbrough, 2004).

Pore forming activity

Pore forming activity was determined by examining haemolysis of

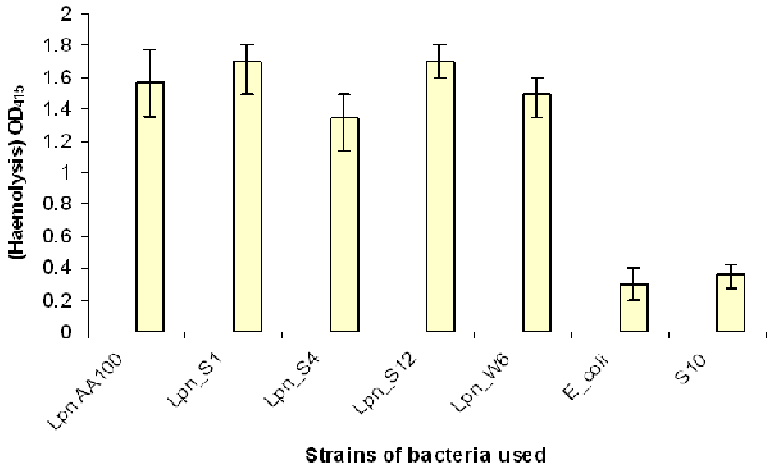

Figure 1. Pore-forming activity of Legionella spp. as determined by contact-dependent haemolysis of sRBC measured as A415. These data are representative of at least three independent experiments performed in triplicate. The absence of error bars indicates very small standard deviations that could not be displayed.

sheep red blood cells (sRBC) by suspected Legionellae isolates as previously described (Alli et al., 2003). Briefly, sheep red blood cells (sRBC) were washed three times by centrifugation for 10 min at 500 x g until a clear supernatant was seen; the cells were then counted with a haemocytometer. Reactions were set up in a final volume of 1 ml PBS with final concentrations of 1 x 10^8 sRBC ml^{-1} and 2.5 x 10^9 bacterial ml^{-1} and incubated at 37°C for 2 h. At the end of incubation period, centrifugation was done at 500 x g for 5 min. Tubes were then observed for haemolysis. Negative control for haemolytic assay was set up using ***E. coli.***

Polymerase Chain Reaction (PCR)

PCR for part of the ***dot/icm*** region was carried out on all suspected isolates that were oxidase, hippurate hydrolysis, and gelatine liquefaction positives. Briefly, the culture lysate obtained by boiling colonies of suspected organism in 200 µl of distilled water in a 1.5 ml Eppendorf tube for 10 min and chilled was used. The 522 bp of the ***dot/icm*** region was amplified in a 30 µl PCR reaction volume containing $MgCl_2$, Taq DNA polymerase (New England Biolab, USA) , 1 µM of Lpn_dot_F1 (GCAATCTTCAGTCCTGGGAG) and 1 µM of Lpn_dot_B1 (TGCTGCTCTTGTTGTGCCA) in a Gene Amp PCR system 9700 (Applied Biosystem, UK) using the following cycling parameters: 94°C for 2 min 1 cycle; 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, for 35 cycles. The positive control was included by using the DNA obtained from ***L. pneumophila*** AA100. Thereafter, the PCR products were analysed on 0.7% agarose gel in Tris Borate EDTA buffer solution, the image was taken with Syngene gel documentation system (Syngene, UK).

Statistical analysis

Chi square was calculated using EpiInfo version 6 where P value less than 0.05 was considered to be significant.

RESULTS

In a survey of the environment containing water (both man made and natural water bodies) in Osogbo, Nigeria, for ***L. pneumophila***, a total of 313 samples was collected, of which 165 (52.7%) were from air conditioner water systems, 98 (31.3%) were from streams and 50 (16%) were from the wells.

Out of the 313 water samples cultured, 179 (57.2%) samples yielded growth while 134 (42.8%) samples yielded no growth. From air conditioner, 81 (49%) samples yielded growth while 84 (51%) samples yielded no growth; from well water 66 (67.3%) samples yielded growth while 32 (32.7%) yielded no growth; and from stream 32 (64%) samples yielded growth while 18 (36%) samples yielded no growth. The suspected bacterial colonies were put through systematic identification of Legionellae by sub-culturing on both BCYE agar medium without supplement (cysteine and ferric chloride) and BCYE agar medium with supplement including antibiotics. This strategy pruned down the number of bacterial isolates down to 5, of which all of them were Gram negative rods exhibiting both catalase and oxidase activities with hippurate hydrolysis and gelatine liquefaction – a biochemical result suggestive of ***Legionella*** spp. Alli et al. (2003) have shown before that pore forming activity within the genus ***Legionella*** is associated with ***L. pneumophila*** and ***L. spirintensis***. It was in view of this that pore forming activity was tested for in all the 5 suspected legionellae isolates by performing haemolysis assay in which sheep red blood cells were used, the result (Figure 1) showed that all exhibited pore forming activity that was very close to that of ***L. pneumophila*** AA100 that was used as a positive control. Further identification was carried out at molecular level by doing polymerase chain reaction for specific detection of 0.52 kb fragment of the dot/icm region of ***L. pneumophila***; this revealed that all the five isolates had the 0.52 kb

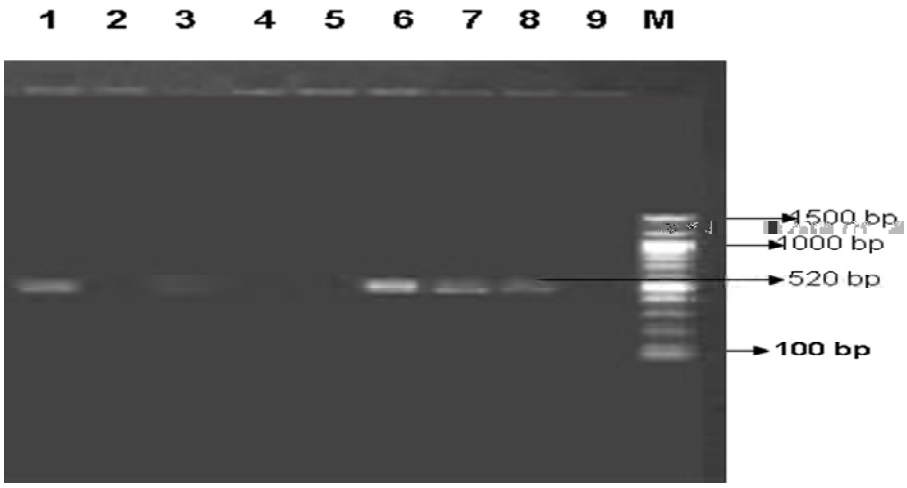

Figure 2. PCR for detection of 0.52 kb region of the dot/icm of ***L. pneumophila***. The PCR products obtained after PCR were run on 0.7% agarose gel as described in the materials and methods. Lanes 1-7: PCR for the colony lysates from suspected legionellae; lane 8: PCR product for AA100 ***L. pneumophila*** DNA as positive control; lane 9: PCR product for water as negative control and lane 10: 100 bp DNA size marker.

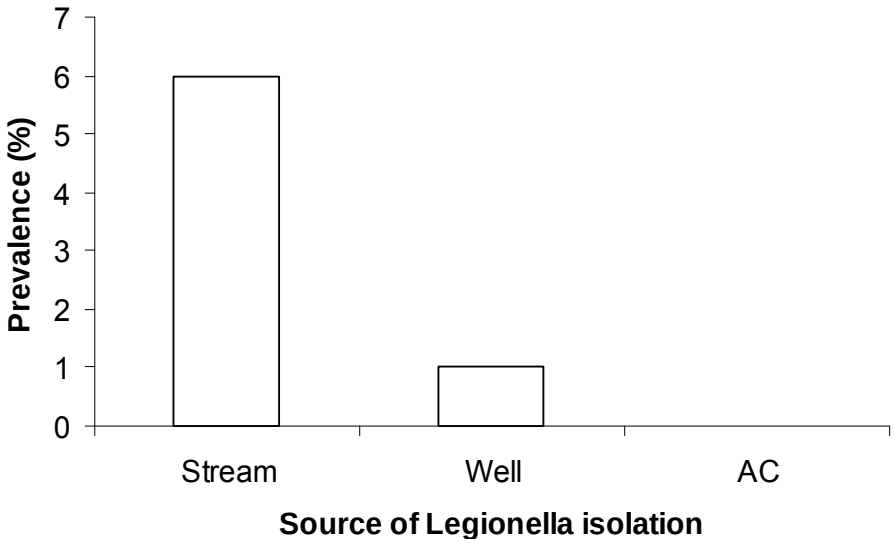

Figure 3. The prevalence of ***L. pneumophila*** in different sources of water in Osogbo, Nigeria.

fragment of the ***dot/icm*** region of ***L. pneumophila*** as shown in Figure 2, indicating that all the 5 isolates (Lpn_S1, Lpn_S4, Lpn_S12, and Lpn_W6) were ***L. pneumophila***. The prevalence of ***L. pneumophila*** in stream samples was calculated to be 8% (4/50) while the prevalence in water from the well was found to be 1.02% (1/98) (Figure 3). There was significant difference in the rate of isolation of ***L. pneumophila*** between stream and well water samples (Chi square, $P < 0.05$). Surprisingly, ***L. pneumophila*** was not isolated in any of the air conditioner water system samples.

DISCUSSION

Legionellae are readily found in natural aquatic bodies with some species been recovered from soil (Steele et al., 1990). Studies have shown that Legionellae are present in all segments of community water supplies, water treatment facilities (Erdogan and Arslan, 2007; Kuroki et al., 2009). The aquatic environment of ***Legionella*** also includes man made habitats such as cooling towers, evaporative condensers, whirlpool spas, decorative fountains, air conditioner water systems, and

potable-water distribution systems. Our study showed that Nigeria is not exempted from the list of countries where ***Legionella*** are found. Moreover, it also showed that the most virulent species of ***Legionella*** (***L. pneumophila***) is present. ***L. pneumophila*** was shown to be prevalent (3.4%) in natural aquatic habitats ground-water (well) and surface water (stream) compared to and man-made (water of condensation from air conditioner water system) in this study.

No reported isolation or detection of Legionellae in countries in Africa with the exception of South Africa. The only evidence available suggesting that Legionellae may be present in Nigeria was the seroprevalence study carried out by Sixl et al. (1987) where the seroprevalence to ***L. pneumophila*** was put at 9% among the blood donors in Abeokuta and Minna (Sixl et al., 1987). Recently, Olukanni et al. (2006) reported the isolation of ***Legionella*** in textile effluent using nutrient agar (Olukanni et al., 2006) and this is doubtful because legionellae require special medium to grow. Our study showed for the first time the reported isolation of ***L. pneumophila*** in aquatic environments (surface water and groundwater) with full identification of the isolates by both biochemical and molecular methods. It means these environments pose a risk in contracting Legionellae especially to immunocompromised individuals. The use of selective media like BCYE agar medium has proven to be very important in the isolation of Legionellae in this study as demonstrated by other researchers in this field (Azara et al., 2006; Bartie et al., 2003). Legionellae have been shown to be very exact in its nutritional demand and this might be the main reason for the non reported isolation of Legionellae in Nigeria and some other countries in Africa. It is also not a pathogen that is routinely checked in respiratory tract infection. The only country that reported the outbreak of Legionellae in Africa including the environmental isolation is South Africa. The general awareness of this organism in their environment may be responsible for the reported isolation of Legionellae in the hospital settings including dental clinics and the communities outside the hospital. Our experience in the use of BCYE agar medium also showed that the medium alone was not sufficient for the selective isolation of Legionellae because environmental organisms seemed to come through despite the incorporation of antibiotics that selectively inhibit the growth of competing microflora of the environments including fungi. This study is in line with other studies where the BCYE with antibiotics was used in the detection and isolation of Legionellae from the environments (Lin et al., 1999; Stout et al., 2003).

Identification of Legionellae could be problematic because these organisms have limited metabolic activities when compared to ***Enterobacteriacea*** and other non-fastidious organisms. This study made use of a simple schema where it exploited the inability of the organism to grow in the absence of cysteine cum iron and the ability to grow in the presence of cysteine and iron as the screening point. This alone narrowed the number of initial isolates to manageable number of isolates that were systematically identified using Gram stain, biochemical tests such as gelatine liquefaction and hippurate hyrolysis. These biochemical tests combined with demonstration of pore forming activity of ***L. pneumophila*** helped in narrowing down to ***L. pneumophila***. Pore forming activity has been described as one of the virulence traits of ***L. pneumophila*** (Alli et al., 2003) which is best demonstrated by haemolysis assay using sheep red blood cells. Combining this haemolysis assay and the ability to amplify the 0.52 kb portion of the dot/icm region of ***L. pneumophila*** helped in the final identification. The demonstration of the 0.52 kb region of the dot/icm also indicates that the pathogenic island is present in Nigeria isolates of ***L. pneumophila***, which means the organism is capable of causing infection and disease if the organism is inhaled in large number by individuals.

In this study, isolates from stream water showed the highest recovery of ***L. pneumophila*** as shown in Figure 3, followed by well water isolate. This high prevalence in stream can be attributed to the interaction of ***L. pneumophila*** with free-living amoeba which facilitates multiplication of the bacteria in their natural environ-ment. Although no air conditioner water sample yielded growth of Legionellae, compared to only one that was positive for well; well was regarded as the 2nd implicating reservoir of ***Legionella***. The non isolation of Legionellae from air conditioner water systems can be attributed to being an artificial man-made that is devoid of amoeba that is supportive of Legionellae growth unlike stream and well that are natural habitats for Legionellae and amoebae***.*** Air conditioner water system has been implicated as a high yield recovery of ***Legionella.*** In a survey carried out on the detection of ***Legionella*** in Germany, 15 water samples from cooling towers of air-conditioning systems at hospitals, authorities, schools, and factories were collected and ***Legionella*** was isolated from about 2 of the air-conditioners investigated on in that study area, thus giving a prevalence of 3.3% (Dermitzel et al., 1992). In our survey, none of the 165 water samples from air-conditioning systems was positive for ***L. pneumophila*** giving a prevalence of 0%. Thus the prevalence in Germany is higher than that of ours. A similar study carried out in Iran, 30 out of 132 (22.72%) hospital water samples collected from different sources were positive for ***L. pneumophila*** using PCR technique of which air conditioner water sample gave a high yield positivity of 10 out of the 30 positives (Hosseini et al., 2008). The study showed air conditioner as being the most implicated source of Legionellae, in contrast to our study where air conditioner was not implicated as a source of ***L. pneumophila*** infection***.*** The inability to isolate ***L. pneumophila*** from air conditioning systems in this study could be attributed to undulating and epileptic power supply in Nigeria. When power failure occurs, air

conditioning systems stops functioning, air conditioning pipes/outlets get dried up, and ***Legionella*** yield within pipe/outlets is greatly reduced because a lot of the bacteria would have died***.*** Notwithstanding, ***L. pneumophila*** was detected in both streams (surface water) and wells (ground water). This study is comparable to a study conducted in Netherlands where PCR technique was used for detection of legionellae in groundwater (well) and surface water (stream); surface water showed a high yield of ***Legionella*** organism compared to groundwater water samples (Wullings and van der Kooij, 2006).

In this study, the total prevalence of ***L. pneumophila*** from environmental water bodies (streams and wells) in Osogbo, Nigeria was calculated to be 3.4% in relation to 148 water samples collected. Its detection is an indication of the presence of ***Legionella*** in Nigeria. Although the prevalence might be small, its presence leaves Nigerians at risk of contracting the disease, in that people could get infected with the disease from areas where streams and wells are present. Water samples from air-conditioning system obtained from the hospital environment were implicated in being contami-nated with ***L. pneumophila***, the most pathogenic strain and it has been shown that cooling towers of air-conditioning systems have been implicated for nosocomial legionellosis (Dermitzel et al., 1992). Recent studies, documenting the genetic identity of clinical and environmental isolates have proven that local outbreaks of legionnaire`s disease may indeed originate from contaminated cooling towers (Hosseini et al., 2008). ***Legionella*** residents within biofilm are a particular problem in cooling tower systems. ***Legionella*** was also detected in well water which serves as a means of water supply in Nigeria. Well water is fetched directly for use in bathing, washing, and for other domestic chores. It is also pumped directly, and run through pipes to store water into overhead tanks to supply to man-made fountains, which generates aerosols. In some rural areas and villages, water from the streams is used for bathing, and cooking. These practices make Nigerians to be at a higher risk in contracting aerosols contaminated with the bacteria. The study concludes that ***L. pneumophila*** is present in water bodies in Nigeria. Translation of the presence of Legionellae in water bodies to risk of contracting Legionellae infection is remained to be seen.

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Full Length Research Paper

Isolation and identification of *Bacillus* strains with antimycobacterial activity

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Tuberculosis is the principal cause of death worldwide due to an infectious disease. The resurgence of tuberculosis, followed by the increase in prevalence of infections caused by nontuberculous mycobacteria (NTM), as well as the multi-drug resistance of mycobacteria to the majority of currently available antibiotics, have encouraged research for new antimycobacterial agents. Soil and water samples from different Moroccan biotopes, have led to the isolation of four bacterial strains (*M*, *R*, *G* and *S*), showing an inhibitory effect on mycobacterial growth. This effect was shown to be due to secreted substances in the growth medium. From subsequent analysis it was concluded that these strains produced different active substances. Sequencing of the 16S rRNA showed that these isolates belong to the genus *Bacillus*. The active substance from isolate *M*, showed the more important inhibitory effect on mycobacterial growth. It precipitated with ammonium sulfate and lost all activity when treated with Proteinase K, revealing its protein nature.

Key words: Tuberculosis, mycobacteria, antimycobacterial agents, ***Bacillus***.

INTRODUCTION

Tuberculosis is a major public health problem (Musser, 1995; Berthet et al., 1999; Rossetti et al., 2002; Chen et al., 2011). Nevertheless, as of 1952, with the discovery of effective antituberculosis agents and the begining of chemotherapy, the implementation of BCG (Bacille Calmette Guérin) vaccination programs (Ann Ginsberg, 2002; Bonnaud, 1996), and the improvement of living conditions, the decline in tuberculosis was considerable in industrialized countries (the rate of infection dropped as much as from 200/100 000 inhabitants in 1900 to less than 10/100 000 inhabitants in 1980) (Rastogi et al., 2001). However, since 1986, there has been a worldwide resurgence of tuberculosis (Newton et al., 2000) to which the HIV epidemic has undeniably been a major contributor, as individuals with the immunological deficiency are more apt to develop the disease (Chin and Hopewell, 1996; Iredia et al., 2011). According to the World Health Organization (WHO) report on global tuberculosis control, the number of new cases was estimated at 9.2 million in 2006, or 139 cases per 100,000 inhabitants (WHO, 2009). The number of deaths from tuberculosis in 2006 was estimated at 1.7 million of which 0.2 million occurred in HIV infected individuals. Developing countries continue to be the most effected by tuberculosis with a mortality rate of nearly 40% as compared to 7% in industrialized nations (Emile, 1996; Dharmarajan et al., 2007).

Two resistant forms of tuberculosis include multidrug-resistant (MDR-TB) and extensively drug-resistant (XDR-TB). MDR-TB is caused by ***Mycobacterium tuberculosis*** strains resistant to at least the two major first-line TB drugs, isoniazid (INH) and rifampicin (RIF). XDR-TB is caused by an MDR-TB strain also resistance to at least one of the three second-line injectable TB drugs (capreomycin, kanamycin or amikacin) in addition to any one of the fluoroquinolones. In 2007, the worldwide estimate was of 500 000 cases of MDR-TB; 85% of these were from 27 countries (of which 15, from the European region). At the end of 2008, 55 countries and territories had reported at least one case of XDR-TB (WHO, 2009).

In Morocco, 500 to 1000 people die each year from tuberculosis. According to the last figures from the

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Ministry of Health, the Kingdom registered 25,500 new cases in 2007, or 82 per 100,000 inhabitants. Seventy percent of these patients were between 15 and 45 years of age (Ben Cheikh et al., 1996), mainly from the population residing in the most heavily populated urban areas. The sanitary services often receive patients in an advanced stage of disease. The delay in diagnosis not only endangers the patient but contributes to tuberculosis transmission as, without adequate treatment, an individual with active disease can infect on the average from 10 to 15 people in a one year time span. Also, inadequate treatment, either in dosage or duration, may result in acquired drug resistance to antituberculosis drugs.

In today's context, new drugs are a priority to better the control of mycobacterial infections and contribute to solution the problem of the emergence of strains resistant to antituberculosis agents. The purpose of this investigation consists in the search for novel substances with an antimycobacterial effect from the Moroccan microbial flora, which has not been extensively explored in this respect.

MATERIALS AND METHODS

Mycobacterial strains

All tests were carried out on non-pathogenic mycobacteria. These include: ***Mycobacterium aurum*** A^+: a rapid growing scotochromogenic species, with a generation time of 6 h, having a similar spectrum of drug susceptibility to ***M. tuberculosis*** (Chung et al., 1995).

Mycobacterium smegmatis MC^2 155: a rapid growing non-pigmented, thermophilic species with a generation time of 3 h.

Isolation of microorganisms secreting substances with antimycobacterial effect

Several samples (water, soil and water) were taken from different Moroccon ecological niches. The samples were kept under agitation for two hours. The supernatant was recovered after sedimentation of the heavier particles. Serial dilutions (of 10^{-1} to 10^{-5}) were carried out in sterile Luria Bertoni (LB) broth. An aliquot of 70 µl of each dilution was spread on LB-agar previously inoculated with ***M. aurum*** A^+ or ***M. smegmatis*** cultures having an optical density at 600 nm ($D.O_{600nm}$) of 0.3. After incubation at 37°C for 48 h, colonies surrounded by an inhibition zone were purified. The four isolates thus obtained were designated ***M***, ***R***, ***G*** and ***S***.

To confirm that the antimycobacterial effect of these isolates was due to secreted substances their cultures were filtered and the filtrate was tested on the mycobacteria using the well or diffusion method. Briefly, the strains were cultures in 50 ml of LB broth, for 48 h at 37°C with agitation; the cultures were then centrifuged at 6000 rpm for 5 min to remove bacteria in suspension. The supernatant was recovered and filtered using 0.45 µm porosity filters. The filtrate obtained was tested by depositing 100 µl in wells cut out from solid media in plates previously inoculated with 100 µl of mycobacterial culture (***M. aurum*** or ***M. smegmatis***) at $D.O_{600\ nm}$ = 0.3. The plates were incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone was reported. The inhibition tests were repeated three times for each strain.

The control used corresponded to a culture filtrate from ***Escherichia coli*** prepared under the same conditions. 100 µl of this filtrate was deposited in the wells prepared in plates previously inoculated with mycobacterial culture (***M. aurum*** or ***M. smegmatis***) at $D.O_{600\ nm}$ = 0.3. The plates were incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone was reported.

Activity spectrum of the isolates

In order to get an idea on the spectrum of activity, the antimicrobial effect of the isolates under study was evaluated against the following bacteria: ***M. aurum*** A^+, ***M. smegmatis*** MC^2 155, ***E. coli DH5α***, ***Bacillus subtilis***, ***Staphylococcus haemolyticus*** (Hassi et al., 2007).

A few colonies from young cultures of each isolate were deposited at the center of a plate previously inoculated with 100 µl of microbial culture (for ***E. coli, B. subtilis*** and ***S. haemolyticus*** the $D.O_{600\ nm}$ was at 0.5, for ***M. aurum*** and ***M. smegmatis*** the $D.O_{600\ nm}$ was at 0.3). The plates are incubated at 37°C for 48 h. After incubation, the presence or absence of an inhibition zone is reported. The tests were repeated three times for each strain.

Confrontation test between the studied isolates

A confrontation test between the studied isolates was carried out in order to determine if these bacteria produced the same active substance or not. For this, A few colonies from young cultures of each isolate were deposited at the center of a plate previously innoculated with 100 µl of culture of another of the isolates under study at $D.O_{600\ nm}$ = 0.5. The plates were incubated at 37°C for 24 h. After incubation, the presence or absence of an inhibition zone was reported. The confrontation test was repeated three times for each strain.

Identification of the isolates

The molecular identification of the isolates was carried out by polymerase chain reaction (PCR) amplification and sequencing of the 16S rRNA gene. In order to classify a microorganism within a genus or as a species, a number of criteria must be respected. The molecular definition of genus states that 16S rRNA sequence similarity should be superior or equal to 97%. Similarity superior to 99% indicates identical species; on the other hand, no identification results if the similarity is inferior to 97% (Drancourt et al., 2000).

DNA for the molecular studies was extracted from a young LB broth culture of the isolates (24 h). A 1 ml aliquot of the culture was transferred to a 1.5 ml microtube (Eppendorf, XXX) and centrifuged at 6000 rpm for 5 min. The pellet was resuspended in 100 µl of sterile distilled water, frozen at -20°C for 30 min, then heated at 95°C for 3 min. This thermolysis procedure was repeated twice in order to burst a large number of cells. After centifugation at 7000 rpm for 10 min, 2 µl of the supernatant (containing the DNA) were used in the amplification reaction (Rodrigues Cavalcanti et al., 2007).

For, amplification, universal primers, fD1 (5' AGAGTTTGATCCTGGCT CAG 3') and Rs16 (5' TACGGCTACCTTGTTACGACTT 3'), used in the identification of bacteria by sequencing of 16S rDNA were selected (Weisberg et al., 1991). The reaction mix was prepared in a final reaction volume of 20 µl and contained: 4 µl of Taq buffer (5x), 1.2 µl of $MgCl_2$ (25 mM), 4 µl of dNTPs (1 mM), 2 µl of fD1 (10 µM), 2 µl of Rs16 (10 µM), 0.2 µl of Taq polymerase (5 U/µl), 4.6 µl of pure H_2O and 2 µl of the DNA. The of amplification conditions consisted in an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C

Table 1. Antimycobacterial effect of the filtrate from the isolated strains.

Filtrate from strain	Diameter of the inhibition zone (cm) [a]	
	Effect against MC^{2} [b]	Effect against A^{+} [c]
M	2.75 ± 0.15	3.22 ± 0.12
R	2.29 ± 0.21	2.71 ± 0.11
G	2.21 ± 0.22	2.56 ± 0.16
S	1.78 ± 0.17	2.22 ± 0.18
E. coli	0	0

[a] Tested on Luria Bertoni agar, [b] ***MC^{2}: M. smegmatis***, [c] ***A^{+}: M. aurum***.

for 30 s, annealing at 55°C for 45 s and extention at 72°C for 1 min 30 s; final extension was performed at 72°C for 10 min. The amplified fragment of approximately 1.5 kb was visualized by electrophoresis using a 1% agarose gel. Two amplification controls were used, a negative control (T-) corresponding to a no template control, and a positive control (T+) corresponding to the 16S rDNA gene of ***B. subtilis.***

For sequencing, PCR products were purified using a PCR Product Purification Kit (JETquick, Genomed) as described by the manufacturer. Amplification was carried out using 2.7 µl of the DNA product. The sequencing reaction mixture contained 2 µl BigDye V 1.1 and 0.25 µl of the primer (forward or reverse), PCR grade water was added for a final reaction volume of 10 µl. Amplification conditions were the following: initial denaturation at 96°C (3 min), 35 cycles of denaturation at 96°C (20 s), annealing at 60°C (5 s) and extension at 60°C (4 min). All sequences were determined in an automated DNA capillary sequencer AbiPrism 3130XL Genetic Analyzer (Applied Biosystems, Foster City, USA) using BigDye™ Terminator Cycle Sequencing Ready reaction Kit (PE Biosystems, Applied Biosystems, Foster City, USA). Two sequencing reactions were performed using the two primers used for PCR amplification.

The sequences of were analyzed using the data base of the National Center for Biotechnology Information (NCBI) and the BLAST N program.

Other than sequencing of the 16S rRNA gene, the molecular identification of the isolates under study was complemented using conventional preliminary tests: macroscopic observation, Gram stain and growth at 50°C (Meyer et al., 2004).

Determination of the protein nature of the active substance of one of the isolates

Precipitation of the active substance of the isolate by ammonium sulfate

The precipitation protocol used was described by Shimei and collaborators for the purification and characterization of antimicrobial peptides (Subpeptin JM$_4$-A et JM$_4$-B), produced by ***B. subtilis*** JM$_4$ (Shimei et al., 2005). Briefly, 100 ml of LB broth was inocularted with the selected strain and incubated at 37°C under agitation for 48 h. After incubation, it was centrifuged at 6000 rpm, at 4°C, for 5 min. The supernatant was recovered and 52.3 g of ammonium sulfate were progressively added under agitation. The mixture was left overnight under agitation at 4°C. The precipitate was centrifuged at 10000 rpm, at 10°C, for 20 min. The pellet was re-suspended in 200 µl of buffer (potassium phosphate KH_2PO_4 50 mM, pH = 6).

The suspension obtained was dialyzed against the same buffer at 4°C for 12 h. The dialisate was filtered using a 0.45 µm de porosity filter. The effect of the dialisate was then tested (three times) against ***M. aurum*** and ***M. smegmatis*** using the well method.

The controls used in this experiment were the LB broth (with no inoculums) and the LB broth inoculated with ***E. coli***. These controls were precipitated by ammonium sulfate, using the same protocol used for the precipitation of the active substance of the strain under study. The effect of the dialisate of these two controls was tested (three times) against ***M. aurum*** and ***M. smegmatis*** using the well method.

Sensitivity of the isolate's protein precipitate to Proteinase K

A 100 µl volume of the filtered dialisate was added to 40 µl of a Proteinase K solution (1 mg/ml). The mixture was homogenized and incubated at 37°C for 3 h. The effect of the Proteinase K treated dialisate, was tested (three times) against ***M. aurum*** and ***M. smegmatis*** using the well method. The control was a solution of Proteinase K at the same concentration.

RESULTS

Isolation of microorganisms secreting substances with antimycobacterial effect

Four bacterial strains (***M***, ***R***, ***G*** and ***S***) were isolated from the samples analyzed. They exerted growth inhibition against the indicator mycobacterial strains as seen by the formation of inhibition zones (Table 1). The inhibitory effect of the active substance from each strain was relatively more important against ***M. aurum*** than ***M. smegmatis***. The ***E. coli*** filtrate, used as control, did not present any antimycobacterial activity.

Activity spectrum of the isolates

Results show that the isolates under study have an antimicrobial effect against Gram positive bacteria, Gram negative bacteria, and mycobacteria (Table 2).

Confrontation test between the studied isolates

In order to determine if the isolated strains produce the same active substances, confrontation tests were carried out between the various isolates. Results show that each isolate exerted an antimicrobial effect against the

Table 2. Activity spectrum of the isolates.

Strains	Inhibitory effect against [a,b]				
	M. aurum (mycobacteria)	*M. smegmatis* (mycobacteria)	*E. coli* (Gram-)	*B. subtilis* (Gram+)	*S. haemolyticus* (Gram+)
M	+	+	+	+	+
R	+	+	+	+	+
G	+	+	+	+	+
S	+	+	+	+	+

[a] Tested on Luria Bertoni agar. [b] +: presence of an inhibition zone.

Table 3. Confrontation between the isolates.

Strain deposited at the center of the plate	Strain spread over the plate [a]			
	M	R	G	S
M	-	+	+	+
R	+	-	+	+
G	+	+	-	+
S	+	+	+	-

[a] Tested on Luria Bertoni agar. +: presence of an inhibition zone. -: absence of an inhibition zone.

indicator strain as could be seen by the formation of an inhibition zone around the colonies deposited at the center of the plate (Table 3).

Identification of the isolates

According to the identification criteria described, results from the sequence analysis, indicated that the isolates under study belong to the genus ***Bacillus*** (Table 4). Concordantly, the isolates were Gram+ and capable of growth at 50°C.

Determination of the protein nature of the active substance of isolate M

Isolate M, which showed a more important activity than that of the other isolates, was selected to study the sensitivity of the isolate's protein precipitate to Proteinase K. The precipitate of the active substance of the isolate inhibited mycobacterial growth creating an inhibition zone around the wells. This activity was eliminated upon treatment by Proteinase K (Table 5). There were no inhibition zones around the wells, with the test controls (LB broth and LB broth inoculated with ***E. coli***).

DISCUSSION

Numerous microorganisms are used in industry in the production of metabolites: enzymes, antibiotics, amino acids, vitamins, etc. The main microorganismes used by industry are Mycetes. However, some bacterial genus, such as ***Streptomyces*** and ***Bacillus***, are also of industrial value (Xiaofeng et al., 2005). The production of anti-microbial compounds is known of an important number of bacteria. This phenomenon corresponds to a defensive mechanism of these bacteria, producing antibiotics, organic acids and lytic agents such as lysozymes. Furthermore, several types of protein exotoxins and bacteriocines are produced (Mota et al., 2004).

Since the 1950's, antibiotherapy is the major defensive means against microbial infections. Amongst industrial fermentations, the production of antibiotics is one of the most important sectors. Screening for strains producing natural antibiotics has led to the production of first generation antibiotics. The majority of antibiotics of microbial origin are produced by actinomycetes and Bacillus (Duval, 1989, Xiaofeng et al., 2005). Bacteria of the genus Bacillus produce a variety of antimicrobial agents, although none used as antituberculosis agents. These are mainly peptides, lipopeptides and phenolic derivatives (Mota et al., 2004). The large majority of peptides are antibiotics. Others are known as antifungal, antitumoral and cytotoxic drugs (Sachtowakyama Fumiy Ishikawa and Kuni, 1984). As examples we can mention the synthesis of iturin by ***B. subtilis***, bacitracin by ***Bacillus licheniformis***, tyrothricin by ***Bacillus brevis***, and polymyxine by Bacillus polymyxa (Duval, 1989).

In this investigation, water and soil and water samples from Morocco led to the isolation of four bacterial strains (M, R, G and S) which inhibited growth of ***M. smegmatis*** and ***M. aurum***. Their antimycobacterial activity was

Table 4. Identification of the isolates.

Isolates under study	Bacterial species showing a high degree of sequence similarity with the isolates under study	Size of the sequenced fragment using primer Rs16 [b] (pb)	% of similarity obtained using primer Rs16	Size of the sequenced fragment using primer fD1 [b] (bp)	% of similarity obtained using primer fD1
M	***Bacillus*** sp. (FR773880.1)[a]	500	100	561	100
	Bacillus subtilis (HQ678671.1)[a]		100		100
	Bacillus amyloliquefaciens (HQ668178.1)[a]		100		100
R	***Bacillus*** sp. (HQ728329.1)[a]	609	100	618	100
	Bacillus subtilis (HQ694434.1)[a]		100		100
	Bacillus amyloliquefaciens (HQ179100.1)[a]		100		100
G	***Bacillus*** sp. (GU361655.1)[a]	478	100	563	100
	Bacillus subtilis (HQ323421.1)[a]		100		100
	Bacillus amyloliquefaciens (HM597236.1)[a]		100		100
S	***Bacillus*** sp. (HQ603746.1)[a]	535	100	623	100
	Bacillus subtilis (HQ678662.1)[a]		100		100
	Bacillus amyloliquefaciens (HM992829.1)[a]		100		100

[a]Access number National Center for Biotechnology Information (NCBI) database. [b]bp: base pairs.

Table 5. Sensitivity of the active substance from isolate M to Proteinase K.

Protein precipitate	Diameter of the inhibition zone (cm) [a]			
	Precipitate not subject to Proteinase K treatment		Precipitate treated by Proteinase K	
	Effect on MC^{2} [b]	Effect on A^{+} [c]	Effect on MC^{2}	Effect on A^{+}
Bacillus (isolate M) [d]	2.8 ± 0.15	3.3 ± 0.21	0	0
E. coli	0	0	-	-
LB broth [e]	0	0	-	-

The control used was a solution of proteinase K. This control showed no antimycobacterial activity.- :Test not performed since the precipitate not subject to the Proteinase K treatment showed no effect.[a] Tested on Luria Bertoni agar.[b] MC^{2}: ***M. smegmatis***.[c] A^{+}: ***M. aurum***.[d] ***M***: ***Bacillus***. [e] LB: Luria Bertoni broth.

demonstrated by the occurrence of growth inhibition zones around the colonies, resulting from the diffusion in the LB agar growth medium of the bioactive substances produced by the isolate. The study of the activity spectrum of the isolates showed that these exert an antimicrobial

activity against Gram positive and Gram negative bacteria as well as mycobacteria.

Inhibition zones were also produced when using the isolate filtrates. The absence of mycobacterial growth (***M. smegmatis*** and ***M. aurum***) around the wells containing the filtrate, confirmed that the antimycobacterial effect observed was due to diffusion in the agar medium of secreted substances. The inhibitory effect of the active substance from each of the strains was comparatively more active against ***M. aurum*** than ***M. smegmatis***. This important inhibition could be due to the fact that M. aurum has a longer generation time than ***M. smegmatis***, which would permit a longer diffusion time for the inhibitor. As the filtrates from the studied isolates are active against ***M. aurum***, it is highly probable that they will also be active against ***M. tuberculosis*** since these two mycobacteria present the same antibiotic susceptibility profiles (Chung et al., 1995).

Ammonium sulfate is widely used to precipitate water soluble proteins. It is one of the most common methods used to concentrate active substances of protein nature secreted by microorganisms in liquid medium. The active substance from the isolate showing the more important antimycobacterial activity was shown to precipitate by ammonium sulfate. The precipitate was also rendered inactive by Proteinase K. These results indicated the isolate's activity was due to a protein substance.

Sequencing of the 16S rRNA gene is presently the most commonly used molecular approach in bacterial phylogeny (Woese et al., 1990). It has led to the creation of large databases (Maidack et al., 1996; Van de Peer et al., 1999), whose use has allowed the identification of many environmental microorganisms as well as others that have been impossible to grow in culture (Relman et al., 1992; Strous et al., 1999). Using this methodology, the isolates were identified as Bacillus strains. These strains are Gram+ bacilli resistant to 50°C, confirming their classification in the genus Bacillus. The confrontation test showed that these isolates produced different active substances, which suggests that they are different strains although all from the genus Bacillus. The active substances could be antibiotics since most natural antimicrobial agents of bacterial origin are mainly synthesized by Bacillus and actinomycetes.

Since mycobacteria can infect macrophages, the effect of the active substances isolated will be studied ex vivo. The effect will also be studied against pathogenic bacteria, namely those responsible for nosocomial infections. Finally, the substances shown to be active ***ex vivo*** (macrophages infected by mycobacteria) will be purified and their structures determined.

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Full Length Research Paper

In vivo and *in vitro* protein profiling in *Acacia nilotica* (L.): A nitrogen fixing tree

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Present investigation was the first attempt which deals with the *in vivo* and *in vitro* comparative study of protein level in *Acacia nilotica* L., a nitrogen fixing tree. Protein was investigated in callus, seed, leaf and stem by means of SDS-PAGE. For obtaining the *in vitro* explant, the seeds were cultured on ½ MS medium under the *in vitro* condition. The cotyledonary nodal explants were taken from the *in vitro* seedlings and cultured in the MS medium supplemented in combination of 2, 4-D (0.4 mg/l) with BAP (0.25 mg/l). Though some differences were observed in the protein contents of *in vivo* and *in vitro* samples, the data proved that protein content in callus was higher than the seed following leaf and stem. In this study it was found that *A. nilotica* contained several protein bands of molecular weight 54.3, 44.1, 42.7, 40.1, 35.6, 31.2, 28.6, 24.7 and 19.5 kDa. These results indicate that the intensity of protein bands was high in *in vitro* sample compared to *in vivo* samples.

Key words: SDS-PAGE, ***in vitro***, ***in vivo***

INTRODUCTION

Acacia nilotica L. is native to the drylands of tropic Africa. It is found in India, Australia and Africa. ***Acacia nilotica*** L. belongs to the family leguminoscae, commonly called as Babool or Kikar. Due to their ability of restore soil fertility, it is known as nitrogen fixing tree. Acacia species are a possible source of protein for human use (Bukhari, 2002). Acacias obtain their nitrogen from ground water rather than from atmosphere, but can produce more crude protein per ha than many grain crops (Prakash et al., 2001). At the molecular level, from the last century, many researches have been done on Acacia species. While, Protein and enzyme analysis of seeds in Acacia species (Ali, 1994). Seed protein analysis in Acacia species (Akkad, 2004), RAPD analysis in Acacia species (Rashmi et al., 2004), Genetic variability Analysis in ***Acacia nilotica*** L. (Ndir et al., 2008) and Extraction of DNA in ***Acacia nilotica*** L. (Sablok et al., 2009). However, no research has been done in Acacia species for comparion of protein profile ***in vitro*** and ***in vivo***. Although, other medicinal plant species were examined for their protein ***in vivo*** and ***in vitro viz***. ***Bacopa monnieri*** (Mohapatra and Rath, 2005); ***Boerhaavia diffusa*** (Sharma, 2006).

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MATERIALS AND METHODS

Establishment of aseptic seedlings

Mature seeds of ***Acacia nilotica*** L. were collected from Sirsi (Haatoj) District, Jaipur, Rajasthan. Prior to surface sterilization, seeds were treated in boiled water at 60°C for about 30 min and then soaked in distilled water for about 24 h. Then they were kept under running tap water for about 10 to 15 min followed by washing with 1% (v/v) Rankleen (Ranklem-India) for 2 min and rinsed with double distilled water for three times. Prior to inoculation, sterilized seeds were again sterilized with 0.1% (w/v) aqueous HgCl2 for about 2 min followed by 2 to 3 rinsing with double distilled water in Laminar Air flow cabinet. These sterilized seeds were inoculated on half strength (Murashige and Skoog) MS salts medium in cultured bottles. After 7 to 10 days, seeds germinated and gave rise seedlings. These ***in vitro*** seedlings were used as source of explants.

Callus induction and maintenance

The Murashige and Skoog (MS) medium was prepared by adding 3% sucrose as a carbon source and 0.8% (w/v) agar as a solidifying agent. ***In vitro*** cotyledonary node (1.0 cm) of 20 day old

Table 1. Protein profiling with intensities in ***Acacia nilotica*** L.

Marker molecular weight (KDa)	Leaf	Stem	Callus	Seed
61.5	-	-	+	+
60.9	+	-	++	++
58.1	-	+	+	+
43.9	-	-	+	+
42.3	+	+	++	+
40.7	+	-	+++	+++
35.6	+++	+++	+++	-
31.2	-	+	+	+
28.5	+++	+++	+++	+++
24.7	+	-	+	+
19.5	+	++	++	++

Strong intensity =+++, moderate intensity =++, weak intensity =+, Absent= -.

seedlings inoculated as explants for callus induction on MS medium supplemented with a series of 2, 4-D (0.4 mg/l) combination with BAP (0.2 mg/l).

The pH of medium was adjusted to 5.8 ± 0.2 before autoclaving at 1210 C for 15 min at 15 lb/in2. 20 ml of molten agar medium was poured into a culture bottle and plugged with nonabsorbent cotton. All cultures were incubated in 16 h /8 h photoperiod under light intensity of 50 µE/ m^2/s provided by cool, white and fluorescent light at 25 ± 2°C with 55% relative humidity. Each treatment performed using eight replicates and the experiment was repeated at least thrice.

Protein content determination

Leaves and stem collected from disease free and healthy plant of ***Acacia nilotica*** L. The specimen was authenticated by the department of Botany, University of Rajasthan and the voucher specimen (Voucher No. RUBL 20432) was deposited for future reference in the Botany Department Herbarium. Leaves and stem were used as an ***in vivo*** sample for comparing protein content with green, friable calli from cotyledon obtained after 28 days.

For protein estimation these samples were lyophilized, macerated in 80% ethanol and elucidated by the method of Lowry et al., (1951). Protein in the unknown sample was estimated at 660 nm using bovine serum albumin as standard and expressed per gm fresh weight basis.

Analysis of protein profile by SDS-PAGE

Electrophoresis has become a useful tool for the characterization of plant proteins. Protein profiles were studied by sodiumdodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). A vertical slab gel apparatus as described by Studier (1973), Desatronic 3000/200 power supply and Frigostat, West Germany, were used during the electrophoretic work. In SDS-PAGE, proteins are treated with sodium dodecyl sulfate (SDS) before electrophoresis so that the charge density of all proteins is made roughly equal. When these samples are electrophoresed, proteins are separated according to mass. The protein bands were visualized by transilluminator and photographs were taken for comparison of results.

RESULTS AND DISCUSSION

During the present set of experiment, the total protein estimated in callus, ***in vivo*** leaves, stem and seed (Figure 1) after that to find out the molecular weight of the total protein these were subjected to SDS-PAGE analysis. The proteins were found to be composed of a total of 33 bands ranging from 14.4 to 66.2 KDa were recognized (Figure 2). Protein profiles further showed variability on the basis of presence or absence and intensities of protein bands with banding pattern (Table 1).

SDS-PAGE is considered as a reliable method of genetic characterization because electrophoretic patterns of the protein fractions are directly related to the genetic background of the proteins and can be used to certify the genetic make-up (Rehana et al., 2004). In order to estimate the variability at genetic level, SDS-PAGE banding pattern of the gel using total protein was investigated.

Overall out of 33 protein bands, molecular weights 19.5, 28.5 and 42.3 KDa shown same protein banding pattern in callus , ***in vivo*** leaves, stem and seed samples but with variation in intensities. Left behind protein banding pattern exhibited a considerable range of variability with regard to their mobilities and intensities. Callus exposed at protein molecular weight ranging from 14.4 to 66.2 KDa. Seed only lacking at the molecular weight 35.6 KDa but leaves absent in 31.2, 58.1 and 61.1 KDa. Whereas, stem missing in the molecular weight of 24.7, 40.7, 43.9 and 60.9 KDa. From this we concluded that the highest numbers of protein bands were observed in callus followed by seed, leaves and stem. Regarding this experiment, no similar and contrary results were available in ***Acacia nilotica*** L. and Acacia species, but in other plants, analogous results were reported in ***Artemisia vulgaris*** (Kumar and Ranjitha, 2009), ***Glycine max*** (L.) Merr (Radhakrishnan and Ranjitha, 2009), and ***Plumbago zeylanica*** L. (Rout et al., 2010). No research has been done contrary to these results. This paper presents and sheds light on the ***Acacia nilotica*** L. proteins which grow on the nile banks of Delta region and in arid regions. Therefore, very much attention should be given to arid species since they are under severing threats

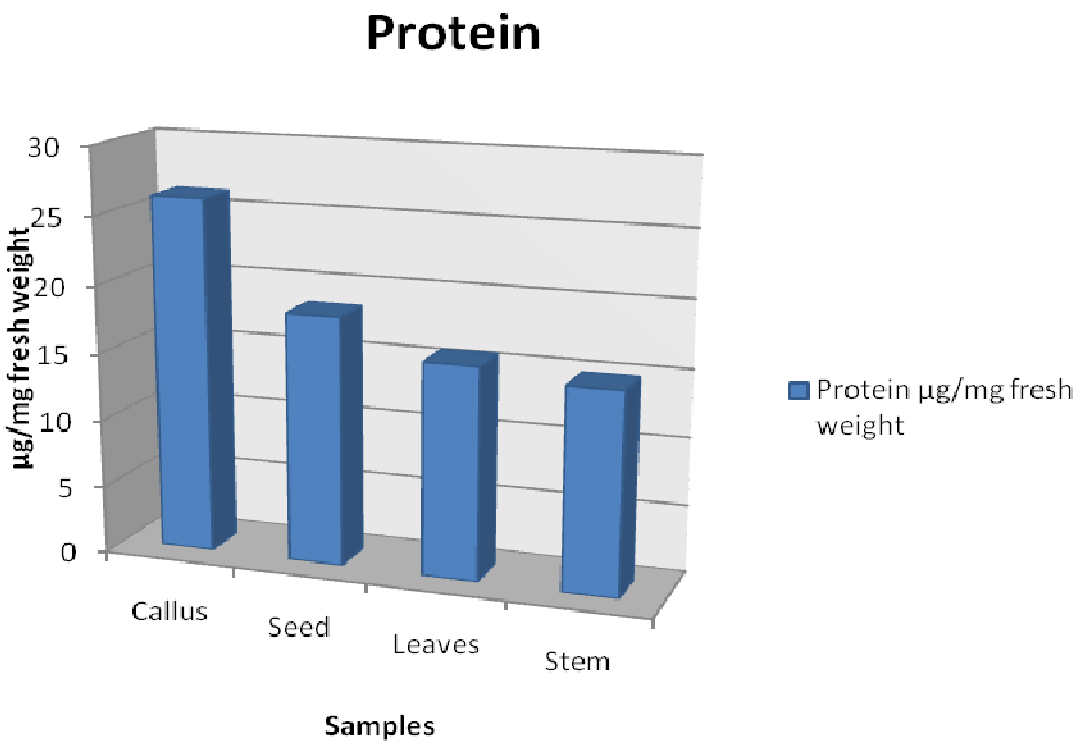

Figure 1. Protein estimation in ***Acacia nilotica*** L.

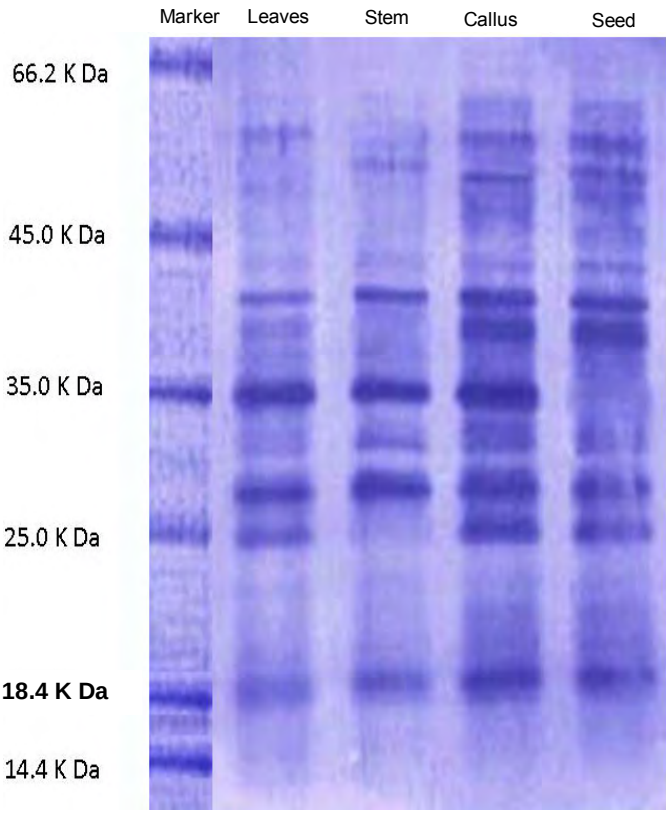

Figure 2. Protein analysis via. SDS-PAGE in ***Acacia nilotica*** L.

these valuable plants which showed some signs as an important resource for desert ecosystem and arid environment.

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Full Length Research Paper

Antibiotic susceptibility and toxins production of *Staphylococcus aureus* isolated from clinical samples from Benin

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A wide range of clinical samples were screened for identification of *Staphylococcus aureus*, their antibiotic sensitivity profile and the production of different leucotoxin and epidermolysins was evaluated. Out of 2,040 biological samples (collected from pus, urine, sperms, genital, catheter and blood of hospitalized and extra-hospital patients) screened, 123 pure cultures of *S. aureus* colonies were isolated. 48.78% of *S. aureus* were resistant to methicillin (MRSA), while 78% of them were isolated from extra-hospital patients. The *S. aureus* isolated from urines, pus and blood produced Panton and Valentine leukocidin (PVL) toxin, while the leucotoxin lukE-lukD was exclusively encountered by *S. aureus* isolated from pus samples. None of the bacterial colony isolated produced epidermolysin toxins A and B. In addition, 3.25% of MRSA and 8.13% of methicillin sensitive *S. aureus* (MSSA) produced PVL respectively. Our results indicated high frequency rate of MRSA in extra-hospital screened samples isolated from various types of infection. This high resistance rate combined with toxin production increases the virulence of *S. aureus* colonies and put therefore at risk the life of the patients in developing countries where auto-medication is not controlled. There is the need to instruct the population in order to avoid further widening of MRSA territory.

Key words: *Staphylococcus aureus*, Panton-Valentine leukocidin (PVL), antibiotic, infection, methicillin-resistant *Staphylococcus aureus* (MRSA), Benin.

INTRODUCTION

The animal organisms are often subject to various parasites. These microorganisms are generally viable at the expense of their hosts, while causing various diseases. The parasitic infectious agents are either diseases. The parasitic infectious agents are either specific pathogenic bacteria inducing clinically defined and physio-pathologically specific diseases, or opportunistic bacteria expressing their pathogenicity by exploiting the host physiological deficiency and by taking

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advantage of the modification of its environment. *Staphylococcus aureus* is one of the most frequently isolated human pathogenic bacteria in the community and hospital infections (Chambers, 1997; Deresinski, 2005; Friedman et al., 2002; Naimi et al., 2003). It is therefore one of the most devastating and widespread disease causing bacteria in hospital epidemiology due to its ability to produce a wide range of toxins and adhesion factors (Baba-Moussa et al., 2008; Foster and Höök, 1998).

The control of *S. aureus* causing diseases heavily relies on intensive use of antibiotic drugs. However, as a result of increasing use of antibiotics, the pathogenic bacteria become more easily resistant to a wide range of these drugs (Kim et al., 2004; Shittu and Lin, 2006). For instance, a year after the introduction of methicillin as an efficient antibiotic in 1960 (Durand et al., 2006), *S. aureus* has been reported to naturally resist this antibiotic (Deresinski, 2005; Ayliffe, 1997; Jevons, 1961). The emergence of methicillin-resistant *S. aureus* (MRSA) is greatly facilitated by the horizontal transfer of the bacterial virulence factors. Thus, MRSA have been reported in Europe, America (Deresinski, 2005; Ayliffe, 1997) and recently in Africa (Bell and Turnidge, 2002; Hayanga et al*.,* 1997; Klugman, 1998). Later, MRSA cases have been reported in extra-hospital environments notably among the community with no contact to hospitals (Vandenesch et al., 2003; Zetola et al., 2005; Ho et al*.,* 2007; Kuehnert et al*.,* 2006). In contrast to the nosocomial colonies, the community acquired MRSA (CA-MRSA) are most sensitive to almost all antibiotics with the exception of the β-lactam antibiotics (O'Brien et al*.,* 2005). In Benin (West Africa) for example, this case of CA-MRSA emergence has also been reported (Makoutodé et al*.,* 1994). However, relatively few data exist from epidemiological studies on the variability of toxins produced by *S. aureus* colonies as well as their resistance profiles to a wide range of antibiotics in this country.

Therefore, in order to establish the trends of *S. aureus* resistance to antibiotics from clinical samples in Benin, we endeavored in this work to screen various clinical samples collected from Microbiology laboratory of the National Teaching Hospital Center, Hubert Koutoukou Maga (NTH-HKM), the biggest hospital center of Benin and subsequently characterize the isolated bacterial colonies. The ultimate objective of this study was to establish the toxin profile and the antibiotic resistance ability of the clinically isolated *S. aureus*.

MATERIALS AND METHODS

Sample collection and *S. aureus* identification

The sample collection was performed for a 4-month period (September 27, 2010 to January 27, 2011) from 2,040 hospitalized and extra-hospital patients admitted at the laboratory of Bacteriology of the National University Hospital Center (CNUH) of Cotonou (Benin). In this study, we focused our effort on isolating *S. aureus* in pure culture. In this regard, only samples more than 90% of bacterial colonies identified as *S. aureus* have been considered for further investigation. For epidemiological data analysis, we took into account every patient's clinical data (age, sex, site, hospitalization etc.) and the origin of the bacterial sample collection. The bacterial identification was done through morphological characterization. Gram stain test, catalase activity, production of DNase and coagulation of rabbit lyophilized plasma were tested according to the manufacturer instruction (Bio Mérieux, France).

Antibiotic susceptibility of *S. aureus* colonies

In order to study the effect of antibiotics on the isolated *S. aureus*, we tested the susceptibility or resistivity of *S. aureus* colonies on agar plates containing 20 different antibiotics. The disc diffusion method was employed on Mueller Hinton agar plates (Bio-Rad-Diagnostic Pasteur, Marnes la Coquette, France) according to the recommendations of the Antibiotic Committee of French Society of Microbiology (CA-SFM). Nitrofurantoin antibiotic were only tested against urine samples. Determination of methicillin resistance was performed by the disk diffusion method on Mueller Hinton agar with 5% (wt/v) NaCl at 37°C for 24 h.

Screening and characterization of bacterial toxins

S. aureus isolates are well known to produce different toxins. They were screened for the production of different leucotoxins such as Panton-Valentine leukocidin ((PVL: LukF-PV et LukS-PV)), leucotoxin LukE-LukD and epidermolysins A (ETA) and B (ETB) using the Ouchterlony method also known as radial gel immunodiffusion (Gravet et al., 1998) in the presence of specific purified anti-leucotoxins and anti-epidermolysin rabbit antibodies .

Statistical analysis

For statistical analysis, biological replicate experiments were conducted throughout this study. Software Microsoft office Excel 2007 was used for the treatment of data. Software Epi Info 6 versions 6.04 (Center for Disease Control and Prevention, Atlanta, GA, U.S.A.) easily allowed the statistical test of χ^2 used in the comparison studies to determine the meaning of every factor of bacterial virulence. The test was considered statistically significant at $P<0.05$.

RESULTS

Prevalence of *S. aureus* isolated according to samples origin

The 2,040 analyzed samples were composed of 30 urethral samples, 162 vaginal samples, 93 sperm samples, 89 pus samples, 17 catheter samples, 122 blood samples and 1527 urinal samples. In total, 123 colonies of *S. aureus* were isolated from the 2,040 independents samples analyzed. On the other hand, 24 colonies of *S. aureus* were co-isolated with other enterobacteria (*Escherichia coli*, *Klebsiella pneumoniae*, Enterococcus, *Pseudomonas aeruginosa*, Acinetobacter spp., *Enterobacter cloacae*, *Morganella morganii*, β hemolytic Streptococcus, hemolytic Bacillus Gram

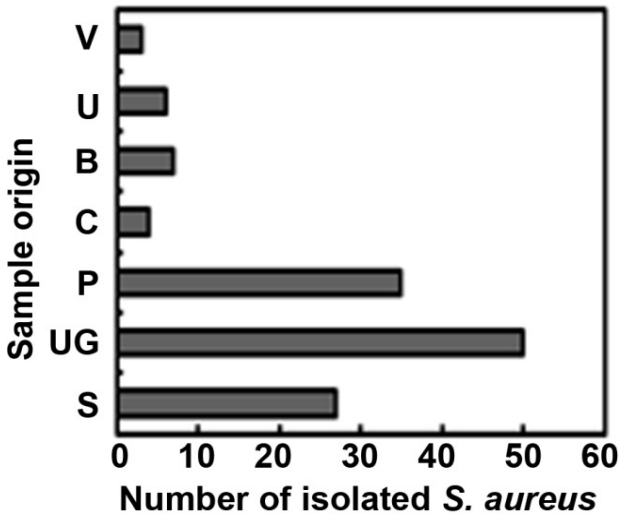

Figure 1. *S. aureus* distribution based on sample provenance. V, Vaginal sample; U, urinal sample; B, blood sample; C, catheter sample; UG, urogenital sample; S, sperm sample.

negative) and/or yeasts (*Candida albicans*). Since we needed to consider only pure cultures of *S. aureus* were not considered in this study.

A prevalence of 6% of *S. aureus* was observed from the entire samples collected. The patient concerned represented 61 and 21% of *S. aureus* isolates originated from hospitalized patients, while the rest (79%) were isolated from samples collected from the community (extra-hospital) patients. *S. aureus* were classified according to their biological provenance (blood, catheters, the pus, the urines, the vaginal samples, the urethral samples and the sperms) (Figure 1). Interestingly, we found the urine samples to be the most contaminated with 33.33% of *S. aureus* colonies followed by pus accounting for 28.45%. The vaginal samples proved to be the least contaminated with 2.44% of *S. aureus*. These infections are induced by the bacterial proliferation, invasion and destruction of local tissues resulting in local and systemic inflammatory response.

Susceptibility studies of *S. aureus* to antibiotics

The antibiotic tests revealed that 118 out of the 123 (95.93%) bacterial colonies were resistant to Penicillin G. Interestingly; the MRSA represented 48.78% of the whole isolates (Table 1). On the other hand, pristinamycin was found to have the greatest inhibiting effect on the *S. aureus* colonies with only 3.25% of bacterial resistance recorded for this antibiotic. In addition, nitrofurantoin antibiotic were specifically efficient against *S. aureus* originated from urinary infections with only 9.75% of bacterial resistance recorded for Nitrofurantoin treatment (Table 2). Out of the 60 *S. aureus* colonies resistant to methicillin (MRSA), 78% (47/60) were from community (extra-hospital) origin (Figure 2A). As depicted in Figure 2B, we represent the distribution pattern of the 60 MRSA based on their origin. Interestingly, out of the 41 *S. aureus* colonies isolated from urine samples, 22 (53.65%) of them were MRSA, 42.85% MRSA come from the pus, 59.26% from sperm samples, 33.33% from genital samples, 14.28% from blood samples and 75% from catheter samples.

Production of toxins by *S. aureus* colonies

Altogether, the PVL was produced by 14 *S. aureus* isolates out of the 123 bacterial colonies identified (11.38%). Among positive blood cultures, 57.14% (4/7) produced PVL, and 7.31% (3/39) urinal samples produced PVL. However, the leucotoxin lukE-lukD was leucotoxin lukE-lukD was produced by 28% (5/35) of the pus isolated colonies (Table 3). On the other hand, no *S. aureus* was isolated among produced epidermolysin ETA and ETB (Table 3).

DISCUSSION

Our study, revealed a prevalence of 6% of *S. aureus* isolated from various samples assessed from the laboratory of Microbiology of the CHU-HKM, Benin. These bacteria were isolated from almost all the types of samples considered in this study. This result is not surprising because of the opportunistic and ubiquitous nature of *S. aureus* (Nauciel and Vilde, 2005). *S. aureus* is the fourth most common hospital-acquired pathogen among older adults, following *E. coli, P. aeruginosa,* and Enterococci. The *S. aureus* isolates from genital samples represented 40.65% of the entire bacteria identified. This is similar to 43% (in Benin) and 40.71% (in Casablanca, Morocco) previously obtained results (Baba-Moussa et al., 1999; Elazhari et al., 2009). Surprisingly, these values are higher than those recorded in developed countries (~0.1 to 2%) (Foster and Höök, 1998). For instance, out of 15,074 infections of all origins, the frequency of urinary infections is 15% and only 2.5% are linked to *S. aureus* in Europe and USA (Fluit et al., 2001).

The strong percentage of *S. aureus* in urogenital infections in Benin can be explained by a passive presence of these germs in the urogenital region (Randrianirina et al*.,* 2007). In addition, this can also be due to the lack of hygiene, the unfavorable climatic conditions and the lack of clean water. In our study, *S. aureus* was also isolated from blood sample. The presence of *S. aureus* in the blood confirms its capacity to provoke septicemia in human. *S. aureus* caused similar proportions of both community-onset (18%) and nosocomial (21%) bloodstream infections (Diekema et al*.,* 2003). In a more recent series, staphylococcal species were the second most common pathogen

Table 1. *Staphylococcus aureus* resistance profile to a wide range of antibiotics

Antibiotics (AB)	Number of colony R (N = 123)	Percentage of AB resistance (%)
Penicillin (Pen G)	118	95.93
Oxacillin (OXA)	60	48.78
Amoxicillin (AMX)	85	69.10
AMX + clavulanate (AMC)	68	55.28
Cefotaxime (CTX)	112	91.05
Cefuroxime (CXM)	102	82.93
Cefixime (FOX)	98	79.67
Ciprofloxacin (CIP)	59	47.96
Ofloxacin (OFX)	51	41.46
Spiramycin (SP)	38	30.89
Lincomycin (L)	29	23.58
Pristinamycin (PT)	4	3.25
Erythromycin (E)	44	35.77
Trimethoprim sulfametoxazole (SXT)	78	63.41
Chloramphenicol (C)	38	30.89
Gentamicin (GEN)	52	42.27
Tobramycin (TM)	64	52.03
Kanamycin (K)	70	56.91
Netilmicin (NET)	51	41.46

Table 2. Antibiotic resistance profile of *S. aureus* according to the types of samples.

Antibiotic	Urine (%)	Pus (%)	Sperm (%)	Genital (%)	Blood (%)	Catheter (%)
Pen G	92.68	97.14	100	88.88	100	100
OXA	53.65	42.85	59.25	33.33	14.28	75.00
AMX	65.85	68.57	74.07	66.66	57.14	100
AMC	53.65	51.42	66.66	55.55	28.57	75.00
CTX	88.48	91.42	100	100	100	100
CXM	88.48	80.00	96.29	77.77	57.14	100
FOX	88.48	77.14	96.29	66.66	28.57	100
CIP	28.17	40.00	55.55	55.55	28.57	75.00
OFX	41.46	51.42	33.33	22.22	14.28	100
SP	19.51	42.85	25.92	11.11	71.42	50.00
L	17.07	40.00	14.81	0.00	14.28	75.00
PT	7.31	0.00	3.70	0.00	0.00	0.00
E	39.02	31.42	25.92	55.55	42.85	50.00
SXT	68.29	51.42	66.66	66.66	57.14	100
C	21.95	40.00	29.62	44.44	0.00	75.00
FT	9.75	NA	NA	NA	NA	NA
GEN	28.17	34.28	55.55	33.33	14.28	25.00
TM	53.65	40.00	62.96	66.66	42.85	50.00
K	63.41	45.71	66.66	66.66	28.57	50.00
NET	46.34	31.42	22.22	55.55	28.57	50.00

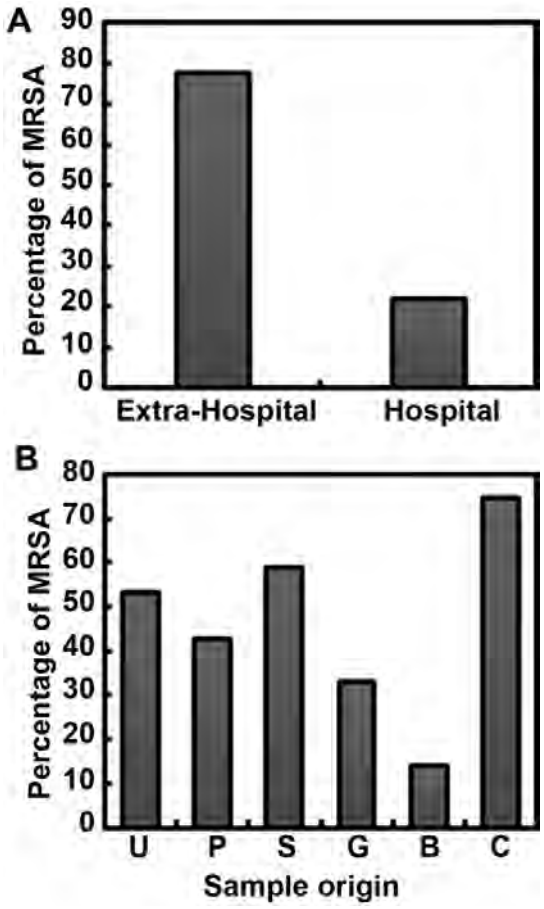

Figure 2. Distribution of methicillin-resistant *S. aureus* (MRSA) based on sample provenance. (A): MRSA registered from extra-hospital and hospital based samples. (B): MRSA registered according to the type of samples. U, Urinal sample; P, pus sample; S, sperm sample; G, genital sample; B, blood sample; C, catheter sample.

Table 3. Toxin profile of *S. aureus* according to the type of samples.

Toxins produced by *S. aureus*	Urine (41)*	Pus (35)*	Sperm (27)*	Genital (9)*	Blood (7)*	Catheter (4)*
PVL (%)	7.31	20	0	0	57.14	0
ETA (%)	0	0	0	0	0	0
ETB (%)	0	0	0	0	0	0
LukE-LukD (%)	0	14.28	0	0	0	0

*Number in parenthesis represents the number of *S. aureus* isolated from indicated biological samples.

(after *E. coli*) reported among older patients hospitalized with bacteremia that was acquired either in the community or hospital (McBean and Rajamani, 2001).

As revealed in Table 1, the *S. aureus* isolates displayed a wide range of antibiotic susceptibility to antimicrobial. Our findings are in agreement with previously reported data on the effects of antibiotics on clinically isolated *S. aureus* (Shittu and Lin, 2006; Denton et al., 2008, Elahari et al., 2009). The bacterial colonies were highly resistant to the AMC, AMX, FOX, CTX and CXM antibiotic family. This might be due to the fact that certain classes of antibiotics are easily accessible and frequently used by

the patients without medical prescription in Benin. The self medication mediating bacterial resistance to antibiotics has been widely reported in Africa (Sow et al., 1993; Kesah et al., 2003; Randrianirina et al., 2007; Diekema et al., 2001; Elazhari et al., 2009). Several MRSA colonies were lately described in France, but the epidemiology of MRSA is poorly understood (Dauwalder et al., 2008). We observed about 48.78% of MRSA from hospitalized patients and about 78% of MRSA from community patients. This high antimicrobial resistance profile might be due to auto-medication of the community patients. This bacterial resistance occurrence is highly reduced (36% in Ireland in 1999, with only 14.28% of MRSA from blood sample) where auto-medication is not allowed (McDonald et al., 2003).

The pathogenicity of *S. aureus* colonies has been related to a wide range of toxin productions. In agreement with our previous report, 11.3% of *S. aureus* isolated in this study produced PVL (Baba-Moussa et al., 1999). The PVL producing *S. aureus* are classically associated with primitive skin infections, notably the furuncles (Couppie et al., 1994; Durupt et al., 2007). In our study, only 4.06% of bacterial colonies tested produced the leucotoxin lukE-lukD (Table 3). These lukE-lukD producing *S. aureus* were all isolated from the pus. This weak rate of lukE-lukD production is not surprising if we assume that lukE-lukD plays an important role in the occurrence of diarrheic infections (Gravet et al., 1998). In fact, 93.6% of *S. aureus* isolated from older patient concerned by post-antimicrobial diarrhea produced lukE-lukD toxin (Gravet et al., 2001). In our study, no *S. aureus* colony produced epidermolysins A and B (ETA and ETB). However, sample screening studies done in Guyane proved that 93% of *S. aureus* originating from impetigo infections produced epidermolysin (Couppie et al., 1998). In addition, 3.25% of *S. aureus* tested produced PVL as well as leucotoxin lukE-lukD. The PVL and lukE-lukD producing *S. aureus* were only isolated from pus samples, suggesting that these toxins target different cell types such as the polynuclears, neutrophils, the monocytes and the macrophages that can easily be found in this type of infection (Gravet et al., 1998).

Until recently, cases of *S. aureus* resistant to penicillin M and sometimes to other antibiotics have been rarely recorded outside the hospitals or medical centers. Our study shows a strong prevalence of community based *S. aureus* colonies that are resistant to methicillin. Evidently, this bacterial germ has nowadays widened its infectious territories. The infections linked to MRSA outside hospital environment have been frequently reported in the USA (Fridkin et al., 2005). However, a screening study conducted at the Centers for Disease Control and Prevention (CDC) appearing in the New England Journal of Medicine, revealed that MRSA infections have been frequently reported among people that have never been admitted in hospital or in contact with hospital environment in a period of one year. Our study shows a variability of toxins produced by *S. aureus* isolated from various types of infections. Interestingly, the production of toxins combined with antibiotic resistance of the bacteria can easily result into disease complication or even death of the patients if appropriate antibiotics are not administered in time.

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Full Length Research Paper

Bioaccumulation of cesium-137 and cobalt-60 from solid cellulosic-based radioactive waste simulates by *Plurotus pulmonarius*

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Solid cellulose-based radioactive wastes (CBW) constitutes in some cases, about 70% of the total solid low and intermediate level organic wastes originated from peaceful applications of nuclear technology in various fields of our life. Cesium-137 and cobalt-60 represent two of the most important radioisotopes spiking these waste categories. Both are serious contamination concerns due to their high energy gamma ray emitting (Cs-137 = 0.662 MeV and Co-60 = 1.17 and 1.33 MeV), besides ^{137}Cs is considered as one of long-lived isotopes ($T_{1/2}$ = 30.5 years). In this part of work, laboratory scale attempts were performed to follow bioaccumulation of Cs-137 and/or Co-60, found separately or together in a mixture of some solid CBW simulates. The process is based on the capability of *Pleurotus pulmonarius* to biodegrade the solid CBW simulates achieving acceptable weight reduction for the waste as well as reasonable bioaccumulation of the two isotopes from the spiked mixture, within their cells. Up to 134.95 and 41.1 kBq/kg (based on the dry weight of mushroom) were accumulated from Cs-137 and Co-60 respectively within a period of 54±3 days. It is worth mentioning that more than 54% weight reduction percent for the solid CBW simulates was acquired only due to a single cultivation process. Based on the data so far obtained, the bioremediation process for solid CBW based on the *P. pulmonarius* bioactivity seems to be simple, effective, and economical and can work where the other process cannot be applied.

Key words: Biological treatment, mushroom, ***Pleurotus*** spp., cellulosic waste, radiocesium, radiocobalt, gamma irradiation.

INTRODUCTION

The accumulation of solid radioactive wastes and the release of radiocontaminants followed by their subsequent dispersion in the environment are a subject of intense public concern. The major burden on the environment from radioactivity is due to discharged waste streams produced by industrial activities allied to the generation of nuclear power, in addition significant quantities of natural and artificial radionuclides were also released as a consequence of nuclear weapons testing, through accidental release, due to the peaceful applications of nuclear technologies in our life (e.g. medicine, research, industry, agriculture) and finally from the ongoing storage of nuclear materials massed over the past 60 years of nuclear activities (Lioyd and Renshow, 2005). Given the high costs and the technical limitations of current chemical – based approaches, there has been an unprecedented interest in the hope of developing cost effective bioremediation attitudes for processing of hazardous wastes (Lioyd, 2003; Agunbiade et al., 2009). Bioremediation is a natural process, and is therefore perceived by public as an acceptable waste treatment process. The operation based on capability of micro-organism to biodegraded the waste that accompanied by biostabilization of the contaminants within the living organism cells. The present work was designed to study the capability of ***Pleurotus pulmonarius*** to bioaccumulate Cs-137 and Co-60 during the biodegradation of spiked mixture of cellulose-based organic solid waste simulate. The distribution of both radionuclides between cap and stem of the mature fruiting bodies was followed. In

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Table 1. Elemental analysis of solid CBW mixture simulate.

Elements	Concentration (%)
Nitrogen	1.01
Carbon	32.4
Hydrogen	5.37

addition the weight reduction percent as well as biodegradation rate of the CBS were evaluated.

MATERIALS AND METHODS

Solid cellulose based waste (CBW) simulates

Equal weights of three categories of solid cellulose-based materials namely; cotton, paper and protective clothes in additions to polyethylene plastics were mixed together to form a mixture of the waste simulates that subjected to bioremediation process. The elemental analysis of the waste mixture simulate was determined by Flash EA series number 1112 and is represented in Table 1.

Radionuclides and chemicals

Radioactive cesium (Cs-137, $T_{1/2}$ = 30.5 years) and cobalt (Co-60, $T_{1/2}$ = 5.25 years) are most famous radionuclides found in numerous types of radioactive waste. Accordingly, they were used in this study to spike the cellulose – based solid waste simulate. The two radionuclides were purchased from Amersham life science company, England. All other chemicals and solvents used were of Analar grade and used without any further purification steps.

Pleurotus pulmonarius fungi

Biodegradation of the radioactive CBW accompanied with bioaccumulation and biostabilization of radiocontaminats was carried out using ***P. pulmonarius*** fungi (Samy cel3014, France). This strain was acquired from Agricultural Research Center, Giza, Egypt, as mycelia on malt agar medium 2 %w/v malt extract with 1.5% w/v agar.

Preparation of *P. pulmonarius* spawns

Wheat spawn was prepared according to Chang (1982). In 250 ml flask, 50 g wheat grains were added to one gram limestone chalk, suspended in 75 ml distilled water. After autoclaving, this flask was inoculated with mycelial disk (one cm in diameter) of ***P. pulmonarius*** and incubated at 26± 2°C for 15 days, after then the spawns were of well complete growth.

Irradiation of *P. pulmonarius* spawns

To enhance the capability of the microorganism for the biodegradation of solid waste simulates, at the end of incubation period, the spawns of ***P. pulmonarius*** were irradiated for 0.75 kGy doses in gamma radiation cobalt-60 cell at dose rate 1.56 Gy/min. The source of gamma irradiation used for irradiating the spawn of the fungus was cabalt-60 gamma cell 3500. This source is located at Middle Eastern Regional Radioisotopes Centre for the Arab Countries, Giza, Egypt.

Bioremediation of radioactive CBW

Three carrier free radioactive solutions, namely, one for ^{137}Cs, second for ^{60}Co and third is mixture of both radionuclides were prepared and used to spike the CBW substrates, separately. The specific activity of each solution was 165 Becqurel/ml. Ten sets each of 100 g oven dried CBW substrates were also prepared. Each of these CBW sets was enriched by with 4 g wheat brain and 4 g $CaCO_3$ then autoclaved. Four CBW sets were treated with ^{137}Cs solution, another two with ^{60}Co solution and the third two with the mixture of the both radionuclides. The last two sets of CBW substrates were not treated with any radionuclides and were used as control sets. Two sets spiked with ^{137}Cs were inoculated with the irradiated ***P. pulmonarius*** spawn. The rest spiked and also the unspiked (control) sets were inoculated with the non-irradiated spawn of ***P. pulmonarius*** following the method previously published (El-sayyad, 2008). Up to four harvestings were picked up from the various sets. Radionuclides biouptake by mushroom was analyzed by counting the contaminated ground fruiting bodies using Multichannel Analyzer PCA-A (Oxford instrument Inc, USA). Dry weight of the obtained mushroom was also measured. The weight reduction percentages of spiked and unspiked CBW sets were calculated according to Iljin et al. (1999) as follows:

$$\text{Weight reduction percent} = \frac{W_o - W_1}{W_o} \times 100$$

Where: W_o: is the initial weight of oven dried waste substrate (g), W_1: is the remaining oven dried residue of substrate in addition to the fruiting bodies of mushroom (3 harvests).

Also, biodegradation rate (g/day) was calculated according to the following equation:

$$\text{Biodegradation rate (g/day)} = \frac{W_o - W_1}{t}$$

Where: t is the time (day) taken up to the last harvest.

Photographs were taken to follow the growth of mushroom on the radioactive CBW substrate spiked with ^{137}Cs /or ^{60}Co and weight reduction effect.

Theoretical background

The ability of microorganisms including actinomycetes, cyanobacteria and other bacteria, algae, fungi, and yeast to bioaccumulate heavy metals and radionuclides from their external environment was investigated by many authors (Zajic and Chiu, 1972; Gadd and Griffiths, 1978; Brost- Pauwels, 1981; Shumate and Strandberg, 1985; Gadd, 1986a,b). Despite of the apparent simplicity of the biological technique for treatment of hazardous waste (that is, growing the microorganism on a media containing radioactive materials), yet to obtain reliable results within ***in vivo*** system is a big problem and more work is needed to establish this technique.

Studies that carried out by Francis (1998) and El-sayyad (2008) on the mechanisms of bioremediation of solid cellulosic waste simulates under various treatment conditions have been resulted in

Table 2. Bioaccumulation of Cs-137 from solid radioactive cellulose - based waste simulate by ***P. pulmonarius****.

Flushing	Uptake (Bq)	Uptake (Bq/g**)	Uptake (%)	
			Stem	Cap
1st flushing	328.1	32.40	30.3	69.7
2nd flushing	68.6	26.49	30.0	70.0
3rd flushing	168.1	76.06	19.0	81.0
Total uptake	564.8	134.95	26.8	73.2

*Non-irradiated spawns were inoculated, **on the basis of dry weight of mushroom.

Table 3. Bioaccumulation of Co-60 from solid radioactive cellulose- based waste simulate by ***P. pulmonarius****.

Flushing	Uptake (Bq)	Uptake (Bq/g**)	Uptake (%)	
			Stem	Cap
1st flushing	52.15	15.80	54.10	45.90
2nd flushing	43.40	4.48	51.10	48.80
3rd flushing	16.30	20.80	42.30	57.70
Total uptake	111.85	41.08	51.20	48.80

*Non-irradiated spawns were inoculated, ** on the basis dry weight of mushroom

the development of two operations:

1. Removal of the contaminants from the waste categories by the mushroom accompanied with biodegradation of the organic solid substrate and detectable reduction in waste volumes and weights was recorded.
2. Biostabilization of radionuclides and toxic materials compositions existing in the waste materials.

The biostabilization process of radionuclides found in the wastes is accomplished by exploiting the unique metabolic capabilities of microorganism. The radionuclides are biosolubilized by fungi directly through reductive dissolution enzymes or indirectly due to the production of organic acids metabolites during the biodegradation of the organic moiety of the wastes (Francis, 1998). The radionuclides released into the growing medium are biostabilized through the enzymatic reductive process e.g. bioprecipitation, biosorption and bioredistribution within the microorganism. The biouptake of radionuclides is claim to be depend on: the microorganism species, the surrounding environment and the physico- chemical properties of the substrates. For another point of view, metal biouptake in living system, may be energy dependent intercellular mechanism and may be a sequence of increased membrane permeability with a resultant exposure of further bind sites within the cell (Abdel- Hafez, 1999).

RESULTS AND DISCUSSION

The factors affecting the biouptake and biotransfer of radiocontaminants by mushroom are claimed to be: the concentration of total and available radionuclide in the wastes, pH-value and growing conditions in additions to the mushroom's trophic group e.g. biological family, genus and species (Kaduka et al., 2006).

CBW substrates were spiked with radioactive solution labelled with cesium-137 or cobalt-60 or mixture of both radionuclides. Mushroom spawns were inoculated on the spiked substrate. Up to four flushing were harvested periodically (36±4 days for the 1st harvest. The 2nd, 3rd and 4th harvests were picked up after 7±2, 15 and 21±2 days after the 1st harvest, respectively) and the radioactive contents in both stems and caps of the mature fungi were measured. At the end of the experiments the dry weights of the harvested fruiting bodies as well as of the remaining substrates were recorded.

The results presented in Table 2 illustrated the total biouptake of Cs-137 by ***P. pulmonarius*** from radioactive CBW spiked by radiocesium only. It is clear from the data obtained that:

1. The radiocesium biouptake at the first and third flushing is greater than that at the second one. It is worth mentioning, also, that the biouptake of Cs-137 at the end of the first flushing is still the greatest.
2. The radiocesium accumulated mainly in the cap of the fruiting bodies compared to that in the stem.
3. The total biouptake of Cs-137 at the end of the three flushing is 134.95 Bq/g (basis on the dry weight of fungi). By this figure ***P. pulmonarius*** shows high bioaccumulation capability for radiocesium relative to some other reported fungi. For example, according to Marten et al**.** (1996) the highest concentration of Cs-137 recorded in mushroom were 16.6 and 41.8 kBq/kg (based on organism dry weight) for ***Lactarius*** sp. and ***Cortinarius*** sp., respectively.

Similar trend was recorded for the biouptake of Co-60 form its radioactive CBW substrate (Table 3). The total

Table 4. Bioaccumulation of Cs-137&Co-60 from solid radioactive cellulose- based waste simulate by ***P. pulmonarius****.

Flushing	Uptake (Bq)	Uptake (Bq/g *)	Uptake (%)	
			Stem	Cap
1st flushing	188.9	81.86	38.5	61.5
137Cs	136.3	59.26	23.5	48.7
60Co	52.6	22.8	15	12.9
2nd flushing	99.10	36.02	20.50	79.50
137Cs	87.80	31.90	15.70	72.90
60Co	11.30	4.10	0.80	0.60
Total uptake	288	117.88	32.20	67.70

*Non-irradiated spawns were inoculated, ** on the basis dry weight of mushroom.

biouptakes of Co-60 at each of three flushing were 15.8, 4.48 and 20.8 Bq/g, (basis on the dry weight of mushroom) respectively. However, it should be noted that the total biouptakes of Cs-137 was greater than that compared to that of Co-60 under the same growing conditions and during parallel harvesting periods. Also, it is clear from Table 3 that slight differences in the biodistribution of radiocobalt between the stem and cap in the mature mushroom was recorded. The data represented in Table 4 described the total uptake of Cs-137 and Co-60 from radioactive CBW, spiked with solution containing the two radionuclides, by ***P. pulmonarius.***

At the end of the first flushing the total activity of 81.86 Bq/g bioaccumulated (based on the dry mass of ***P. pulmonarius)*** while was greater than that at end of the second period. It should be pointed that mushroom failed to produce any fruiting bodies after the second harvesting. It is clear, also, from the Table 4 that the biouptake of Cs-137 is usually greater than that of Co-60 even from substrate spiked with their mixture in the two harvesting periods.

In spite of that cesium-137 is long lived biotoxic radionuclides (Franta and Vanara, 1987). Yet, there were big differences in the bioremoval rates of both radiocontaminants (that is, Cs-137 and/or Co-60) from the spiked substrates under the same cultivation methodology and conditions. This may attributed to the high solubility of cesium compared to cobalt and hence it is easily filtrated to the organism cells. It should be also noted that, the bioaccumulation activities of the microorganisms decreased after the first flushing and they regenerated their abilities after the second harvesting again.

Hence, the third flushing characterized by high biouptake compared to the second. Hence reactivation the colonies of the fungi by inoculating fresh mushroom spawn periodically seems to be essential to keep the bioaccumulation process of the radionuclide in a continuous.

However, it is worth mentioning that the of both cesium and cobalt radionuclides could be detected up to the third harvesting within the period of 54±3 days. This could be supported by the proposal that mushrooms are characterized by acceptable resistance to environmental factors including metal toxicity and highly extreme irradiation conditions. Identical conclusion was reached by Fomina et al. (2006) and El-sayyad (2008).

Figure 1 illustrates the radioactive biodistribution in the stems and the caps of the fruiting bodies of mushroom through the successive flushing during the biodegradation of the CBW radioactive waste substrate. It is clear that Cs-137 was accumulated mainly in cap of ***P. pulmonarius*** and increase in the third flushing compared to the first and second ones (from≈70% up to more than 80% from substrate spiked with Cs-137 only). On contrary the radiocesium bioaccumulation in the stem was lower and decreased from the first two harvesting to the third one reaching 30.3% and down to 19.0%. On the other hand slightly differences between the accumulation of Co-60 in both stem and caps from substrate spiked with Co-60 only was recorded.

It is worth mentioning that the bioaccumulation of both Cs-137 and Co-60 from CBS substrate spiked with the two radionuclides increase in the caps in the second flushing to reach 79.50%. On contrary the bioaccumulation of both radionuclides in the stem drops from 38.5 to 20.5% at the end of the second flushing. Even so the bioaccumulation of Co-60 increased in caps with time while it decreased in cap simultaneously.

The variation in the biodistribution of Cs-137 and Co-60 between cap and stem of mature fruiting bodies referred to the behaviour of each radionuclide within the organism. Cesium is very soluble element and hence it smoothly transpired to cap and bioconcentrated there. On contrary, cobalt may be needed for some enzymatic activities in the organism e.g. alkaline phosphatase (Wolfe and Hoehamer, 2003). Also, according to Bilgrami and Verma (1974) it was found that fungi have the ability to synthesize vitamin B-12 (coblamins) and cobalt is

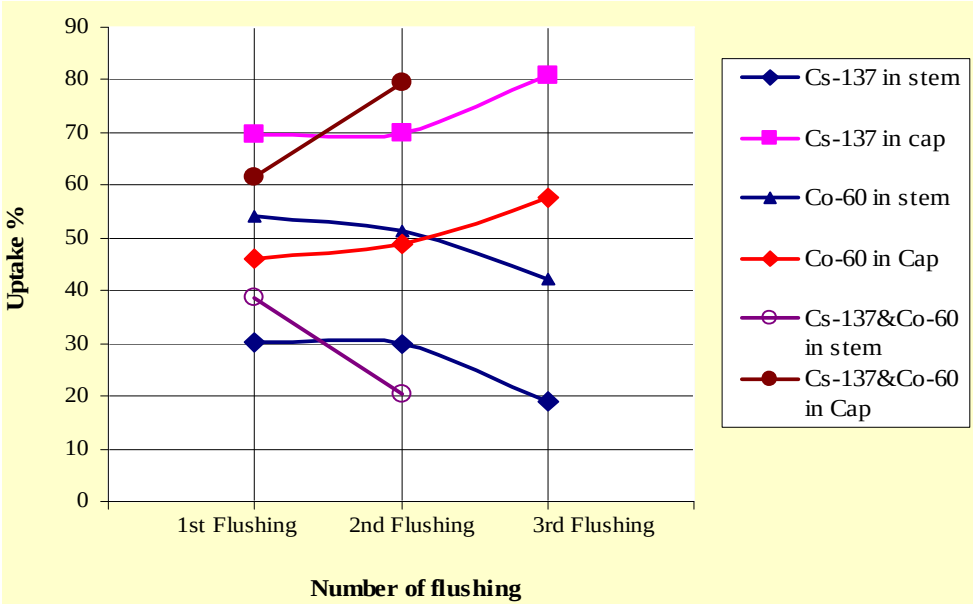


Figure 1. Bioaccumulation of cesium-137 and/or Co-60 by ***P. pulmonarius*** from radioactive cellulosic mixture waste simulate.

Table 5. Effect of irradiation pretreatment of the ***P. pulmonarius*** spawn on the bioaccumulation radicesium-137 from the soiled cellulosic mixture substrate spiked with radiocesium only.

Flushing	Bioaccumulation of cesium-137 Bq/g dry fruiting bodies	
	Non-irradiated	Irradiated spawn*
1st flushing		
Stem	24.5	47.5
Cap.	37.3	87.2
2nd flushing		
Stem	19.3	25.95
Cap.	31.6	40.9
3rd flushing		
Stem	30.6	43.5
Cap.	102.5	116.5
4th flushing		
Stem	-	93.04
Cap.	-	97.13

*Total irradiation dose was equal to 0.75 KGy.

known to be essential for this compounds. Therefore, it is nearly consumed by whole mushroom and hence there is slightly difference in its biodistrebution between cap and stem.

Table 5 described the effect of exposing the ***P. pulmonarius*** spawn to total irradiation dose equal to 0.75 KGy before its inoculation on cellulosic mixture substrate spiked with cesium-137. The bioaccumulation of radiocesium from that compost recorded slightly higher values compared to that of non-irradiated spawn. It is indeed quite striking to observe also that up to fourth flushing were harvested. This confirms again the earlier obtained results on the enhancement in the bioactivity of mushroom due to the irradiation treatment. However, it

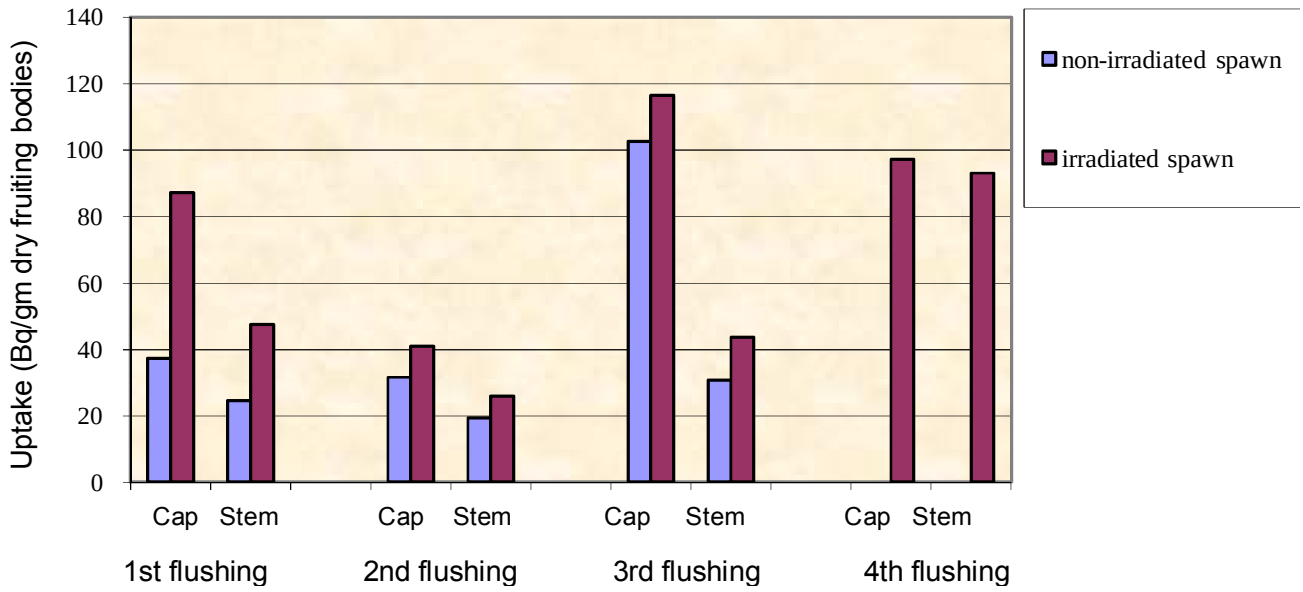


Figure 2. Effect of irradiation treatment of the ***P. pulmonarius*** spawn on the bioaccumulation of radiocesium-137 from spiked solid cellulose mixture waste substrate.

Table 6. Weight reduction percentage and degradation rats (g/day) for solid cellulose- based waste simulate by ***P. pulmonarius***.

CBW	Weight reduction (%)	Degradation rats g/day
Unspiked	42.9	0.72
Spiked with Cs-137	58.0	0.97
Spiked with Co-60	54.5	0.91
Spiked with both Cs-137 and Co-60	58.6	0.97

should be mentioned that, for the irradiation treatment of spawn before inoculation, the bioaccumulation of radio-cesium-137 is also higher in the caps compare to that in stem Figure 2. The same result were reached for the non- irradiated spawn (Table 4).

More than 15% weight reduction percentage was achieved in biodegrading the spike CBW compared to that of unspike one (Table 6). On the other hand, weight reduction percentages of the solid radioactive CBW degraded by ***P. pulmonarius*** is slightly varied based on the types of radiocontaminats. The degradation rate (g/day) for the solid cellulose- based waste exhibited similar trend (Table 6).

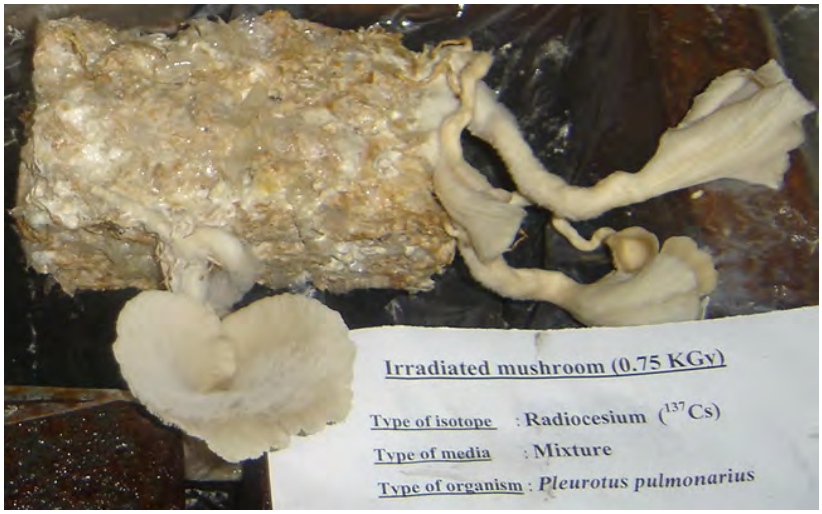
It should be noted that the weight reduction and degradation rate values for spiked substrates were highly comparable to that of non-spiked ones, which may refer to the internal irradiation doses received by the micro-organisms due to the two gamma emitters (Cs-137 and Co-60) found in the substrate (Figure 3a to c). These received doses enhance the capability of mushroom for the degradation of CBS. Comparable tendency was obtained in our previous published works (El-sayyad, 2008).

Conclusion

Studies on the bioremediation of some radioactive cellulose based waste simulates, in experimental ***in vivo*** conditions, had resulted in development of two treatment operations:

1. Biodegradation of the solid organic moieties in the waste, through their consumption as a source of carbon and energy by fungi and consequently acceptable weight and volume reduction figures was reached.
2. Simultaneous, biostabilization of radiocontaminants found in the waste, within the organism cells.

Irradiation of the ***P. pulmonarius*** spawn before their cultivation enhances the bioaccumulation of Cs-137 and up to four flushing were collected. The work provides an introduction for cost effective, natural hope and hype environmentally friendly clean up technology based on



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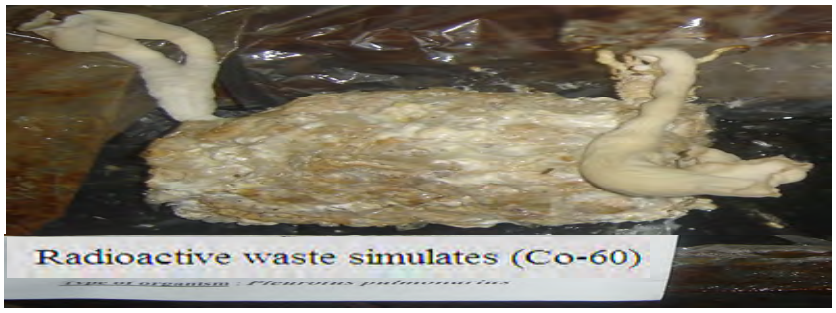

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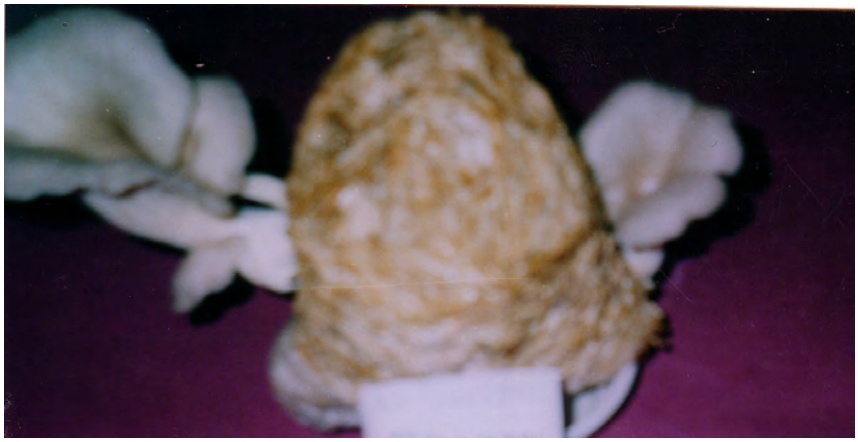

c

Figure 3. ***P. pulmonarius*** cultivated on mixture of wastes contaminated with radiocesium and radiocobalt. (a) Mixture of waste contaminated with Cs-137, (b) Mixture of waste contaminated with Co-60, (c) Non-contaminated mixture of waste (control).

the capability of ***P. pulmonarius*** to process mixture of some categories of solid organic cellulose based hazardous wastes.

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Full Length Research Paper

Effect of Avid® on the synnema-like formation of *Aspergillus flavus* grown on Czapek medium

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The effect of Avid® on the induction of synnema-like structure of *Aspergillus flavus* (61 strains) grown on various types of agar media was investigated. Other related species of *Aspergillus* within the section *Flavi*, *A. parasiticus* (14 strains), *A. nomius* (14 strains), *A. psuedotamarii* (2 strains), *A. tamarii* (20 strains) and *A. oryzae* (5 strains), were also evaluated for comparison. When Avid® was added into five different solid media (Czapek, V8, *Aspergillus flavus* and *A. parasiticus*, corn meal and potato dextrose agar) at 0.004% (v/v), only the *A. flavus* strains (all 61 tested) were able to produce synnema-like structures, and only in the Czapek agar medium containing Avid®. The effect of carbon (sucrose, glucose and starch) and nitrogen ($NaNO_3$, urea and peptone) sources in Czapek medium containing Avid® on the induction of a synnema-like structure was also determined, with synnema-like structure induction being found in all three carbon sources but only in $NaNO_3$ as the nitrogen source. All the three of the inert solvents in Avid® (mineral oil, butylated hydroxytoluene and n-methylpyrrolidone) did not induce synnema-like structure formation, indicating that abamectin, an active ingredient of Avid® together with $NaNO_3$ likely play important roles in the induction of synnema-like structure of *A. flavus*.

Key words: ***Aspergillus flavus***, Avid®, synnema-like structure.

INTRODUCTION

Aspergillus flavus is a fungus in ***Aspergillus*** subgenus ***Circumdati*** section ***Flavi***. This species can be divided into two groups based on their aflatoxin production, the aflatoxigenic group (with the ability to produce aflatoxins) and non-aflatoxigenic group (without ability to produce aflatoxins) (Chang and Hua, 2007). Aflatoxigenic ***A. flavus*** strains are able to produce aflatoxins B (AFB1 and AFB2) and some strains also produce aflatoxins G (AFG1 and AFG2) (Horn and Greene, 1995). This species is of interest since it can be found in several economically important crops including corn, cotton, peanut and tree nuts, in the field or during storage and is more frequent than other aflatoxigenic strains, such as ***A. parasiticus***, ***A. nomius*** and ***A. pseudotamaraii*** (Rodriguez and Mahoney, 1994).

Avid® is a miticide or insecticide used to control mites or insects in a wide range of important crops (Van Der Geest et al., 2000). It is composed of the active ingredient, abamectin, and three inert solvents, mineral oil,

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butylated hydroxytoluene (BHT) and n-methylpyrrolidone (Syngenta Crop Protection, Inc., Greensboro, NC, USA). Avid® has been added in microbial culture media to prevent contamination caused by mites or insects associated with crop samples, and thus proved a useful practice for isolation of microorganisms from crop samples.

In our primary observation, when ***Aspergillus*** spp. were cultivated on Czapek agar plates containing Avid®, it was observed that ***A. flavus*** strains, but not other species, could form synnema-like structures. Therefore, in this research, the effect of Avid® on the induction of the synnema-like structure were evaluated in 61 strains of ***A. flavus*** growing on various types of solid culture media. Other related species of ***Aspergillus*** within the section ***Flavi*** (***A. flavus***, ***Aspergillus parasiticus***, ***Aspergillus nomius***, ***Aspergillus psuedotamarii***, ***Aspergillus tamarii*** and ***Aspergillus oryzae***) were included for comparison.

MATERIALS AND METHODS

Fungal cultures

Fifty-five strains of ***Aspergillus flavus***, 12 strains of ***A. parasiticus***, 12 strains of ***A. nomius***, and 18 strains of ***A. tamarii*** were obtained from the Fungi Section, Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. Six reference strains of ***A. flavus*** (NRRL 3357, NRRL 21882, TX 9-8, F3W4, Af 53, and Af 13) were obtained from Department of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA, USA. To strains of each of ***Aspergillus parasiticus*** (SRRC 75 and SRRC 143-A), ***A. nomius*** (SRRC 362 and SRRC 375), ***A. tamarii*** (SRRC 99 and SRRC 1088) and ***A. pseudotamarii*** (SRRC 2420 and SRRC 2428) plus four strains of ***A. oryzae*** (SRRC 302, SRRC 480, SRRC 2079, and SRRC 2085) were obtained from USDA-ARS, Southern Regional Research Center, New Orleans, LA, USA.

Media and culture conditions

Each ***Aspergillus*** species / strain was grown on V8 medium (5.0% (v/v) V8 juice and 2.0% (w/v) agar) in the dark at 30°C for 7 days. Spores were harvested from these cultures using sterile water and the suspension was adjusted to 10^5 spores per ml. A spore suspension of each ***Aspergillus*** species (5 µl per plate) was inoculated onto solid media and incubated in the dark at 30°C for 7 days for morphological observation.

To investigate the effect of Avid® (0.15 EC, Norvatis Crop Protection, Greensboro, NC, USA) on the synnema-like formation of ***A. flavus***, and the other related species in the section ***Flavi***, each fungal isolate was grown on Czapek, V8, AFPA (Aspergillus flavus and parasiticus agar) (Pitt et al., 1983), Corn Meal Agar (CMA, Difco, USA) and Potato dextrose Agar (PDA, Difco, USA), with or without 0.004% (v/v) Avid®, incubated in the dark at 30°C for 7 days. To determine the effect of the Avid® concentration on the synnema-like formation, Avid® at six different concentrations (0.002, 0.004, 0.008, 0.02, 0.05 and 0.01 % (v/v)) was added into the culture medium. Carbon and nitrogen sources in the culture medium were varied so as to include one of each three carbon sources (sucrose, glucose and starch) and three nitrogen sources ($NaNO_3$, urea and peptone). The three inert solvents in Avid®, mineral oil, butylated hydroxytoluene (BHT) and n-methylpyrrolidone (0.004% v/v), were also tested for their ability to induce a synnema-like structure by incorporating them into the Czapek medium. The colonies of fungi were observed daily for morphological changes. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

When ***Aspergillus*** spp. were grown on different kinds of solid culture media (Czapek, V8, AFPA, CMA and PDA) with or without Avid® at 0.004% (v/v), all cultures grew well and produced conidia regularly. However, only ***A. flavus*** (of all 61 strains) grown on Czapek medium containing Avid®, developed the synnema-like structures (fused white conidiophores bearing yellow to green conidia) after 3 days of incubation at 30°C in the dark (Figure 1). When this species was grown on Czapek media without Avid® or on the four other media tested with or without Avid®, the fungus did not produce a synnema-like structure (Figure 2). In this study the miticide clearly did not affect the growth of ***Aspergillus*** spp. However, Avid® might be able to decrease the germination, vegetative growth, and conidial number of the entomopathogenic fungus, ***Beauveria bassiana***, (Oliveira and Neves, 2004). Although not commonly found, the formation of synnema by ***Aspergillus*** section ***Flavi*** was previously reported in a mutant of ***A. flavus*** (NRRL 29254) and some strains of ***A. caelatus*** which was induced by the regulation of carbon and nitrogen sources in the culture media (McAlpin, 2001; 2004).

All strains of ***A. flavus*** could grow and form synnema-like structures at different level concentrations of Avid®, ranging from 0.002 to 0.01% (v/v), added into Czapek media. Thus, Avid® at this concentration range, can induce the formation of a synnema-like structure of ***A. flavus***. The formation of synnema-like structure of ***A. flavus*** was not affected by the three carbon sources tested, with all strains of ***A. flavus*** showing no difference in colony morphologies when they grew on Czapek media containing Avid® using either sucrose, glucose, or starch as the sole carbon source, and they all produced synnema-like structures. However, with respect to the nitrogen source, all ***A. flavus*** strains could form synnema-like structures only when grown on Czapek media containing Avid® with $NaNO_3$ as the sole nitrogen source. Urea and peptone were not able to induce synnema-like structure in ***A. flavus***. The results support the absence of synnema-like structure of ***A. flavus*** strains grown on V8, AFPA, CMA, and PDA media since they contain either urea or peptone as a nitrogen source. The role of nitrogen source in morphological changes of ***Aspergillus***

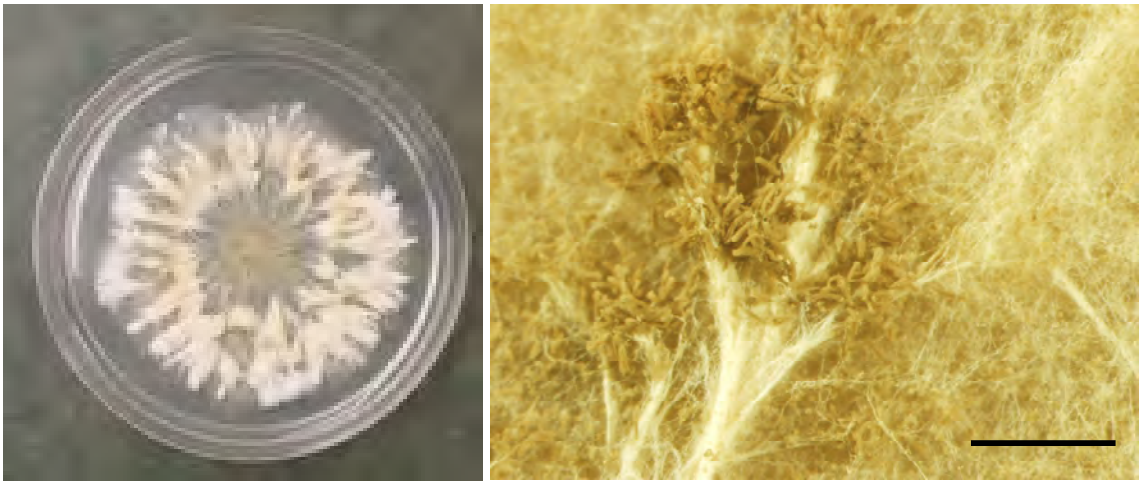

Figure 1. Colony of ***A. flavus*** NRRL 3357 (A) and its synnema-like structure (B). The fungus was grown on Czapek medium containing 0.004% (v/v) Avid® incubated at 30 °C in the dark for 7 days. (bar = 1 mm.)

Figure 2. Colony ***A. flavus*** NRRL 3357 (A) and its typical conidia (B) The fungus was grown on Czapek medium without Avid® incubated at 30°C in the dark for 7 days. (bar = 1 mm.)

has previously been reported. $NaNO_3$ was shown to be an excellent nitrogen source for synnema/sclerotium formation while ammonium sulfate inhibited growth, sporulation and synnema/sclerotium formation of ***A. caelatus*** and a mutant strain of ***A. flavus*** (McAlpin, 2001; 2004).

In order to clarify which ingredient in Avid® could induce the synnema-like structure of ***A. flavus***, the inert solvents in Avid®, mineral oil, butylated hydroxytoluene (BHT) and n-methylpyrrolidone, were separately added in Czapek medium to the same concentration as that of 0.004% Avid®. All ***A. flavus*** strains failed to produce synnema-like structures when grown on Czapek medium containing any of these three inert solvents. Thus, unless a corequirement of two or more of the inert solvents is required; the result suggests that synnema-like structure of this species was induced by abamectin, an active ingredient in Avid®.

In conclusion, the results presented here show that Avid® could induce synnema-like structure of ***A. flavus*** growing on Czapek medium. None of the other ***Aspergillus*** species tested could form this structure when grown on this medium containing Avid®. The miticide could induce synnema-like structure at a wide

concentration range (from 0.002 to 0.01% (v/v)). $NaNO_3$ in Czapek medium together with abamectin in Avid® were found to play important roles in the induction of synnema-like structure of ***A. flavus***. The results open up the possibility of using Avid® in a selective culture medium to help distinguish ***A. flavus*** from the other ***Aspergillus*** in the section Flavi.

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Full Length Research Paper

Toxicity of essential oil from *Artemisia argyi* against *Oryzaephilus surinamensis* (Linnaeus) (Coleoptera: Silvanidae)

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The repellent, fumigant effect and contact toxicity of essential oil extracted from *Artemisia argyi* (Asteraceae: Artemisia) plant against *Oryzaephilus surinamensis* (Linnaeus) (Coleoptera: Silvanidae) was investigated. The *A. argyi* oil exhibited strong repellent, fumigant effect and contact toxicity against *O. surinamensis* which progressively increased with increased exposure dosage. Repellency percentage of *A. argyi* oil against *O. surinamensis* adults reached IV grade after 48 h exposure period at the dosage of 0.40 µl/cm^2, the corrected percentage mortality for fumigant toxicity reached more than 97% at the dosage of 160 µl/l air, and the current population inhibition percentage (CPI) and F_1 progeny population inhibition percentage (PPI) reached 77.38 and 96.06% at the dosage of 0.80 µl/g, respectively. The results suggest that the *A. argyi* oil has great potential for effectively controlling *O. surinamensis*.

Key words: Natural product, ***Artemisia argyi***, toxicity, ***Oryzaephilus surinamensis,*** stored grain insect.

INTRODUCTION

The ***O. surinamensis*** (Linnaeus) (Coleoptera: Silvanidae) is one of the most serious pest insects of stored cereal grains and flour throughout the world. Currently, intensive use of phosphine and other synthetic insecticides for the control of stored products pests has resulted in serious problems including insecticide resistance, environment contamination, unacceptable pesticide residues in food, lethal effects on non-target organisms, and so on (White and Leesch, 1995; Jovanović et al., 2007). Development and implementation of alternative control strategies and integrated pest management systems have recently been considered to be the only solution to combat these increasing pesticide-resistant insect pests. Recent research has focused on natural product alternatives for pest control in developing countries and for organic food production in industrialized countries (Isman, 2006, 2008; Liu et al., 2007; Rajendran and Sriranjini, 2008).

Many essential oils and their constituents have been studied to possess potential as alternative compounds to currently used insect-control agents (Shaaya et al., 1997; Huang et al., 2000; Lee et al., 2004; Boekea et al., 2004; Cosimi et al., 2009; Nerio et al., 2009). In previous studies, ***Ailanthus altissima*** bark oil (Lü and Wu, 2010), and ***Ocimum gratissimum*** oil and its constituents (Ogendo et al., 2008) were shown to be repellent and fumigant against ***O. surinamensis*** population. The LC_{50} value in fumigant toxicity of the essential oil of aerial parts of ***Agastache foeniculum*** for adults of ***O. surinamensis*** was 18.781 Kl/l (Ebadollahi et al., 2010). The essential oil of ***Thymus vulgaris*** gave 100% mortality of ***O. surinamensis*** at 2,000 and 3,000 ppm (Nesci et al., 2011). The essential oil from ***Artemisia scoparia*** Waldst et Kit had significant fumigant and repellent activity against three stored product insects, ***Callosobruchus maculatus*** (Fab.), ***Sitophilus oryzae*** (L.), and ***Tribolium castaneum*** (Herbst) (Negahban et al., 2006), and the essential oil from ***Artemisia sieberi*** Besser also had potent fumigant activity against the above three stored product insects (Negahban et al., 2007). ***A. argyi*** (Asteraceae: Artemisia) plants are found growing wild and abundantly throughout the temperate zones in China, and its dry plants have ever often been used to control repel mosquitoes and other insects. Here, the paper describes a laboratory study to evaluate the potential

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bioactivity of essential oil from ***A. argyi*** plants against ***O. surinamensis***.

MATERIALS AND METHODS

Insects

The test insects were obtained from laboratory stock cultures maintained in the dark in incubators without exposure to any insecticide at 27±2°C and 75±5% relative humidity at the Institute of Stored Product Insects of Henan University of Technology. The food media used was wheat flour, rolled oats and yeast (6:3:1, w/w/w). Healthy and consistent developed adult insects (1-2 weeks old) were randomly chosen for tests.

Preparation of the essential oil

The ***A. argyi*** plant was collected in Henan, central China, June 2010, dried at room temperature, ground to fine powder. Each 50 g of the powder was extracted by Soxhlet method with 250 ml anhydrous diethyl ether at 40°C until the distilled liquid was colorless. The solvent was evaporated under vacuum in a rotary evaporator. The essential oil was stored in airtight fuscous glassware in a refrigerator at 4°C.

Repellency bioassay

The repellent effect of the ***A. argyi*** oil against ***O. surinamensis*** adults was evaluated using the area preference method. Test areas consisted of Whatman No.1 filter paper cut in half (Φ12.5 cm). An aliquot of 3.07, 6.14, 12.28 and 24.56 µl of the ***A. argyi*** oil dissolved in 1 ml acetone (analytical purity) was evenly applied on a half-filter paper disc using a micropipette corresponding to dosages of 0.05, 0.10, 0.20 and 0.40 µl/cm^2, and the other half of the remaining filter paper was treated with 1 ml acetone alone and used as control. The treated and control half discs were air-dried for about 10 min to evaporate the solvent completely. Full discs were subsequently remade by attaching treated halves to untreated halves with clear adhesive tape. Each remade filter paper disc was tightly fixed on the bottom of a 12.5 cm diameter petri dish daubed with polytetrafluoroethylene (PTFE) on the inside wall to avoid the insects escaping. Then 30 unsexed adult insects of each species were released separately at the center of the filter paper disc and the petri dishes were subsequently covered and kept in incubator at 27±2°C and 75±5% relative humidity. Each treatment was replicated 5 times and the number of insects present on the control (N_c) and treated (N_t) areas of the discs was recorded after 12, 24, 48, 72 h, respectively.

Percentage repellency (PR) values were calculated as follows:

$$PR = [(N_c - N_t) / N_c]100\%$$

The mean percentage repellency value was calculated and assigned to repellency classes (Juliana and Su, 1983) from 0 to V: class 0 (PR< 0.1%), class I (PR=0.1–20%), class II (PR=20.1–40%), class III (40.1–60%), class IV (60.1–80%), class V (80.1–100%).

Fumigant activity

Fumigation bioassay of the ***A. argyi*** oil without grain was carried out with 30 unsexed adult insects exposed in a 250 ml glass flask sealed with a rubber stopper. An aliquot of 0, 5, 10, 20 and 40 µl of the ***A. argyi*** oil respectively dissolved in 1 ml acetone (analytical purity) was evenly applied on a Whatman No.1 filter paper strip (7×9 cm) corresponding to dosages of 0 (as a control), 20, 40, 80 and 160 µl/l air based on the flask volume, which was dried in air for 10 min and then fixed on the stopper by a staple at one end. The stopper was tightly stuffed in the flask to make the filter paper suspend in the top of the flask, and care was taken to avoid the filter paper contacting the flask inside wall. The flask was placed in the incubators at 27±2°C and 75±5% relative humidity. Five replicates were conducted. The number of dead insects was recorded after 48 h.

The procedure of fumigation bioassay of the ***A. argyi*** oil with grain was same as the above except that the flask held 20 g wheat and 2 g rolled oats.

Contact toxicity of *A. argyi* oil, F_1 progeny production in grains

An aliquot of 0, 20, 40 and 80 µl of the ***A. argyi*** oil dissolved in 1 ml acetone (analytical purity) was evenly mixed with 100 g wheat and 5 g food media in a 350-ml flask corresponding to dosages of about 0 (as a control), 0.20, 0.40 and 0.80 µl/g. Then 30 unsexed adult insects were exposed in the flask tightly sealed with plastic film. The flask was placed in the incubators at 27±2°C and 75±5% relative humidity. The number of living adult insects and the number of F_1 progeny larvae was recorded after 15 d, respectively. Five replicates were conducted.

The current population inhibition percentage (CPI) and F_1 progeny population inhibition percentage (PPI) was calculated using the following formula:

$$CPI = [(Cn - Tn) / Cn]100$$

$$PPI = [(P_c - P_t) / P_c]100$$

where Cn is the number of living adults in the control flask, Tn the number of living adults in the treated flask, P_c is the number of F_1 progeny larvae in the control flask, and P_t is the number of F_1 progeny larvae in the treated flask.

Statistical analysis

For the above tests, and the percentage mortality was corrected by the Abbott (1925) formula. The percentage mortality was determined and transformed to arcsine square-root values for analysis of variance (ANOVA). Treatment means were compared and separated by Scheffe's test at ***P*** = 0.05 (SAS Institute, 1994).

RESULTS

Repellency bioassay

The ***A. argyi*** oil had potent repellent activity against ***O. surinamensis*** adults (Figure 1), and the repellency value significantly increased with increased exposure dosage (df=3, P<0.05). The repellency class reached IV grade after 48 h exposure period at the dosage of 0.40 µl/cm^2.

Fumigant activity

The ***A. argyi*** oil showed strong fumigant activity against ***O. surinamensis*** adults under both Flasks with grain and Flasks without grain (Table 1). The fumigant toxicity

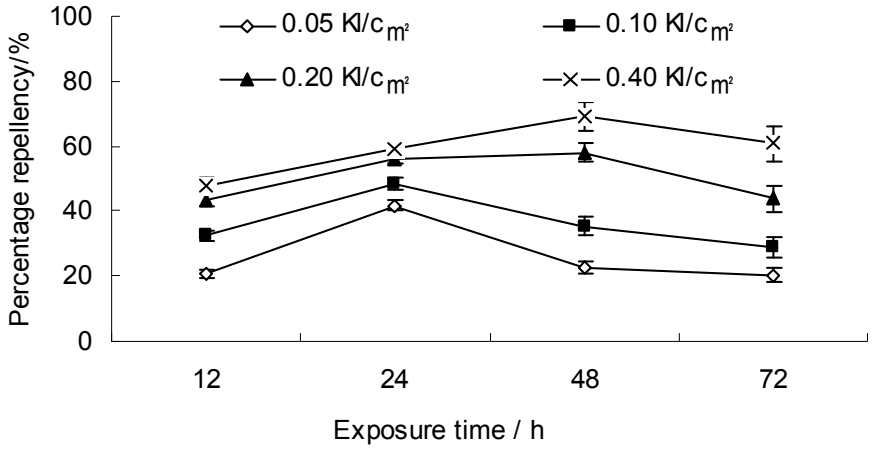

Figure 1. Repellent activity of the ***A. argyi*** oil against ***O. surinamensis*** adults.

Table 1. Fumigant activity of ***A. argyi*** oil against ***O. surinamensis*** adults.

Dosage (µl/l air)	Corrected mortality (%)	
	Flasks with grain	Flasks without grain
20	43.26±2.36^{c}	48.21±5.33^{c}
40	67.52±5.32^{b}	73.66±3.55^{b}
80	89.63±1.86^{a}	95.38±3.86^{a}
160	97.56±3.47^{a}	100.00±0.00^{a}

Each datum in the table is mean ± SE. The data in a column followed by different letters indicate significant difference tested by Scheffe's test at $P = 0.05$.

significantly increased with increased exposure dosage with the corrected percentage mortality reached 97.56 and 100.00% at the dosage of 160 µl/l air under both Flasks with grain and Flasks without grain, respectively (df=3, P<0.05).

Contact toxicity of *A. argyi* oil, F_1 progeny production in grains

Contact activity of ***A. argyi*** oil against ***O. surinamensis*** in grains progressively increased with increasing exposure dosage (df=3, P<0.05). Specially, the CPI and PPI reached 77.38 and 96.06% at the dosage of 0.80 µl/g, respectively (Table 2).

DISCUSSION

Previous research testified that plant-derived essential olis exhibited strong toxic effects on ***O. surinamensis*** (Tripathi et al., 2000; Negahban et al., 2006, 2007; Ogendo et al., 2008; Ebadollahi et al., 2010; Lü and Wu, 2010). Our results also clearly showed that ***A. argyi*** oil exhibited strong toxic activity against ***O. surinamensis***, which testified that ***Artemisia*** essential oil has great potential to control stored product insects (Tripathi et al., 2000; Negahban et al., 2006, 2007; Wang et al., 2006), and were similar to the activity of essential oil from ***A. scoparia*** (Negahban et al., 2006) and ***A. sieberi*** (Negahban et al., 2007) against three stored product insects, ***C. maculatus*** (Fab.), ***S. oryzae*** (L.), and ***T. castaneum*** (Herbst). These results suggest that ***A. argyi*** oil had huge potential as a repellent or a fumigant for the effective control of ***O. surinamensis***. Meanwhile, the effect of ***A. argyi*** oil is safe to consumers because it has been used in many pharmaceutical preparations in traditional Chinese medicine.

Thus, it is necessary to investigate the bioactivity of ***A. argyi*** oil and their pure constituent level along with structure–activity relationships against different developed stages of the major stored grain insects in the future. In addition, a chlorpyrifosmethyl resistant strain of ***O. surinamensis*** has been found to have cross-resistance to ***Eucalyptus*** essential oil and 1,8-cineole fumigant activity (Lee et al., 2000), which has been led to some essential oil-degrading enzymes induced by chlorpyrifosmethyl (Lee, 2002). Therefore, whether the ***O. surinamensis*** will produce resistance to ***A. argyi*** oil and their pure constituent should also be further investigated.

Table 2. Contact toxicity of ***A. argyi*** oil against ***O. surinamensis*** in grains.

Dosage (μL/g)	The number of living adults	The number of living F_1 progeny larvae	Current population inhibition rate (%)	F_1 progeny population inhibition rate (%)
0	28.92±1.83[a]	25.38±2.45[a]	/	/
0.20	23.28±1.67[b]	15.45±1.89[ab]	19.50±4.21[b]	39.12±3. 28[b]
0.40	13.45±1.15[b]	7.65±1.21[b]	53.49±3.16[b]	69.86±5.42[a]
0.80	6.56±0.85[c]	1.00±0.67[b]	77.38±3.22[a]	96.06±6.85[a]

Each datum in the table is mean ± SE. The data in a column followed by different letters indicate significant difference tested by Scheffe's test at ***P*** = 0.05.

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Full Length Research Paper

Bioactivity of *Hydnora africana* on selected bacterial pathogens: Preliminary phytochemical screening

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Medicinal plants contain a variety of chemical substances with important therapeutic properties that can be utilized in the treatment of human diseases. *Hydonora africana* is used in folklore remedies for the treatment of diarrhoea, dysentery, kidney and bladder complaints among other ailments; hence we assessed the *in vitro* antimicrobial activity of this plant against three bacterial species (*Helicobacter pylori* ATCC 43526*, Helicobacter pylori* PE 252C*, Staphylococcus aureus* NCTC 6571 *and Aeromonas hydrophila* ATCC 35654). The agar well diffusion method was used to determine the susceptibility of bacterial strains to crude extracts of the plant. The minimum inhibitory concentration (MIC_{50}) and minimum bactericidal concentration (MBC) of the active crude extracts were determined by the microdilution test. Ciprofloxacin (0.0125 mg/mL) was used as positive control. The presence of phytochemicals was also assessed using standard methods. Results were analyzed statistically by the one-way ANOVA test. *Hydnora africana* demonstrated antimicrobial activity against all the organisms with a mean zone diameter of inhibition ranging from 0 to 22 mm. The MIC_{50} of the extracts ranged from 0.078 to 2.5 mg/mL and MBC ranged from 0.78 to 25 mg/mL. Phytochemical assay revealed the presence of alkaloids, tannins, flavonoids, saponins and steroids in the extracts. It is concluded that *H. africana* may contain compounds with therapeutic activity.

Key words: *Hydnora africana,* medicinal plant, minimum inhibitory concentration, minimum bactericidal concentration, phytochemicals.

INTRODUCTION

Plants have a great potential for producing new phytochemicals with profound antimicrobial activity against human pathogens. According to the world health organization (WHO), more than 80% of the world's population depends on traditional medicine for their primary health care needs (Duraipandiyan et al., 2006). There is an upsurge of resistant microbial strains to conventional antimicrobials necessitating the need for a search and development of new drugs to circumvent the problem (Panda et al., 2009).

Hydnora africana is a parasitic plant which is predominant in the dry and semi-arid parts of the Succulent Karoo, Eastern Cape Karoo and the dry coastal thickets between the Eastern Cape and KwaZulu-Natal Provinces of South Africa (Asfaw et al., 1999). The plant belongs to the family *Hydnoraceae* (Musselman, 1991). It is renowned for its healing properties, and has been used by traditional medicine practitioners to treat aliments such as diarrhea, dysentery, kidney and bladder complaints (Van and Gericke, 2000). For example, infusions are used as face wash to treat acne by the Xhosa people of South Africa (Van and Gericke, 2000). More than 12 *Hydnora* species have been described with antimicrobial properties (Bolin and Musselman, 2009). However, there is paucity of data in South Africa on its antimicrobial activity as well as its chemical constituents, hence the present investigation against different Gram-positive and negative bacterial pathogens. *Helicobacter pylorus is* a Gram-negative, microaerophilic bacterium that causes

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duodenal ulceration, chronic gastritis and gastric cancer. (Ndip et al., 2008). An increasing number of infected individuals are found to harbour antibiotic-resistant strains (Ndip et al., 2008; Tanih et al., 2010). The emerging resistance to antibiotics, especially metronidazole and amoxicillin limits their use in the treatment of infections (Smith et al., 2001; Tanih et al., 2010). *Aeromonas hydrophila* is a heterotrophic, Gram-negative, rod shaped bacterium, mainly found in areas with a warm climate. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated water (Villari et al., 2003). It causes gastroenteritis, cellulitis, myonecrosis and eczema. Its increasing resistance to agents such as chloramphenicol, florenicol, tetracycline, sulfonamide, nitrofuran derivatives, and ciprofloxacin used to eliminate and control impending infection requires attention (Wang et al., 2011). *Staphylococcus aureus* is a facultative anaerobic, Gram-positive coccus and the most common cause of staphylococcal infections (Cosgrove et al., 2009). It causes a range of illnesses from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia and sepsis (Kluytmans et al., 1997). The treatment of choice for *S. aureus* infection is penicillin; but in most countries, penicillin-resistance as well as MRSA is extremely common (Neely and Maley, 2000; Nkwelang et al., 2009). This study therefore documents the antimicrobial activity of *H. africana* against the selected bacterial pathogens, and a preliminary assessment of the possible phytochemicals responsible for its action.

MATERIALS AND METHODS

Bacterial strains

The following standard strains of bacteria *Aeromonas hydrophila* ATCC 35654, *S. aureus* NCTC 6571, *Helicobacter pylori* ATCC 43526 and a local metronidazole-resistant strain of *H. pylori* PE 252C isolated in our laboratory (Tanih et al., 2010) were used. Cultures of the other organisms were resuscitated and maintained on nutrient agar slants at 4°C (Cheesbrough, 1982; Cowan and Steel, 2004), while *H. pylori* was suspended in 20% glycerol and stored at -80°C until used.

Preparation of plant extracts

Hydnora africana was selected based on ethnobotanical information and preliminary data obtained in our laboratory. It was identified in collaboration with botanists at the University of Venda, Limpopo Province, South Africa where voucher specimens (BP04) have been deposited.

The method described by Ndip et al. (2008) to prepare extracts was employed with modifications. The plant was harvested, air dried for 2 weeks and ground to fine powder using a blender (ATO MSE mix, 702732, England). Organic solvents including methanol, ethanol, acetone, ethyl acetate (100%) and water were used for extraction. Briefly, the dried plant material (2.8 kg) was macerated in five fold excess of the solvent in extraction pots such that the level of the solvents was above that of the plant material. The slurry was put in a shaker incubator (Edison, N.J., USA) regulated at room temperature (RT) for 48 h then centrifuged at 300 rpm for 5 min (Model TJ-6 Beckman, USA) and filtered using filter papers of pore size 60^{Å}.The process was repeated twice for a total of three extractions (Okeleye et al., 2010). The combined extracts was concentrated in a rotavapor (BUCHI R461, Switzerland) and transferred to labelled vials and allowed to stand at RT to permit evaporation of residual solvents. A 3 gram sample of each plant extract was used for the preliminary bioassay, and 3 g kept in the extract bank for subsequent use. Stock solutions were prepared by dissolving the extracts in 10% Dimethyl Sulphoxide (DMSO).

Antibacterial susceptibility test

The agar well diffusion technique was employed as previously described by Dastouri et al. (2008). For *H. pylori*, Columbia base agar was prepared following the manufacturer's instructions, supplemented with 7% defibrinated horse blood and Skirrow's supplement (Oxoid, UK) while for *A. Hydrophila* and *S. aureus*, Muller-Hilton agar (Oxoid, UK) was prepared following the manufacturer`s instructions. A 0.5 McFarland standard was prepared by the method of Koneman et al. (1992), and 5 mL put into a sterile test tube. An inoculum of each microorganism was prepared from subcultures of the bacterial suspension and estimated to contain 10^8 colony forming units. Four to five colonies of the same morphological type were picked, emulsified in 0.9% physiological saline and used to evenly inoculate specific agar plates depending on the microorganisms. Wells were cut in each agar plate with a 6 mm cork borer. About 100 µL of the different concentrations (200, 100, 50 mg/mL) of the extract were put separately into each well, in each plate. Ciprofloxacin (0.0125 mg/mL) was used as a positive control. The plates were incubated at 37ºC for 24 h for *A. hydrophila* and *S. aureus* and 3 to 5 days for *H. pylori* under microaerophilic conditions (Anaerocutt, Baringstoke, England); the diameter of the zone of inhibition was measured and recorded in millimeters. The experiment was repeated 2 times for each strain.

Determination of minimum inhibitory concentration (MIC_{50})

MIC_{50} determination was carried out by the microdilution test method in 96 well plates as earlier described (Banfi et al., 2003; Njume et al., 2010) with slight modifications. Briefly, Two-fold dilutions of the most potent extracts and antibiotic (ciprofloxacin) were prepared in the test wells in complete Brian Heart Infusion (BHI) broth (Oxoid, UK); the final extracts and antibiotic concentrations ranged from 0.0024 to 5 mg/mL. Twenty microlitres of each bacterial suspension was added to 180 µL of extract containing culture medium. Control wells were prepared with culture medium and bacterial suspension only. Also included was culture medium and extract only at different concentrations. An automatic ELISA micro plate reader (Model 680, Bio-Rad, Japan) adjusted to 620 nm was used to measure the absorbance of the plates before and after 24 h incubation. The absorbencies were compared to detect an increase or decrease in bacterial growth. The lowest concentration of the test extract resulting in inhibition of 50% of bacterial growth was recorded as the MIC.

Determination of minimum bactericidal concentration (MBC)

To determine the MBC, 0.2 mL of the contents of the MIC was serially diluted tenfold in 0.9% physiological saline (Ndip et al., 2007). A loop full was taken from each tube and inoculated onto BHI agar plates. The MBC was recorded as the lowest concen-

tration of the extract that gave complete inhibition of colony formation of the test bacteria at the latter cultivation.

Phytochemical screening of the extracts

A small portion of the dry extract was subjected to phytochemical test using previously established methods (Akinpelu et al., 2009) to test for alkaloids, tannins, flavonoids, steroids and saponins.

Test for alkaloids

Exactly 0.5 g of the plant extract was dissolved in 5 mL of 1% HCl on steam bath. A millilitre of the filtrate was treated with drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids.

Test for tannins

About 1 g of the extract was dissolved in 20 mL of distilled water and filtered. Two to three drops of 10% $FeCl_3$ were added to 2 mL of the filtrate. The production of a blackish-blue or blackish-green colouration was indicative of tannins. To another 2 mL of the filtrate was added 1 mL of bromine water. A precipitate was taken as positive for tannins.

Test for flavonoids

A 0.2 g of the extract was dissolved in 2 mL of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration was an indication of the flavonoids.

Test for saponins

Two grams of the extract was boiled in 20 mL of distilled water in a water bath and filtered (Acrodisc syringe filter pall, USA). Aprroximately 10 mL of the filtrate was mixed with 5 mL of distilled water and shaken vigorously for a stable persistent froth. The frosting was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of an emulsion.

Test for steroids

About 0.5 g of the extract was dissolved in 3 mL of $CHCl_3$ and filtered. Concentrated H_2SO_4 was added to the filtrate to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

Statistical analysis

Analysis was performed using the SPSS Version 17.0 (Illinois USA, 2009). The one way ANOVA test was used to determine if there was any statistically significant difference in the zone diameters of inhibition of the different solvent extracts; the MIC_{50} of the extracts and the control antibiotic (ciproxacillin). P-values < 0.05 were considered significant.

RESULTS

Extract yield

Different solvents including ethyl acetate, acetone, ethanol, methanol and water were used for extraction because the type of solvent used may have an effect on the nature of the compounds extracted, the quantity extracted and the resulting bioactivity of the extract. Water extracted the highest quantity followed by methanol, and ethyl acetate the least (Figure 1).

Antimicrobial susceptibility testing

The extracts showed *in vitro* activity against the test bacterial pathogens with the exception of water. The mean zone diameter of inhibition ranged from 0 to 22 mm (Table 1). Acetone, methanol and ethyl acetate were the most active extracts for *S. aureus*, *A. hydrophila* with mean zone diameter of inhibition ranging from 13 to 22 mm, while for *H. pylori,* methanol and ethyl acetate extracts showed activity with mean zone diameter ranging from 14 to 21 mm. The most active crude extracts (methanol and ethyl acetate) against all test microorganisms were statistically significant ($P < 0.05$) compared to all other extracts, but not to ciprofloxacilin ($P > 0.05$) the positive control, with mean zone diameter ranging from 14 to 17 mm. DMSO used as negative control, showed no activity. An inhibition zone of ≥ 12 mm was chosen as representative of bacterial susceptibility to the extracts. The breakpoint of ciprofloxacin (0.05 mg/mL) was taken at 21 mm (CLSI, 2008).

MIC and MBC determination

The active extracts were further assayed to determine their MIC_{50} and MBC against the bacterial pathogens. Although only methanol and ethyl acetate extracts showed activity against *H. pylori*; methanol, acetone, ethanol and ethyl acetate extracts were active against *S. aureus* and *A. hydrophila*. Subsequently methanol and ethyl acetate extracts were used for the determination of MIC_{50} and MBC for *H. pylori* (ATCC 43526 and PE 252C) and methanol, acetone, ethanol and ethyl acetate extracts were used to determine MIC_{50} and MBC for *S. aureus* and *A. hydrophila*. The MIC_{50} and MBC ranged from 0.078 to 2.5 mg/mL and 0.78 to 25 mg/mL, respectively for all studied microorganisms (Tables 2 and 3), while for ciprofloxacin it ranged from 0.0098 to 0.078 mg/mL and 0.78 to 0.098 mg/mL, respectively.

The Gram positive bacterium (*S. aureus*) was most susceptible to *H. africana* compared to the Gram negative bacteria (*A. hydrophila and H. pylori*). However, there was no statistically significant difference ($P > 0.05$) between the MIC_{50} and MBC of the different solvents against Gram negative and Gram positive organisms.

Phytochemical analysis

Phytochemical analysis of the four extracts is

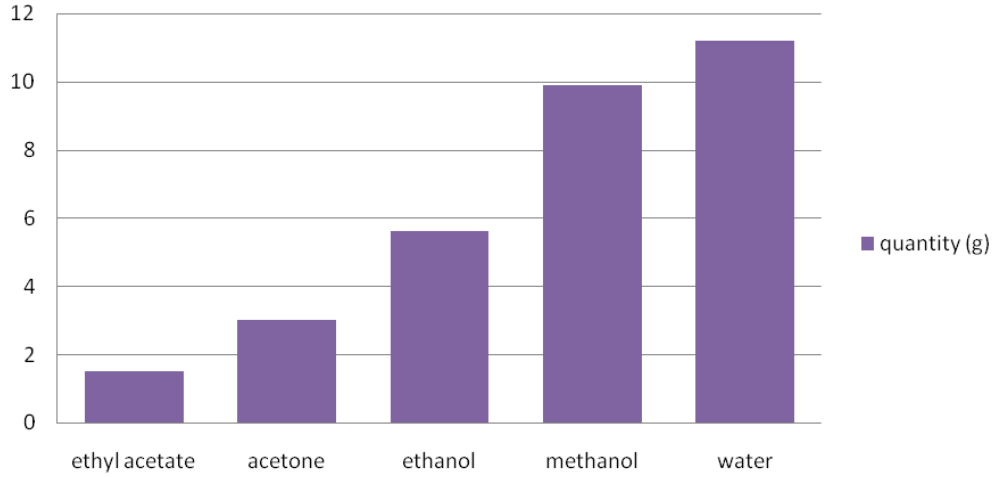

Figure 1. Quantity (grams) of *H. africana* flower extracted with different solvents. X-axis shows the different solvents used for extraction and Y-axis shows quantity extracted in grams.

Table 1. Antibacterial activity of extracts of *H.africana* against selected bacterial pathogens.

	Zone diameter at different concentration (mm)*																
	Methanol			water			Acetone mg/mL			Ethyl acetate			Ethanol			Cipro	
SBP	200	100	50	200	100	50	200	100	50	200	100	50	200	100	50	0.025	0.0125
S.a	17±2.1	21±2.1	22±2.1	0	0	0	20±0.7	19±1.4	22±3.5	16±0.7	18±0.7	19±0.7	14±1.4	16±1.4	17±1.4	17±0.7	17±0.7
A.h	17±0.7	16±0.7	15±1.4	0	0	0	17±2.1	17±1.4	18±1.4	14±1.4	13±1.4	15±0	13±0.7	16±1.4	14±0.7	17±1.4	17±1.4
H.p1	20±0.7	16±1.4	15±1.4	0	0	0	0	0	0	15±2.8	17±1.4	14±1.4	0	0	0	14±1.4	14±1.4
H.p2	17±2.1	21±2.1	18±0.7	0	0	0	0	0	0	16±0.7	19±1.4	17±0.7	0	0	0	15±0.7	15±0.7

SBP, selected bacterial pathogens; *S.a*, *S. aureus*; *A.h*, A. *hydrophila*; *H.P*1, *H. pylori* 43526; *H.p*2, *H. pylori* PE 252C; Cipro, ciprofloxacin; *, experiment was repeated twice and zone of inhibition recorded as mean zone diameter ±SD. Sensitivity zone ≥ 12 mm.

Table 2. MIC_{50} of different solvents extract of *H. africana* and the antibiotic against selected bacterial pathogens.

	Extracts and Antibiotic (mg/mL)				
SBP	**Methanol**	**Acetone**	**Ethanol**	**Ethyl acetate**	**Ciproxacillin**
S. aureus	0.625	0.156	0.156	0.078	0.0098
A. hydrophila	0.3125	0.156	–	0.078	0.0098
H. pylori43526	2.5	ND	ND	1.25	0.078
*H. pylori*252C	–	ND	ND	2.5	0.078

SBP, selected bacterial pathogens; –, MIC_{50} values not within susceptible range; ND, not determined.

Table 3. MBC (mg/mL) of different solvent extracts of *H. africana* and antibiotic against selected bacterial pathogens.

SBP	Extracts/ Antibiotic (mg/mL)				
	Methanol	Acetone	Ethanol	Ethyl acetate	Ciproxacillin
S. aureus	6.25	1.56	1.56	0.78	0.098
A. hydrophila	3.125	1.56	_	3.125	0.098
H.pylori43526	25	_	_	12.5	0.78
*H.pylori*252C	ND	_	_	ND	0.78

SBP, selected bacterial pathogens; –, MBC values not within susceptible range; ND, not determined.

Table 4. Phytochemical constituents of different solvent extracts of *H.africana.*

Phytochemicals	Solvent extracts			
	Methanol	Acetone	Ethanol	Ethyl acetate
Alkaloids	+++	++	+++	++
Saponins	+++	++	++	+++
Tannins	+++	+++	+++	+++
Flavonoids	+++	+++	+++	+++
Steroids	+++	+++	+++	+++

+++, Present in large quantity; ++, Present in moderate quantity.

in Table 4. The results revealed the presence of the following secondary metabolites: alkaloids, saponins, tannins, steroids and flavonoids, based on colour, heamolysis, turbidity, layers, emulsification and precipitation following the reactions.

DISCUSSION

The phytoconstituents of various plants have longed been known and their antimicrobial properties have been widely reported (Nostro et al., 2000; Roy et al., 2006). The antimicrobial activities of plant extracts have been linked to the presence of some bioactive compounds. These secondary metabolites also serve to protect the plants against bacterial, fungal and viral infections (De and Ifeoma, 2002; El-Mahmood and Amey, 2007). These bioactive compounds are known to work synergistically to produce various effects on human and animal subjects (Amagase, 2006). However, most reports on *H. africana* have focused mainly on the morphology of the plant, while information on its activity against hospital based pathogens is scanty (Bolin et al., 2009).

The extraction of active compounds from plant material and their activitiy depends on the type of solvent used in the extraction process (Parekh et al., 2005; Majhenic et al., 2007). In this study, it was observed that plant extractions with organic solvents provided stronger antibacterial activity than extraction with water. This study confirms the results of previous studies, which reported that water is not a suitable solvent for extraction of antibacterial compounds from medicinal plants compared to organic solvents, such as methanol (Karaman et al, 2003; Moniharapon and Hashinaga, 2004; Parekh et al, 2005; Majhenic et al, 2007).

S. aureus and A. hydrophila were the most susceptible organisms to all solvent extracts except water, with mean zone diameter of inhibition ranging from 13 to 22 mm. Moreover *H. pylori* (43526 and PE 252C) were only susceptible to methanol and ethyl acetate with mean zone diameter that ranged from 14 to 21 mm. This variation in activity may be due to the solvent used for extraction, e.g., methanol and ethanol are used for alkaloid extraction; acetone for flavonoids and steroids and ethanol may also be used for sterols, polyphenols, and tannins (Büssing, 1996). However, lack of activity demonstrated by water extracts *in vitro* against the organisms, does not necessarily imply that they would demonstrate weak activities *in vivo*.

In line with the findings of this study, another study had demonstrated very potent antibacterial activity of *Hydnora abyssinica*. In their study, Saadabi and Ayoub (2009) screened crude extracts of the family *Hydnoraceae* and reported potent antibacterial activity against common pathogenic Gram-negative and positive bacteria including *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*.

In the present study, MIC_{50} and MBC recorded for *H. africana* against all studied microorganisms ranged from 0.078 to 2.5 mg/mL and 0.78 to 25 mg/mL, respectively. However, the methanol extract showed no inhibition at MIC_{50} against *H. pylori* PE 252C. The MIC results confirm earlier findings by Nariman et al. (2004) who documented MIC ranges of 0.0037 to 2 mg/mL. Also, MIC values of

0.0625 to 0.5 mg/mL have been documented for East African medicinal plants against similar bacterial pathogens (Fabry et al., 1996). A Gram positive bacterium (*S. aureus*) was most susceptible to *H. africana* compared to the Gram negative bacteria (*A. hydrophila and H. pylori*). Most plants extracts are active against Gram positive bacteria; this has been attributed to the fact that the cell wall of Gram positive bacteria is easier to penetrate than the Gram negative bacteria which contains an outer membrane with a lipopoly-sacharide layer which is impermeable to certain antibiotics and antibacterial compounds (Fennell et al., 2004).

Phytochemical analysis of the extracts of *H. africana* revealed the presence of alkaloids, saponins, tannins, flavonoids and steroids (Table 4). These phytochemical compounds are known to be biologically active and thus aid the antimicrobial activities of plants. Alkaloids were one of the phytochemical compounds identified in this study. Most common biological properties of alkaloids are toxic against cells of foreign organisms, anti-inflammatory, anti-asthmatic, and anti- anaphylactic properties (Ganguly and Sainis, 2001; Staerk et al., 2002) and may be responsible for the observed activity.

The presence of flavonoids in crude extract of *H. africana* is important since they have been reported to exhibit antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic, antioxidant, antitrypanosomal and antileishmanial properties (Ferguson, 2001; Hodek et al., 2002). Flavonoids in human diet may reduce the risk of various cancers, coronary heart diseases as well as preventing menopausal symptoms (Xu et al., 2000; Hodek et al., 2002; Tasdemir et al., 2006). Saponins and tannins were also reported in this study. Saponins are responsible for numerous pharmacological properties and are known to produce inhibitory effects on inflammation (Estrada et al., 2000). Tannins exert antimicrobial activities by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells (Njume et al., 2009). Herbs that have tannins are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003). Motar et al. (1985) revealed the importance of tannins for the treatment of inflamed or ulcerated tissues. Tannins were observed to have remarkable activity in cancer prevention (Li et al., 2003). Steroidal compounds were also present in the crude extracts of *H. africana;* they have drawn much interest in pharmacy due to their relationship with such compounds as sex hormones (Okwu, 2001). The findings of our study demonstrated the *in vitro* activities of the crude extracts of *H. africana* and provide preliminary evidence for the use of this plant in traditional medicine. This plant may provide new lead molecules, which could become starting materials for the synthesis of new drugs. Further study using bioassay-guided fractionation is necessary to isolate and characterize active compounds of the plant.

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Full Length Research Paper

Characterization of pathogenic or non-pathogenic *Enterococcus faecalis* isolated from lambs from Xinjiang, a remove North-west province of China

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The majority of the 11 pathogenic *Enterococcus faecalis* from lambs developed encephalitis and 45 non-pathogenic *E. faecalis* from intestinal and respiratory microbiota of healthy lambs were belonged to Streptococcus serotype D. Haemolytic study revealed that 8 of 11 pathogenic stains had stable haemolyticus; 8/30 strains of intestinal normal microbita and 3 of 15 strains from respiratory system showed unstable haemolyticus. Of 11 pathogenics *E. faecalis*, 8 of 9 virulence factor genes were detected in all the strains; 5 of 11 expressed Esp, CylA, Asa1, Ace, efa, EF0591 and EF3314 simultaneously and 1 of 11 expressed GelE; Two of 11 did not express any of the 9 virulence factor genes. Among 30 strains isolated from the intestinal microbita, only one had 2 (GelE, EF3314) and one had 3 (GelE, EF3314 and Asa1) of the 9 virulence factor genes. The homology of these 3 common virulence gene fragments (GelE, EF3314 and Asa1) was more than 95% between *E. faecalis* from GenBank and intestinal microbita and 96% when comparing the *E. faecalis* isolated from intestinal microbita and from those of pathogenic strains. Antibiotic sensitivity study indicated that all of the 11 pathogenic strains were resistance to a variety of antibiotics in various degrees. In comparison, Only 2 strains from normal flora were resistance to individual antibiotics. *In vivo* challenge study showed that all of the 11 the pathogenic strains could lead to the death of mice, whereas none of the isolates from normal flora could cause the death of the experimental animals.

Key words: Biochemical characteristic, *Enterococcus faecalis*, lamb,virulence factor gene.

INTRODUCTION

Enterococcus faecalis are an important part of normal flora in humans and animals. It is the second regular bacteria, following to *E. coli* as ecological agents (Yuan and Fu, 2003; Drahovska et al., 2004). Recent studies have confirmed that the pathogenicity of *E. faecalis* is an important nosocomial infectious pathogen after *Staphylococcus* in the aerobic Gram-positive *cocci* (Schaberg et al., 1991). *E. faecalis* today is ranked second to third in frequency among bacteria isolated from hospitalized patients (Kayse, 2003). Treatment for *E. faecalis* is difficult due to the possession of a large number of virulence factors and drug resistant. So the study on *E. faecalis* has become one hot point in medical research (Giridhara Upadhyaya et al., 2009; Katie Fisher, 2009). There are also reports of infection in livestock and poultry in the veterinary practice, but the relationship between pathogenic and normal flora *E. faecalis* and its pathogenic mechanisms are not yet very clear.

We have 11 isolated *E. faecalis* from Lamb encephalitis recently, but the relationship between pathogenic strain and normal flora isolates from healthy lamb is unclear.

So our purpose is to make a comparative study on different sources of *E. faecalis* from lamb, such as culture characteristics, biochemical features, hemolytic characterristics, drug susceptibility, animal corresponding pathogenicity and the types of virulence factor genes and

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the fragments nucleotide sequence, which can provide information for pathogenic mechanism of animal *E. faecalis* infection.

MATERIALS AND METHODS

Reagents

TaqDNA polymerase and DNA gradient ladders were obtained from Shanghai Sangon Bio-Engineering Company. *Streptococcus* Grouping Kit was obtained from the France BioMerieux Inc. Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Engeering Material Co., Ltd. China. Antibiotics were purchased from Hangzhou Tianhe Microorganism Regent Co., Ltd China.

Isolation of *E. faecalis*

Eleven pathogenic *E. faecalis* were isolated from lamb brains which had developed encephalitis and were identified. Thirty and fifteen *E. faecalis* of normal flora were isolated from intestinal microbiota and respiratory microbiota of healthy lambs, respectively.

Growth characteristics of isolates

The purified *E. faecalis* were inoculated on ordinary nutrient agar plate, *Streptococcus* selective blood agar medium, LB blood agar medium, and incubated at 37°C under 10% CO_2 for 24 h.

Culture characteristics of the isolates

A single bacteria colony was picked up from blood agar plate after 24 h and was inoculated in LB broth with 5% fetal bovine serum with or without 10% CO_2 in order to test the requirement for oxygen. To test the tolerance to temperature, the isolates were inoculated in LB broth with 5% fetal bovine serum at either 40°C or 10°C for 24 ~ 48 h. To test the tolerance to high-salt or high-alkali, the isolates were inoculated in LB broth containing 5% fetal bovine serum with either 6.5% NaCl or pH 9.6 at 37°C for 24 ~ 48 h.

Biochemical characterization of the isolates

Isolates from blood agar after 24 h were identified by VITEK-AMS32 Bacterial Biochemical Identification System. Hemolysis to sheep red blood cells were determined by plate assay (PA) (Li and Li, 1999). Serotype of the isolates was identified by use of *Streptococcus* Grouping Kit.

Antimicrobial susceptibility test (disk diffusion method)

15 to 20 mm zone of inhibition is high-sensitive, 10 to 14 mm is the medium-sensitive, 10 mm below is low- sensitive, 0 mm is non-sensitive according the standards adopted from United States Committee for Standardization of Clinical Trials. Antibacterial drugs include gentamicin, tetracycline, norfloxacin, streptomycin 2000, penicillin vancomycin, nitrofurantoin, chloramphenicol, rifampicin and erythromycin.

Mice and infection

Group of 3 Kunming out-bred mice, 6 to 8 weeks old, female, were obtained from Xinjiang Medical University, China. For infection study, all of the 11 pathogenic strains, 10 randomly picked strains of intestinal isolates and 10 stains of respiratory isolates were injected and was infected with intra-peritoneally (i.p.) with 3×10^8 bacteria per mouse in Martin broth. Control group was injected with LB broth alone.

Detection of 9 types of virulence factor genes and sequence analysis

Virulence factor genes include haemolysin activator (Cyl A), gelatinase (GelE), enterococcal surface proteins (Esp), endocarditis antigen (EfaA), collagen-binding protein(Ace), aggregation substances(Asa373 and Asa1), another 2 protein EF0591 and EF3314. The PCR primers synthesized according to reference (Roberta et al, 2004) were showed in Table 1. and normal flora strains reference Molecular Cloning (Sambrook and Russell, 2002) with a slight modification. The homologous of DNA fragments were analyzed with ClustalX sequence analysis software.

RESULTS

Growth and cultivation characteristics of isolates

Most of the *E. faecalis* isolated from the three different sources were long-chain and Gram-positive coccus in 2 kind of liquid cultures (Figure 1). In aging cultures they sometimes became Gram-negative. Colonies were round, smooth, moist, medium-sized with neat edge in blood agar LB plate after anaerobic culture. They also produced the colonies which are colorless, transparent, round, surface wet, slightly smooth, neat edge, like tip in *Streptococcus* select agar medium. Those isolates almost can not grow or grow poorly in the ordinary medium and make *Streptococcus* enrichment medium become slightly turbid and a small amount of flocculent precipitate at the bottom of tube. All strains can grow at 45°C and 10°C and also grow in 6.5% NaCl or pH 9.6 LB broth with serum. These features are consistent with characteristics of *E. faecalis.*

Biochemical characteristics of isolates

Biochemical reactions of 45 strains from normal flora were the same as that of 11 pathogenic strains when assayed with VITEK-AMS 32 systems. Butyl-diphenyl-pyrazole dione (PYR) test is positive; Cyclic Adenosine monophosphate test, Optochin test and bacitracin inhibition test are negative; These isolates can ferment glucose and produce L-lactic acid mainly; Catalase test is negative, but some strains can produce false catalase; Benzidine test is negative. Eight of 11 pathogens produce β-hemolysis in sheep blood agar, which is relatively stable after continuous passage. Eight of 30 strains from intestinal microbiota and 3 of 15 strains from respiratory microbiota also produce hemolysis, but these characteristics disappear after continuous passage. The results of serotypes showed that 8 of 11 were type D, 1 of

Table 1. Information of PCR primers for detection of different virulence factor genes.

Name of virulence vector genes	Sequence of primer	Length of Segment (bp)	GenBank accession No.	Location
Esp	TTG CTA ATG CTA GTC CAC GAC C GCC TCA ACA CTT GCA TTG CCG A	932	AF034779	1217-1249
GelE	ACC CCG TAT CAT TGG TTT CAG CAT TGC TTT TCC ATC	405	M37185	762-1163
CylA	GAC TCG GGG ATT GAT AGG C GCT GCT AAA GCT GCC CTT AC	688	AD1CLYL	6656-7344
Asa1	CCA GCC AAC TAT GGC GGA ATC CCT GTC GCA AGA TCG ACT GTA	529	SFPASA1	3122-3651
Asa373	GGA CGC ACG TAC ACA AAG CTA C CTG GGT GTG ATT CCG CTG TTA	619	AJ132039	3094-3713
Ace	GGA ATG ACC GAG AAC GAT GGC GCT TGA TGT TGG CCT GCT TCC G	616	AF159247	160-776
EfaA	GCC AAT TGG GAC AGA CCC TC CGC CTT CTG TTC CTT CTT TGG C	688	EFU03756	312-1000
EF0591	AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C	844	NC_004668	99-1003
EF3314	AGA GGG ACG ATC AGA TGA AAA A ATT CCA ATT GAC GAT TCA CTT C	566	NC_004668	35-601

Note: Primer references (Roberta et al., 2004).

Figure 1. Gram staining of the pathogenic *E. faecalis s* cultivated in broth（100×）The *E. faecalis* were isolated from brains of infected lamb. They were cultured in broth for 18 h. The bacteria were stained long-chain and Gram-positive coccus.

11 is type G, 2 of 11 is not identified in pathogens; Twenty-four of 30 are type D, 6 of 30 are not identified from intestinal microbiota; Fifteen of 15 are type D from respiratory microbita.

Antibiotic susceptibility test

Eleven pathogenic isolates were highly sensitive to nitrofurantoin and moderately sensitive to chloramphenicol,

Table 2. Antibiotics susceptibility test of the *E. faecalis* isolated from the three sources.

Antibiotic category		Nitrofurantoin	Chloramphenicol	Rifampicin	Vancomycin	Norfloxacin	Penicillin	Tetracycline	Streptomycin	Gentamicin	Erythromycin
					Pathogenic strains						
	1-11	S	S	S	S	R	R	R	R	R	R
					Intestinal strains						
E. faecalis strains from lambs	1-29	S	S	S	S	S	S	S	S	S	S
	30	S	S	S	S	S	S	R	R	S	R
					Respiratory strains						
	1-14	S	S	S	S	S	S	S	S	S	S
	15	S	S	S	S	S	R	S	S	S	S

Table 3. *In vivo* study of the pathogenicity of the stains isolated from different sources. Each of the *E. faecalis* strains isolated from 3 sources was infected into three mice (see Material and Methods). By 24 h post-infection, the numbers of the dead mice were recorded. No more death of the mice was observed after 24 h.

	Numbers of mice died / numbers of mice infected										
Infected with pathogenic strains	2/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	1/3	2/3	1/3
Infected with intestinal strains	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	
Infected with respiratory strains	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	

rifampicin and vancomycin. These isolates were resistant to norfloxacin, penicillin, tetracycline, streptomycin, gentamicin and erythromycin to various degrees. The rest of strains from normal flora were sensitive to the other antibiotic except that 1 of 30 strains from intestinal microbiota was resistant to tetracycline, erythromycin and streptomycin, 1 of 15 strains from respiratory microbita is resistant to penicillin (Table 2).

Experimental infection of mice

All mice infected with pathogenic strains appeared apathetic and back hair handstand 16 h after infection. Of 33 mice infected with the 11 pathogenic strains (3 mice per strain), 25 of 33 the mice died within 24 h post-infection. The mice infected with normal flora strains appeared temporary apathetic and loss of appetite, but they gradually recovered after 12 h. The control group looked normal during the experiment (Table 3 and Figure 2).

Results of virulence factor genes

Nine virulence factor genes were listed in Table 1. Our gene analysis showed that 8 of 9 virulence factors were tested in the 11 pathogenic *E. faecalis*. Among the 11 pathogenic *E. faecalis*, five took Esp, CylA, Asa1, Ace, efa, EF0591 and EF3314 virulence factors genes, one strain had GelE only. Two strains did not take any of the 9 virulence factor genes (Figure 3 and Table 4). Of the 30 *E. faecalis* isolated from intestinal microbiota, three out of the nine virulence factor genes were detected in all of them. One of the normal strains had virulence factor genes GelE and EF3314 and one took virulence factor genes GelE, EF3314 and Asa1. Of the 15 isolated from respiratory microbita, none of the virulence factor genes were detected among them (Figure 4 and Table 4). The Homology of amplified fragments of three common virulence factor genes is 99.53, 96.2 and 99.12%, respectively between normal flora and pathogenic *E. faecalis*. Similarly, the homology from normal flora *E. faecalis* is 98.03, 95 and 99.3% compared to the information of GenBank from medical the cultural and biochemical characteristics of *E. faecalis* from normal flora were basically the same as that of pathogens except that the individual biochemical characteristics of individual strains were instable. These results were basically consistent with biochemical characteristics of *E. faecalis*. Haemolyticus is an important virulence indicator of certain bacteria. Eight of 11 pathogenic stains showed signifi-

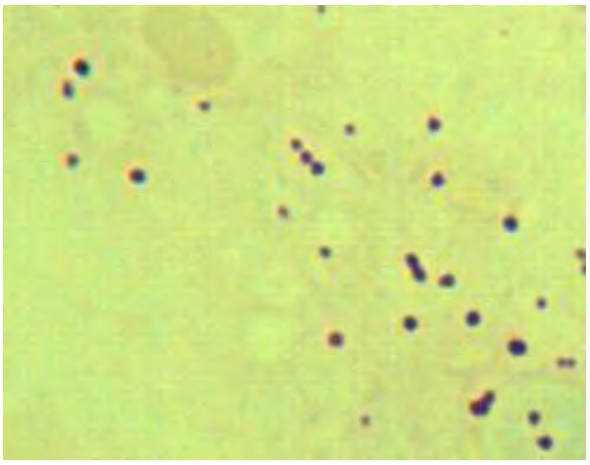

Figure 2. Wright's staining of the *E. faecalis* in encephalon of dead mice infected with pathogenic strains（100×). The brain smear of the infected mice was subject to Wright's staining. The *E. faecalis* were scattered or a very short strain including 2 to3 bacteria in brain smear of mice infected with pathogenic strains.

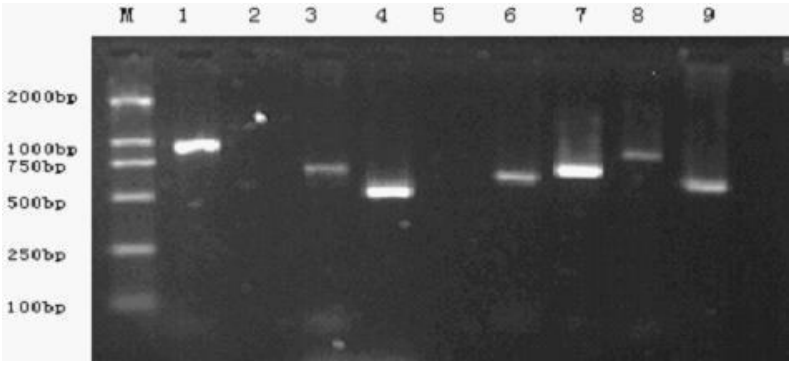

Figure 3. Virulence factor genes detected in stains No. 4 of pathogenic strains by PCR. M: Marker DL2000；1: Esp; 2: GelE; 3: CylA; 4: Asa1; 5: Asa373; 6: Ace; 7: Efa; 8: EF0591; 9: EF3314. Nine virulence facter genes from the No. 4 pathogenic strains were amplified with PCR and the products were analysed by gel electrophoresis in 0.8% (w/v) agarose gel.

Table 4. Detection of virulence factor genes by PCR. Nine virulence factor genes were tested in *E. faecalis* from 3 sources of lambs. Pathogenic strains have more combination of virulence factors gene than that of normal flora.

Virulence factors gene	Pathogenic strains											Intestinal strains			Respiratory strains
	1	2	3	4	5	6	7	8	9	10	11	1	2	3-30	1-15
Esp	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-
GelE	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-
CylA	-	+	+	+	+	+	+	-	-	+	-	-	-	-	-
Asa1	+	+	+	+	+	+	+	-	-	+	-	-	+	-	-
Asa373	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ace	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-
efa	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
EF0591	-	+	+	+	+	+	+	-	-	+	-	-	-	-	-
EF3314	+	+	+	+	+	+	+	-	-	+	-	+	+	-	-

Note: + mean there is the virulence factor gene; - mean there is no the virulence factor gene.

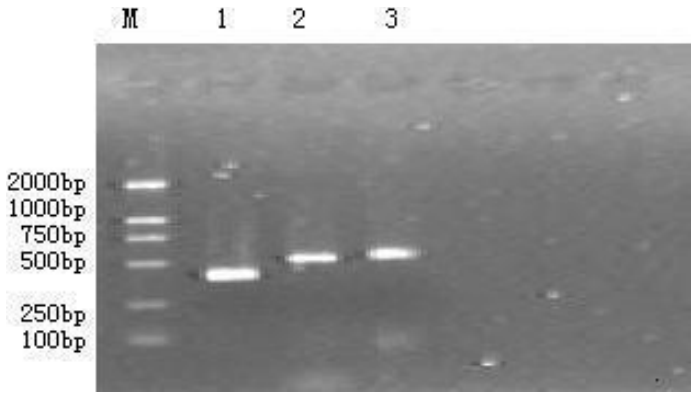

Figure 4. Virulence factor genes GelE, Asa1, and EF3314 were detected in stain No. 2 of the non-pathogenic stains isolated from the intestine by PCR. M: DL2000; 1: GelE; 2: Asa1; 3: EF3314. Nine virulence facter genes from the No. 2 non-pathogenic stains were amplified with PCR and the products were analysed by gel electrophoresis in 0.8% (w/v)agarose gel.

Table 5. The homology (%) of the 3 common virulence factor gene fragments from normal flora are compared among pathogenic *E. faecalis* and Genbank.PCR products of 3 common virulence factor gene fragments (GelE, Asa1 and EF3314)cloned into vector, and sent them to Sangon Bio-Engineering Company for sequencing, then the homologous of DNA fragments were analyzed with ClustalX sequence analysis software.

	***E. faecalis* from normal Flora**		
	GelE	**Asa1**	**EF3314**
E. faecalis from GenBank	98.03	95.00	99.30
Pathogenic *E. faecalis*	99.53	96.20	99.12

cant hemolysis, and this feature did not disappear upon passages. While only 8 of 30 strains of normal flora showed hemolytic clinical isolates (GelE（M37185, Asa1（X17214）, and EF3314（NC004668) (Table 5).

DISCUSSION

The results of biochemical characteristics showed that properties and it's hemolytic disappeared after the limited passages. Experimental infection of mice also verified this. All these evidences indicated that hemolytic of *E. faecalis* still was an indispensable factor in lambs encephalitis caused by *E. faecalis*.

The mechanism of drug resistant in *E. faecalis* is more complex, which include natural resistance, acquired drug resistance and multi-drug resistance (Zhang et al., 2001; Li and Zhang, 2004). Susceptibility test results indicated that the pathogenic strains were resistant to most antibiotics in various degrees,and individual strains from normal flora is also resistance to certain antibiotics, the reason for these phenomena in *E. faecalis* may be due to the complete manifestation of natural resistance and acquired drug resistance under the pressure of a large number of antibiotics used in clinic. Pathogenic strains were resistant to streptomycin, gentamicin and erythromycin. Interestingly, there were also individual strains of normal flora *E. faecalis* which were resistant to tetracycline, erythromycin and streptomycin, suggesting that drug resistance of *E. faecalis* was very universal. Researchers had confirmed that some drug resistant plasmid could be transferred each other among different sources *E. faecalis* or between *E. faecalis* and other bacteria (Launay et al., 2006; Simjee et al., 2006; Jacobsen et al., 2007). Multi-drug resistance genes could also be horizontally transferred between humans and poultry (Lim et al., 2006). Antibiotics are used widespread in animal husbandry and agriculture, which may increase the transmitted opportunity of *E. faecalis* drug resistance, including the *E. faecalis* in normal flora of humans and animals, these situation may give some *E. faecalis* from normal flora more opportunity to become pathogenic strains. Therefore, one should be very cautious when come to use any antibiotics in human medicine and veterinary practice.

Pathogenicity island usually was found in *E. faecalis*, there were several virulence factor genes in those pathogenicity island, different virulence factor genes had different roles in the course of infectious disease (Heikens et al., 2007, 2008; Dupont et al., 2008). Some

researchers found that *E. faecalis* from different sources had different combination of virulence factor genes in medical clinic (Mannua et al., 2003; Roberta et al., 2004). The pathogenicity of *E. faecalis* from clinical isolates was more virulent than from that animal manure and dairy products. Our study indicated that virulence factor genes could be detected both in *E. faecalis* causing lambs encephalitis and in normal flora *E. faecalis*. But pathogenic isolates had more combination of virulence factor genes than the isolates of normal flora. This was basically consistent with the results which Roberta reported (Roberta et al., 2004). Our study showed that EF3314 and GelE appear in normal flora *E. faecalis* simultaneously, Maria also reported that GelE can be easily detected in *E. faecalis* from dairy (Lopes et al., 2006). All these suggested that GelE has a high frequency in the non-pathogenic *E. faecalis*. Sequencing results showed that sequence homology of these 3 virulence factor gene fragments in normal flora *E. faecalis* (GelE, Asa1 and EF3314) was over 95 and 96% compared with corresponding sequence from GenBank and from *E. faecalis* causing lamb encephalitis. These results showed that the 3 virulence factor gene fragments had high homology among 3 *E. faecalis* from different sources of lamb. It suggests to us once again that there is a close relationship among *E. faecalis* from veterinary clinic, medical clinic and animal normal flora. We still do not know clearly the detailed mechanisms how *E. faecalis* from normal flora becomes pathogenic strains.

It is well known that *E. faecalis* is commonly used as microbial agent that has played a positive role on prevention of intestinal infectious diseases and regulation of microbiota homeostasis balance in recent years (Garcia et al., 2004). But we should also realize that *E. faecalis* as an ecological agent may artificially increase the opportunity to delivery the drug resistance and transfer virulence factor gene in the process of using these ecological agent considering its particularity.

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Full Length Research Paper

Seroprevalence of *Mycoplasma gallisepticum and Mycoplasma synoviae* infection in the commercial layer flocks of the Centernorth of Iran

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This survey was carried out to determine the seroprevalence of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) infections in commercial layer farms in Centernorth of Iran. A total of 2000 serum samples were collected from 40 commercial layer flocks (50 samples/ flock) mostly > 40-week-old. Sera tested by serum plate agglutination (SPA) method using commercial MG antigen and MS antigen. Positive reactions retested by SPA on 1:8 dilution and the flocks with more than 10% positive reactions considered positive serologically. The results showed that 4 of 40 (10%) flocks were MG positive and the rest (90%) were negative. About MS, 17 of 40 (42.5%) flocks were positive and 33 of 40 (57.5%) were negative. Sera tested for MG were: 125 of 2000 (6.25%) positive, 1821 of 2000 (91.05%) negative and 54 of 2000 (2.7%) suspect. Sera for MS were: 371 of 2000 (18.55%) positive, 1617 of 2000 (80.85%) negative and 12 of 2000 (0.6%) suspect. Statistical analysis showed a positive correlation between MS positive flocks and strain (LSL flocks compared to Hy-Line flocks) and capacity of the farms significantly (P<0.05). It seems that the seroprevalence of *MS* in the commercial layer flocks of the Centernorth of Iran is high.

Key words: ***Mycoplasma gallisepticum, Mycoplasma synoviae,*** seroprevalence, serum plate agglutination test.

INTRODUCTION

Avian Mycoplasmosis caused mainly by ***Mycoplasma gallisepticum*** (MG) and ***Mycoplasma synoviae (MS),*** can cause considerable economic losses in chicken such as chronic respiratory disease, reduce weight gain and meat quality and increase FCR and mortality in broiler, tremendous drop in egg production in layers and increase embryo mortality in breeders (Stipkovits and Kempf, 1996; Kleven and Noel, 2008; Ley, 2008). The disease may be transmitted horizontally and vertically and remains in the flock constantly as subclinical form (Bradbury and Jordan, 2003). It has been shown that an association between infection of the oviduct with MS and the eggshell apex abnormalities (EAA) characterized by a roughened shell surface, shell thinning, increased translucency, cracks and breaks (Feberwee et al., 2009). In the last years, there have been reported a high prevalence of avian mycoplasmosis and increasing of MS infection, possibly due to the growth of large flocks within small geographically areas, under inadequately biosafety conditions (Avakian and Kleven, 1990; Keleven and Noel, 2008). This aspect may lead to the need of reevaluating the control strategies, introducing surveillance programs and vaccination which is one of the effective approaches to control and prevention of mycoplasmosis. In order to define the necessity of vaccination, the rate of contamination to this pathogen should be determined. Among serological tests, the serum plate agglutination (SPA) test is very simple, rapid, and sensitive and can be used for the detection of MG & MS antibodies under field conditions (Kleven, 1998). The present study was carried out to determine the seroprevalence of MG and MS

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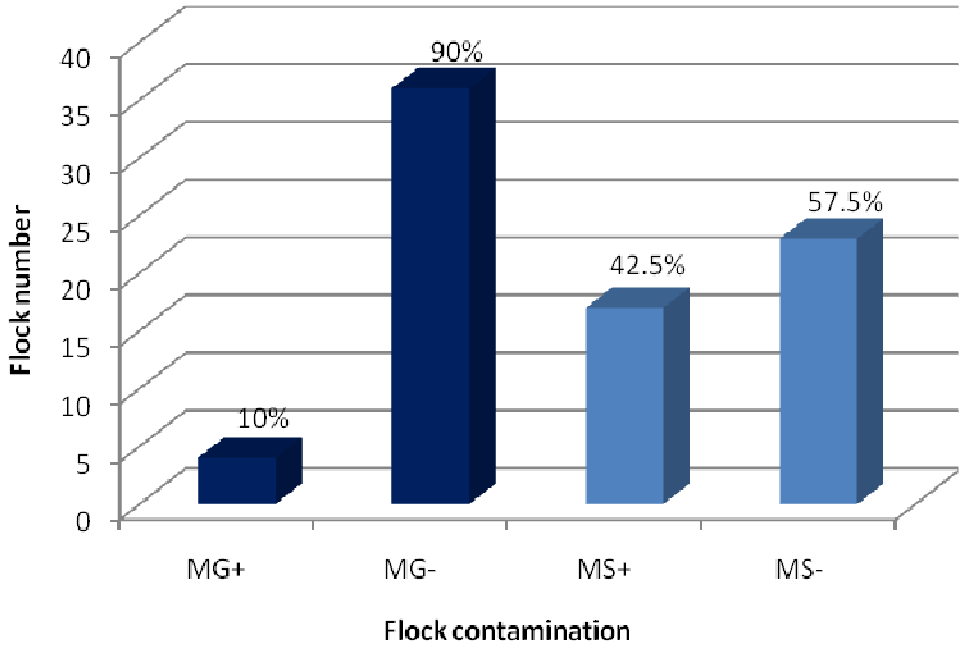

Figure 1. Seroprevalence of MG and MS on the commercial layer flocks in the Center north of Iran.

infection in the chickens of selected model commercial layer farms of the Centernorth of Iran and detect the correlation between positive farms and breeding variables.

MATERIALS AND METHODS

Sampling

A total of 2000 serum samples were collected from 40 commercial layer flocks, mostly above 40 weeks old of age (50 serum samples from each flocks), which were randomly selected in the different areas of two important provinces (in view of poultry production) in Centernorth of Iran during January to May 2010.

Data collection

Some information about qualitative variables (geographical area of flocks, commercial strain of layer type, breeding system, antibiotic consumed during previous three mounts, present of maternal antibodies against MG and MS at one-day-old of age, any vaccination against MG and MS administered in their parent flocks) and quantitative variables (farm capacity, egg production rate, house number, flock age) were gathered from each farms to be calculated for correlation.

Serum plate agglutination (SPA) test

The SPA test was done with crystal violet stained ***M. gallisepticum*** antigen (Nobilis® MG Antigen, Intervet International Co., Holland) and ***M. synoviae*** antigen (Soleil®-MS Antigen, Ceva International Co, France). For this test 25 µl of antigen and 25 µl of chicken sera were placed side by side with a sampler on a glass plate and mixed properly by stirring with small tooth pick followed by gentle rocking. Results were read within 2 min. In positive cases granules formed slowly which was seen during rocking, but in negative case no such granules formed within two minutes. Positive reactions retested by SPA on 1:8 dilution and those sera which were positive in the first SPA test but showed negative reaction in the second SPA test were considered suspect. The flocks with more than 10% positive reactions were considered positive serologically based on the suggestion of Kleven and Bradbury, (2008).

Data analysis

Statistical analysis was done by SPSS soft ware (Version 16) and T-Test for calculation of correlation between MG and MS positive flocks and breeding variables.

RESULTS

The results showed that 4 of 40 (10%) flocks were MG positive and the rest (90%) were negative. About MS, 17 of 40 (42.5%) flocks were positive and 33 of 40 (57.5%) were negative. Sera tested for MG were: 125 of 2000 (6.25%) positive, 1821 of 2000 (91.05%) negative and 54 of 2000 (2.7%) suspect. Sera for MS were: 371 of 2000 (18.55%) positive, 1617 of 2000 (80.85%) negative and 12 of 2000 (0.6%) suspect (Figures 1 and 2). Statistical analysis was done by SPSS soft ware (version 16) and T-Test to calculate the correlation between MG and MS positive flocks and breeding variables. Among qualitative variables, only the strain of layer flock was significant

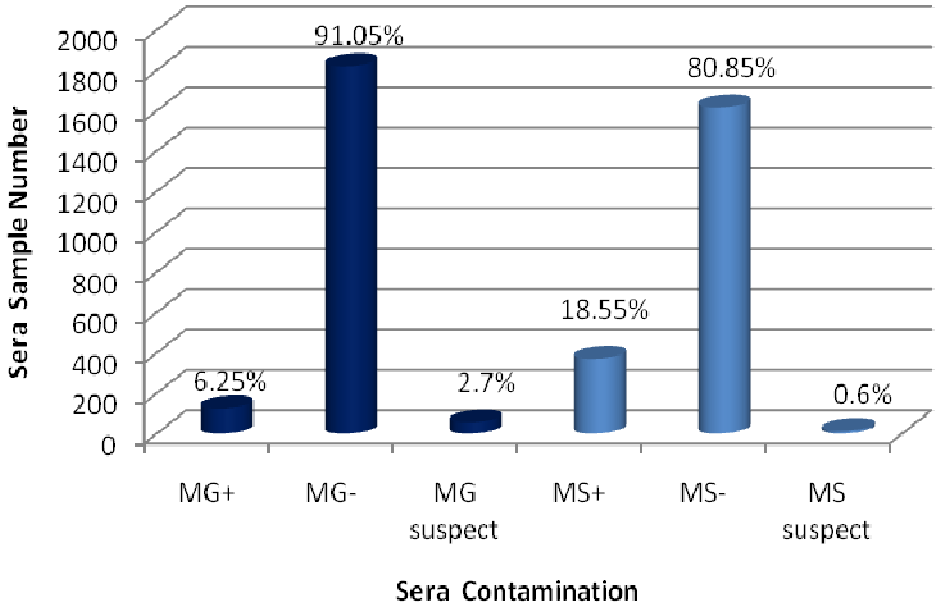

Figure 2. SPA reaction against MG &MS on sera samples from commercial layer flocks in the Centernorth of Iran.

correlation. Rate of MS contamination in the flocks with L.S.L strain, was more than flocks with Hy-Line strain significantly ($P<0.05$). Of quantitative variables, two variables of house number and farm capacity were significant. Rate of MS contamination in the farms which had more house and high capacity were higher than the other significantly ($p<0.05$). Due to low rate of MG positive flocks, correlation between positive flocks and breeding variables was not calculated.

DISCUSSION

The present study was done in order to determine the seroprevalence of ***M. gallisepticum*** and ***M. synoviae*** infections in the commercial layer flocks in the Centernorthern of Iran by serum plate agglutination (SPA) test as a screening test. According to the results, the seroprevalence of MG in the studied areas was low; only 4 of 40 (10%) flocks were positive, whereas seroprevalence of MS was high (42.5%) and there were a significant correlation between MS positive flocks and flock strain, farm capacity, house number of farm. Therefore, the commercial layer flocks which were L.S.L strain (compared to Hy-Line), had more capacity and more houses seemed to be more sensitive to MS infection. It seems that intensive nature of poultry farming provides opportunity for recycling of the pathogens due to population density. Because of low rate of MG positive farms, correlation between positive farms and breeding variables was not calculated. Seroprevalence of MG in the different areas of Bangladesh by SPA test reported 58.9% (Sarkar et al., 2005) in breeder poultry farms and 49.50 % in broiler and 66.50% in layer (Barua et al., 2006), with the highest and lowest prevalences in winter and summer seasons respectively. These findings were in close agreement with the previous results reported by Alam et al. (2003) in Bangladesh, Kelly et al. (1994) in Zimbabwe, Shah-Majid (1996) in Malaysia, Pandey and Hasegawa (1998) in Zambia, Mushi et al. (1999) in Botswana and Chakraborty et al. (2001) in India who reported the seroprevalence of mycoplasmosis are higher in winter and for this reason we collected the sera mostly in cold season.

In our study, seroprevalence of MS was more than MG. It seems that MS is widely spread (Kleven and Noel, 2008). Buim et al. (2009) showed that mycoplasmas was isolated from 72.7% of poultry farms in Brazil by multiplex PCR with predominance of MS. Suzuki et al. (2009) reported seroprevalence of MS was 53% by Elisa test with the high rate of MS infection. In the Germany, 84% of commercial layer flocks during laying period were positive for mycoplasmas in PCR test, while 75% turned out positive for MS and all were negative for MG (Köhn et al., 2009). Feberwee et al. (2008) showed that the seroprevalence of MS in commercial poultry by SPA was 73% in Dutch. Kapetanov et al. (2010) reported that the overall seroprevalence of the MG and MS of the flocks in Serbia was 9.01 and 47.49% in 2000 and was 11.59 and 22.17% in 2009 by SPA and Elisa tests respectively and concluded that seroprevalence of MS was decreased , versus the MG increased.

The age of flock that we chose, was over 40-week-old. In the epidemiological study of MG and MS in Romania

by ELISA test, Botus et al. (2008) showed that high seroprevailence (80%) of MG and MS was detected from poultry older than 36 weeks. We use SPA test based on the suggestion of Kleven and Bradbury, (2008) SPA test is quick, relatively inexpensive and sensitive and can be widely used as an initial screening test for flock monitoring and serodiagnosis. Comparison with the other serological tests, SPA is more sensitive than Elisa and HI, but less specific than them (Ley, 2008). However, the SPA test is prone to false positive results and non-specific reactions (Avakian et al., 1993; Czifra et al., 1993; Abdelmoumen and Roy, 1995; Osman et al., 2009). Certain nonspecific SPA reactions may be reduced by diluting the serum (Ross et al., 1990), so in the present study positive reactions retested by SPA on 1:8 dilution. Comparison of culture, PCR, and different serologic tests (SPA, HI and ELISA) for detection of MG and MS infections, Feberwee et al. (2005) showed that it is not advisable to rely completely on only one test (system) and Ewing et al. (1996) suggested that PCR should be considered as confirmatory test for SPA and ELISA for MG and MS infections.

Conclusion

It is concluded that the seroprevalence of MS infection in the commercial layer farms of the Centernorth of Iran, in contrast to MG infection, is high. However, the use of live MS and MG vaccines as a prevention strategy in commercial layer flocks needs more studies, and molecular identification should be used for the completion of these findings.

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Full Length Research Paper

Molecular characterization of *Citrus tristeza* virus strains in Peninsular Malaysia

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Sixty Malaysian *Citrus tristeza* virus (CTV) isolates were characterized by bi-directional polymerase chain reaction (BD-PCR) and restriction fragment length polymorphism (RFLP) analysis of their coat protein (CP) gene. In BD-PCR analysis, 392-bp fragments were amplified from seven isolates. The other 53 isolates produced only 320-bp fragments. RFLP patterns of RT-PCR products of CP gene digested with HinfI restriction enzyme were similar to I-IV, VI-VII and two new groups. Isolates AMK1, AMJ12, AMT38, AMT39 and AMT43 could not be classified when they were compared to any standard CTV digest pattern. These isolates produced a unique restriction pattern with two fragments of 210 and 300 bp and isolate AMI61 produced different restriction pattern with three fragments of about 100, 270 and 300 bp. Therefore these isolates were designated as Groups IX and X. These results suggest that CTV populations in Malaysia contain new genetic variants.

Key words: *Citrus tristeza* virus (CTV), strains, coat protein (CP) gene, restriction fragment length polymorphism (RFLP), bi-directional PCR, Malaysia.

INTRODUCTION

Citrus is a very ancient crop that occurs since over 4000 years ago (Mukhopadhyay, 2004). It is an important commercial fruit crop worldwide with a total production of 105.4 million tons, which is grown in tropical and subtropical regions of the world (Olivares-Fuster et al., 2003; Mohan Jains and Priyadarshan, 2009). The origin and diversity center of citrus and its related genera is considered to be Southeast Asia, possibly ranging from Northeastern India eastward through the Malay Archipelago (Mohan Jains and Priyadarshan, 2009).

Citrus tristeza virus (CTV) is a worldwide distributed Closterovirus (family Closteroviridae), which causes one of the most economically important diseases of citrus in the world (Bar-Joseph et al., 1989; Che et al., 2001; Gowda et al., 2009; Lair et al., 1994; Narvaez et al., 2000; Satyanarayana et al., 2001). CTV particles are flexuous and threadlike with a size of 2000×10-12 nm (Huang et al., 2004; Jiang et al., 2008; Ruiz-Ruiz et al., 2007) and are composed of a positive sense, single stranded genomic RNA about 20 kb in size (Che, et al., 2002; Hilf, et al., 1995; Huang, et al., 2004; Ruiz-Ruiz, et al., 2007) that contains 12 open reading frame (ORFs) (Che et al., 2002; Fagoaga et al., 2005; Narvaez et al., 2000; Satyanarayana et al., 2001) and encode at least 19 proteins (Che et al., 2002; Che et al., 2001; Huang et al., 2004; Satyanarayana et al., 2001). Two of these proteins are capsid proteins of 25 and 27 kDa which comprise about 95 and 5% of the virus coat, respectively (Bar-Joseph et al., 1989; Jiang, et al., 2008; Roy, et al., 2005; Ruiz-Ruiz et al., 2007). This closterovirus is a phloem limited virus and is transmitted by aphids in a semi-persistent manner (Bar-Joseph et al., 1989; Brown et al., 1988; Genc 2005; Gottwald et al., 1999). *Toxoptera citricida* and *Aphis gossypii* are the most efficient vectors (Bar-Joseph et al., 1989; Brlansky et al., 2003; Brown et al., 1988; Gottwald et al., 1999; Roy et al., 2005).

The virus is genetically and biologically diverse and the virus isolate, citrus cultivar, rootstock, time of infection and environmental conditions can affect symptoms (Huang et al., 2004; Satyanarayana et al., 2001). A complex range of symptoms are produced under field

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conditions. There are three economically devastating field symptoms caused by CTV, including death and decline, stem pitting (Broadbent et al., 1996; Garnsey et al., 1987; Genc, 2005; Gmitter et al., 1996; Satyanarayana et al., 2001) and seedling yellows (Ruiz-Ruiz et al., 2007). Decline and death of most citrus species grafted on sour orange (*Citrus aurantium* L.) which are caused by most isolates of CTV can be avoided by using CTV resistant or tolerant rootstocks (Dominguez et al., 2000). In citrus growing areas, where sever isolates of CTV are common, coat protein (CP) mediated resistant transgenic plants (Dominguez et al., 2002; Febres et al., 2008) and cross protection with mild strains (Lin et al., 2002) can reduce yield losses (Dominguez et al., 2000). This needs quick and sensitive methods for differentiation of mild strains from virulent strains. It is possible to detect both severe and mild strains of CTV in the same plant simultaneously using bi-directional PCR (Jiang et al., 2008). CTV isolates were first classified into seven p25/HinfI groups. Groups IV and V produced mild symptoms while others produced sever symptoms in the indicator plants (Gilling et al., 1993). Later another group was found in china and defined as Group VIII. This isolate produced no symptoms in indicator plants (Jiang et al., 2008). We reported the detection of CTV in the asymptomatic and symptomatic citrus samples by molecular techniques and the association of CTV with diseased citrus in Peninsular Malaysia (Ayazpour et al., 2011). However, there was not enough information about CTV and its p25/HinfI groups in Malaysia, so this research was performed to characterize CTV isolates in Malaysia.

MATERIALS AND METHODS

Sampling

Samples were randomly collected from 340 asymptomatic and symptomatic citrus trees throughout peninsular Malaysia. Mature shoots and leaves of citrus plants were collected from eastern, western, southern and northern branches of each tree and mixed for the test. Their infection with CTV was checked by ELISA. For BD-PCR and RFLP analysis, sixty positive samples including *Citrus aurantifolia, Citrus sinensis, Citrus maxima, Citrus reticulata, Citrus hystrix, Citrus microcarpa and Fortunella sp*. were selected from Selangor, Pahang, Johor, Terengganu, Perak and Kedah states (Table 1).

Nucleic acid extraction from citrus tissues

Total RNA was extracted from shoot barks, midribs and petioles. About 0.2 g of tissues was pulverized in liquid nitrogen with mortar and pestle and collected in a 1.5 ml sterile microtube. Each sample was suspended in 400 µl TES buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 2% w/v SDS) and 400 µl phenol/chloroform/isopropanol (25/24/1) and was shaken vigorously for ten minutes. After centrifugation (14000 rpm) for ten minutes, the supernatant was treated with 200 µl ethanol (99.8%) in a new tube and used for total RNA extraction by RNeasy mini kit (Qiagene) according to the manufacturer's instructions. The extracted RNA was used as a template for amplification of the CP gene of CTV.

Primers

Primer pair CP1 (5′-ATG-GAC-GAC-GAA-ACA-AAG-AA-3′)/ CP2 (5′-TCA-ACG-TGT-GTT-GAA-TTT-CC-3′) were used for amplification of the complete CP cistron (672 bp) of CTV (Jiang et al., 2008). The internal sense primer CP3 (5′-TTTGGACTGACGTCGTGTT-3′) and the internal anti sense primer CP4 (5′-TTACCAATACCCTTAGAATTAT-3′) were used for differentiation of CTV severe and mild strains (Huang et al., 2004). The expected sizes of PCR products with primer sets CP2/CP3 and CP1/CP4 were 320 and 392 bp, respectively.

cDNA synthesis and polymerase chain reaction amplification

cDNA was synthesized using RNA extracted from citrus tissues as template and CP2 as primer. The total reaction volume was 40 µl, which contained 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 0.2 mM each of the four dNTPs, 1 µm CP2, 1 µm CP4, 20 U reverse transcriptase and 18.75 µl extracted RNA. First, RNA and primer were mixed gently and heated for 10 min at 65°C and then immediately cooled on ice. Then other materials were added and the contents were mixed gently and incubated at 25°C for 10 min, 42°C for 60 min and 72°C for 10 min, respectively. Preparation of cDNA for BD-PCR was done as above, but CP2 and CP4 were used as primers. PCR amplification was performed in 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.05 mM each of the four dNTPs, 2 mM $MgCl_2$, 0.3 µM of each primer (CP1, CP2), 1.25 U Taq DNA polymerase (iNtRON Biotechnology) and 1 to 4 µl of RT mixture. For BD-PCR, the mixture was the same, except that 0.3 µM of each primer, CP1, CP2, CP3 and CP4, was added and RT mixture has been made with CP2 and CP4 as primers. The PCR cycling profile was one cycle at 94°C for five min, followed by 35 cycles of 94°C for 30 s, 56°C for one min, and 72°C for one min, with a final extension step at 72°C for 10 min. PCR amplified fragments were separated in 1.2% agarose gel in Tris-borate (TBE) buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3). After electrophoresis, the gels were stained in 0.5 µg/ml ethidium bromide and analyzed using BIO imaging system (Syngene). A 100 bp DNA Ladder (Fermentas) was used as a nucleic acid marker.

Restriction fragment length polymorphism of CP gene

The PCR amplified products of the CP gene of different CTV isolates were digested with the restriction enzyme HinfI to differentiate CTV strains and evaluate their variation in peninsular Malaysia. Each digestion reaction was composed of 20 µl of PCR product, 20 U of the restriction enzyme, and 2 µl of 10 × digestion buffers in a total volume of 32 µl. The digestion reaction was performed at 37°C for 90 min and the products were separated by electrophoresis in a 3% agarose gel and visualized after staining with ethidium bromide.

Sequencing and Phylogenetic analysis of the CP gene of CTV

Fifty six isolates of CTV were chosen for sequencing of their CP genes. The amplified products of approximately 672 bp for the complete CP gene were sequenced commercially (NHK BIOSCIENCE SOLUTIONS, Korea). A multiple sequence alignment was performed by using Clustal W 1.6 (Thompson et al., 1994) and

Table 1. Host and GenBank accessions of CTV isolates in peninsular Malaysia.

Isolates	Host	Accession number
AMC2	*Citromelo*	HQ012375
AMC13	*Citrus reticulata*	HQ012378
AMC18	*Citrus sinensis*	HQ012380
AMI61	*Citrus microcarpa*	HQ012381
AMI62	*C. microcarpa*	HQ012382
AMj1	*C. microcarpa*	HQ012383
AMJ12	*C. microcarpa*	HQ012384
AMJ31	*Citrus aurantifolia*	HM131219
AMK1	*C. aurantifolia*	HQ012385
AMK8	*C. microcarpa*	HQ012386
AMK10	*C. aurantifolia*	HQ012387
AMK11	*C. aurantifolia*	HQ012388
AMK17	*C. aurantifolia*	HQ012389
AMK19	*C. aurantifolia*	HQ012390
AMK22	*Citrus hystrix*	HQ012391
AMK25	*C. hystrix*	HQ012392
AMK27	*C. hystrix*	HQ012393
AMK30	*C. hystrix*	HQ012394
AMK35	*C. microcarpa*	HQ012395
AMK42	*C. aurantifolia*	HQ012396
AMKu2	*C. microcarpa*	HQ012397
AMM11	*C. reticulate*	HQ012398
AMM14	Lemon	HQ012399
AMM18	*C. aurantifolia*	HQ012400
AMM20	*C. aurantifolia*	HQ012401
AMM22	*C. aurantifolia*	HQ012402
AMM28	Lemon	HQ012403
AMM29	*C. aurantifolia*	HQ012404
AMSB2	*C. microcarpa*	HQ012405
AMSB3	*C. hystrix*	HQ012406
AMSB7	*C. microcarpa*	HQ012407
AMT1	*C. aurantifolia*	HQ012408
AMT3	*C. aurantifolia*	HQ012409
AMT4	*C. aurantifolia*	HQ012410
AMT5	*C. aurantifolia*	HQ012411
AMT7	*C. aurantifolia*	HQ012412
AMT8	*C. aurantifolia*	HQ012413
AMT9	*C. aurantifolia*	HQ012414
AMT12	*C. microcarpa*	HQ012415
AMT14	*C. hystrix*	HQ012416
AMT15	*C. microcarpa*	HQ012417
AMT21	*C. hystrix*	HQ012418
AMT27	*C. aurantifolia*	HQ012419
AMT28	*C. hystrix*	HQ012420
AMT31	*C. hystrix*	HQ012421
AMT32	*C. hystrix*	HQ012422
AMT35	*C. microcarpa*	HQ012423
AMT36	*C. aurantifolia*	HQ012424
AMT37	*C. aurantifolia*	HQ012425
AMT38	*C. aurantifolia*	HQ012426
AMT39	*C. aurantifolia*	HQ012427

Table 1. Contd

AMT40	*C. aurantifolia*	HQ012428
AMT41	*C. microcarpa*	HQ012429
AMT42	*C. aurantifolia*	HQ012430
AMT43	*C. aurantifolia*	HQ012431

a phylogenetic tree was constructed by MEGA software Version 4 (Tamura et al., 2007), using neighbor-joining method with 1000 bootstrap replications. GenBank accessions and the sequence sources of the CTV CP genes used for RFLP and construction of Phylogenetic tree are listed in Table 1.

RESULTS

CTV strains differentiated by bi-directional PCR

Bi-directional (BD) PCR was done based on two sets of strain-specific primers for CP encoding region to identify mild and sever strains in the samples. The 392-bp fragment was amplified only from AMK19, AMK27, AMC18, AMM11, AMM20, AMM28 and AMJ12. This means that only 11.67% of total samples produced the 392-bp fragment. Of these seven samples, only AMM28 produced a 392-bp fragment, while the other six samples produced both 320 and 392-bp fragments. The other 53 samples produced only the 320-bp fragment, accounting for 88.33% of total samples. These results suggest that 88.33% of the analyzed plants were infected with sever strains, 1.67% with mild strain and 10% were infected with both sever and mild strains (Figure 1).

RFLP profiles of the CP gene from different CTV isolates

672 bp amplified fragments of CTV isolates using CP1 and CP2 primers of the CP gene were digested with the restriction enzyme HinfI. Digestion of the PCR products of CTV isolates revealed a high sequence divergence between CTV isolates (Figure 2). According to the previous defined RFLP groups (Gilling et al., 1993; Jiang et al., 2008), the restriction enzyme HinfI created digestion patterns of Groups I to IV, VI to VII and two other new groups. Isolates AMK1, AMJ12, AMT38, AMT39 and AMT43 produced a unique restriction pattern with two fragments of about 220 and 300 bp. These results were confirmed by virtual digesting of their sequences in computer software (Bikandi et al., 2004). These isolates were designated as Group IX. Isolate AMI61 alone produced another restriction pattern with three fragments of about 100, 270 and 300 bp. This isolate was designated as Group X. AMT31 and AMT36 were placed in RFLP Group I, AMK25 in Group II, AMC2, AMJ31, AMSB2 and AMSB7 in Group III and AMM28 in Group IV. AMT1, AMT3, AMT5, AMT7, AMT9, AMT21, AMT27, AMT32, AMT34, AMT35, AMT42, AMT44, AMM14, AMM18, AMM22 and AMM29 indicated the same restriction sites and were placed in RFLP Group VI. Isolate AMT41 did not produce any restriction pattern. None of the CTV isolates produced the restriction pattern of groups V or VIII. The remaining 34 CTV isolates had mixed infections. Among these, the restriction pattern of Group VI was the most frequent (67.24%) followed by Group III (38.33%), Group IV (25%), Group II (15.52%), Group I (12.07%), Group VII (8.62%), Group IX (8.62%) and Group X (1.72%), respectively (Table 2).

Phylogenetic analysis

Phylogenetic tree of these isolates in comparison with seven isolates from other countries showed that these isolates were clustered into two separate clades (Figure 3). Most of the Malaysian CTV isolates were placed in Clade 1. Clade 1 was separated into two subclades, comprising all Malaysian isolates in Subclade 1-1 and the isolates Bangalore, NUagA and ML12 in Subclade 1-2. Considering that these tree samples are from South Asia, can conclude that most isolates are close to another CTV isolates of Sought Asia. Some CTV isolates of Malaysia were clustered in Clade 2. Isolates T36 and NZRB and five isolates from Malaysia were placed in Subclade 2-1; while isolates T30 and T385 and other five Malaysian isolates were close together and placed in Subclade 2-2.

DISCUSSION

Based on the BD-PCR results, seven isolates of CTV comprising AMK19, AMK27, AMC18, AMM11, AMM20, AMM28 and AMJ12 were characterized as mild strains. According to Table 1, these mild strains were found in all citrus species; therefore there is no differentiation between hosts of mild and severe strains. The HinfI restriction fragment patterns revealed that the Malaysian CTV population structure is complex. By using the HinfI restriction fragment patterns for separating mild strains from sever strains, the Group IV was identified as mild strain (Gilling et al., 1993), so the 15 isolates recognized as Group IV (AMT8, AMT15, AMT28, AMK1, AMK8, AMK17, AMK19, AMK22, AMK27, AMK42, AMC18, AMM11, AMM20, AMM28 AND AMJ12), were either mild strain or complexes of mild and severe strains. By

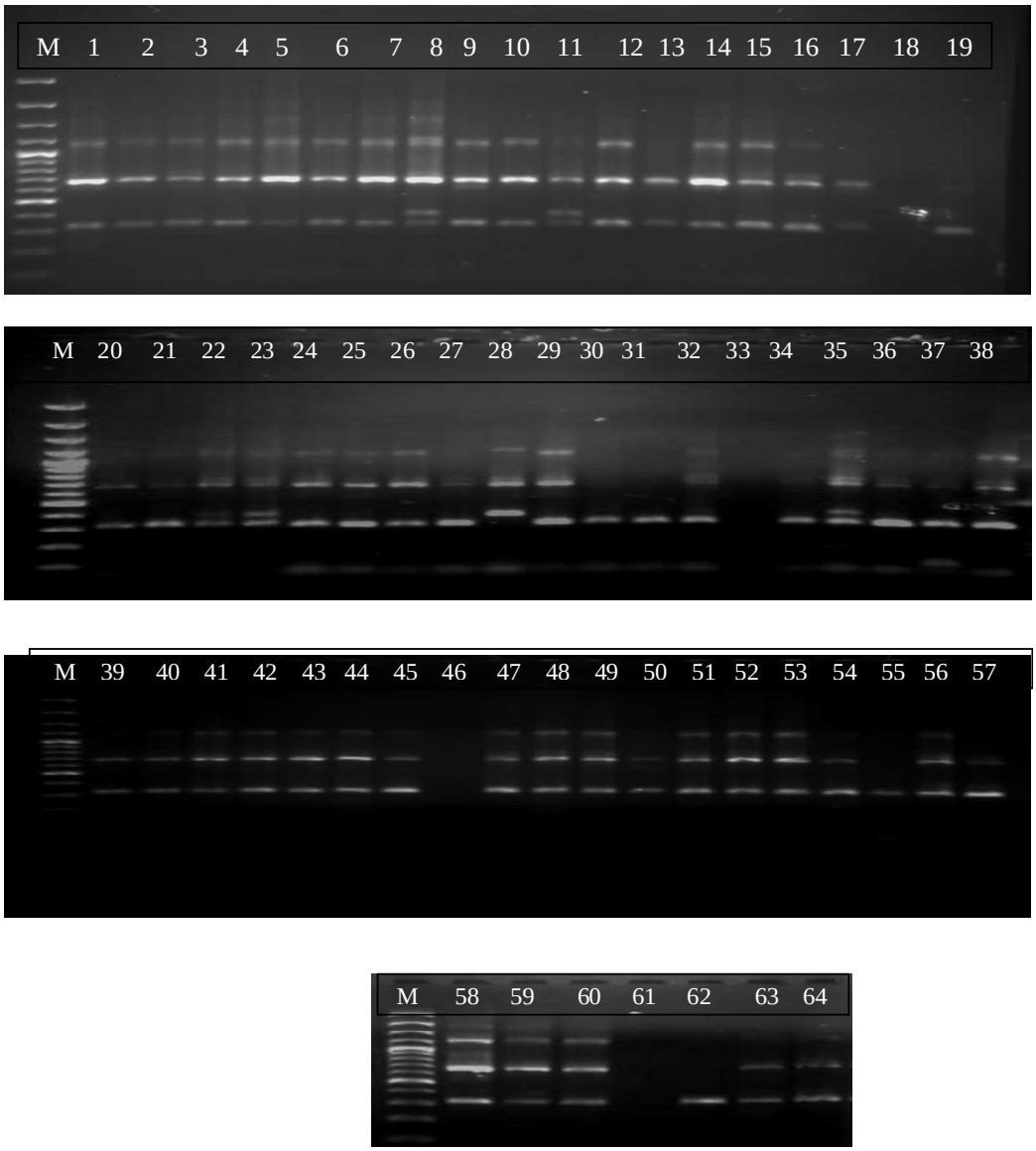

Figure 1. Amplification of partial CP gene from CTV Malaysian isolates with CP_1, CP_2, CP_3 and CP_4 primer pairs). M, molecular marker 100bp, 1- AMK1, 2-AMK5, 3-AMK8, 4-AMK10, 5-AMK11, 6-AMK16, 7-AMK17, 8-AMK19, 9-AMK22, 10-AMK25, 11-AMK27, 12-AMK30, 13-AMK35, 14-AMK42, 15-AMSB2, 16-AMSB3, 17-AMSB7, 18-WATER, 19-L, 20-AMC2, 21-AMC13, 22-AMC18, 23-AMM11, 24-AMM14, 25-AMM18, 26-AMM20, 27-AMM22, 28-AMM28, 29-AMM29, 30-L, 31-AMKu1, 32-AMKu2, 33-water, 34-AMJ1, 35-AMJ12, 36-AMJ31, 37-AMI61, 38-AMI62, 39-AMT1, 40-AMT3, 41-AMT4, 42-AMT5, 43-AMT7, 44-AMT8, 45-AMT9, 46-water, 47-AMT12, 48-AMT14, 49-AMT15, 50-AMT21, 51-AMT27, 52-AMT28, 53-AMT31, 54-AMT32, 55-AMT36, 56-AMT37, 57-AMT38, 58-AMT35, 59-AMT39, 60-AMT40, 61-water, 62-AMT41, 63-AMT42, 64-AMT43.

comparing these two methods for separating mild strains from severe strains, it is perceived that BD-PCR cannot recognize all mild strains. On the other hand, the HinfIrestriction fragment patterns method verified the results of BD-PCR, so we recognize this method more efficient. In China Jiang et al. (2008) also detected only two mild isolates of CTV by BD-PCR method and seven mild isolates with RFLP method. These results are consistent with our observations in Malaysia.

Isolates AMK1, AMJ12, AMT38, AMT39 and AMT43 produced a unique restriction pattern with fragments of 210 and 300 bp. On the other hand, isolate AMI61 produced another restriction pattern with three fragments of about 100, 270 and 300 bp. These patterns appeared neither in the seven groups defined by Gilling et al. (1993), nor in the group defined by Jiang et al. (2008). To our knowledge, this is the first report on the presence of these restriction sites in a CP gene of any CTV isolate.

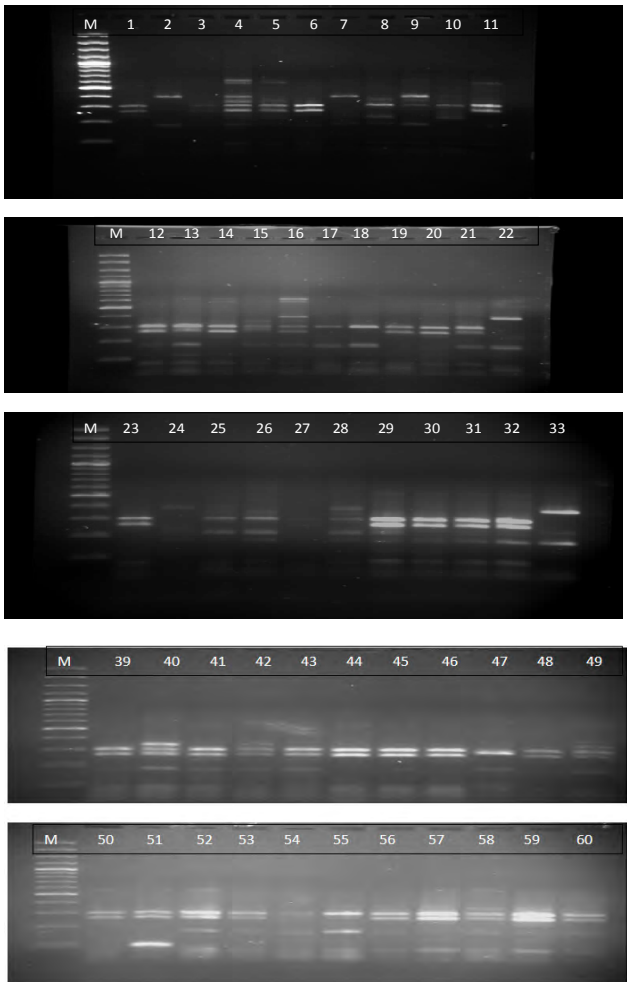

Figure 2. RFLP profiles of CP gene of 60 CTV isolates from Peninsular Malaysia. M-Molecular marker 100bp, 1-AMT1, 2-AMT14, 3-AMT21, 4-AMT28, 5-AMT32, 6-AMT34, 7-AMT36, 8-AMT37, 9-AMT40, 10-AMT42, 11-AMT44, 12-AMK8, 13-AMK19, 14-AMK22, 15-AMK27, 16-AMK30, 17-AMC2, 18-AMM20, 19-AMM22, 20-AMT8, 21-AMT15, 22-AMT31, 23-AMT7, 24-AMT36, 25-AMT38, 26-AMT39, 27-AMT41, 28-AMT43, 29-AMK1, 30-AMK5, 31-AMK10, 32-AMK11, 33-AMK25, 34-AMM11, 35-AMJ12, 36-AMSB2, 37-AMSB3, 38-AMSB7, 39-AMT3, 40-AMT4, 41-AMT5, 42-AMT12, 43-AMT27, 44-AMT35, 45-AMM14, 46-AMM18, 47-AMM28, 48-AMM29, 49-AMK12, 50-AMT9, 51-AMI61, 52-AMI62, 53-AMC13, 54-AMJ1, 55-AMJ31, 56-AMK16, 57-AMK17, 58-AMK35, 59-AMK42, 60-AMC18.

Table 2. Restriction patterns of the CP gene from 60 isolates created by HinfI digestion and frequency of each pattern.

Isolate	I	II	III	IV	VI	VII	VIII	IX	X
AMT1					+				
AMT3					+				
AMT4		+			+				
AMT5					+				
AMT7					+				
AMT8				+	+				
AMT9					+				
AMT12		+			+				

Table 2. Contd.

AMT14	+								
AMT15			+	+	+				
AMT21					+				
AMT27					+				
AMT28	+			+	+	+			
AMT31	+								
AMT32					+				
AMT34					+				
AMT35					+				
AMT36	+								
AMT37		+				+			
AMT38								+	
AMT39		+						+	
AMT40		+				+			
AMT41									
AMT42					+				
AMT43	+		+					+	
AMT44					+				
AMK1			+	+	+			+	
AMK5			+		+				
AMK8			+	+	+				
AMK10			+		+				
AMK11			+		+				
AMK12			+		+	+			
AMK16			+		+				
AMK17				+	+				
AMK19			+	+	+				
AMK22				+	+				
AMK25		+							
AMK27		+		+	+				
AMK30	+		+		+				
AMK35		+	+		+				
AMK42				+	+				
AMC2			+						
AMC13			+		+				
AMC18			+	+	+				
AMM11			+	+		+			
AMM14					+				
AMM18					+				
AMM20			+	+					
AMM22					+				
AMM28				+					
AMM29					+				
AMJ1			+		+				
AMJ12	+	+	+	+				+	
AMJ31			+						
AMSB2			+						
AMSB7			+						
AMI61					+				+
AMI62			+		+				
Frequency and % of each group	7 (12.07)	9(15.52)	23 (38.33)	15 (25)	39 (67.24)	5(8.62)	0(0)	5 (8.62)	1 (1.72)

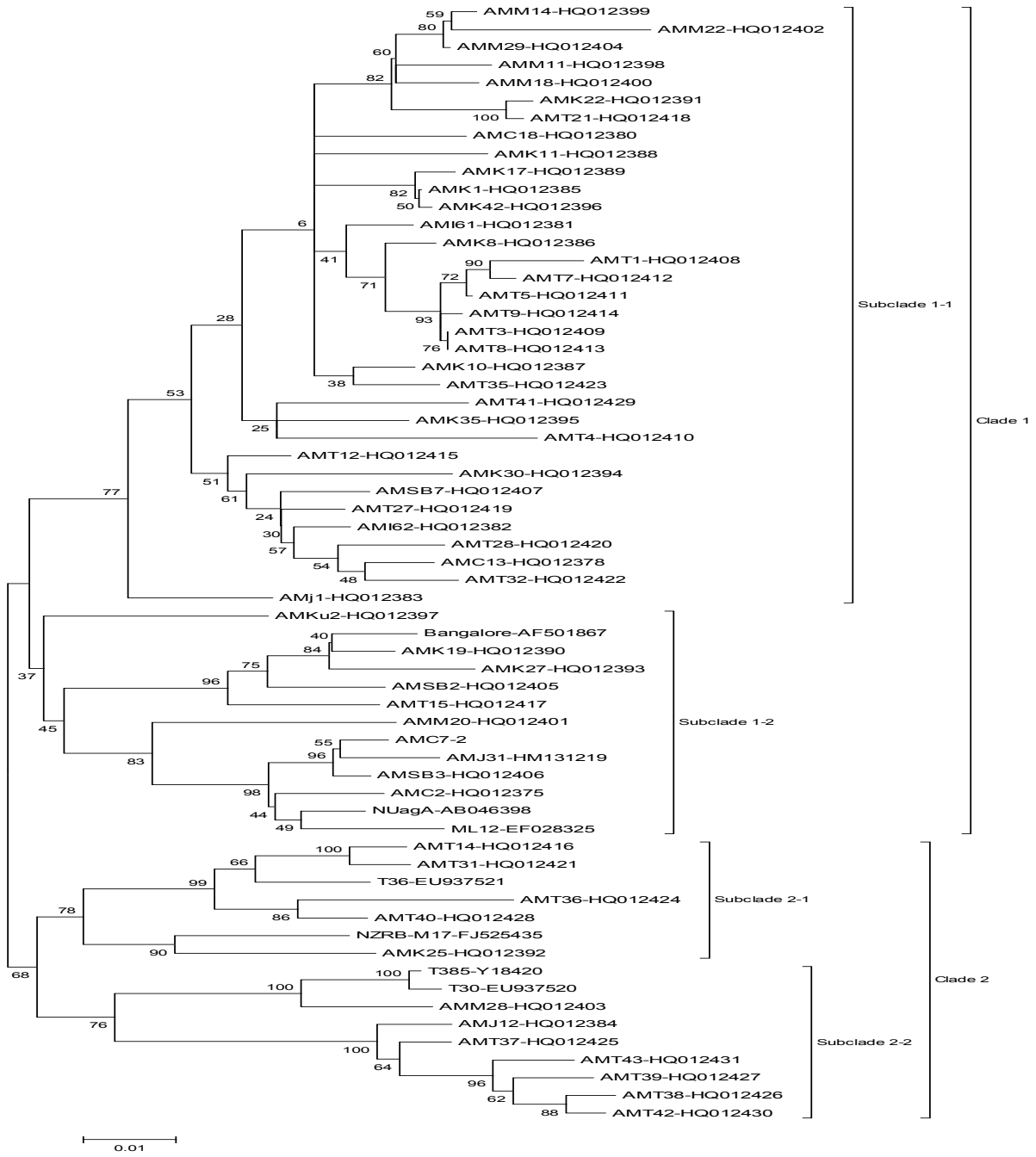

Figure 3. Neighbor-joining tree with bootstrap values of 63 isolates, 56 samples in this work and 7 isolates published in the GenBank.

According to these results we can conclude that CTV populations in Malaysia contain new genetic variants.

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Full Length Research Paper

Killing kinetics and bactericidal mechanism of action of *Alpinia galanga* on food borne bacteria

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The aim of this study was to explore the killing kinetics and bactericidal mechanism of action of *A. galanga* against food borne bacteria in order to promote this plant as a source of natural preservative. The comparison on antibacterial power was firstly done among its essential oil and the crude extracts obtained from various extracting solvents. The essential oil showed the extremely strongest antibacterial activity and more effective killing activity against Gram negative than Gram positive food borne bacteria. The kinetic time of the oil for complete bactericidal action against *E. coli* was 40 min whereas that of gentamicin was 120 min. The scanning electron microscopy (SEM) of bacterial morphology after exposed to the oil showed bacterial membrane destruction. It was concluded that the essential oil is the highest potential part of *A. galanga* against food borne bacteria. Its killing kinetics against Gram negative bacteria was extremely faster than a broad spectrum antibiotic gentamicin. Its mechanism of bactericidal action was along with the bacterial membrane disruption and malfunction leading to cell death.

Key words: *Alpinia galanga*, antibacterial, mechanism of action, essential oil, killing kinetics.

INTRODUCTION

Certain food borne diseases are originated from contaminated food by different strains of bacteria. These bacteria are not only directly harmful as pathogens but also cause food deterioration and leaching of the toxic substances to human body. Food safety therefore becomes a highly important issue for both consumers and food industries due to the rising number of case reports on food associated infections. A good manufacturing practice has been introduced in food industries in order to control the pathogen level in food products (Fan et al., 2009; Grob et al., 2009). The most effective way to minimize food contaminated by microorganisms is to add an effective antimicrobial agent or a so-called preservative, into food products. The substances used as preservatives nowadays are both from chemical synthesis and natural source. The later, for example, the use of potential edible plants is having more increasing interest by consumer because of its less toxic and the feeling of natural sense.

Various plants have shown their potential on antimicrobial action (Akinpelu and Onakoya, 2006; Hernandez et al., 2007; Karaman et al., 2003; Vuddhakul et al., 2007). Some have been used for treatment of infectious diseases caused by pathogenic micro-organisms (Oke et al., 2009; Voravuthikunchai et al., 2004). *Alpinia galanga*, a plant in family Zingiberaceae is widely distributed in the tropical area. It has been used as a medicine for curing stomachache in China and Thailand (Yang and Eilerman, 1999). Under the names “Chewing John”, “Little John to Chew”, and “Court Case Root”, it is used in African-American folk medicine and hoodoo folk magic (Yronwode, 2002). *A. galanga* fresh rhizome has characteristic fragrance as well as pungency and used as essential component in many Asian food products. It was reported that the crude extracts of *A. galanga* have antioxidant and antimicrobial activities against certain microorganisms (Habsah et al., 2000; Mayachiew and Devahastin, 2008). Janssen and Scheffer (1985) reported that the monoterpenes in the essential oil from fresh galangal rhizomes contain an antimicrobial activity against *Trichophyton mentagrophytes.*

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The ethanol crude extract of *A. galanga* was reported to have inhibitory effect against *Staphylococcus aureus* (Oonmetta-aree et al., 2006). However, the antibacterial data of this plant against food borne bacteria was not enough to be ensured of using this plant as natural preservative. Moreover, the knowledge deeply on its antibacterial kinetics and mechanism of action against any bacteria is very rare. In order to promote *A. galanga* as a natural source of food preservative, various strains of common food borne Gram positive and Gram negative bacteria should be tested.

The purpose of this study was to investigate the antibacterial activity of the rhizome of *A. galanga* against food borne bacteria. We attempted to study in depth the antibacterial activity of the most active extract of *A. galanga* on minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), killing kinetics and the mechanism of action against the tested bacteria.

MATERIALS AND METHODS

Plant materials

Rhizomes of *A. galanga* (6 to 12 months age) cultured in the northern part of Thailand were collected. A voucher specimen was deposited at the Herbarium of Faculty of Pharmarcy, Chiang Mai University, Thailand. Fresh rhizomes were used for extraction of the essential oil. Dried rhizome powder was used for preparation of crude extracts. It was prepared by slicing the fresh rhizomes into small pieces and drying at 60°C for 48 h. The dried rhizome was ground into fine powder to be ready for solvent extraction.

Essential oil extraction

The fresh rhizomes were chopped and subjected to hydro-distillation for 6 h using a clevenger apparatus to obtain the essential oil fraction. The essential oil obtained was dried using anhydrous sodium sulphate and then stored in an airtight light resistant bottle at 4°C until further test.

Gas chromatography-mass spectrometry (GC-MS) analysis of the essential oil

The essential oil of *A. galanga* freshly obtained from hydro-distillation was subjected to gas chromatography-mass spectrometry (GC-MS) analysis performed on agilent 6890 gas chromatography coupled to electron impact (EI, 70 eV) with HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) supplied by HP, USA (30.0 m × 250 μm, i.d. 0.25 μm film thickness). The oven temperature was programmed to increase from 100 to 280°C at a rate of 3°C/min and finally held isothermal for 10 min. The carrier gas was helium introduced at a rate of 1.0 ml/min. Diluted sample of 1.0 μl was injected manually and the split ratio was adjusted to 40:1. GC–MS analyses were performed using a Thermo Finnigan-TRACE GC (Waltham, Massachusetts, USA) coupled with a TRACE MS plus (Waltham, Massachusetts, USA) (EI 70 ev) of the same company.

Identification of essential oil constituents

The components of *A. galanga* essential oil were identified by comparison of their mass spectra with those of NIST98 library data of the GC–MS system and Adams libraries spectra, as well as by comparison with the compounds' elution order with their retention indices reported in the literature (Adams, 2001). Retention indices of the components were determined relative to the retention times of a series of n-alkanes with linear interpolation.

Preparation of the crude extracts

Dried powder of *A. galanga* was separately weighed and macerated in a different-polarity solvent, that is, hexane (non-polar), ethyl acetate (semi-polar), and ethanol (polar) for 4 cycles at room temperature. Each cycle lasted 7 days with 1 h mechanical stirred everyday. The filtrates of the same solvent from each macerated cycle were pooled. The solvent was removed under reduced pressure at 45°C by using a rotary evaporator. The weight of the resulting extracts was measured and stored in dark bottles at 4°C until use.

Microbial strains

The food borne microorganisms used in this study were composed of 7 reference strains, that is, *Escherichia coli* ATTCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 14028, *Salmonella typhi* DMST 5784, *Listeria monocytogenes* DMST 1730, *Shigella sonii* DMST 561 and three field strains of *E. coli*. Tryptic soy broth (TSB) or tryptic soy agar (TSA) from Merck, Darmstadt, Germany was used for culturing the bacteria. All strains were stored at -20°C in glycerol and regenerated twice before tested.

Screening for antimicrobial activity

A comparative antimicrobial potency of *A. galanga* essential oil and its three crude extracts was studied by using the disc diffusion method according to Najjaa et al. (2007) and Arias et al. (2004) with minor modification. Briefly, single colony of the test bacteria were transferred into TSB and incubated overnight. Three milliliters of each culture were mixed with 100 ml of melted TSA at about 45°C and poured onto the surfaces of an agar plate containing 2% agar. The sample test solution was loaded onto a sterile filter paper 8-mm disc (Advantec, Tokyo, Japan) to obtain a final sample deposition of 20 mg. Negative control discs were similarly prepared using distilled water and pure solvents. Each loaded disc was placed on the aforementioned bacterial culture plates and incubated at 37°C for 18 to 24 h. A disc of 0.8 mg gentamicin was used as a positive control. Inhibition zones (including the diameter of disc) were measured and recorded.

Minimum inhibitory and bactericidal concentrations

The determination of MIC and MBC of *A. galanga* essential oil was carried out by a broth dilution method (Yu et al., 2004). Tween 20 was used to solubilize the extracts. All tests were performed in TSB. Serial doubling dilutions of the oil was prepared in a 96-well microtiter plate ranged from 0.05 to 200 mg/ml. The final concentration of each strain was adjusted to 4×10^4 cfu/ml. Plates were incubated at 37°C for 24 h. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity. To determine MBC, broth was taken from each well and incubated in Tyramide Signal Amplification (TSA) at 37°C for 24 h. The MBC was defined as the lowest concentration of the essential oil at which incubated microorganism was completely

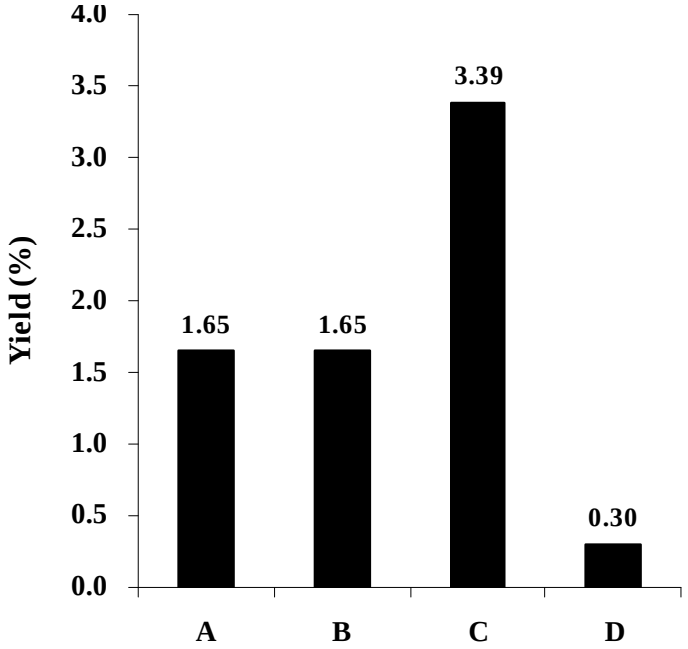

Figure 1. The yield of *A. galanga* rhizome extraction; hexane extract (A), ethyl acetate extract (B), ethanol extract (C) and essential oil (D).

killed. Each test was performed in three replicates. Gentamicin was used as a positive control.

Study of bacterial killing kinetics

In this study, 5 reference strains of bacteria; *L. monocytogenes* DMST 1730, *S. aureus* ATCC 25923, *S. typhi* DMST 5784, *S. sonii* DMST 561, *E. coli* ATCC 25922 and 3 field strains of *E. coli* were used as the test microorganisms. The killing kinetics of *A. galanga* essential oil were conducted at the oil concentrations equal to MBC of the bacterial strains. Bacterial cells were grown to logarithmic phase during 1 h pre-incubation in fresh broth prior to the addition of the essential oil solution. A bacterial concentration between 6 and 8 log cfu/ml was used. The cultures were then incubated in a shaker (Julabo, Allentown, PA) at 37°C for certain period of time or until no viable cells were found. Viable counts were determined by plating 50 µl of known dilutions of the culture samples on to TSA. Cell count plates were incubated for up to 48 h before any were considered as having no growth. Plates with 30 to 300 colonies were used for cfu counts. Log cfu was plotted against time for construction of the bactericidal kinetic curves. Gentamicin was used as a positive control.

Bacterial morphology

The bacterial morphology before and after exposed to *A. galanga* essential oil was examined by using scanning electron microscopy (SEM). Sample preparation for SEM was done as follows: The bacterial suspension before or after certain time of exposure to the oil was dropped into a filter membrane and air dried. Next, the bacteria were fixed with 2.5% glutaraldehyde in Phosphate buffered saline (PBS) and rinsed with the same buffer solution. Subsequently, the fixed bacteria were stained with 1% OsO_4 in PBS for 1 h and dehydrated with different mixtures of water and ethanol. The membrane was coated with gold and analyzed with SEM.

RESULTS

Yield of extraction

In hydro-distillation of the fresh *A. galanga* rhizomes yielded the essential oil of 0.30% v/w. For solvent extraction of the dried rhizome, it gave the crude extracts with various yields depending on the extracting solvents used. It was found that the most non-polar solvent hexane gave the crude extract with the highest yield of 3.39% w/w (Figure 1).

Chemical composition of the essential oil

By using chromatographic procedure, twenty seven compounds, representing 93.06% of *A. galanga* essential oil was identified. Quantitative and qualitative analytical results by GC–MS were shown in Table 1.

Antibacterial activity of the extracts

The growth inhibition zones of *A. galanga* essential oil in comparison with its crude extracts from different solvents against two common food borne bacteria, *E. coli* and *S. typhimurium* were presented in Table 2. The essential oil showed the highest antibacterial potency and was selected for further investigation on determination of MIC and MBC which more strains of food borne bacteria were used. The results were demonstrated in Table 3. It was found that *A. galanga* essential oil had strong bactericidal activity against *E. coli*, *S. aureus, S. sonii*, and *S. typhi* which MIC and MBC of the oil against each strain demonstrated the same value of 4.0, 8.0, 2.0, and 2.0 mg/ml respectively. The MIC of the oil against *L. monocytogenes* was 2.0 mg/ml demonstrating bacteriostatic effect whereas the bactericidal activity against this strain was 4.0 mg/ml.

Bacterial killing kinetics

The result in this study was comparative kinetic bactericidal action between *A. galanga* essential oil and a broad spectrum antibiotic gentamicin at the concentration of their MBC. The kinetic bactericidal action against Gram negative and Gram positive bacteria expressed as time killing curves was shown in Figures 2 and 3, respectively.

Bacterial morphology study

Under the SEM investigation of the bacterial cells morphology after various time of exposure to the essential oil, it was found that the bacterial cell was rapidly shrunk within 10 min. The morphology of normal bacteria in comparison with the shrink cells was shown in Figure 4(a-b). After shrinking, the cells were erupted and

Table 1. Main chemical composition of *A. galanga* essential oil.

Component	%Area	RT[a]	RI[b]
Limonene	29.64	3.32	1041
Gamma-terpinene	1.22	3.56	1058
Alpha-terpinolene	0.44	3.99	1087
1-Undecene	0.20	4.09	1093
(-)-Borneol	0.72	5.48	1166
Para-cymen-8-ol	3.06	6.03	1189
Alpha-terpineol	0.20	6.25	1198
Z-Citral	1.23	7.50	1248
(-)-Bornyl acetate	0.39	8.51	1282
Piperitenone	33.31	10.56	1349
Alpha-cubebene	0.15	10.78	1355
Decaoic acid	1.31	11.55	1377
Beta-elemene	1.91	12.30	1398
Alpha-gurjunene	0.20	12.83	1414
Trans-beta-caryophyllene	3.38	13.39	1431
Trans-beta-farnesene	0.42	15.44	1487
Beta-selinene	0.46	15.56	1490
Delta-selinene	0.31	15.71	1494
Pentadecane	5.62	15.95	1500
Alpha-amorphene	3.01	16.50	1517
7-Epi-alpha-Selinene	0.83	16.65	1521
Trans-gamma-Bisabolene	2.25	16.79	1525
Alpha-Cadinol	0.61	21.08	1661
Gamma-Selinene	0.40	21.62	1681
Beta-Bisabolene	0.84	22.21	1702
Apiol	0.65	22.45	1708
Alpha-trans-Bergamotol	0.30	27.46	1828

[a)] Retention times; [b)] Retention indices.

Table 2. Bacterial inhibitory zone of *A. galanga* crude extracts and its essential oil using the disc diffusion method (n=3).

Sample	Inhibition zone (mm)*	
	***E. coli*ATCC 25922**	***S. typhimurium*ATCC 14028**
Ethanol extract	NZ	NZ
Ethyl acetate extract	9.0±0.1	7.8 ±0.4
Hexane extract	NZ	NZ
Essential oil	10.2 ± 0.4	9.5 ± 1.0
Gentamicin	16.3 ± 0.9	10.4 ± 1.2

* NZ : no inhibition zone.

destroyed into pieces. Most of cell destruction occurred within 40 min.

DISCUSSION

Microbial contamination is a major cause of deterioration as well as the loss of quality and safety of food. Severity of pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks of food-borne diseases. The Gram-positive bacterium *Staphylococcus aureus* is mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Mylotte et al., 1987).

Table 3. MIC and MBC of *A. galanga* essential oil in comparison with gentamicin obtained by the broth dilution method (n=3).

Bacterial Strain	MIC (mg.mL^{-1})		MBC (mg.mL^{-1})	
	Essential oil	Gentamicin	Essential oil	Gentamicin
E. coli ATCC 25922	4	16	4	16
S. aureus ATCC25923	8	8	8	16
S. sonii DMST 561	2	8	2	16
S. typhi DMST5784	2	8	2	8
L. monocytogenes DMST 1730	2	4	4	8

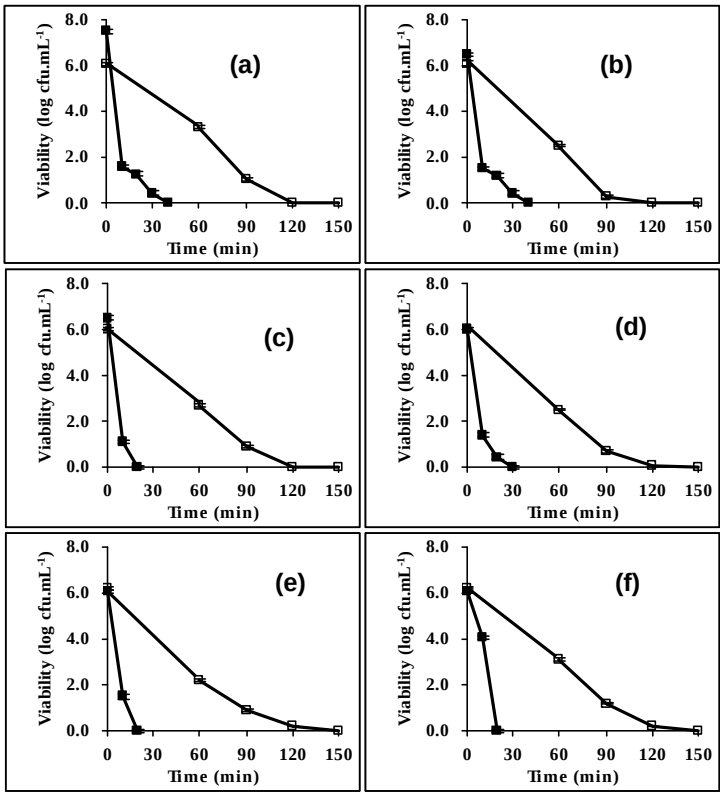

Figure 2. Killing kinetic time of *A. galanga* essential oil (■) and gentamicin (□) against *E. coli* ATCC 25922 (a), *E. coli* field strains (b-d), *S. typhi* DMST 5784 (e), and *S. sonii* DMST 561 (f) (n=3)

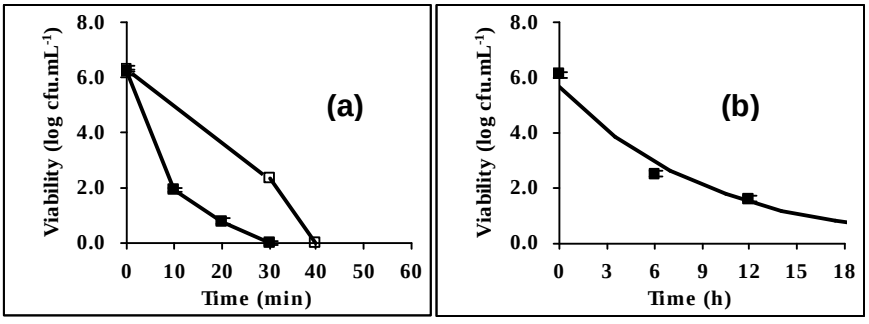

Figure 3 Figure 3. Killing kinetic time of *A. galanga* essential oil (■) and gentamicin (□) against *L. monocytogenes* DMST 1730 (a) and *S. aureus* ATCC 25923 (b) (n=3

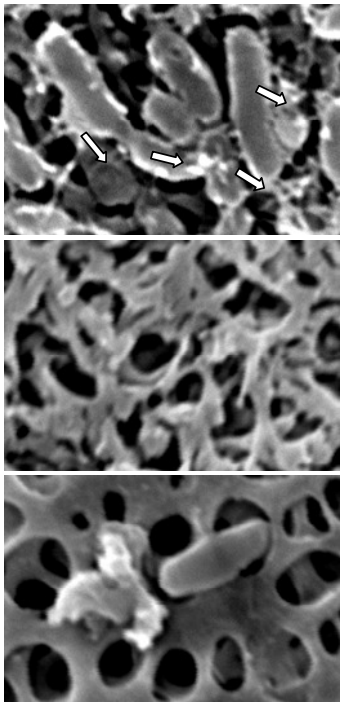

Figure 4. Morphology of *E. coli* ATCC 25922 after 5 min (a) and 40 min (b) exposed to *A. galanga* eseential oil (Arrows point to the lesions of cell membrane destruction) and the comparison of cell normal morphology (c) at the initial (right) and final (left) of exposure

Listeria monocytogenes is responsible for the severe food-borne illness, listeriosis, which has been recognized to be one of the emerging zoonotic diseases during the last two decades (Farber, 2000). The Gram-negative bacterium *Escherichia coli* is present in human intestines and causes urinary tract infection, coleocystitis or septicaemia (Singh et al., 2000). With the increase of bacterial resistance to antibiotics, the use of potential plant extracts represents a concrete alternative for infection control or for the preservation of food. *A. galanga* is one of the plants possesses high antimicrobial power and has been used by local people for a long time on the treatment of infectious diarrhea. However, such practice is in the lack of a sufficient scientific basis. This present study was attempted to provide more valuable scientific data base of this plant.

As seen, the yield of the extracts obtained by different solvent as well as different method of extraction was different. The amount of crude extract obtained by hexane was approximately 2-folds higher than those extracted by ethyl acetate and ethanol indicating that the rhizome of *A. galanga* contains large quantity of non-polar compounds. The yield of essential oil is the least when compared with the three crude extracts. It was reported previously by Ibrahim et al. (2009) that the yield of essential oil obtained from *A. conchigera*, a small galangal, was 0.16% v/w. From this present result, it demonstrated that *A. galanga*, which so called big galang -a, possesses higher essential oil content than a small galanga.

The essential oil consisted mainly of two cyclic terpenes; piperitenone (33.3%) and limonene (29.64%). As far as our literature survey could ascertain, there was only one report on the chemical composition of the essential oil of *A. galanga* grown in Malasia (De Pooter et al., 1985). Of the compounds identified previously which represented 83 to 93% depending on its method of preparation, the two main compounds were different from our results. This is possibly due to the fact that the rhizomes were grown in different regions, which may have caused the differences in their chemical composition.

The result of antibacterial activity demonstrated that for the crude extracts, *S. aureus* was more sensitive to the ethanol and hexane extracts than *E. coli* and *S. typhimurium*. These results were in substantial agreement with the previous study of Oonmetta-aree et al. (2006) which reported that ethanol extract of *A. galanga* had inhibitory effect against *S. aureus* but not be able to inhibit *E. coli.* The ethyl acetate extract showed less inhibitory effect against *E. coli* and *S. typhimurium* than the essential oil. This extract exhibited slightly higher inhibitory activity against the Gram positive *S. aureus* whereas the essential oil showed obviously the strongest inhibition against the Gram negative *E. coli* and *S. typhimurium*. These results demonstrated the extremely high power of *A. galanga* essential oil on both Gram negative and Gram positive bacterial tested strains. It has been reported that the essential oil of *Zingiber offinale*, a plant which belongs to the same family as *A. galanga*, showed no inhibitory action against *E. coli* (Singh et al., 2008). The result of our study presented the advantage of *A. galanga* oil that had ability to inhibit many Gram negative bacteria including *E. coli* and *S. typhimurium*. It is also obviously seen that *A. galanga* essential oil had stronger bactericidal activity than the broad spectrum antibiotic gentamicin demonstrating that the oil contained compounds with high antibacterial activities. Important to mention, *A. galanga* essential oil had a strong activity against Gram negative bacteria which are known for their insensitivity against by many antibacterial agents (Conejo et al., 2008; Jeon et al., 2008; Johnson et al., 2008). Moreover, all tested strains are concerning as food contaminations and are the causes of food borne diseases. Consequently, *A. galanga* essential oil showed a promising natural food preservative to minimize bacterial growth in food products. Our results also indicated the high potential of *A. galanga* essential oil to inhibit food borne pathogens of the field strains which tend to be antibiotic resistant strains.

It was seen that the essential oil of *A. galanga* killed all tested bacteria faster than gentamicin. The results revealed that within 10 min *A. galanga* essential oil killed

E. coli ATCC 25922 about 6 log cfu.mL^{-1} whereas in the same period of time gentamicin killed the bacteria less than 1 log cfu/ml (Figure 2 a). Further, the time for complete killing of *E. coli* ATCC 25922 by *A. galanga* essential oil was only 40 min whereas that of gentamicin was 120 min. The same fast kinetic killing results of *A. galanga* oil were observed in three field strains of *E. coli* as shown in Figure 2(b to d). It was reported that the crude extract of *Azadirachta indica* was the potent antibacterial; however it could not kill *E. coli* after 24 h exposure (Okemo et al., 2001). Therefore, it could be indicated that the essential oil of *A. galanga* had greater bactericidal activity than the other plants investigated previously. In addition, the other Gram negative bacteria included *S. typhi* DMST 5784 and *S. sonii* DMST 561 also showed highly sensitive to *A. galanga* oil.

This bacterial sensitivity expressed in a significantly decreased in numbers of bacteria after short exposure to the oil as shown in Figure 2(e-f) respectively. The kinetic bactericidal action of *A. galanga* essential oil to Gram positive bacteria demonstrated that within 10 min, *A. galanga* essential oil decreased about 5 log cfu/ml of *L. monocytogenes* DMST 1730 whereas approximately not more than 1 log cfu/ml were decreased by gentamicin. Moreover the time required for *A. galanga* oil on complete killing of the bacteria was only 30 min. Therefore, *A. galanga* essential oil was more potential for complete killing *L. monocytogenes* DMST 1730 than gentamicin. The earlier kinetic study of tea tree oil on pathogenic bacteria by LaPlante showed that tea tree oil could not completely kill the tested Gram positive bacteria within 24 h (LaPlante, 2007). Our results demonstrated the higher potency of *A. galanga* oil on killing Gram positive bacteria. The kinetic study in *S. aureus* ATCC 25923 revealed a slightly decrease in numbers of bacteria after exposed to the essential oil and the complete killing time was more than 24 h. These results indicated that *A. galanga* essential oil was more effective on killing Gram negative than Gram positive bacteria.

The SEM study was done to examine the change in bacterial morphology after different time exposure to the essential oil in order to indicate the possibility of mechanism of antibacterial action of *A. galanga* essential oil. As our results demonstrated that among the tested bacteria, *E. coli* showed the most sensitive to *A. galanga* oil. Therefore *E. coli* ATCC 25922 was selected to investigate its morphology under the SEM. *A. galanga* oil showed obviously cytological modification to *E. coli* cells. The appearance of the destroyed cells structure was obviously seen as shown in Figure 4(c). This could be considered that *A. galanga* oil had high affinity to interact with the lipopolysaccharide on bacterial cell membrane. The interaction altered the structure of cell membrane. The shrinkage of cell morphology was important evidence on cell membrane alteration caused by the oil. This result led to a malfunction of bacterial cell membrane on normal permeability. The leakage of bacterial essential intracellular components caused cell lyses and final death in a short period.

Conclusion

The use of natural antimicrobial agents is gaining interest due to consumer and producer awareness on health problems. This paper shows that the essential oil of *A. galanga* had strong bactericidal activity against both Gram negative and Gram positive bacteria but more potent in Gram negative strains. The MIC and MBC values of the oil against Gram negative bacterial were substantially lower which showed higher potency and the kinetic bactericidal action was extremely faster than gentamicin. SEM results suggested that the mechanism of *A. galanga* oil on antibacterial action was through a modification of bacterial cell membrane leading to a permeability malfunction of the membrane. The results from this study suggested that the essential oil of *A. galanga* is a promising natural potential food preservative.

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Full Length Research Paper

Isolation, characterization and fingerprinting of some chlorpyrifos- degrading bacterial strains isolated from Egyptian pesticides-polluted soils

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Five bacterial isolates (B-CP5- B-CP6 - B-CP7- B-CP8- B-CP9) were isolated from pesticides-contaminated soil in Egypt. The capability of these isolates to degrade chlorpyrifos was investigated using enrichment mineral salt (MS) medium containing chlorpyrifos. Two different PCR-based techniques, RAPD-PCR and PCR-RFLP for amplified16S rRNA fragment were used to conduct genetic fingerprinting and obtain specific molecular markers for the studied isolates. The isolates exhibited substantial growth in mineral salt medium supplemented with 100-300 mg/L chlorpyrifos as a sole source of carbon and energy. Based on their morphological, cultural and biochemical characters, the isolates have been identified as *Pseudomonas stuzeri, Enterobacter aerogenes, Pseudomonas pseudoalcaligenes, Pseudomonas maltophila* and *Pseudomonas vesicularis* respectively. *Pseudomonas stuzeri* was the most potent degrader strain. Five specific markers for this strain were determined. The highest genetic similarity was observed between CP8 and CP7 (66%), while the lowest genetic similarity was detected between CP8 and CP6 (37%). All isolates had the same pattern after digestion of 16S rRNA amplified fragment with two restriction enzymes (EcoRI and AluI) except *Enterobacter aerogenes,* which generate two monomorphic bands at 420 and 130 bp, respectively. In conclusion, the strain *Pseudomonas stuzeri* could be used to clean up the areas contaminated with Chlorpyrifos. Obtained molecular markers might be used for identifying and tracking the most potent bacterial isolate. The used PCR techniques represent a powerful tool and could be used for rapid typing of this strain.

Key words: Pesticide, chlorpyrifos, biodegradation, 16S rRNA, RAPD-PCR, *Pseudomonas* sp.

INTRODUCTION

Recently the use of microbes for effective detoxify, degrades and removal of toxic chlorinated compounds from contaminated soils has emerged as an efficient and cheap biotechnological approach to clean up polluted environments (Strong and Burgess, 2008). Chlorpyrifos (CP) is one of the most widely used organophosphorous insecticide. Chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol (TCP) are the two potent transformation products of chlorpyrifos (Bhagobaty et al., 2007). Unlike other organophosphorous compounds, CP has been reported to be resistant to enhanced degradation since its first use in 1965. Although CP has been reported to be degraded co-metabolically by bacteria in liquid media, several attempts to isolate CP-degrading bacteria from agricultural soil have not been successful (Mallick et al., 1999; Racke et al., 1990). Singh et al. (2004) isolated the first CP-degrading bacterium, it was Enterobacter B-14, which hydrolyzed CP to diethylthiophosphate DETP and TCP, and utilized DETP for growth and energy. Two recently isolated CP-degrading bacteria, *Stenotrophomonas* sp. and *Sphingomonas* sp., could also utilize CP as the sole source of carbon and phosphorous, but they did not degrade TCP (Yang et al.,

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2006; Li et al., 2007). DNA-based analyses can contribute significantly to the characterization of bacteria that have been successfully isolated from the polluted environments. RAPD-PCR approach has been shown to be useful in fingerprinting and distinguish between microbial strains within a species (Nowrouzian et al., 2001). An excellent target for bacterial identification and phylogenetic characterization is 16S rRNA gene, the 16S rRNA gene is universally distributed and highly conserved (Woese, 1987). Due to its conserved nature and ease of manipulation, it has been extensively used to establish accurate identification of bacterial isolates. Cleavage of PCR-generated 16S rRNA gene amplicons by a restriction enzyme(s) (RE) results in differentiation by 16S rRNA PCR-RFLP technique. This procedure has been used extensively as a method for bacterial species identification (Wilson et al., 1995; Marshall et al., 1999; Conville et al., 2000; Steinhauserova et al., 2001; Bayoumi et al., 2010). The present study aims to isolate, characterize and fingerprinting of chlorpyrifos degrading bacteria from Egyptian pesticides-polluted soils as well as obtain specific molecular markers of the most potent chlorpyrifos degrading bacterial isolates. In addition examine the effects of various environmental factors which affect the bacterial biodegradation potentiality of chlorpyrifos.

MATERIALS AND METHODS

Pesticide used

Chlorpyrifos (CP) (95%purity) was obtained from Dow-agroscience Co.

Media

Mineral salts (MS) medium enriched with Chlorpyrifos as a sole source of carbon and energy was used as a routine medium for isolation and characterization of different species of chlorpyrifos utilizing bacteria and was prepared as the followings: Solution (A) contained in (g/L): KNO_3, 0.5; KH_2PO_4, 0.68; NaH_2PO_4, 1.79; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.35; $CaCl_2.2H_2O$, 0.4; and agar-agar, 15.0 for solid medium and distilled water up to 1000 ml. Chlorpyrifos was added by 100 to 300 mg/L. Solution (B) trace salt solution (100g / ml): $FeSO_4.7H_2O$, 0.1; $ZnSO_4$, 0.1; $MnSO_4$, 0.1; $CuSO_4$, 0.1, $CaCl_2.2H_2O$, 0.1; H_3PO_4, 0.1, and distilled water up to 100 ml/L. The solid and liquid mineral salts media were supplemented with yeast extract and glycerol (3.0 g/ml) or (3.0 ml/l) respectively for obtaining enrichment media.

Study site

Four Chlorpyrifos polluted soil samples were collected from various agricultural fields belong to Cairo and Giza Egyptian governorates.

Reagent used

The 4-aminoantipyrine was used to measure the production of phenolic compounds in culture solution. It was prepared as following: (1) 5 mM Fe $(NH_4)_2(SO_4)_2$ solution, (2) 100 mM α-ketoglutarate solution (3) 40 mM 4-aminoantipyrine solution. This reagent used for detection of phenolic products of the enzymatic cleavage of 2,4-D and related compounds. 2,4-D α-ketoglutarate deoxygenase, cleaves the phenoxy ether bond of o,o-diethl o-(3,5,6-trichloro-2-pyridyl) phosphothionate (Chlorpyrifos), to form 2,4-trichlorophenol TCP. The red color resulting from a 2 mg/l of TCP solution is capable of being visually distinguished from the control (remains bright yellow).

Isolation of cp-degrading bacteria by enrichment and screening

One gram of each soil sample was added to 99 ml MS medium supplemented with yeast extract and glycerol containing 0.1 ml CP in 250 ml flasks. The suspension was incubated at 30°C with shaking at 100 rpm for 10 days; 10 ml of the culture broth was transferred after 10 days to 90 ml of the fresh media. After the fourth enrichment transfer to fresh medium containing CP as sole source of carbon and energy, the media was supplemented with 1.5% agar and incubated at 30°C for 7 days. Bacterial isolates grown on chlorpyrifos-containing agar were subjected to morphological, cultural and biochemical studies by the aid of Berge's Manual of Determinative Bacteriology (Holt et al., 1994). All bacterial isolates were screened based on the formation of degrading haloes around the bacterial colonies and potential isolates were obtained and tested for their potential degrading ability of CP.

Parameters controlling cp biodegradation by CP5

Eight parameters were investigated for studying CP biodegradation by CP5 on MS medium supplemented with CP as sole source of carbon and energy viz. incubation period (1, 2, 3, 4, 5, 6 and 7 days), different CP concentrations (1, 2, 4, 6, 8,10, 20,40, 60, 80 and 100 ml/L); inoculum size (0.5, 1, 2, 4, 8, 10 ml/100ml); incubation temperatures (25, 30, 35, 40 and 45°C); different carbon sources (D-Glucose, D-Fructose, D-Mannose, D-Ribose, Maltose, Arabinose, L-Rhamnose, Sucrose, Lactose, and Starch). All different tested carbon sources were applied as equimolecular weight located in 1% (w/v) sucrose except poly-sorbite and starch which were applied by 1%; different pH values (5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9) using 1 N HCl and 1 N NaOH; different nitrogen sources (sodium nitrate, magnesium nitrate, potassium nitrate, ammonium chloride, ammonium nitrate, EDTA, urea and peptone); under static and shaking conditions. After each parameter, 4-aminoantipyrene reagent was used to measure the production of phenolic compounds.

Molecular genetics fingerprinting

Genomic DNA extraction

Genomic DNA was extracted from five studied isolates using Easy Quick DNA extraction kit (Genomix) following the manufacturer's instructions.

Random amplified polymorphic DNA (RAPD)

RAPD-PCR

PCR reactions were carried out using sixe arbitrary 10-mer primers (Operon Tech., Inc.). PCR reactions were conducted using 2x superhot PCR Master Mix (Bioron; Germany) with 10 Pmol of each

Table 1. List of used primers, their nucleotide sequences, total number of bands, monomorphic and polymorphic bands produced by six primers.

Primer code	Prime sequence	Isolate					Total bands	Amplified bands	Monomorphic bands	polymorphic bands
		CP5	CP6	CP7	CP8	CP9				
A2	5'-TGCCGAGCTG-3'	11	11	11	14	13	60	17	7	10
A3	5'-AGTCAGCCAC-3'	4	11	7	8	7	37	13	6	7
B3	5'-CATCCCCCTG-3'	12	9	13	10	12	56	15	8	7
B5	5'-TGCGCCCTTC-3'	7	10	8	7	8	40	18	4	14
C3	5'-GGGGGTCTTT-3'	8	7	6	8	6	35	10	8	2
C5	5'-TGCGCCCTTC-3'	11	7	9	9	11	47	11	6	5
Total		53	55	54	56	57	275	84	39	45

6 different arbitrary 10-mer primers. The codes and sequences of these primers are listed in Table 1. The 25 Kl reaction mixture was (10 Pmol. of each primer, 30-50 ng of DNA template and 12.5 Kl of 2x superhot PCR Master Mix). The PCR protocol was initial denaturation, 94°C for 2.5 min and 35 cycles of subsequent denaturation, 94°C for 45 s; annealing temperature, 37°C for 30 s; extension temperature, 72°C for 2 min and final extension, 72°C for 10 min. PCR products were analyzed on 10 x 14 cm 1.5% agarose gel electrophoresis with DNA ladder standard 100 bp (Jena Bioscience, Germany)for 30 min using Tris-borate- EDTA Buffer and visualized by ultraviolet illumination after staining with 0.5 Kg/ml ethidium bromide.

16S RRNA PCR-RFLP analysis

The amplification of 16s rRNA gene was performed with universal primer 968F AAC GCGAAGAACCCTAC and 1401R GCGTGTGTACAAGACCC. The reaction mixture was (10 Pmol. of each primer, 50 ng of DNA template and 12.5 Kl of 2x superhot PCR Master Mix). The PCR program was as follow; 1 cycle at 95°C, 5 min; 35 additional cycles consisting of 95°C 1 min, 63°C 1 min, and 72°C 1 min and 72°C 10 min as post PCR reaction time. The amplified DNA fragments were digested with two restriction enzymes (EcoRI and. AluI) separately as described by the manufacturer (Jena Bioscience, Germany). The digested DNA fragments were separated on 2.5% agarose gel electrophoresis with DNA ladder standard 100 bp (Jena Bioscience, Germany), stained with 0.5 Kg/ml ethidium bromide, visualized on a UV Transilluminator, photographed by Gel Doc. System and analyzed with software data analysis for Bio- Rad Model 620 USA.

Statistical analysis

The presence / absence RAPD and 16S rRNA PCR-RFLP data were analyzed using the SPSS-PC programs of (Nie et al., 1975). Pair-wise comparisons between strains were used to calculate the genetic similarity values (F) derived from Dice similarity coefficient.

RESULTS

Five different bacterial isolates were selected on the basis of production of halo zone around the bacterial colonies and growth in high concentrations of CP (100-300 mg /L). Morphological, cultural and biochemical parameters were determined and illustrated in the Table 2. Degrading bacterial isolates were characterized and identified as *Pseudomonas stuzeri* (B-CP5), *Enterobacter aerogenes* (B-CP6), *Pseudomonas pseudoalcaligenes* (B-CP7), *Pseudomonas maltophila* (B-CP8) and *Pseudomonas vesicularis* (B-CP9), by consulting Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984) and Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). *Pseudomonas stuzeri* (B-CP5) was selected as the most potent CP- utilizing bacterial isolate.

Parameters controlling the Chlorpyrifos biodegradation by *Pseudomonas stuzeri* B-CP5

Data recorded in Table 3 showed that, when the *Pseudomonas stutzeri (*B-CP5) was grown on MS medium supplemented with 0.3 ml/L Chlorpyrifos as sole source of carbon and energy proved to be capable of grown with 7 days as optimum incubation period, preferred Chlorpyrifos concentration 0.1-0.35 ml/L, maximum inoculum size (0.5 ml), optimum temperature (30°C), optimum pH (7), favorite sugar (fructose) and preferred nitrogen source (ammonium nitrate) under shaking rotation of (100 rpm). Below and above optimal incubation period, Chlorpyrifos concentrations, inoculum size, temperature, pH, the phenolic compounds production decreased gradually. Fructose only was induced in biodegradation of Chlorpyrifos whereas all tested carbon sources failed to induce the production of phenolic compounds. Ammonium nitrate was exhibited the most preferd nitrogen source whereas other tested nitrogen sources failed in biodegradation of Chlorpyrifos.

Molecular genetics characterization

RAPD and 16S rRNA PCR-RFLP

RAPD and 16S rRNA PCR-RFLP techniques were used to conduct the genetic fingerprinting, construct the genetic relationship and determine genetic distance between the studied isolates and identify specific molecular markers for most potent isolate. An informative profile was obtained (Figures 1 and 2). The six used primers produced multiple band profiles with a variable number

Table 2. Characteristics of Chlorpyrifos-degrading strains isolated from contaminated Egyptian soils.

Test	CP5	CP6	CP7	CP8	CP9
Gram's reaction	–	–	–	–	–
Cell shape	Coccobacilli	Short rods	Short rods	Short rods	Short rods
Methyl red	+	+	+	+	+
Motility	+	+	+	+	+
Spores	–	–	-	-	–
Catalase	+	+	+	+	+
KoH (3%)	+	+	+	+	+
Oxidase	+	+	+	+	+
Methyl red	+	+	+	+	+
Levan formation	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Voges-Proskauer test	–	-	–	–	–
Nitrite reduction	–	–	–	–	–
Fermentation of glucose	+	+	+	+	+
Fermentation of mannose	+	+	+	+	+
Fermentation of starch	–	–	–	+	+
Fermentation of arabinose	–	–	–	–	–
Fermentation of fructose	–	–	–	–	–
Fermentation of lactose	–	–	–	–	–
Fermentation of mannitol	–	–	–	–	–
Fermentation of rhamnose	–	–	–	–	–
Fermentation of sucrose	–	+	–	–	–
Production of amylase	+	+	+	–	+
Production of pectinase	+	–	–	–	–
Production of cellullose	+	+	+	+	–
Production of gelatinase	–	–	+	+	+
Production of H_2S	+	–	–	–	–
KCN resistance	–	–	–	+	–

Table 3. Parameters controlling the biodegradation of Chlorpyrifos by *Pseudomonas stuzeri* –B-CP5.

Incubation periods (days)	Phenolic compounds µg/ml	CP Concentration (ml/L)	Phenolic compounds µg/ml	Inoculum size (ml/l)	Phenolic compounds µg/ml	Temperature (ºC)	Phenolic compounds µg/ml	pH	Phenolic compounds µg/ml
1	0.024	0.1	0.256	0.1	0.199	20	0.123	5	0.0
2	0.045	0.15	0.233	0.2	0.298	25	0.199	5.5	0.0
3	0.084	0.2	0.21	0.4	0.244	30	0.229	6	0.199
4	0.122	0.25	0.188	0.5	0.20	35	0.21	6.5	0.25
5	0.157	0.3	0.132	1	0.142	40	0.12	7	0.299
6	0.190	0.35	0.122	2	0.121	45	0.11	7.5	0.312
7	0.234	0.4	0.02	2.5	0.122	50	0.11	8	0.12
8	0.20	0.45	0.01	5	0.1			8.5	0.12
9	0.14	0.5	0.01					9	0.11

and molecular weights of amplified DNA fragments (Table 1). Different polymorphic and monomorphic markers were obtained across RAPD profiles (Table 4 and 6). As regards genetic relationships, data presented in (Table 5) showed that, the highest genetic similarity was between CP8 and CP7& (66%), while the genetic similarity between CP8 and CP6 was the lowest (37%). All isolates gave the same pattern after digestion with two

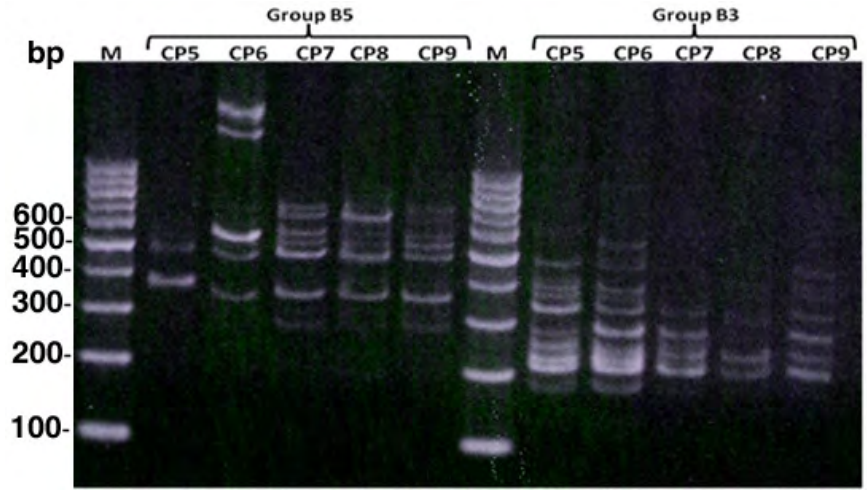

Figure 1. RAPD profile of soil bacterial isolates (CP5, CP6, CP7, CP8 and CP9) revealed from RAPD B5 primer (Group B5) and B3 primer (Group B3).

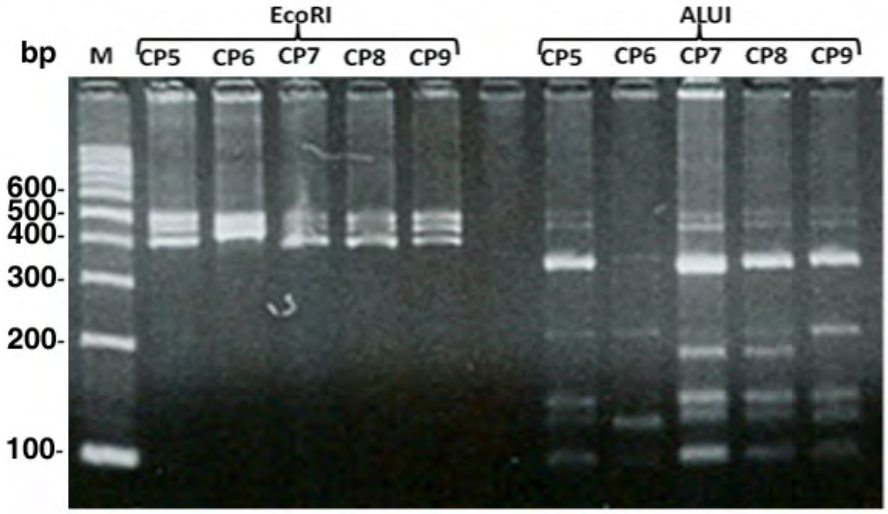

Figure 2. Restriction analysis of a 16SrRNA gene fragment amplified by PCR after digestion with *EcoR1* and *ALU1.*

restriction enzymes (*EcoRI* and *AluI*) separately except CP6 isolate (Figure 2).Two monomorphic bands were obtained (200 and 500 bp) among five bacterial isolates after digestion. Two specific bands for CP6 were identified (420 and 130 bp) with *EcoRI* and ALUI respectively (Figure 2).

DISCUSSION

Obtained molecular genetic data documented that the studied isolates belongs to two different genera as showed in Figure 3. The dendrogram showed phylogenetic tree which divided into two main groups. The first main group consisted of two subgroups. The first subgroup contains CP7, CP8 and CP9 isolates. The closest genetic distance was found between CP7and CP8 isolates, which were first clustered together and then with CP9 isolate. The second subgroup of the first main group includes CP5 isolate. On the other hand, the second group includes CP6 isolate. These two genera have focused on its wide range of diverse degrading capabilities and potential application in bioremediation. This is might be due to the great genetic diversity among these two genera which confirmed by different pattern of amplification and restriction enzyme digestion based on RAPD and PCR-RFLP. Chlorpyrifos has been reported to be degraded co-metabolically by bacteria, which needs extra carbon sources (Richins et al., 1997; Mallick et al., 1999). (Singh et al., 2004) reported isolation of

Table 4. Specific markers for each chlorpyrifos degrading bacterial isolate across RAPD-PCR analysis.

Primer	Bacterial strain					Total
	CP5	CP6	CP7	CP8	CP9	
A2		5(350,520,780,850,870bp)		1(580 bp)	1(320bp)	7
A3		4(230,280,630,680bp)		1(430bp)	1(850bp)	6
B3	2(680,550 bp)	4(500,530,630,700 bp)		2(580,620 bp)		8
B5	1(600 bp)	3(150, 620, 670 bp)				4
C3	2(280,680 bp)	4(480,520,620,740 bp)			2(270, 800bp)	8
C5		6(250,280,320,430,550,740 bp)				6
Total	5	26	0	4	4	39

Table 5. Similarity coefficient values among the five studied bacterial isolates.

Isolates	CP 5	CP 6	CP7	CP8	CP 9
CP 5	1.00				
CP 6	0.40	1.00			
CP 7	0.44	0.44	1.00		
CP 8	0.53	0.37	0.66	1.00	
CP 9	0.54	0.40	0.55	0.62	1.00

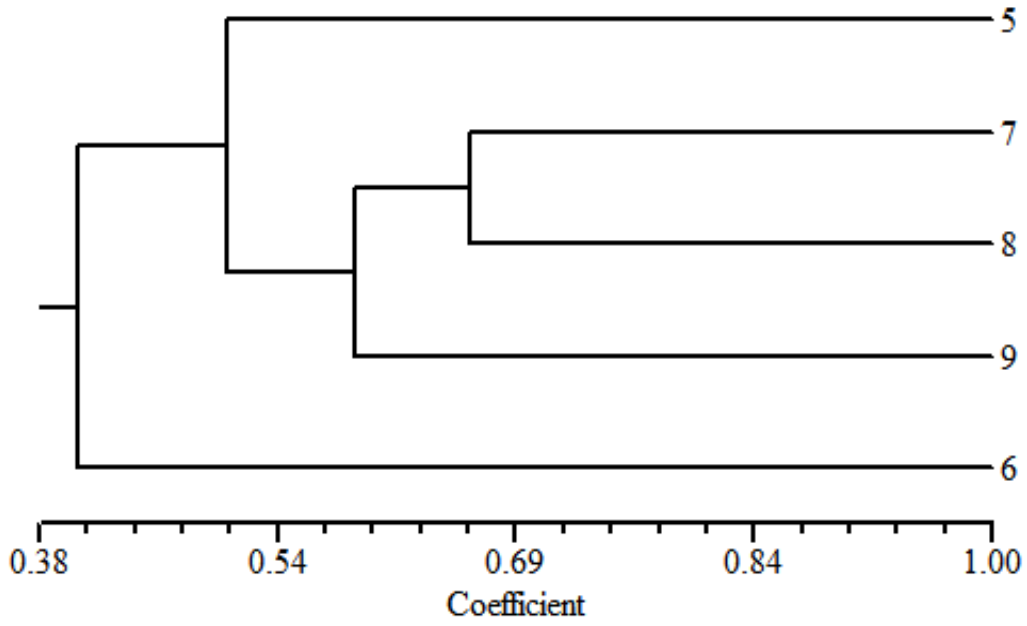

Figure 3. Dendrogram demonstrating the relationships among the five studied bacterial isolates.

Enterobacter sp. B14, which can use CP for the supply of carbon and phosphorous sources, which it stopped degrading CP in the presence of other carbon sources. However, strain *Pseudomonas stuzeri* (B-CP5) shows a more rapid degradation in the presence of an additional fructose as carbon source. This indicates that CP could also be degraded co-metabolically by *Pseudomonas stutzeri* (B-CP5), which might signify the environmental adaptation of this bacterium. *Pseudomonas stutzeri* (B-CP5) was distinguished and characterized by three random primers (B3, C3 and B5) among six used primers. Primer B3 generate two polymorphic bands (550 and 680 bp), C3 obtain two polymorphic bands (280 and 680 bp) while B5 release one polymorphic band (600 bp).These polymorphic bands considered as specific markers for *Pseudomonas stutzeri* with the three mentioned primers Table 4. On the other hand CP6 belongs to other genera so it was generate 26 polymorphic bands

Table 6. Specific markers for bacterial isolate across RAPD and16SrRNA PCR-RFLP analysis.

Primer	Isolate	Specific marker (M.W. bp)
C5	CP6:*Enterobacter aerogenes*	250, 280, 320, 430, 550 and 740
B3	CP5: *Pseudomonas stuzeri*	550 and 680
	CP6:*Enterobacter aerogenes*	500, 530 and 680
	CP8:*Pseudomonas maltophila*	520, 580 and 630
C3	CP5: *Pseudomonas stuzeri*	280 and 680
	CP6:*Enterobacter aerogenes*	480, 520, 620 and 740
	CP9:*Pseudomonas vesicularis*	270 and 800
B5	CP5: *Pseudomonas stuzeri*	600
	CP6:*Enterobacter aerogenes*	150, 620 and 750
A2	CP6:*Enterobacter aerogenes*	350, 520, 780, 850 and 870
	CP8:*Pseudomonas maltophila*	580
	CP9:*Pseudomonas vesicularis*	320
A3	CP6:*Enterobacter aerogenes*	230, 280, 630 and 680
	CP8:*Pseudomonas maltophila*	430
	CP9:*Pseudomonas vesicularis*	850
EcoRI	CP6:*Enterobacter aerogenes*	420
AluI	CP6:*Enterobacter aerogenes*	130

among six used random primers and two polymorphic bands (420 and 130 bp) with EcoRI and AluI, respectively. These polymorphic bands considered as specific markers for *Enterobacter aerogenes*.

Chlorpyrifos has been reported to be degraded in liquid media by *Flavobacterium* sp. (Sethunathan et al., 1973), *Pseudomonas diminuta* (Serdar et al., 1982) and *Arthrobacter* sp. (Mallick et al., 1999), which were initially isolated to degrade other organophosphate compounds. However, these microorganisms do not utilize CP as a source of carbon but as a co-metabolite (Singh et al., 2003; Yang et al., 2006). Recently a *Stenotrophomonas sp*. has been isolated, which is capable of degrading both CP and TCP (Yang et al., 2006; Lakshmi et al., 2008)

In conclusion, among the five studied isolates *Pseudomonas stuzeri* (B-CP5) was the most potent isolate to degrade the Chlorpyrifos. The results of this study demonstrate the usefulness of the RAPD-PCR analysis for detecting DNA polymorphism in bacterial isolates and establishing the relationships among different isolates. The majority of random primers used gave distinctly reproducible patterns in the entire isolates studied. However, primers are varied in the extent of information. Primers B5 and A2 revealed highly polymorphic patterns. Whereas, less polymorphic products were generated by C3 primer. Some DNA fragments were apparently similar in size among the studied isolates, whereas others were unique to a particular isolate. Therefore, different polymorphic and monomorphic bands were given and some of monomorphic considered as specific markers for identifying and tracking these bacterial isolates.

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Full Length Research Paper

Differential diagnosis of *Entamoeba* spp. in gastrointestinal disorder patients in Khorramabad, Iran

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Differential diagnosis of *Entamoeba* spp. has great clinical and epidemiological importance. *Entamoeba moshkovskii* cysts and trophozoites are morphologically indistinguishable from *Entamoeba dispar* and *Entamoeba histolytica*. This study was carried out for the first time to detect *Entamoeba* spp. in stool samples by using molecular method from April 2010 to December 2010 in Khorramabad, Iran. A total of 862 fecal specimens were collected from patients having abnormal gastrointestinal symptoms and who were referred to the health care centers of Khorramabad. Out of 862 stool samples, 16 (1.86 %) showed the presence of *E. histolytica/E. dispar/E. moshkovskii* cysts by microscopic examination. Consequently, single-round PCR was carried out to differentiate the *Entamoeba* spp. OF the sixteen samples that were microscopically positive, 1 (6.25%) was *E. moshkovskii*, and 15 (93.75%) were *E. dispar*. Our results, along with those of other similar study conducted in different parts of Iran, reveal that *E. dispar* is more prevalent.

Key words: *Entamoeba histolytica, Entamoeba dispar, Entamoeba moshkovskii,* single-round PCR, Iran.

INTRODUCTIONS

Amoebiasis is still one of the main health problems in tropical and sub tropical regions with low health and economic level (Simonetta et al., 2003). Since microscopic diagnosis in differentiation of these species has a low sensitivity and accuracy, so we need much more accurate methods for differentiation of these species (Nazemalhosseini-Mojarad et al., 2010a).

For a tenth of people, *E. histolytica* become an invasive disease and one hundred thousand people die because of amoebiasis disease which makes it the second fatal protozoan disease in the world after malaria (WHO, 1997; Diamond and Clark, 1993).

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Studies have shown that much infection in the world is for *E. dispar* (Heckendorn et al., 2002; Clark, 1998). Also studies in Iran confirmed that prevalence of *E. dispar* is much more than *E. histolyica* (Hooshyar et al., 2004; Nazemalhoseini-Mojarrad et al., 2007; 2010a).

Entamoeba moshkovskii cysts and trophozoites are morphologically indistinguishable from those of the non-pathogenic *Entamoeba dispar* and *Entamoeba histolytica* that is causative agent of amoebiasis and can therefore confound annotation of stool microscopy (Ali et al., 2003). The organism is mainly free-living amoeba that rarely infects humans (Clark and Diamond, 1991; Clark and Diamond, 1997). A high incidence of *E. moshkovskii* infections was reported in stool specimens from Bangladesh and Australia and Turkey (Ali et al., 2003; Foteder et al., 2008; Tanyuksel et al., 2007). Three *E. moshkovskii* infections and one simultaneous infection

with *E. moshkovskii* and *E. dispar* have also been reported in Iran (Nazemalhosseini-Mojarad et al., 2010a; Solaymani–Mohammadi et al., 2006). An expert consultation on amoebiasis in Mexico City recommended the collection of accurate new data on the prevalence of *Entamoeba* species for planning rational control strategies (WHO, 1997). Differential diagnosis of *E. histolytica, E. dispar and E. moshkovskii* has great clinical and epidemiological importance (Nazemalhosseini-Mojarad et al., 2010a). One of the most important advantages of this differentiation is avoiding unnecessary treatment with anti-amoebic chemotherapy and decreasing economic cost, side effects and drug resistance (WHO, 1997; Diamond and Clark, 1993). This study was carried out for the first time to detect *E. moshkovskii, E. histolytica* and *E. dispar* in stool samples from patients clinically suspected to have gastrointestinal infections by using of single–round PCR in Khorramabad, Lorestan province, Iran.

MATERIALS AND METHODS

Study area and clinical samples

The descriptive study was conducted from April 2010 to December 2010 in Khorramabad which is located between valleys of Zagros Mountain at the west of Iran. A total of 862 fecal specimens were collected from patients having abnormal gastrointestinal symptoms (such as diarrhea, abdominal pain, nausea and flatulence) who were referred to the health care centers of Khorramabad, Iran. The stool samples were examined microscopically by using direct slide smear, lugol's iodine, formalin–ether concentration, and trichrome staining. The suspected samples were stored at -20°C for later use.

DNA preparation

Genomic DNA was extracted directly from stool specimens were microscopically positive by using a QIAamp® DNA Stool Kit (QIAGEN) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until PCR amplification.

PCR reaction

A single–round PCR reaction and set of primers were used as described previously (Hamzah et al., 2006). The sequence of the forward primer used was conserved in all three *Entamoeba* spp., but the reverse primers were specific for apiece. The expected PCR products from *E. histolytica, E. dispar* and *E. moshkovskii* were 166 bp, 752 bp, and 580 bp, respectively (Hamzah et al., 2006).

Amplification of each species–specific DNA fragment started with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min (Hamzah et al., 2006). PCR products were visualized with ethidium bromide staining after electrophoresis on 1.5% agarose gels. DNA isolated from axenically grown *E. histolytica KU2*, *E. dispar AS 16 IR* and *E. moshkovskii Laredo* (ATCC accession no. 300 42) (Nazemalhosseini-Mojarad et al., 2010a) were used as positive controls. The study was approved by Medical Research Ethics Committee of Lorestan University of Medical Sciences.

RESULTS

Out of 862 stool specimens, 359(41.65%) of whom were female and 503(58.35%) male, 16(1.86%) showed the presence of *E. histolytica/E. dispar/E. moshkovskii* cysts by microscopic examination. After DNA extraction, the single-round PCR was carried out to differentiate the *Entamoeba* spp. of the sixteen samples that were microscopically positive, 1 (6.25%) was *E. moshkovskii* and 15 (93.75%) were *E. dispar*. Infection of *E. histolytica* was not observed in this study. Amplification produced fragments of 752bp and 580bp corresponding to the expected products from *E. dispar* and *E. moshkovskii*, respectively (Figure 1).

DISCUSSION

E. histolytica, E. dispar, and *E. moshkovskii* are morphologically indistinguishable from each other, except that trophozoites containing ingested red blood cells are more likely to be *E. histolytica* (Hamzah et al., 2006; Haque et al., 2008; Parija and Khairnar, 2005). Because previous studies have shown that *E. moshkovskii* could infect humans, identification of this protozoan parasite to the species level has importance (Ali et al., 2003). *E. moshkovskii* from human samples has already been reported in Bangladesh, India, Australia, Turkey, and Iran (Ali et al., 2003; Tanyuksel et al., 2007; Nazemalhosseini-Mojarad et al., 2010a; Solaymani–Mohammadi et al., 2006; Parija and Khairnar, 2005; Fotedar et al., 2007a, b). 109 stool samples from preschool children in Bangladesh were tested by PCR, 17 (15.6%) were positive for *E. histolytica*, 39 (35.8%) positive for *E. dispar*, 23 (21.1%) were positive for *E. moshkovskii* infection (Ali et al., 2003). In another study 746 stool specimens from patients with gastrointestinal disorder in India were screened, prevalence of *E. dispar*, *E. moshkovskii* and *E. histolytica* were 8.8, 2.2 and 1.7%, respectively (Parija and Khairnar, 2005).

This is the first study to detect *Entamoeba* spp. in stool samples from patients clinically suspected to have gastrointestinal infections in Khorramabad, Lorestan province, Iran. Our results, along with those of other similar study conducted in different parts of Iran, reveal that *E. dispar* is much more prevalent than *E. histolytica* (Nazemalhosseini-Mojarad et al., 2010a, b; Hooshyar et al., 2004; Nazemalhoseini-Mojarrad et al., 2007). From central, southern, and northern Iran in both urban and rural areas 16,592 stool samples were collected and results showed that the average prevalence of infection with *E. histolytica/E. dispar* is 0.78, 4.6 and 3.9%, respectively (Hooshyar et al., 2004).

Also PCR-RFLP analysis showed that 92.1% were *E.*

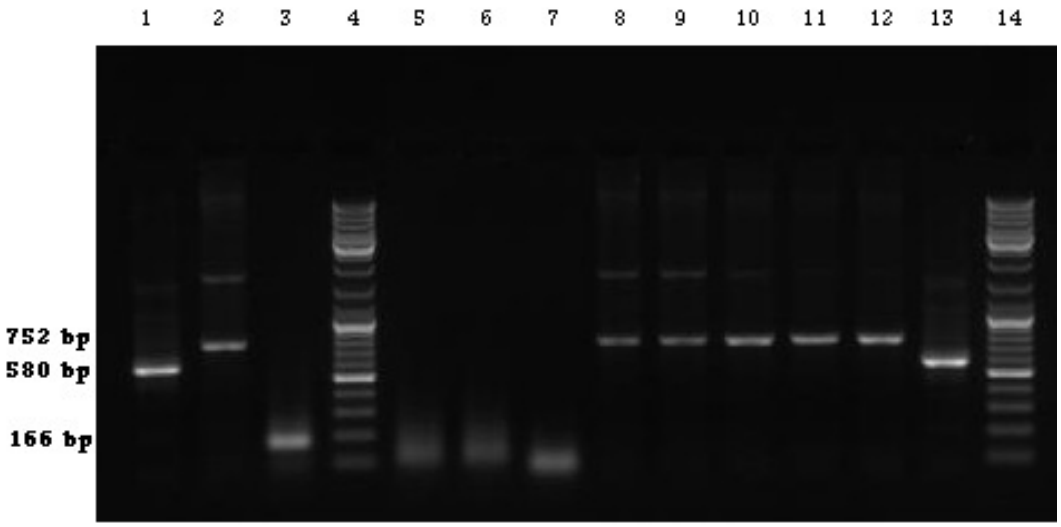

Figure 1. Agarose gel electrophoresis of Entamoeba species using single–round PCR.
Lane 1: *E. moshkovskii* – positive isolate; Lane 2: *E. dispar* – positive isolate; Lane 3: *E. histolytica* – positive isolate; Lane 4, 14: 100bp DNA ladder marker; Lane 5: *E. dispar* negative control; Lane 6: *E. moshkovskii* negative control; Lane 7: *E. histolytica* negative control; Lane 8-12: *E. dispar* isolates ; Lane 13: *E. moshkovskii* isolate.

dispar and only 7.9% were *E. histolytica* and/or mixed infections. The actual prevalence of *E. dispar* and *E. histolytica* in Iran was reported to be 92.7 and 7.3%, respectively (Hooshyar et al., 2004). Similar studies conducted in different countries revealed that the prevalence of *E. dispar* infection is much more than *E. histolytica* (Heckendorn et al., 2002; Clark, 1998). *E. dispar* was estimated that is the cause of 90% of infections in humans with *Entamoeba* spp. (Tanyuksel and Petri, 2003). On the other hand, in some parts of the worlds, such as Mexico and Japan the prevalence of *E. histolytica* infection is high (Petri et al., 2000; Tachibana et al., 2000). Also we found one *E. moshkovskii* isolate in 16 PCR-positive samples that was obtained from a dysenteric stool specimen in which common bacterial agents of dysentery by routine stool culture was negative. Viral dysentery is not common in Iran (Nazemalhosseini-Mojarad et al., 2010a), so this finding supports the opinion that *E. moshkovskii* may has a role in the developing of gastrointestinal symptoms (Tanyuksel et al., 2007; Nazemalhosseini-Mojarad et al., 2010a). But it is not clear *E. moshkovskii* caused the observed symptoms. Some other studies have proposed *E. moshkovskii* to be likely an entropathogen in patients presenting with gastrointestinal symptoms (Foteder et al., 2008; Nazemalhosseini-Mojarad et al., 2010a; Parija and Khairnar, 2005). out of 3,825 stool samples were collected from patients with gastrointestinal disorders in Iran, 2 *E. moshkovskii* (3.45%), and one mixed *E. dispar/E. moshkovskii* infection(1.73%) were detected from dysenteric stool specimens by single–round PCR (Nazemalhosseini-Mojarad et al., 2010a). More reliable data on the prevalence and pathogenesis of *E. moshkovskii* infection are needed to discern the potential role of this amoeba as an entropathogen (Tanyuksel et al., 2007). Conversely, some other reports support the commensal nature (Beck et al., 2008). Solaymani–Mohammadi et al. (2006) found one *E. moshkovskii* from apparently healthy person in Iran (Solaymani–Mohammadi et al., 2006).

Several molecular diagnostic tests have been developed for detection and discrimination of the three morphologically indiscernible *Entamoeba* spp. found in humans, such as conventional and real time PCR, nested multiplex PCR , and single–round PCR assay (Hamzah et al., 2006; Fotedar et al., 2007a, b). In the present study, direct DNA extraction from stool was used, which was recommended by the World Health Organization (WHO/PAHO/UNESCO report, 1997).

By using direct DNA extraction, there is no need to culture; moreover this method is very simple, fast and specific. By using direct DNA extraction from stool sample and with carrying out single-round PCR, the detection of infection is so fast. Because of morphological similarities of *Entamoeba* spp. and lack of differentiation of them in stool samples of macroscopic experiments, using single–round PCR which is a specific method, and also using direct DNA extraction of stool specimen which is an easy, fast and specific method is suggested for routine diagnosis of disease and epidemiological studies (Nazemalhosseini-Mojarad et al., 2010a; Hamzah et al., 2006; Clark, 1998).

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Short Communication

First report of black root rot disease (*Thielaviopsis basicola*) of carrot in Saudi Arabia

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During January-February 2010, a black root rot (BRR) disease of carrot (*Daucus carota* L.) was observed in vegetable markets of Riyadh, Saudi Arabia. The recovery of *Thielaviopsis basicola* from local carrot fields indicated its appearance in propagules ranged between 8.32-10.15 (total count/g soil). This report describes the first evidence of black root rot disease of carrot caused by *T. basicola* in vegetable markets of Riyadh (Saudi Arabia).

Key words: Black root rot disease, *Thielaviopsis basicola*, Saudi Arabia, *Daucus carota* L.

INTRODUCTION

Carrot (*Daucus carota* L.) is one of the most fresh market vegetables grown in many countries involving Saudi Arabia (Al-Harbi et al., 1997). Black root rot (BRR) disease of carrot caused by *Chalara elegans* Nag Raj and Kendrick [Syn. *Thielaviopsis basicola* (Ferr.)] is one of the most serious diseases that impede carrot production in fields and hinders development of fresh carrot markets (Punja et al., 1992) in many production countries.

METHODOLOGY

During January-February 2010, a BRR disease of Saudi carrot was observed in vegetable markets of Riyadh, Saudi Arabia. Symptoms include the developed patches of superficial black lesions on the surface of carrot roots [Figure 1B]. These patches are irregular in shape, occur in random pattern [Figure 1B] and are sometimes surrounded by matt white halo [Figure 1C]. The infectious surface area seems to increase in both size and darkness (Black) resulting in covering of all root surface [Figure 1D].

No field symptoms had been noted although the recovery of *T. basicola* from local carrot fields indicated its appearance in propagules ranged between 8.32-10.15 (total count/g soil). The pathogen propagules in the soil used dilution plate agar method on a selective medium of Specht and Griffin (1985). Pathogenicity testing of the isolates demonstrated the role of *T. basicola* in disease incidence. In this connection, it is necessary to mention that, during the last few years, carrot growers have begun to brush carrots before storage to remove the outer peel of the root. Such practice may justify the appearance of BRR as only postharvest. Usually, carrots are stored in polyethylene bags (10 kg in capacity) at 10-15°C for 3-5 days (wholesale markets). Subsequently, it is stored at 15-20°C for 5 days (retail markets). The disease incidence was 3 and 12% for wholesale markets and retail markets, respectively.

Isolation of pathogen

The fungal pathogen was frequently isolated on a selective medium (Specht and Griffin, 1985) from the surface of carrot root carrying ideal symptoms (naturally infected) of BRR disease (Punja et al., 1992). Single hyphal transfers was carried out on both modified RB-M_2 medium (His, 1978) and carrot disc (Anderson and Welacky, 1988), followed with incubation for five days at 21±1°C. The identification of developed mold was carried out according to Ellis (1971).

RESULTS AND DISCUSSION

The mycelium was initially matt white and later became dark black on both agar medium [Figure 2A] and carrot disc [Figure 2B]. The blackening of the mycelia growth resulted from abundant production of chlamydospores (aleuriospores) which were noticed microscopically in

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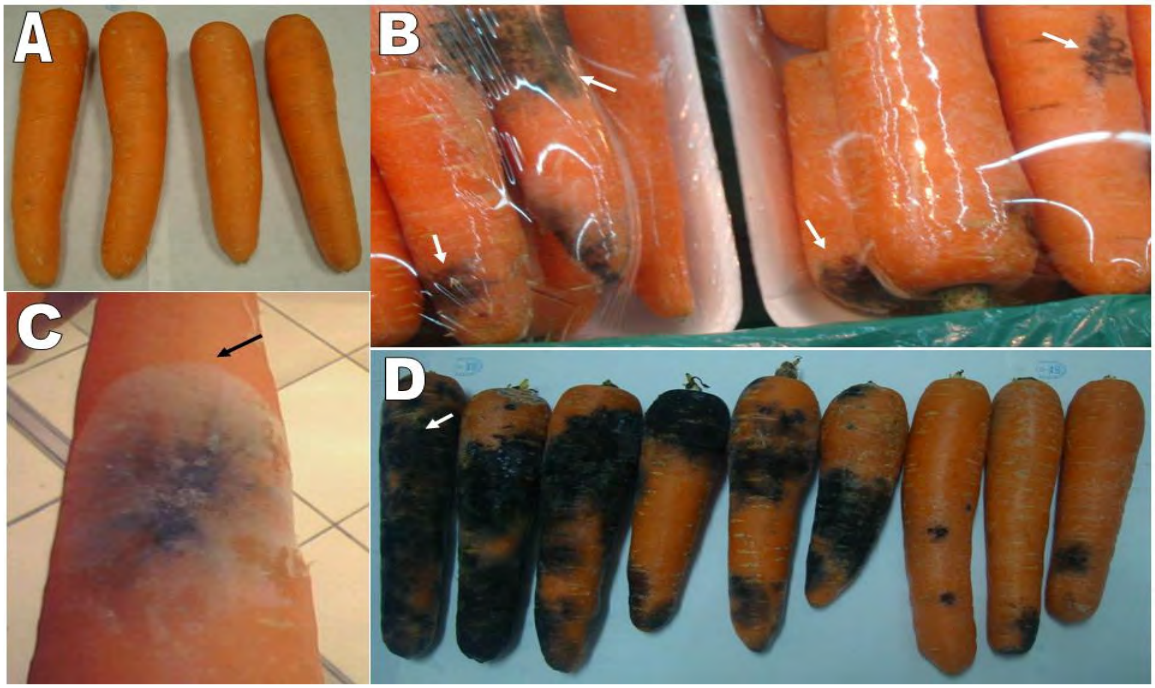

Figure 1. Development of post harvest black root rot (BRR) disease of carrot. A) Healthy control carrot root B) Natural infection of carrot root with BRR disease, note irregular black patches in random pattern (arrows) C) Close-up of black patch surrounded by matt white halo (arrow) D) Subsequent development of black patches (increase in both size and blackness) to cover all carrot root surface (arrow).

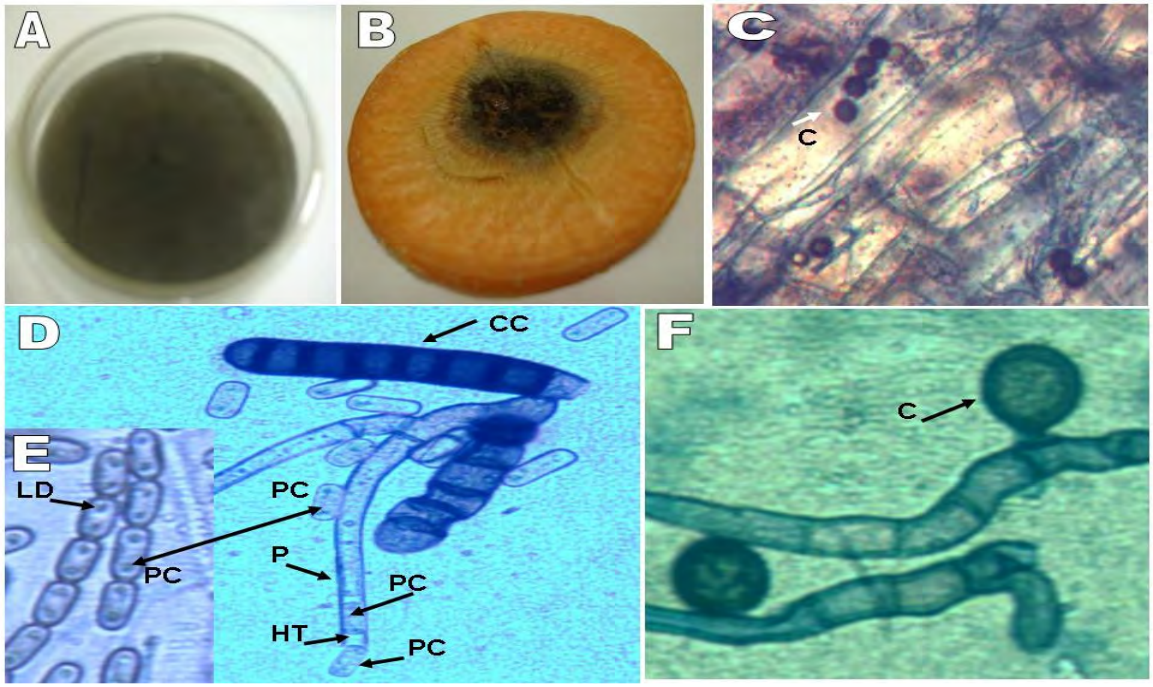

Figure 2. Growth morphology and light micrographs of *Thielaviopsis basicola*. A) Growth of *T. basicola* on modified RB-M_2 medium B) Mold growth on carrot disc C) Dark melanized independent chlamydospores (aleuriospores) [C] of *T. basicola* within the epidermal layer of infected carrot root (arrow) D) Development of cluster of dark-thick walled melanized entire chlamydospores (aleuriospores) [CC] (arrow) E) Phialoconidia [PC] arise from elongate phialides [P] (arrow) and enclosed in hyphal tube [HT] (arrow); Phialoconidia [PC] contain clusters of lipid droplets [LD] at the polar ends (arrow) F) Dark melanized thick wall chlamydospores (aleuriospores) [C] produced singly in lateral pattern (arrow).

epidermal layer of infected carrot root [Figure 2C]. The microscopic examination indicates the presence of two kinds of spores (conidia), phialoconidia [PC] and chlamydospores (aleuriospores) [CC] (Figure 2D). Phialoconidia [PC] was produced from elongated phialides (P) composed of rounded bases with a long narrow cylindrical neck or barrel (Figure 2D). Phialoconidia (PC) was typically rectangular with rounded corners, subhyaline non-septate and contains clusters of lipid droplets (LD) at the polar ends with central clear regions (Figure 2E) where the nucleus is located. Chlamydospores (aleuriospores) showed characteristic dark melanized thick wall. Chlamydospores produced [C] singly in lateral pattern [Figure 2F] or in cluster (chain) pattern of entire conidiogenous cells (CC) (Figure 2D). Based on the symptoms and mycelia characters (colony morphology) with the help of microscopic investigation, the fungus was identified as *T. Thielaviopsis basicola*.

CONCLUSION AND RECOMMENDATION

This report describes the first evidence of black root rot disease of carrot caused by *T. basicola* in vegetable markets of Riyadh (Saudi Arabia). Further investigation focusing on plant disease survey in fields and its control is needed via biologically based strategies, which will be our target in future.

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Short Communication

Detection of inducible clindamycin resistance by an automated system in a tertiary care hospital

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Clindamycin is commonly used in treatment of erythromycin resistant *Staphylococcus aureus* causing skin and soft tissue infections. *In vitro* routine tests for clindamycin susceptibility may fail to detect inducible clindamycin resistance due to '*erm*' genes resulting in treatment failure thus necessitating the need to detect such resistance by a rapid method. In the era of automation, vitek-2 system provides a panel for detection of inducible clindamycin resistance with conjunction of other antimicrobial susceptibility testing. The present study evaluated the performance of a vitek-2 card for detection of inducible clindamycin resistance in a tertiary care hospital. Non-duplicate clinical isolates of hundred *S. aureus* were obtained from various clinical samples. Antimicrobial susceptibility testing was carried out, including detection of clindamycin resistance and methicillin resistance pattern by vitek-2 identification and antimicrobial susceptibility testing (ID/AST) system by using AST-GP67 card. The results were compared to those of D-test as per CLSI guidelines on erythromycin resistant isolates. EPI INFO software, Licensed by CDC Atlanta was used for analysis of data. The sensitivity and specificity for the vitek-2 card was 95.4 and 100%, respectively in comparism to disk approximation test (D-test),. The performance of vitek-2 card was 100% specific and rapid for detection of inducible clindamycin resistance with other antimicrobial susceptibility results.

Key words: Automated system, constitutive macrolide-lincosamide-streptogramin B (MLS_B) phenotype, inducible MLS_B phenotype, methicillin resistance *Staphylococcus aureus* (MRSA), msrA gene (MS phenotype).

INTRODUCTION

Emergence of methicillin resistance in *Staphylococcus aureus* (MRSA) has left us with very few therapeutic alternatives available to treat staphylococcal infections. The macrolide-lincosamide-streptogramin B (MLS_B) family of antibiotics serves as one such alternative, with clindamycin being the preferred agent due its excellent pharmacokinetic properties (Fiebelkorn et al., 2003).

The development of resistance in *Staphylococcus* species to Macrolide, lincosamide and streptogramin B has limited the use of these antibiotics. Macrolide resistance may be due to enzymes encoded by a variety of *erm* genes-MLS_B phenotype and may be constitutive ($cMLS_B$ phenotype) or inducible ($iMLS_B$ phenotype). Another mechanism is active efflux pump encoded by the *mrs* A gene (MS phenotype). The MS and $iMLS_B$ phenotypes are indistinguishable by using standard susceptibility test methods, but can be distinguished by erythromycin-clindamycin disk approximation test (D-test), automated vitek-2 system and demonstration of resistance genes by molecular methods (Steward et al., 2005; Pal et al., 2010).

The aim of this study was to determine

1. The rate of inducible clindamycin resistance in both methicillin-resistant and susceptible strains of *S. aureus* in our hospital as data describing $iMLS_B$ prevalence among *S.* aureus isolates in this region is unknown.
2. To know the sensitivity and specificity of automated vitek-2 system in detecting inducible clindamycin resistance within 8 to 16 h with other antimicrobial susceptibility results in comparison to D-test which will

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Table 1. Distribution of isolates.

S/N	Phenotype	MRSA (%)	MSSA (%)	Total (%)
1	ER-S,CL-S	0	14	14
2	ER-R, CL-R ($cMLS_B$)	8	4	12
3	ER-R,CL-S,D^- (MS)	6	25	31
4	ER-R,CL-S, D + ($iMLS_B$)	28	15	43
5	Total	42	58	100

ER, erythromycin; CL, clindamycin; $cMLS_B$, constitutive resistance to clindamycin; $iMLS_B$,, inducible clindamycin resistance; D^-, negative D-test; D+, positive D-test; MS, MS phenotype.

take 48 h as per CLSI guidelines.

MATERIALS AND METHODS

One hundred isolates of *S. aureus* were recovered from blood, pleural fluid, pus, sputum, tracheal aspirate, and other specimens received in the Department of Microbiology over a period of three and half months from December 2010 to mid March, 2011.

All the *S. aureus* were identified by conventional microbiological methods including colony morphology, gram stain, catalase, and slide coagulase and tube coagulase test.

All the *S. aureus* were subjected to antimicrobial susceptibility testing including detection of inducible clindamycin resistance and cefoxitin screening test by vitek-2 identification and antimicrobial susceptibility testing (ID/AST) system by using AST-GP67 card, which incorporated to the Advanced Expert System (AES), software which validates and interprets susceptibility test results and detects antibiotic resistance mechanisms. The CLSI susceptibility breakpoints were used.

The Vitek 2 AST-GP67 card (bioMe´rieux, Marcy l'E´toile, France) was used according to the manufacturer's recommendations. Briefly, three to five colonies of an 18 to 24 h-old culture of *S. aureus* were inoculated in a 0.45% NaCl solution and adjusted to a concentration equivalent to a 0.5 to 0.63 McFarland standard. The solution was then loaded with the card in the Vitek 2 system. The incubation period was determined by the Vitek 2 system. Two wells are used to detect inducible clindamycin resistance in the Vitek 2 card: One with 0.5 mg of clindamycin/liter and another one with a combination of 0.25 and 0.5 mg of clindamycin and erythromycin/liter, respectively. Both the instrument and the Advanced Expert System (AES) results were considered.

Disk approximation testing (D-test) was performed for each isolate according to Clinical and Laboratory Standards Institute (CLSI) method. A 0.5 McFarland suspension was prepared in normal saline for each isolate and inoculated on Mueller-Hinton agar plate. Clindamycin (CLI)-2 µg and erythromycin (ER)-15 µg disks (HIMEDIA, Mumbai) were placed 15 mm apart edge to edge manually. Plates were incubated at 35°C for 24 h and zone diameters were recorded. Induction test categories were interpreted given as:

1. MS phenotype - Staphylococcal isolates exhibiting resistance to erythromycin (zone size ≤ 13 mm) while sensitive to clindamycin (zone size ≥ 21 mm) and giving circular zone of inhibition around clindamycin was labeled as having this phenotype.
2. Inducible MLS_B phenotype - Staphylococcal isolates showing resistance to erythromycin (zone size ≤13 mm) while being sensitive to clindamycin (zone size ≥ 21 mm) and giving D shaped zone of inhibition around clindamycin with flattening towards erythromycin disc were labeled as having this phenotype.
3. Constitutive MLS_B phenotype - this phenotype was labeled for those Staphylococcal isolates which showed resistance to both erythromycin (zone size ≤ 13 mm) and clindamycin (zone size ≤ 14 mm) with circular shape of zone of inhibition if any around clindamycin.

Statistical analysis

Statistical analysis was performed using EPI INFO software; Licensed by CDC Atlanta was used for analysis of data.

RESULTS

One hundred Staphylococcal isolates included in the present study were tested for antimicrobial susceptibility testing including detection of inducible clindamycin resistance and cefoxitin screening test by vitek-2 identification and susceptibility testing (ID/AST) system by using AST-GP67 card. Of the 100 *S. aureus* isolates, 86% were erythromycin resistant. These isolates when subjected to D test showed that 12% isolates were resistant to both erythromycin and clindamycin indicating constitutive MLS_B phenotype; 74% isolates showed clindamycin sensitivity. Out of these, 43% showed positive D test indicating inducible MLS_B phenotype while 31% gave negative D test indicating MS phenotype (Table 1). The overall percentage resistance for all three phenotypes was as follows:

Inducible clindamycin resistance = 43%
Constitutive clindamycin resistance = 12%
MS Phenotype = 31%

Percentage of inducible clindamycin resistance is higher in MRSA as compared to methicillin susceptible *S. aureus* (MSSA) (p value is 0.0119 which is highly significant, by z-test).

There was no difference between results obtained from the instrument and the advanced expert system (AES). Inducible clindamycin resistance was not detected in two strains that were D-test positive. The specificity was 100% (Table 2).

Table 2. The performance of the vitek-2 card.

Vitek-2 result	No. of isolates with:		Total
	D-test positive	D-test negative	
Positive	41	0	41
Negative	2	57	59
Total	43	57	100

Sensitivity = 95.4%; Specificity = 100%; Negative predictive value (NPV) = 96.6%; Positive predictive value (PPV) = 100%. When these 2 strains were retested, they were still negative with the vitek-2 card for the second time. Again, the results were similar when considering MSSA and MRSA, separately.

DISCUSSION

In the context of increasing prevalence of community-acquired MRSA, alternative drugs to treat skin and soft tissue Infections are needed. Clindamycin appears to be an interesting option because of the availability of an oral formulation, good bioavailability, and distribution in skin and abscesses. (Daurel et al., 2008) Resistance to clindamycin is highly variable in different patient populations (Moore et al., 2008; Schreckenberger et al., 2004) and, if this drug is to be used, rapid susceptibility testing for inducible clindamycin resistance must be available. In order to choose an appropriate method for each laboratory and patient populations, the performance has to be evaluated.

The vitek-2 system was reported to be 98% sensitive in detecting inducible clindamycin resistance in a study (Nakasone et al., 2007) that tested 62 strains of *Staphylococcus* spp. In two other studies, the sensitivity for inducible clindamycin resistance detection was 99% (Griffith et al., 2007; Sharp et al., 2009). In a study (Pal et al., 2010) sensitivity and specificity of vitek-2 system is 93 and 100%, respectively. In the present study, the Vitek 2 card failed to detect inducible clindamycin resistance in 2 strains (negative predictive value of 96.6%. Both of these isolates were from clinical specimens of different patients and were found in the different wards of the hospital. There were no false positive results for inducible clindamycin resistance with the vitek-2 card. So, positive results can be reported without further confirmation with D-test. Our findings indicate that the vitek-2 card would allow 96.6% of isolates to be correctly reported as resistant concomitantly with other antimicrobial susceptibility results. Laboratories that want to reach 100% sensitivity would still have to test erythromycin resistant/ clindamycin susceptible isolates that showed negative results for inducible clindamycin resistance with the vitek-2 card with the D-test.

The use of vitek-2 system in routine Laboratory will enable us in guiding the clinicians regarding judicious use of clindamycin in skin and soft tissue infections as clindamycin is not a suitable drug for positive inducible clindamycin resistance (ICR) isolates while it can definitely prove to be a drug of choice in case negative ICR isolates. Vitek-2 system also provides other therapeutic options of antibiotics along with ICR result.

As per our knowledge, this is the first study in India comparing two methods of detection of inducible clindamycin resistance. We raise the concern about the two strains of *S. aureus* in which h the $iMLS_B$ were not detected with vitek-2 card.

We conclude that it is important for laboratories to be aware of the local prevalence of $iMLS_B$ isolates. On the basis of their data, they can choose which technique to use for detection of inducible clindamycin resistance in routine depending on their local epidemiology and availability of automated vitek-2 system in their institution. This prevalence of $iMLS_B$ may change over time with the emergence of strains with different sensitivity patterns, so periodic surveys should be performed if testing is not routine.

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