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Giesielski SD, Seed TR, Ortiz JC, Melts J (2001). Intestinal parasites among North Carolina migrant farm workers. *Am. J. Public Health.* 82: 1258-1262

Stoy N, Mackay GM, Forrest CM, Christofides J, Egerton M, Stone TW, Darlington LG (2005). Tryptophan metabolism and oxidative stress in patients with Huntington's disease. *N. J. Neurochem.* 93: 611-623.

Mussel RL, De Sa Silva E, Costa AM, Mandarim-De-Lacerda CA (2003). Mast cells in tissue response to dentistry materials: an adhesive resin, a calcium hydroxide and a glass ionomer cement. *J. Cell. Mol. Med.* 7:171-178.

Booth M, Bundy DA, Albonico P, Chwaya M, Alawi K (1998). Associations among multiple geohelminth infections in school children from Pemba Island. *Parasitol.* 116: 85-93.0.

Fransiscus RG, Long JC (1991). Variation in human nasal height and breath, *Am. J. Phys. Anthropol.* 85(4):419-427.

Stanislawski L, Lefevre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A (2003). TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. *J. Biomed. Res.* 66:476-82.

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The **Introduction** should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

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The conclusion should highlight the contribution of the study and its relevance in general medical knowledge

The **Acknowledgments** of people, grants, funds, etc should be brief.

References: Same as in regular articles

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Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

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Review

Applications of recombinant protein therapeutic agents in periodontics contributors

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Accepted 29 March 2013

Based on the improved understanding of cell and molecular biology of periodontal wound healing, the recombinant technology comprising rh growth factors and carrier construct is applied in periodontal regeneration. In 1997, the first recombinant (that is, synthetic) protein therapeutic agent was approved by the US Food and Drug Administration (FDA). In this paper we review the two commercially available recombinant agents, that is, rhPDGF-BB and rh-BMP-2 used for periodontal regeneration.

Key words: Periodontal regeneration, recombinant proteins, gene, tissue regeneration.

INTRODUCTION

According to Murakami and Noda (2000), in normal wound healing multiple cytokines act in concert to regulate the cellular functions of various cell types within and adjacent to a wound in nearly all tissues, including the periodontium. Also, signalling molecules such as growth factors and morphogens are capable of stimulating cellular events. Tissue engineering or recombinant technology could be a more predictable modality, which can modulate the wound healing with supply of abundant growth factors.

Tissue engineering is a relatively new field of reconstructive biology which utilizes mechanical, cellular, or biologic mediators to facilitate reconstruction/regeneration of a particular tissue.

The goal of tissue engineering and regenerative medicine is to promote healing and ideally, true regeneration of a tissue's structure and function more predictably, more quickly, and less invasively than allowed by previous techniques.

TISSUE ENGINEERING TRIAD

An ideal approach to tissue engineering is based on sound principles of developmental and molecular biology

of signal transduction, and of the cell biology of tissue morphogenesis, including the supramolecular assembly of the extracellular matrix. Using tissue engineering, the wound healing progress is manipulated so that tissue regeneration occurs.

This tissue engineering approach to bone and periodontal regeneration combines three key elements to enhance regeneration (Lynch, 2008) (Figure 1).

1. Conductive scaffolds.
2. Signalling molecules.
3. Cells.

Cells are considered as a major component of the tissue regeneration process. Stem/progenitor cells contribute to the regeneration process. It also requires a scaffold or a supportive template which is necessary for the organization of these replicating cells. And in addition, it requires the presence of certain signaling molecules which act as growth and differentiating factors.

Recombinant protein therapeutics

Characteristics include:

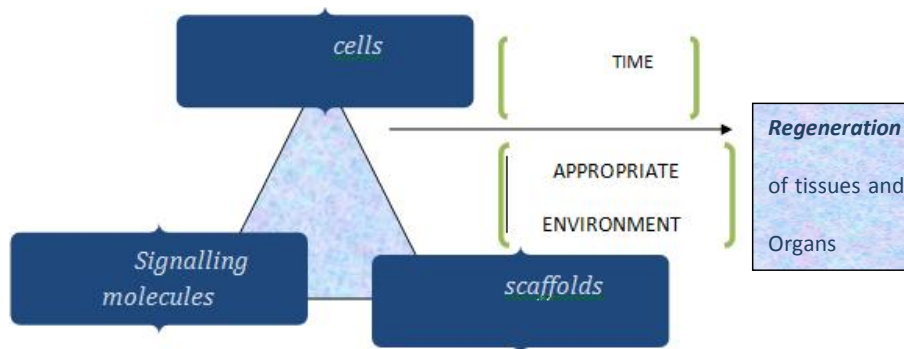


Figure 1. Tissue engineering triad.

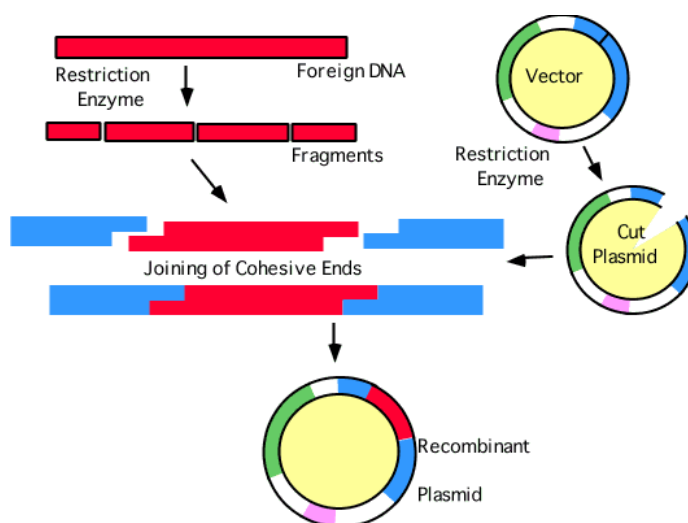


Figure 2. Formation of recombinant protein.

1. Highly concentrated growth modulating molecules. Sutherland and Bostrom, 2007
2. Increased predictability of regenerative results for clinician and patients. Sutherland and Bostrom, 2007
3. Combination products such as regenerative proteins with tissue specific matrices (scaffolds) is the emerging trend.
4. Promising approach to periodontal regeneration.

RECOMBINANT PROTEINS

Derived from: Recombinant DNA.

Recombinant DNA: Is a form of artificial DNA created by either combining 2 or more DNA sequences or inserting it into another DNA strand.

Mechanism/ Procedure

-The gene/ specific DNA sequence from the human cell is selected and isolated.

- Gene is isolated and carefully grafted onto a vector in order to be cloned (the vectors like bacteria are able to grow independently hence are the ideal choice for this purpose).

-Little section of the vector's gene is removed and the gene of interest is implanted into it.

-Bacterial plasmid is the transfected into host cells like yeast/ Chinese hamster ovarian cells/ *Escherichia coli*, capable of large scale growth.

-The grafted gene grows with the vector gene it thereby creating the gene scientists wanted.

- Once it has been created, scientists carefully extract it from them.

-Thus, proteins are synthesized, concentrated purified and packaged in large sterile quantities (Figure 2).

Applications

- i) To diagnose and treat a number of genetic disorders.
- ii) To isolate proteins and for therapeutic purposes.
- iii) To determine gene sequences and mutations.

Table 1. US FDA approved recombinant protein therapeutics.

| Recombinant protein therapeutics | Approved indication |
|---|--|
| rhPDGF-BB (gel) | - Treatment of neuropathic ulcers (Wieman et al., 1998). - No applications in periodontics (Nevins and Giannobile, 2003). |
| rhPDGF-BB (with β tricalcium phosphate) | Treatment of intrabony and furcation periodontal defects and gingival recession associated with periodontal defects (Nevins and Giannobile, 2003) |
| rhBMP-2 (with type 1 collagen sponge) | As an alternative to autogeneous bone graft for sinus augmentations and for localized alveolar ridge augmentations for defects associated with extraction sockets (Boyne and Marx, 1997) |

Table 2. *In vivo* studies.

| Factor | Animal studies | Result | Studies |
|----------------------------|----------------|--|--------------------------|
| PDGF-BB | Beagle dog | Promoted PDL fibroblast proliferation | Wang et al. (1994) |
| | Monkey | New attachment | Giannobile et al. (1996) |
| PDGF/dexamethasone | Monkey | Regeneration | Rutherford et al. (1993) |
| PDGF –BB/ePTFE/citric acid | Beagle dog | Regeneration | Cho et al. (2002) |
| BMP-2 | Baboon | Regeneration | Ripamonti et al. (1994) |
| | Beagle dog | Increase in bone and cementum (rare ankylosis or resorption) | Sigurdsson et al. (1993) |

iv) First applied clinically tube used as recombinant human insulin.

Other medically used recombinant forms include:

- i) rh growth hormone
- ii) rh blood clotting factors
- iii) rh hepatitis vaccine
- iv) rh PDGF
- v) rh BMP 2,7.

RECOMBINANT PROTEINS IN PERIODONTAL REGENERATION

List of recombinant proteins used in various trials of periodontal regeneration include:

- 1) rh PDGF-BB plus rh IGF-1
- 2) rh PDGF-BB plus β -TCP
- 3) rh BMP-2 with type I collagen
- 4) rh bFGF (rh FGF-2)
- 5) rh TGF- β
- 6) rh osteogenic potential 1/ BMP-7
- 7) BDNF
- 8) GDF-5.

To date, only three recombinant growth factor products have been widely *commercialised*, for use in tissue regeneration (Table 1) (Lynch, 2008)

Among these only rh PDGF-BB with tricalcium phosphate and rh BMP-2 with type 1 collagen sponge are used in periodontics.

Review of articles on *in vivo*, *in vitro* and human studies are as shown in Tables 2, 3 and 4, respectively.

RECOMBINANT HUMAN PLATELET DERIVED GROWTH FACTOR (PDGF)–BB

Platelet derived growth factor (PDGF) is a naturally occurring protein found abundantly in bone matrix forms which includes PDGF-AA, PDGFB and PDGF-AB. Major sources include platelets, macrophages, epithelial cells, endothelial cells, smooth muscles and bone matrix.

Significance

- 1. Released locally during clotting by blood platelets at the site of injury.
- 2. Stimulates wound healing response.
- 3. Promotes rapid cellular migration (chemotaxis).

Table 3. *In vitro* studies.

| Factor | Cell type | Result | Studies |
|----------------------------|---|--|--|
| PDGF- BB | Rat PDL cells | Mitogenic effect PDGF>FGF>EGF | Blom et al. (1994) |
| | Human PDL cells | Increased mitogenic activity | Oates et al. (1993) |
| | | An increased proliferation plus chemotaxis: PDGF BB>AB>AA Capable of upregulating collagen synthesis in the extracellular matrix | Boyan et al. (1994) Tomoyuki kawase (2003) |
| | Human gingival fibroblasts | Increased hyaluronate synthesis, blocked inhibitory effects of LPS on cell growth | Bartold and Raben (1992) |
| | | Mitogenic | Piche et al. (1992) |
| | Human PDL and gingival fibroblasts | PDGF alone had a greater proliferation effect | Boyan et al. (1994) |
| | Osteoblasts | Promote chemotaxis, matrix synthesis, and mitogeneis | Canalis et al. (1989) Piches Graves (1992) Hughes and Aubin (1992) |
| | | Involved in maturation and remodelling of newly formed blood vessel, angiogenic and vasculogenic cells might act as important target initially responding to this mitogenic factor | Risau (1997). |
| | | Have direct and indirect effects on bone resorption by the upregulation of collagenase transcription | Rydzziel et al. (2000) |
| | | increase in IL 6 expression in osteoblasts | Franchimont et al. (1997) |
| BMPs | Mesenchymal cells | Accelerated provisional extracellular matrix deposition and subsequent collagen formation | Glenn et al. (2004) |
| | Osteoblasts | Promote osteoblast phenotype | Ripamonti et al. 1994 |
| | | osteopontin and osteocalcin expressed in late stages of osteoblast differentiation | |
| | | Sequential expression of osteopontin and osteocalcin mRNA in the process of ectopic bone formation | Hirota et al. (1994) |
| | | Osteocalcin production depends on BMP-2 concentration | Zhao et al. (2003) |
| | | Upregulate Cbfa1/Runx2 under certain conditions during osteoblast differentiation therefore, this is a candidate down stream target of BMPs , although Smad complexes can also directially interact and activate target genes independently of Cbfa1/Runx2 | Jonk et al. (1998) |
| | Mesenchymal cells | Stimulates osteopontin and osteocalcin. | Lecanda et al. (1997) |
| | | BMP-2,induces the differentiation of undifferentiated cells, 2T9 (osteoblast progenitor cells), in the lineage | Schwartz and Ren (2000) |
| | | Recombinant BMP-2 increased alkaline phosphatase activity and osteocalcin production in the bone marrow stromal cell line | |
| | | Raising the possibility that BMP-2 may be involved in the differentiation of osteoblasts from progenitor cells resident in the bone marrow. | Rosen and Thies (1992) |
| Periodontal ligament cells | No increase in osteopontin or bone sialoprotein within periodontal ligament | Rajshankar et al. (1998) | |

Table 4. Human studies.

| Factor | Defect | Result | Studies |
|---------|---------------------------------|--|--|
| PDGF-BB | Intrabony defect and furcations | Bone fill was seen and there was gain in attachment. | Nevins and Giannobile (2003), Nevins et al. (2005), Nevins et al. (2007) and McGuire et al. (2006) |
| BMP-2 | Intrabony defects | Only clinical attachment was gained | Nevins and Giannobile (2003). |
| | Furcation defects | | |
| | Sinus elevation | Bone fill of about 13.4 mm was obtained | van den Bergh et al. (2000) |

- Promotes cellular proliferation (mitogenesis).
- Promotes regeneration of periodontal tissues including bone, cementum, PDL – (Lynch, 1980).

In 1997, the first recombinant (that is, synthetic) protein therapeutic agent was approved by the US Food and Drug Administration (FDA). The product provides recombinant human PDGF-BB in gel formulation for the treatment of recalcitrant neuropathic dermal ulcers in diabetic patients.

In 2005, rhPDGF-BB + β tricalcium phosphate product was approved for bone and periodontal regeneration and treatment of gingival recession. This product contains approximately 1,000 times higher concentration of PDGF than the level commonly obtained through platelet concentration. (Bowen-Pope et al., 1988, Huang et al., 1983)

rh PDGF-BB is more than 98% pure recombinant protein developed using conventional recombinant expression techniques under highly controlled conditions.

In a landmark study:

- Nevins and Giannobile (2003) showed histological evidence of periodontal regeneration in treated intrabony furcation defects with rh PDGF-BB.
- Nevins (2005) conducted a clinical study on humans with rh PDGF-BB delivered with β -TCP for advanced periodontal osseous defects. Results showed larger gain of CAL, greater bone gain and percentage of defect fill with the combination.
- No adverse affects such as root resorption, ankylosis, inflammation were reported.
- Also, rh PDGF improves bone healing at tooth extraction sites (Cardaropoli, 2003), in patients with diabetes and osteoporosis and in peri-implant bone (Berglundh, 2008).
- Also, McGurie (2006) use PDGF-BB with bone graft material and covered it with collagen membranes in recession sites, thus providing results comparable to CT grafts with no need for second surgical site.

Role of rh PDGF-BB in periodontal regeneration

- It promotes DNA synthesis and chemotaxis in

periodontal ligament cells especially in osteoblastic phenotype (Wang et al., 2004).

- It stimulates collagen and non collagen proteins synthesis (Lynch et al., 1991).

- In cultures of osteoblast-like cells, it downregulates alkaline phosphatase activity and osteocalcin (Zaman et al., 1999).

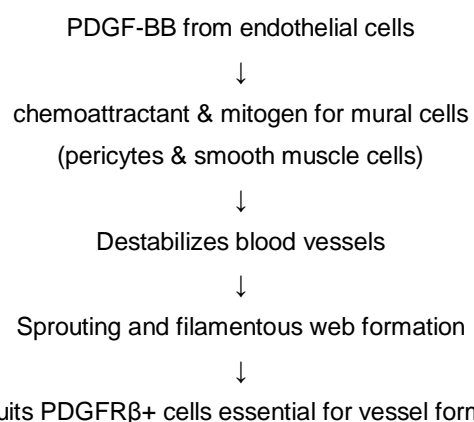
- It enhances demineralised bone matrix induced cartilage and bone formation (Howeels, 1997).

- PDGF increases the pool of osteogenic cells, and cells that will differentiate into cementoblasts and periodontal ligament cells (that is, acts as a chemotactic agent and mitogen); whereas their subsequent differentiation into osteoblasts or chondrocytes is directed by BMP family (Cho et al., 2002; kugimiya et al., 2005), heghhehog proteins, (Murakami and Noda, 2000) and activation of the Wnt- signalling pathway. (Hadjiargyrou et al., 2002)

- It exerts indirect effects on bone regeneration by increasing the expression of angiogenic molecules such as vascular endothelial growth factor (VEGF) (Bouletreau et al., 2002) and hepatocyte growth factor/scatter factor, as well as the proinflammatory cytokine interleukin-6. VEGF is a key molecule in bone regeneration.

Mechanism of action

Genetic models demonstrate that endothelial cell derived PDGF-BB is required to recruit PDGFR- β positive cells and stimulate blood vessel maturation.



Recruits PDGFR β + cells essential for vessel formation

PDGFs can modulate the responsiveness of osteogenic cells to BMPs by increasing the expression of gremlin and IGF signalling. The responsiveness of osteogenic cells to PDGFs can be regulated by the inflammatory cytokine interleukin-1, which inhibits PDGFR α expression in MG-63 cells and human osteoblastic cells

Clinical applications

1. rh PDGF-BB has been used to promote bone regeneration around endosseous implants. Allori et al., 2008
2. FDA has cleared the clinical use of rhPDGF-BB for chronic skin wounds in diabetic patients (Regranex, Ethicon) and for periodontally related osseous defects (GEM 21S, BioMimetic Therapeutics) and rh BMP-2 (InFuse Medtronic Sofamor Danek) for anterior interbody spine fusion, open tibial fractures, sinus elevations, and defects associated with tooth extraction (Lynch 2008).
3. It is noteworthy to consider the therapeutic role for rh PDGF for the compromised bone wound healing in patients with diabetes. It has been shown there is a decrease in cellular proliferation in the fracture callus and a decrease in levels of PDGF transcripts in diabetic rats, suggesting a correlation between PDGF levels and fracture healing response. (Pietrzak and Eppley 2005)
4. In fenestration defects in alveolar bone, recombinant PDGF-BB applied to root surfaces increased proliferation of periodontal ligament, cementoblasts, osteoblasts, perivascular cells and endothelial cells Hollinger et al., 2008.

GEM - 21S GROWTH FACTOR ENHANCED MATRIX

FDA approved components include:

- Synthetic β tricalcium phosphate.
- Highly porous and resorbable.
- Osteoconductive scaffold/ matrix.
- Provides framework for bone growth.
- Aids in preventing collapse of soft tissue.
- Promotes stabilization of blood clot.
- Pore diameter of scaffold \rightarrow 1 to 500 μ m.
- Particle size \rightarrow 0.25 to 1 mm.
- Recombinant PDGF-BB.
- Native protein constituent of blood platelets.
- Causes mitogenesis, angiogenic and chemotactic effects on bone and PDL cells.

Indications

- Intrabony periodontal defects.
- Furcation periodontal defects.
- Gingival recession associated with periodontal defects.

Contra-indications

- Untreated acute infections at surgical site.
- Untreated malignant neoplasm at the surgical site.
- Known hypersensitivity to the product components.
- General contra indications to grafting / surgery.

Warnings

- Various features of recombinant human PDGF (GEM 21s) are yet unknown.
- Interactions with other medications are unknown.
- Carcinogenesis, reproductive toxicity are unknown.
- Effects in pregnant and nursing women are unknown.
- Effects in smokers/ tobacco users are unknown.
- Effects in pediatric patients are unknown.
- Also GEM -21s is intended to be placed in periodontally related defects. Must NOT be injected systemically.
- Radio-opaque in nature and should be considered during evaluation. It is comparable to the radio-opacity of bone initially, diminishes as it is resorbed.

Supply

Each kit consists of:

- One cup containing 0.5cc of β -TCP particles (0.25 to 1 mm).
- One syringe containing solution of 0.5 ml rh PDGF (0.3 mg/ml).

Cost

- 0.5 cc of β TCP / 0.5 ml of PDGF (0.3 mg/ml) \rightarrow \$300.

Directions to use

1. Appropriate sterile conditions should be maintained.
2. β TCP and PDGF are to be mixed. Following a waiting period of 10 min, saturated GEM 21S is placed into the defect.
3. Placement should be with moderate pressure at the level of surrounding bone walls.
4. The kit should not be resterilized/ reused.

Storage

- To be refrigerated at 2 to 8°C.
- β TCP can be stored at room temperature.

Clinical trial

The use of GEM 21 s based on the study by Nevins et

al., (2005) concluded:

1. Dosage of rh PDGF → 0.3 mg/ml.
2. Showed improved periodontal parameters over two years.
3. Resulted in better regeneration in comparison to emdogain.

Advantages

1. Clinical and radiographic benefits of regeneration.
2. Better outcomes than enamel matrix derivatives.
3. Speedy clinical attachment level gains, reduction in gingival recession and improved bone growth.
4. No need for second surgery (as in autogenous bone graft sites).
5. Also, when combining periodontal therapy with rh PDGF and Er:YAG laser, promising results have been shown.

Disadvantages

1. High cost.
2. Various interactions with drugs and systemic health – unknown.
3. Handling difficulties.
4. Mild surgical adverse events – swelling, bleeding, dizziness, difficult breathing, headaches, anaphylaxis.
5. Long term benefits / adverse effects are still unknown.

BONE MORPHOGENETIC PROTEINS (BMPs)

Bone morphogenetic proteins (BMPs) are morphogens and differentiation factors originally isolated from bone matrix based on their ability to induce ectopic bone formation, that is, bone formation de novo where bone does not normally exist, such as in subcutaneous or intramuscular site. Wozney et al., 1988). It should be noted that BMP is a member of TGF- β family. In 1965, Urist showed that crude bone extracts induced new bone in ectopic site in muscle pouch in rat model. He coined the term 'bone morphogenetic protein'. The main sources of BMPs are the bone and kidney cells.

Role

- Act as growth and differentiation factors.
- Act as chemotactic factors/ agents.
- Differentiate stem cells from surrounding mesenchymal cells/ tissue and bone forming cells.
- Also stimulate angiogenesis and migration and proliferation of stem cells.

Recombinant Human BMP-2

RhBMP-2 in combination with a type I bovine collagen sponge has been approved in the US by the FDA for use in spinal fusion, tibial fracture repair, and most recently, as an alternative to autogenous grafts in sinus augmentation and extraction socket grafting procedures in skeletally mature patients. Preclinical results do not support the appropriateness of rhBMP-2 for the treatment of human periodontal defects. Lim et al., 2003) It is an active ingredient in osteoinductive grafts.

The primary activity of rhBMP-2 appears to be differentiating mesenchymal precursor cells into mature osteoblasts and/or chondroblasts. In addition, rhBMP-2 is chemotactic for some osteoblastic-type cells. RhBMP-2 has been shown to induce the complete sequence of endochondral ossification.

Effect on cells in periodontal soft tissue and bone healing

1. Stimulate proliferation and migration of undifferentiated bone cell precursors and induce new bone formation (Sigurdsson, 1993).
2. Helps undifferentiated pleuripotent cells to differentiate into cartilage and bone forming cells (Boden, 2001).
3. Act as chemoattractant for mesenchymal cells
4. Stimulate alkaline phosphatase activity, thus stimulates bone formation. Rosen and Thies, 1992 .
5. Helps in formation of bone matrix (Yasko 1992).
6. Along with bFGF, BMP-2 stimulates angiogenesis Li et al., 2005.

Effect on periodontal ligament cells

1. Stimulate matrix synthesis.
2. Stimulate cementoblast proliferation.
3. Stimulate cementum production.
4. Regulate the proliferation and mitogenesis of the cells of osteoblastic lineage.
5. Stimulate maturation of osteoblastic cells.
6. Stimulate alkaline phosphatase activity, thus in turn stimulating increased bone formation.
7. Induce osteoblastic transformation of stromal cells.
8. Along with basic fibroblast growth factor it stimulates angiogenesis.

Clinical applications

1. Maxillofacial reconstruction (Boyne et al., 2005).
2. Alveolar ridge augmentation (Barboza et al., 2004).
3. Sinus floor augmentation (Boyne and Marx, 1997; Boyne et al., 2005).
4. Implant fixation (Hanisch et al., 2003; Bessho et al., 1999).

Periodontal regeneration

Wikup (2003, 2004) showed significant augmentation of alveolar ridge, used a dome shaped space providing porous expanded PTFE device to create unobstructed space to obviate the compression of rhBMP-2/ ACS. Thus allowing vascularity from gingival connective tissue.

Carrier systems

Several carrier systems have been screened to evaluate their efficacy and biocompatibility with BMPs. Ideal requirements include:

1. Maintaining its structural integrity at the target site.
2. Releasing BMPs in desired concentration over time.
3. Non obstruction of bone formation, thus undergo timely resorption.
4. Should not compromise the physiological and biochemical properties of bone.

Carriers under evaluation

Craniofacial indications in animal models include:

- Hydroxapatite- particulate/ putty formulations
- β Tricalcium phosphate
- Calcium sulphate
- Calcium phosphate
- Calcium carbonate
- Bioglass
- Organic polymers
- Allogenic/ xenogenic collagen preparations
- Absorbable collagen sponge
- Wikup, 2003, 2004 – used rhBMP-2 and ACS with / without ePTFE
- Supra alveolar defects: Need scaffolds with rhBMP-2
- Intrabony defects: May be treated successfully with rhBMP-2 only.

Alternative carrier systems

Wikisjo et al. (2003) investigated rhBMP-2 in calcium phosphate cement matrix.

Indications

- Can be easily shaped to desired contour.
- Provides space for rh BMP to induce bone formation.
- Injectable (for inlay and minimally invasive technology).
- Maxillary sinus augmentation with titanium implants placement.

Clinical applications

- Infuse.

- FDA approved.

Components

- Rh BMP
- ACS- absorbable collagen sponge.
- Is a bovine type I collagen matrix.

Supportive clinical trials

1. van den Bergh et al., (200) reported significant sinus floor augmentation with both rhBMP-2 and rhOP-1.
2. Hanisch et al., (2003) reported re-osseointegration of endosseous implants exposed to peri-implantitis.
3. Jovanovic et al. (2003) established normal physiologic bone formation, osseointegration and long term functional loading of implants.

Clinical indications

- 1) Sinus augmentation.
- 2) Alveolar ridge augmentation:
 - a) Dose: 0.2 to 1.75 mg.
 - b) Inlay (extraction site) - exhibited significant bone formation (Florellini, 2005).
 - c) Onlay (ridge augmentation) showed negligible regeneration (Barboza et al., 2004; Barboza et al., 2000).
- 3) Craniofacial reconstruction:
 - a) Acute / chronic post traumatic discontinuity defects
 - b) Congenital malformations
 - c) Tumour resection defects
- 4) Supports dental implants:
 - a) Significant osseointegration.
 - b) Recently, 'Bone Inductive Implants' → titanium implants with purpose- designed surface serving as a vehicle for rh BMP-1 is being developed.

Exclusion

- Pregnancy.
- Hypersensitivity to the components.
- Infection or tumor.
- Systemic illness.

Possible complications

- Allergic reactions
- Bleeding
- Infection

- Pain, discomfort, swelling, etc.

Drawbacks

- Carrier – ACS→ is vulnerable to tissue compression.
- Less effective for inlay indications (intra-bony defects).
- High cost.
- Possible adverse reactions.
- Poor results in periodontal regeneration. Recent study by Song (2011) suggests reduced collagen synthesis and increased adipogenic differentiation by human PDL cells under rhBMP-2 effect.

GROWTH DIFFERENTIATION FACTOR

- Member of TGF β superfamily.
- Also called cartilage derived morphogenetic protein-1.

Roles

GDF -5,6,7

- In animal studies suggest important regulatory roles in periodontal attachment.

GDF-5

- Plays critical role in mesenchymal cell recruitment inducing cartilage and bone formation, and ligament cell differentiation in morphogenesis.
- Promotes PDL cells proliferation by influencing ECM metabolism (Nakamura et al., 2003).
- Supports and accelerates periodontal tissue formation.
- Shows no evidence of ankylosis or root resorption.
- However, it induces bone regeneration less aggressively as compared to rh BMP-2, BMP-7.

Recombinant forms

Rh GDF-5 + PLGA (polylactideglycolic acid)

- Cortellini Tonetti (2001, 2007) supported minimally invasive regenerative procedures.
- Herbery (2008) reported easy to use in contained and non contained periodontal defects.
- Kimura et al., (2003) demonstrated stimulation of periodontal regeneration.

Rh GDF -5 + β TCP

- Lee (2010) reported potential to support periodontal attachment in one walled intra-bony defects.
- Further long term studies are necessary to confirm uneventful regeneration in human periodontal tissues.

Osteogenic protein-1 (rh BMP-7)

- Approved for bone regeneration in long bone fractures and lumbar spine fusion.
- van den Bergh et al., (2009) reported significant sinus floor augmentation with rh OP-1.
- Has been evaluated for significant sinus floor augmentation procedure with BMP-2.

DISCUSSION

Pros

Recombinant proteins appears to be a promising solution to clinical problems leading to

- rapid periodontal regenerative capability with more predictability.
- optimal compatibility for clinical applications.
- without risk of potential immunological reactions.
- without risk of transmission of infections.

1. rh PDGF- in intra-bony and furcation defects.
2. rh BMP/ ACS- in augmentation of maxillary sinus and alveolar ridge.

-in osseointegration of endosseous implants and re-osseointegration of implants.

Cons

Although promising, the currently available growth factors provide limited clinical benefits. Loop holes include:

- Inappropriate doses.
- Inappropriate delivery systems.
- Expensive.
- Carriers with lacking ability of cell adhesion.
- Carrier systems resorbing untimely to the wound repair process.
- Recombinant technology relies on the inherent ability of transfected cells like yeast/ Chinese hamster cells / *E. coli* which could produce rh GFs of demonstrated biological activity.
- Variable healing responses lead to variable regenerative results.
- Wound healing requires various growth factors (and not just one) to act together to regulate cellular events.
- Recombinant protein therapy offers single growth factor which may be inadequate to achieve the desirable effects eg: rh BMP, rh GDF.

Thus an improved regenerative synthetic product may be synthesized by combining highly concentrated GFs in required amounts, so that the cocktail can successfully regenerate the lost periodontium.

CONCLUSION

Based on the improved understanding of cell and molecular biology of periodontal wound healing, the recombinant technology comprising rh growth factors and carrier construct is applied in periodontal regeneration. Recombinant proteins represent a major evolution in regenerative therapies and have a potential to become a new standard of care broadening the scope of clinical practice. Whereas, still the knowledge in the area needs to be broadened to accept this tissue engineering trend as a regular regenerative therapy.

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Review

Effect of maternal iron status on placenta, fetus and newborn

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Maternal anemia (hypoferriemia) results in increased pre-term and low birth weight deliveries and higher rate of stillbirths. There are irreversible structural alterations in placenta. The transfer of iron to fetus is reduced in spite of gradient in relation to severity of maternal hypoferriemia. The fetal hepatic and brain iron contents were reduced. The brain iron reduction was irreversible on rehabilitation and was associated with irreversible neurotransmitter and their receptor alterations.

Key words: Maternal anemia, stillbirths, placenta.

INTRODUCTION

The outcome of severe pregnancy anemia has been associated with increased incidence of premature births, fetal distress, increased perinatal mortality, and a higher frequency of maternal deaths Nair et al., (1970). In the case of moderate to severe anemia, breathlessness, edema, congestive heart failure and even cerebral anoxia have been observed. 200 anemic pregnant women observed in the University Hospital, Institute of Medical Sciences, Varanasi, showed: reduced gestation; higher incidence of premature labor, preterm, low birth weight and still birth deliveries. These newborns had low apgar score and there were increased number of neonatal deaths. Maternal mortality was 13 out of 200 anemic as compared to 1 in 50 controls. The anemic mothers do not tolerate blood loss during childbirth; as little as 150 ml can be fatal. Normally, a healthy mother during childbirth may tolerate a blood loss of up to 1,000 ml Agarwal (1984).

Current knowledge in the development of iron deficiency

Iron deficiency is an end result of a long period of negative iron balance mainly due to poor dietary availability, rapid growth and blood loss. The pathological stages are:

- a) Pre-latent deficiency: hepatic (Hepatocytes and macrophages), spleen and bone marrow show reduced iron stores (reduced bone marrow iron and serum ferritin).
- b) Latent deficiency: as the bone marrow iron stores become absent, plasma iron decreases and bone marrow receives little iron for hemoglobin regeneration (bone marrow iron absent, serum ferritin < 12ug/L, transferrin saturation < 16% and free erythrocyte protoporphyrin is increased), however, hemoglobin concentration remains

normal.

c) Iron deficiency anemia: this is a very late stage of iron deficiency with progressive fall in hemoglobin and mean corpuscular volume.

Prevalence of nutritional anemia in pregnant women (India)

National studies by the Indian Council of Medical Research (ICMR) Indian Council Medical Research (1989), covering 11 states, reported in 1989, prevalence of anemia by estimating hemoglobin using cyanmethemoglobin method in pregnant rural women as 87.6%, hemoglobin being < 10.9 g/dl. These anemic women were given different doses of iron 60, 120 and 180 mg with 500 ug folic acid daily for 90 days in 6 states; 62% in spite of iron-folate therapy for 3 months, continued to remain anemic Indian Council Medical Research (1992). Thus indicating that short-term treatment as recommended in the National anemia control programme may not be sufficient to control anemia in pregnancy. However, it was observed that birth weight improved and low birth weight deliveries were significantly reduced Agarwal et al., (1991). The administration of higher dose 335 mg of ferrous sulphate and 500 ug of folic acid for 14 weeks as daily dose was found to be effective in control of pregnancy anemia Gomber et al., (2002).

National family Health Survey 1998-99 (NFHS-2) using hemocue method reported prevalence of anemia as 49.7% in pregnant women; 56.4% in breastfeeding non-pregnant women and 50.4% among non-pregnant non-breastfeeding women. Hemocue method estimates higher levels of hemoglobin thus difficult to compare with the other National studies. In 2005, NFHS-3 demonstrated increase in prevalence of anemia, suggesting marginal rise in anemia nation wide NFHS-2 &- 3 India 1998-99-& 2005 (2000).

Nutrition Foundation of India in 2002 to 2003 studied prevalence of anemia in pregnancy and lactation in 7 states (Assam, Himachal Pradesh, Haryana, Kerala, Madhya Pradesh, Orissa, Tamil Nadu). The prevalence of pregnancy anemia was 86.1% (Hb < 7.0 g/dl in 9.5%), and in lactation up to 3 months was 81.7% (Hb < 7.0 g/dl in 7.3%). The interstate differences responsible for differences in prevalence of anemia were particularly related to fertility, women education, nutrition status and occupation, availability of antenatal services and iron folate tablets as possible factors (Agarwal et al., 2006; Sharma and Agarwal 2007).

The Indian Council of Medical Research (ICMR) in 1999 to 2000 conducted District Nutrition Survey in 11 states covering 19 districts pregnancy anemia prevalence was 84.6% (Hb < 7.0 g/dl in 9.9%). The study also found 90% adolescent girls with anemia in these districts Teoteja and Singh (2001). The prevalence as well as severity of anemia during pregnancy and lactation is grave. This is the period when brain cells grow and

neurotransmitters develop, iron is essential for it.

Iron status in pregnancy

This includes:

1. Fetal growth depends, to a large extent, on the availability of iron from the mother.
2. Normal non-pregnant woman needs iron 1.3 mg/day.
3. Total pregnancy need of iron is 1000 mg or more. Absorption rate of 6 mg/day in the last 2 trimesters.
4. 350 mg of iron is lost to the fetus and placenta.
5. 250 mg is lost in blood at delivery. 450 mg is needed to increase the RBC mass. Lastly around 240 mg is lost as basal losses.
6. In cesarean delivery blood loss is almost twice (500 ml). In moderate and severe anemia mother will die if blood loss is >150 ml.
7. During lactation, iron loss is 0.3 mg/day.

Placenta in iron deficiency

Iron transport

Normally 'placental iron transfer' to fetus becomes 3 to 4 times during 20 to 37 weeks of gestation. The placenta traps maternal transferrin removes iron and actively transports it across to the fetus where it becomes bound to fetal transferrin and is distributed to the liver, spleen and other fetal hemopoietic tissues, maintaining higher levels of fetal iron as compared to the mother. Placenta plays an important role in maintaining iron transport to fetus. This process of iron transport is purely a placental function over which mother and fetus has no control, as placenta continues to trap iron even when fetus is removed in animals Fletcher and Suter (1969). The placental trophoblastic membrane appears to act as an effective barrier against the further transport of iron to the fetus. In spite of this efficient protective mechanism, the placental iron content reduces significantly in maternal hypoferrinemia (Agarwal 1984; Singla et al., 1978; Singla et al., 1979; Agarwal et al., 1983). This was an important finding as earlier studies on Swedish and American women had shown that cord iron does not change in iron deficient pregnant women (Vahlquist, 1941; Rios et al., 1975). However, recent studies have confirmed that the maternal anemia affects the placento-fetal unit (Emamghorashi and Heidari 2004; Paiva et al., 2007; Kumar et al., 2008; Lee et al., 2006).

Morphometry and biochemical alterations

Beischer et al (1970) analysed data (from Australia, India, NewGuinea, Singaore and Thailand) and demonstrated that in all the studies, placental weight in maternal

anemia was higher than the control. This increase in placental weight was higher with increasing parity. The placental hypertrophy did not correspond to fetal size and had no correlation with maternal serum protein. Ratten and Beischer (1972) confirmed that the placental weight exceeds the 90th centile in 20% of patients with hemoglobin < 8.2 g/dl and in 13.2% of those with hemoglobin 8.2 to 9.1 g/dl. The placental hypertrophy is postulated to be due to hypoxia, which is supported by evidence of similar phenomenon at higher altitudes. In our studies, maternal anemia was associated with low maternal serum albumin. Both deficiencies were associated with reduced weight and volume of placenta. Placentae in maternal anemia showed reduced number of cotyledons and increase in incidence of ill-defined cotyledons and eccentric attachment of cord. There was increased shrinkage in formalin in pregnancy anemia (Sen and Agarwal 1976; Khanna et al., 1979; Agarwal et al., 1981; Marwah et al., 1979). This reduction in placental weight was due to reduced DNA (cell number), however cell size was increased (weight/DNA). In maternal hemoglobin RNA, content per cell remained constant Agarwal (1991). Placental succinic dehydrogenase activity was decreased, total nicotinamide adenine dinucleotide phosphate (NADP) - dependent isocitrate dehydrogenase (ICDH) was more than NAD + dependant ICDH in severe maternal hypoferritemia; suggesting impaired citric acid cycle Agarwal (1984).

Histology

There was decreased villous vascularity leading to fibrosis with increased endarteritis obliterans reflecting response to hypoxia. There was progressive decrease of surface area and volume of villi per unit volume of blood vessel in relation to degree of anemia; suggesting maturational arrest Agarwal 1984; Marwah et al., 1979; Agboola 1975; Fox 1967). On treatment with iron, there was increase in hemoglobin, cord iron and placental (non-hem iron) and placental shrinkage in formalin reduced. However, the reduced villus vascularity, increased villus fibrosis and endarteritis obliterans in placenta of anemic mother did not reverse. It was postulated that moderate-severe anemia present from the early days of pregnancy induces irreversible structural alteration, as iron is needed in 2nd week of pregnancy for placenta formation Agarwal (1984).

Fetus-newborn

Cord serum iron and hemoglobin were reduced in preterm as well as full term infants of hypoferritemic mothers. There is an increased gradient in presence of maternal iron deficiency for transport of iron from mother to fetus but the transport remains proportionate to the degree of maternal hypoferritemia. The weight of full term singleton babies born of anemic mothers was reduced in

direct relation to hemoglobin level. Similarly, these babies showed a progressive decrease in Apgar scores also Agarwal (1984). Fetal liver iron stores are reduced significantly in maternal hypoferritemia. Normally bigger, the infant, and more advanced the gestational age higher, was the amount of iron in fetal liver, spleen and kidney. The tissue iron content increases steeply in the last 8 weeks of gestation. Infant born before 36 weeks of gestation had half the iron content in hepatic reserve Singla et al., (1985). Breast milk iron content is increased in hypoferritemic mothers, a phenomenon of "Physiological trapping" (Khurana et al., 1970; Franson et al., 1985).

To understand more, a rat model was created with latent iron deficiency (low hepatic iron without change in hematocrit) in pregnancy (Agarwal 2001; Shukla et al., 1989; Taneja et al., 1986; Taneja et al., 1986; Shukla et al., 1989; Mittal et al., 2002).

Fetal brain iron content and neurotransmitters in maternal (rat) latent iron deficiency

Iron as a micronutrient is required for regulation of brain neurotransmitters by altering the pathway enzymatic system. To study iron deficiency, a rat model was developed to create iron deficiency (low hepatic iron) without change in hematocrit Agarwal (2001). In post-weaning rats, iron decreased irreversibly in all brain parts except medulla oblongata and pons. Susceptibility to iron deficiency showed variable reduction in different parts of the brain: corpus striatum, 32%; midbrain, 21%; hypothalamus, 19%; cerebellum, 18%; cerebral cortex, 17%; and hippocampus, 15%. Alterations in brain iron content also induced significant alterations in copper (Cu), zinc (Zn), calcium (Ca), manganese (Mn), lead (Pb) and cadmium (Cd) Shukla et al., (1989).

Fetal latent iron deficiency (Rat) and- brain neurotransmitters

In latent iron deficiency there was irreversible reduction in neurotransmitters: Brain 'glutamate metabolism'-[glutamic acid decarboxylase (GAD), glutamate dehydrogenase (GDH), gamma amino butyric acid (GABA-T)] (Taneja et al., 1986; Shukla et al., 1989):

- a) Marked reduction in levels of brain GABA, L glutamic acid and enzymes for biosynthesis of GABA and L- glutamate like glutamate decarboxylase and glutamate transaminase.
- b) Binding of H3 muscimol at pH 7.5 and 1 mg protein/assay (GABA receptor) increased by 143%, but glutamate receptor binding decreased in the vesicular membranes of latent iron deficient rats by 63% (Agarwal 2001; Mittal et al., 2002).
- c) Brain 'TCA-cycle' enzymes-mitochondrial NAD+ linked

dehydrogenase significantly reduced

d) Brain '5-HT metabolism'- Tryptophan, 5-HT, 5-HIAA significantly reduced.

e) The whole brain and corpus striatum showed reduction in catecholamine, dopamine nor-epinephrine, tyrosine and monoamine oxidase, while tyrosine amino transferase increased in corpus striatum, in spite of reduction in whole brain suggesting that latent iron deficiency induced irreversible neurotransmitter alterations Shukla et al., (1989).

f) Brain 'catecholamine metabolism'- Whole brain-dopamine, neonephrine, tyrosine and TAT significantly reduced in 'corpus striatum', same as in whole brain, except TAT was increased Shukla et al., (1989).

These changes specific to iron deficiency as neurotransmitter alterations in fetal brain due to malnutrition (undernutrition or on diets with limiting amino acids) get normalized partially or completely on rehabilitation (Prasad et al., 1979; Prasad and Agarwal, 1980). The significant effects on neurotransmitter receptors (glutamate mediators) during early stages of iron deficiency clearly indicate the deficits in both excitatory and inhibitory pathways of the central nervous system, showing an important role of iron in brain (Agarwal, 2001).

To test the above findings in humans, babies born of moderate to severely anemic mothers were examined for "impact of iron deficiency on mental functions". The intrauterine growth retarded offspring's of anemic as well as undernourished mothers showed hypotonia in 72% and hypoexcitability in 56% (Bhatia et al., 1979; Bhatia et al., 1980; Agarwal et al., 2002). There was modification of responses in several neonatal reflexes for example, limp posture, poor recoil of limbs, incomplete moro's and crossed extensor responses. Their electroencephalogram (EEG) had shortening of sleep cycle [rapid eye movement (REM) and non-rapid eye movement (NREM)], the reduction was more marked for REM sleep. There was some inter and intra hemispheric asymmetry and abnormal paroxysmal discharges; suggesting dysmaturity of brain (Bhatia et al., 1979; Bhatia et al., 1980).

The above findings were not specific to effects of anemia on mental functions. Therefore effects of anemia (nutrition controlled) on mental functions were then studied in rural children during a period of three years. Mental functions in nutrition controlled 388 rural primary school (6 to 8 year of age), matched for social and educational status were studied by WISC and arithmetic test to assess "Intelligence, attention and concentration". Anemia did not affect intelligence - except subtest-digit span, but in arithmetic test, attention and concentration was poor in anemic children Agarwal et al., (1989).

Effects of iron deficiency and/or anemia on brain

Iron deficiency anemia in infancy has been consistently shown to negatively influence performance in psycho-

motor development. Short-term iron therapy did not improve the lower scores, despite complete hematological replenishment. Neurological maturation was studied in infants 6 months old, including auditory brain stem responses and naptime 18 lead sleep studies. The central conduction time of the auditory brain stem responses was slower at 6, 12 and 18 months and at 4 years, despite iron therapy beginning at 6 months. During sleep-wakefulness cycle, heart rate variability- a developmental expression of the autonomic nervous system, was less mature in anemic infants. This is possibly due to altered myelination of auditory nerves Walter (2003). It has been observed that these changes are resistant to iron therapy in children < 2 years of age with iron deficiency with anemia, but not in older children McCann and Ames (2007). These studies supported earlier findings that brain functions are significantly affected in latent iron deficiency in the brain growth period, and such changes are irreversible. These have serious consequences for example, poor cognition and learning disabilities.

CONCLUSION

The above researches review mainly affects of maternal hypoferrremia on iron status of placenta, cord blood (hemoglobin and ferritin), and fetus (brain and hepatic iron content). The rat model of "latent iron deficiency" showed irreversible brain iron reduction and irreversible neurotransmitter alterations in 'brain growth period'. Once anemia sets in, the additional effects are due to anoxia. Our nation is faced with the problem of iron deficiency that leads to anemia- a clinical condition due to deficiency of many nutrients, mainly iron, folic acid and vitamin B12. Folic acid is essential from prenatal period and its deficiency causes neural tube defects.

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Review

Comparison of different methods for assessing sperm concentration in infertility workup: A review

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Sperm count assessments form the essential component of the diagnostic and prognostic evaluation of male fertility, according to guidelines of WHO (1992). The problem of subjective bias, inter and intra operator variability of reporting is discussed in this paper. The problem of inter operator variability has been improved and reproducibility has been made more objective with the introduction of computer-assisted semen analysis (CASA) protocols. To overcome the stated limitations and achieve objective assessment with a high precision, a new technique called flow cytometry was developed. Different methods for the estimation of sperm concentrations like hemocytometry, spectrophotometry, microcells, plate reader, image analysis and finally flow cytometry are compared and contrasted. Their relative merits and demerits are discussed with a detailed review of literature. Methods for estimation of sperm concentration are discussed in this paper.

Key words: Sperm counts, semen analysis, flow cytometry.

INTRODUCTION

Reproductive biology needs accurate and precise determination of sperm counts to achieve any success rate (Foote et al., 1978; Fenton et al., 1990; Woelders, 1991; Evenson et al., 1993; Donoghue et al., 1996). Microscopic estimation of sperm counts is the oldest and the simplest procedure of the semen analysis. This analysis is performed routinely by toxicology laboratories and by veterinary insemination centers, in addition to human infertility clinics (Graham, 1994; Vetter et al., 1998; Auger et al., 2000). This method of estimation of human spermatozoa suffers from many drawbacks like subjective bias. The subjective aspect when compounded by low sperm counts leads to variability in intra and inter laboratory results (Auger et al., 2000). This problem is discussed in detail in this paper. The problem of inter operator variability has been improved and reproducibility

has been made more objective with the introduction of computer-assisted semen analysis (CASA) protocols (Davis and Katz, 1992; Krause et al., 1993; Holt et al., 1994). But here, small changes in instrument settings and assay conditions were used to affect significantly the objectivity of these measurements. The present review discusses the methods for estimation of sperm concentration.

METHODS FOR ESTIMATION OF SPERM CONCENTRATION

The initial method used in most centers is to estimate the sperm concentration by a hemocytometric count or by a spectrophotometric determination of turbidity of a measured dilution of a sample of semen (Foote et al., 1978; Woelders, 1991; Donoghue et al., 1996). Evenson et al. (1982) have pointed out that many artifacts such as cytoplasmic droplets and other debris can adversely affect

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the accuracy of these measurements. Although electronic sperm cell counters can be used for a rapid estimation of sperm counts, Evenson et al. (1993) have demonstrated that any cellular debris in the same range as the size of a spermatozoon can interfere with the readings. This problem is particularly accentuated in sperm counts made on freeze-thawed semen samples as they contain egg yolk particles, fat droplets and other particulate matter (Parks, 1992; Evenson et al., 1993). Use of hemocytometers for estimation of sperm counts in freeze-thawed samples of semen has not gained much acceptance because of many technical reasons (Freund and Carol, 1964). Evenson et al. (1993) have developed a flow cytometric method of semen analysis in which beads composed of fluorescent microspheres were used. This method met with limited success because of the laborious process of preparation of these beads and the need for highly skilled personnel. Studies have reported (Carlsen et al., 1992; Auger et al., 1995; Irvine et al., 1996; Aitken, 1999) that the quality and counts of spermatozoa is showing downward trend in human semen analysis reports. Sperm count assessments form the essential component of the diagnostic and prognostic evaluation of male fertility, according to guidelines of WHO (1992). Although the report of WHO (1992) had laid down clear cut guidelines for the hemocytometric estimation of sperm counts, Auger et al. (2000) have demonstrated vividly that the results are difficult to compare because of variations between laboratories and between technicians. All these had led to the increased use of flow cytometry to estimate sperm concentrations and to bring concurrent agreement between different laboratories (Eustache et al., 2001; Tsuji et al., 2002).

To overcome the stated limitations and achieve objective assessment with a high precision, a new technique called flow cytometry is utilized (Gledhill et al., 1976; Garner et al., 1986; Morrell, 1991; Parks, 1992; Graham, 1994, 2001). This technique also allows the researcher to examine several different other parameters like plasma membrane integrity (Evenson et al., 1982; Garner and Johnson, 1994, 1995), mitochondrial function (Evenson et al., 1982; Graham et al., 1990; Garner et al., 1997), acrosomal status (Graham et al., 1990; Thomas et al., 1997) and chromatin structure (Evenson et al., 1980). A flow cytometer is an easy instrument to operate now in clinical use for the estimation of sub populations of lymphocytes and in the stem cell laboratories world wide. Garner et al. (1994) reported a protocol to assess sperm concentrations with fluorescent microspheres popularly called beads. They assessed sperm viability by flow cytometry using a modified SYBR 14 and propidium iodide (PI) method. This protocol can differentiate live, dead and moribund spermatozoa of different species of mammalian and avian semen.

Efforts are being made to correlate the fertility success ratio in bulls and bears with that of sperm viability. Kroetsch et al. (2009) have shown that there is significant variation in the fertility ratio of semen samples obtained from the same male animal but in different ejaculates. Matson (1997) argued that the chance of selecting the best ejaculate (from the same animal) depends first and foremost on the precision of the semen analysis. He therefore concluded that simultaneous estimation of sperm concentration and viability results in more accurate prediction of success rates. If these two estimations were performed on different samples or on different instruments or by different personnel, the reports and the results may not be that accurate.

RESULTS AND DISCUSSION

We now make a comparative analysis of different methods to estimate the sperm concentrations that were used in many infertility centers worldwide. Of these, the first three are conventional methods and the remaining

novel methods are as follows:

- 1) Hemocytometry method
- 2) Spectrophotometry method
- 3) Microcell analysis
- 4) Fluorescent plate reader
- 5) Image analysis
- 6) Flow cytometer

Hemocytometry method

Hemocytometry is the oldest, well established 'gold standard' in all cell count estimations including sperm counts. Seman et al. (1996) had exposed threadbare that hemocytometer readings are prone to wide variations and imprecise readings. Mahmoud et al. (1997) have showed that different models of hemocytometers also contribute to observer variation. Prathalingam et al. (2006) have used Thoma hemocytometers since they observed in the previous studies of having less cyclic voltametric (CV) compared to other models (Christensen et al., 2005). Although their estimated CV is less than that reported by others, Prathalingam et al. (2006) had reported that the hemocytometer turned out to be the third most imprecise method to estimate sperm concentrations. As hemocytometry is laborious for routine use and prone to observer bias, Cooper et al. (1992) made an attempt to automate and at the same time capture images from the hemocytometer loaded with fluorescent labeled spermatozoa.

Spectrophotometric method

It is routinely used in many of the artificial insemination laboratories throughout the world in estimating sperm counts. The results obtained from this method are very well verifiable. Hansen et al. (2002) have reported that the CV by spectrophotometric method is 6.3%, whereas Prathalingam et al. (2006) reported the results as 4.1%. This method has the advantage of completing the estimation quite rapidly but the problem is that the equipment needs frequent calibration and maintenance. Unlike other methods that estimate particulate matter, spectrophotometry uses a procedure of colour estimation of the given solutions. So the absorption reading of the cell suspension varies with time and so the time frame within which the estimation is performed is critical for this procedure. This element reduces the objective value of this test, particularly when repeat estimations are performed on the same sample. Prathalingam et al. (2006) had suspected that this element could have influenced the CV value of this test in their study.

Lu et al. (2007) had given the data shown in Table 1 comparing to methods of sperm cell counting. They had used 60 semen samples to load the upper and lower chambers of 3 hemocytometers and the right and left

Table 1. Sperm counts obtained by hemocytometer and cell – VU devices.

| Chamber No. | Hemocytometer No. | | | Cell – VU No. | | |
|-------------|-------------------|-----------|-----------|---------------|-----------|-----------|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| Lower/Left | 50.8±18.8 | 58.2±37.4 | 50.5±33.7 | 52.8±35.9 | 61.5±50.2 | 54.5±49.6 |
| Upper/Right | 49.0±18.2 | 56.0±34.8 | 50.6±32.5 | 50.4±33.8 | 59.4±46.3 | 50.5±43.0 |
| CV | 0.925 | 0.969 | 0.988 | 0.968 | 0.994 | 0.996 |

Table 2. A correlation matrix indicating the r value of each method compared.

| Method | Flow cytometry | Hemocytometry | Image analysis | Microcells | Plate reader | Spectrophotometry |
|-------------------|----------------|---------------|----------------|------------|--------------|-------------------|
| Flow cytometry | * | | | | | |
| Hemocytometry | 0.99 | * | | | | |
| Image analysis | 0.90 | 0.90 | * | | | |
| Microcells | 0.96 | 0.96 | 0.86 | * | | |
| Plate reader | 0.91 | 0.91 | 0.86 | 0.83 | * | |
| Spectrophotometry | 0.99 | 0.99 | 0.90 | 0.97 | 0.88 | * |

sides of 3 cell-VU counting slides.

Fluorescent plate reader method

Spermatozoa were labeled with a fluorescent dye and loaded on a hemocytometer. The resultant image was analyzed with the help of software. Gravance et al. (2000) had performed some pioneering work on this method and reported that the use of the image analysis program has generated a higher CV than that obtained with hemocytometers. Though these results surprised many researchers, it was surmised that it could be because of the lower number of spermatozoa that were counted in these initial studies. In view of the above discussion, Prathalingam et al. (2002) had optimized the protocol before initiating the study. The optimal concentration of spermatozoa was initially estimated to be 2.5×10^6 cells/ml. When the same concentrations of spermatozoa were used for the hemocytometer and for this method, the software program was unable to distinguish cells within clusters. This probably led to errors in calculations. But the use of this software made the analysis much more rapid. It had become a well known fact that the use of plate reader and a software program made the simultaneous measurements of several ejaculates possible. Prathalingam et al. (2006) have opined that the higher CV could be improved by increasing the area under analysis instead of using specimens with higher sperm concentrations. Moreover, a fluorescence plate reader could be used as a low cost alternative to flow cytometry by allowing large number of ejaculates to be processed. Prathalingm et al. (2002) had concluded that further refinement of the protocols are needed for this method as this procedure had the double advantage of counting the number of spermatozoa as

well as assessing the viability of the sperms.

Pranthalingam et al. (2006) made a comparative analysis of different methods. They had estimated 100 samples of semen of different species and gave the data comparing the results from different methods of estimation of sperm concentrations as shown in Table 2.

Microcells analysis

Microcells have the added advantage of assessing the motility of spermatozoa simultaneously with the measurements of cell counts. Tomlinsen et al. (2001) have reported significantly low sperm counts with microcell method as compared to those obtained by hemocytometers ($p = 0.11$). Sokol et al. (2000) reported a close correspondence between the readings obtained by hemocytometer and with microcells ($r = 0.88$). Prathalingam et al. (2002) have made a study on bull semen and cautioned while extrapolating the results to humans because human semen contains more debris and less sperm concentrations and lower rates of motility per ejaculates. They also have reported a higher CV for microcells as compared to hemocytometers, a finding that is in correspondence with Tomlinson et al. (2001) and Brazil et al. (2004b).

A common problem encountered by many researchers doing comparative studies of estimates of sperm counts is that they were dealing with an unknown number of spermatozoa in each sample, so there was no standard sample to compare with the test sample. Latex beads of known numbers were used by some researchers (Seaman et al., 1996; Brazil et al., 2004a) as a control to measure the concentrations of spermatozoa in test semen samples. When combined with an unknown number of spermatozoa, the advantage of these latex beads is that their numbers can be estimated accurately by

gravimetric method as long as their properties are constant and uniform. These latex beads can be stored and used repeatedly for an extended period of time and this allows comparison of sperm counts against a given reference of consistent bead numbers (Accubead, Hamilton Thorne, Beverly, Mass). Although their use has improved readings, variability across studies persisted as the number of beads did not match in different batches ($18 \times 10^6 \pm 2.5 \times 10^6$ beads/ml; $35 \times 10^6 \pm 5 \times 10^6$ beads/ml; Accubead). Mahmoud et al. (1997) have reported an increased CV for bead and sperm counts when the semen was mixed with latex beads and estimated with an improved Neubauer Chamber.

CONCLUSION

This review had examined the merits and demerits of several common methods of estimation of sperm concentrations in current use. The oldest, the commonest and the one considered as the 'gold standard' for long is the hemocytometric method. The flow cytometer is the latest, sophisticated and the most precise method developed till date. The spectrophotometer is the second most precise method and is very commonly used for the estimation of sperm counts of several non-human species. But it may not be the ideal procedure for a clinical laboratory as the volume of semen and sperm concentration is low for humans. Although flow cytometric procedure is gaining ground in many research laboratories, a preliminary sperm count assessment with a different procedure is recommended by many researchers to ensure that an adequate dilution of semen for the flow cytometer is achieved. The optimal sperm concentration is considered to be about 250×10^3 sperms/ml for the flow cytometer to give the best results. It is also considered to be one of the essential precaution that the sperm concentration and flow rate are adjusted to ensure that there will be no 'missed events'.

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Review

Medicinal values of garlic: A review

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Garlic products are used as sources of medicine in many ways in human beings in their day today life. As a result, researchers from various disciplines are now directing their efforts towards discovering the medicinal values of garlic on human health. The main interest of researchers in the medicinal values of garlic is its broad-spectrum therapeutic effect with minimal toxicity. Garlic extract has antimicrobial activity against many genera of bacteria, fungi and viruses. Garlic contains a higher concentration of sulfur compounds which are responsible for its medicinal effects. The chemical constituents of garlic have also been investigated for treatment of cardiovascular disease, cancer, diabetes, blood pressure, atherosclerosis and hyperlipidaemia and highly praised by several authors. Therefore, this paper is reviewed to inspire and impress the young researchers about the medicinal values of garlic.

Key words: *Allium sativum*, immunity booster, antibacterial, antifungal, antiviral, anticancer

INTRODUCTION

Natural products of animals, plants and microbial sources have been used by man for thousands of years either in the pure forms or crude extracts to treat many diseases (Parekh and Chanda, 2007). Garlic (*Allium sativum* L.) is one of those plants that were seriously investigated over several years and used for centuries to fight infectious diseases (Onyeagba et al., 2004). The taxonomic position of garlic and related genera had been a matter of controversy for long period of time. The most recent classification scheme of garlic was class Liliopsida, subclass Liliidae, superorder Liliianae, order Amaryllidales, family Alliaceae, subfamily Allioideae, tribe Allieae and genus *Allium* which is mainly based on the sequences of nuclear ribosomal DNA (Friesen et al., 2006).

The early Egyptians used garlic to treat diarrhea and its medical power was described on the walls of ancient temples and on papyrus dating to 1500 BC (Bradley, 1992). It was used by Greek physicians Hippocrates and Galen to treat intestinal and extra-intestinal diseases; ancient Japanese and Chinese used it to treat headache, flu, sore throat and fever. In Africa, particularly in Nigeria, it is used to treat abdominal discomfort, diarrhea, otitis

media and respiratory tract infections (Jaber and Al-Mossawi, 2007). In Europe and India, it was used to treat common colds, hay fever and asthma. Garlic is nicknamed as Russian penicillin for its widespread use as a topical and systemic antimicrobial agent; it is commonly used in many cultures as an excitement and reputation of healing power (Timbo et al., 2006).

POTENTIALLY ACTIVE CHEMICAL CONSTITUENTS OF GARLIC

Garlic contains at least 33 sulfur compounds, several enzymes and the minerals germanium, calcium, copper, iron, potassium, magnesium, selenium and zinc; vitamins A, B1 and C, fiber and water. It also contains 17 amino acids to be found in garlic: lysine, histidine, arginine, aspartic acid, threonine, swine, glutamine, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tryptophan and phenylalanine (Josling, 2005). It has a higher concentration of sulfur compounds than any other *Allium* species which are responsible both for garlic's pungent odor and many of its medicinal

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effects. One of the most biologically active compounds in garlic is allicin (diallyl thiosulfinate or diallyldisulfide). The most abundant sulfur compound in garlic is alliin (S-allylcysteine sulfoxide), which is present at 10 and 30 mg/g in fresh and dry garlic, respectively (Lawson, 1998). Typical garlic food preparation such as chopping, mincing and crushing disturbs S-allyl cysteine sulfoxide and exposed it to the allinase enzymes, then quickly converted it to diallyl thiosulfinate, which give off garlic's characteristic aroma. The allinase enzyme responsible for diallyl thiosulfinate conversion becomes inactivated below a pH of 3.5 or with heating (Pedrazza-Chaverri et al., 2006). Although allicin is considered the major antioxidant and scavenging compound, recent studies showing that other compounds may play stronger roles; such as polar compounds of phenolic and steroidal origin, which offer various pharmacological properties without odor and are also heat stable (Lanzotti, 2006).

ROLE OF GARLIC IN HEALTH

Garlic can rightfully be called one of nature's wonderful plants with healing power. It can inhibit and kill bacteria, fungi, lower (blood pressure, blood cholesterol and blood sugar), prevent blood clotting, and contains anti-tumor properties. It can also boost the immune system to fight off potential disease and maintain health (Abdullah et al., 1988). It has the ability to stimulate the lymphatic system which expedites the removal of waste products from the body. It is also considered an effective antioxidant to protect cells against free radical damage. It can help to prevent some forms of cancer, heart disease, strokes and viral infections. Garlic alone can provide us with over two hundred unusual chemicals that have the capability of protecting the human body from a wide variety of diseases. The sulfur containing compounds found in garlic afford the human body with protection by stimulating the production of certain beneficial enzymes (Mansell and Reckless, 1991).

Treat cardiovascular disease

Disorders of the heart and the circulatory system claim more lives than any other diseases. It is the obstruction or clogging of the coronary arteries which causes more deaths than any other factors. The arteries, which supply the heart with blood and oxygen, become increasingly narrower as plaque builds up over time. When blood supply becomes restricted, a certain portion of the heart is deprived of oxygen and leads to heart attack. The two greatest means of heart disease are high blood pressure and high blood serum cholesterol levels; which are directly impacted by the therapeutic action of garlic. The relevant role of garlic in coronary heart disease was done on rabbits and found that even pre-existing atherosclerotic deposits and lesions could actually be reversed if garlic was consistently consumed (Bordia, 1981).

From a study conducted in India, 432 coronary artery patients were randomly grouped into two groups and half of them were supplied with garlic juice in milk, whereas the other group patients were not supplied with garlic juice. The result showed that within the three years of the study time, nearly twice as many patients had died in the group not supplied with garlic juice (Yeh et al., 2006). It is well reported to scavenge oxidants, increase superoxide dismutase, catalase, glutathione peroxidase, glutathione levels, inhibit lipid peroxidation as well as it reduces cholesterol synthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA. It has been shown to reduce platelet aggregation, arterial plaque formation, decrease homocysteine, lower blood pressure, and increase microcirculation. It may also help prevent cognitive decline by protecting neurons from neurotoxicity and apoptosis, thereby preventing ischaemia or reperfusion-related neuronal death and by improving learning and memory retention (Borek, 2006).

Reduces high blood pressure/hypertension

Garlic has probably been most popularized as a complementary therapy for blood pressure control (Capraz et al., 2006). A recent *in vitro* study has confirmed that, the vasoactive ability of garlic sulfur compounds whereby red blood cells convert garlic organic polysulfides into hydrogen sulfide, a known endogenous cardio-protective vascular cell signaling molecule (Benavides et al., 2007). Using 2400 mg garlic tablet containing 31.2 mg allicin has high dose reduced diastolic pressure by 16 mmHg after 5 h of administration (McMahon and Vargas, 1993). A meta-analysis made on pooled data from 415 patients showed also reduction of 7.7 mmHg diastolic pressure (Silagy and Neil, 1994).

As natural blood thinner

Platelets and fibrin play great role in blood clotting and higher amount of fibrin in blood can cause heart attack. Garlic constituents can reduce fibrin formation and also help reduce the fibrin existing in the blood even better than aspirin (Fukao et al., 2007). Ajoene, a sulfur compound found in garlic seems to be responsible for its anti-clotting effect; but ajoene is only viable at room temperature or above, it is not present in raw or freeze-dried garlic. It is believed that the addition of garlic to a diet can help to increase the breakdown of fibrin from 24 to 30% in people (Ernst, 1994).

As natural immunity booster

With the arrival of frightening viral diseases like HIV/AIDS, boosting immunity system is receiving a new attention. Because these types of diseases have no

effective cures or treatments, strengthening the body's ability to fight off infection has become even more important. Garlic has abundant sulfur containing amino acids and other compounds that seem to initiate increased activity in the immune system (Lau et al., 1991). It is one of the impressive conductors of the body's immune system; which stimulates immune function by making macrophages or killer cells more active. We are constantly beaten by inadequate nutrition, cigarette smoke, physical injury, mental tension and chemical pollution. In light of the enormous pressures, which our immune systems sustain, supplemental nutrients like garlic are clearly needed (Salman et al., 1999). Its remarkable content of germanium alone offers excellent immune stimulation. In addition to germanium, garlic contains thiamine, sulfur, niacin, phosphorous, and selenium (Morioka et al., 1993).

Preliminary studies in humans, using an alliin standardized garlic powder preparation, have demonstrated positive effects on immunoreactions and phagocytosis. In aged subjects, the administration of 600 mg garlic powder per day for 3 months induced significant ($p < 0.01$) increases in the percentage of phagocytosing peripheral granulocytes and monocytes when tested *ex vivo* for their ability to engulf *Escherichia coli* bacteria. Another human study was conducted with an unrefined garlic extract (5 to 10 g/day) which was given to HIV/AIDS patients. For the seven patients who completed the 12 weeks study, there was a major increase in the natural killer cells activity from a seriously low mean value (Abdullah et al., 1988).

In USA, trials in HIV/AIDS patients have demonstrated the enhancement of natural killer cells activity using garlic extracts; and Chinese studies with viral infections in bone marrow transplant patients have demonstrated a "potent antiviral activity". A double blind placebo controlled survey using a 100% alliin yielding supplement has reported that alliin can reduce the occurrence of the common cold and recovered from symptoms (Josling, 2001).

Atherosclerosis and hyperlipidaemia

Health claims advertizing garlic's universal ability to lower cholesterol level and decrease lipid peroxidation in order to inhibit plaque formation. *In vitro* studies clearly have shown that, it has an ability to suppress low density lipoprotein (LDL) and an increased resistance of LDL to oxidation (Lau, 2006). Results from controlled human studies are mixed with studies performed in the early 1990's and was showing effective results. As more researches were conducted newer processes to extract garlic, recent study of 15 hypercholesterolemia patients evaluated a material produced from garlic fermented with the mold *Monascus pilosus*. This preparation significantly reduced serum total cholesterol and low density lipoprotein cholesterol levels when checked at 2 and 4

weeks after treatment beginning. The level of triglycerides had a tendency towards reduction in hypertriglyceridemic patients as well, whereas high density lipoprotein cholesterol was unchanged (Sumioka et al., 2006). After 60 days of supplementation, low-density lipoprotein, serum triglyceride and very low density lipoprotein, were reduced by 21, 37, and 36.7%, respectively (Jeyaraj et al., 2006).

Prevents diabetes

A number of animal studies support the effectiveness of garlic in reducing blood glucose in streptozotocin-induced as well as alloxan-induced diabetes mellitus in mice. Most of the studies showed that garlic can reduce blood glucose level in diabetic mice and rabbits (Ohaeri, 2001). A study was conducted to evaluate oral administration of garlic extract for 14 days on the level of serum glucose, total cholesterol, triglycerides, urea and uric acid, in normal and streptozotocin-induced diabetic mice. The result of the study showed significant decrease ($p < 0.05$) in serum glucose, total cholesterol, triglycerides, urea, uric acid, aspartate amino transferase and alanine amino transferase levels, while increased serum insulin in diabetic mice, but not in normal mice. From a comparison study made between the action of garlic extract and glibenclamide, it was shown that the antidiabetic effect of the garlic was more effective than the glibenclamide (Eidi et al., 2006).

Anticancer

Of the many favorable actions of garlic, inhibition of the growth of cancer is perhaps the most prominent. It has several synergistic effects that either prevent or possibly may fight cancer. The action of garlic has been attributed to stimulate immune effector cells including T-cell and natural killer cells. Numerous epidemiological, clinical and laboratory studies have demonstrated that, garlic has a great role in cancer prevention especially in relation to digestive tract cancers. Human population studies have shown that, regular intake of garlic reduces the risk of esophageal, stomach and colon cancer. This was thought to be due to the antioxidant effect of alliin in reducing the formation of carcinogenic compounds in the gastrointestinal tract (Galeone et al., 2006).

Dutch research in the Netherlands cohort study found a significant decrease in the development of stomach cancer in those consuming garlic close relatives of onions (Dorant et al., 1996). Garlic reduces the risk of patients with prostate cancer, especially those with localized disease. Men in the higher of two intake categories of total *Allium* vegetables (>10.0 g/day) had a statistically significant lower risk of prostate cancer than those in the lowest category (<2.2 g/day). Similar comparisons

between categories showed reductions in risk for men in the highest intake categories for garlic specifically. The reduced risk of prostate cancer was independent of body size, intake of other foods and total calorie intake and was more pronounced for men with localized prostate cancer than with advanced prostate cancer (Hsing et al., 2002). Prostate specific antigen serum markers had significant decreases during short term ingestion, but returned to baseline after 4 weeks (Mehraban et al., 2006).

A very important epidemiological study for Americans has been published in which the intake of 127 foods (including 44 vegetables and fruits) was determined in 41,387 women (ages 55 to 69) followed by a five year monitoring of colon cancer incidence. The most striking result of this "Iowa Women's Health Study" was the finding that garlic was the only food which showed a statistically significant association with decreased colon cancer risk. For cancers anywhere in the colon, the modest consumption of one or more servings of garlic (fresh or powdered) per week resulted in a 35% lower risk, while a 50% lower risk was found for cancer of the distal colon (Steinmetz et al., 1994).

Dermatologic applications

A study examined 43 persons for their topical use of two different garlic extracts for wart and corn treatment. Of these persons, 15 volunteers utilized a water extract of garlic, while 23 volunteers applied lipid extract to appropriate areas twice a day. Five controls applied only a neutral solvent. All lipid extract volunteers experienced complete resolution of wart and 80% of corn within one to two weeks. The water extract seemed to be less potent, with complete dissolution of smaller warts and corns, and only partial dissolution of larger ones. Controls showed no improvement from baseline. The lipid extract did cause some burning, redness, blistering and skin darkening, which was resolved after conclusion of use (Dehghani et al., 2005).

Antimicrobial

The antimicrobial properties of garlic were first described by Pasteur (1958), and since then, many researches had demonstrated its effectiveness and broad spectrum antimicrobial activity against many species of bacteria, viruses, parasites, protozoan and fungi (Jaber and Al-Mossawi, 2007). Garlic is more effective with least side effects as compared to commercial antibiotics; as a result, they are used as an alternative remedy for treatment of various infections (Tepe et al., 2004). Out of the many medicinal plants, garlic has an antimicrobial property which protects the host from other pathogens highlighting the importance of search for natural antimicrobial drugs (Bajpai et al., 2005; Wojdylo et al.,

2007). Previously conducted researches confirmed that garlic is not only effective against Gram positive and Gram negative bacteria but also possess antiviral and antifungal activities (Tsao and Yin, 2001).

Antiviral

Garlic and its sulfur constituents verified antiviral activity against coxsackievirus species, herpes simplex virus types 1 and 2, influenza B, para-influenza virus type 3, vaccinia virus, vesicular stomatitis virus, human immunodeficiency virus type 1 and human rhinovirus type 2. The order of compounds found in garlic for virucidal activity was, ajoene > allicin > allyl methyl thiosulfanate > methyl allyl thiosulfanate; no activity was found for the polar fractions, alliin, deoxyalliin, diallyl disulfide, or diallyl trisulfide. Several laboratory tests have shown that garlic is an effectual treatment for both the influenza B virus and herpes simplex virus. Two independent researchers in Japan and Romania have found that garlic is able to protect living organisms from the influenza virus (Tsai et al., 1985). Most recently, a double blind placebo controlled study has shown significant protection from the common cold virus. As conducted by The Garlic Centre, published in *Advances in Therapy*, this is the first serious work to show prevention, treatment and reduction of re-infection benefits from taking Allimax Powder capsules once daily (Josling, 2001).

Antibacterial

Garlic extract inhibits the growth of Gram positive and Gram negative bacteria, such as *Staphylococcus*, *Streptococcus*, *Micrococcus*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Pseudomonas*, *Shigella*, *Salmonella*, *Proteus*, and *Helicobacter pylori* (Tsao and Yin, 2001). Its antibacterial activity is mainly due to the presence of allicin produced by the enzymatic activity of allinase on alliin. Allicin is considered to be the most potent antibacterial agent in crushed garlic extracts, but it can be unstable, breaking down within 16 h at 23°C (Hahn, 1996). However, the use of a water-based extract of allicin stabilizes the allicin molecule due to the hydrogen bonding of water to the reactive oxygen atom in allicin or there may be water soluble components in crushed garlic that destabilize the molecule (Lawson, 1996). The disadvantage of this approach is that allicin can react with water to form diallyl disulphide, which does not exhibit the same level of antibacterial activity of allicin (Lawson and Wang, 1996).

Antifungal

Ajoene is an active compound found in garlic which plays a great role as topical antifungal agent (Ledezma and

Apitz-Castro, 2006). Garlic has been shown to inhibit growth of fungal diseases as equally as the drug ketoconazole, when tested on the fungi *Malassezia furfur*, *Candida albicans*, *Aspergillus*, *Cryptococcus* and other *Candida* species (Shams-Ghahfarokhi et al., 2006). A report from a Chinese medical journal delineates the use of intravenous garlic to treat a potentially fatal and rare fungal infection of the brain called *Cryptococcus meningitis*. In the report, the Chinese compared the effectiveness of the garlic with standard medical treatment which involved a very toxic antibiotic called Amphotericin-B. The study revealed that, intravenous garlic was more effective than the drug and was not toxic regardless of its dosage (Lemar et al., 2007).

A study found that *Candida* colonies were substantially reduced in mice that had been treated using liquid garlic extract. The study also revealed that garlic stimulated phagocytic activity. This implies that infections such as *Candida* may be controlled because garlic stimulates the body's own defenses. Garlic oil can be used to treat ring-worm, skin parasites and warts if it is applied externally. Lesions that were caused by skin fungi in rabbits and guinea pigs were treated with external applications of garlic extract and began to heal after seven days (Sabitha et al., 2005).

Antiparasitic

Many herbalists worldwide recommend garlic as a treatment for intestinal parasites. In some cultures, children infested with helminthes are treated with enemas containing crushed garlic. One of the traditional Chinese medical treatments for intestinal diseases is an alcoholic extract of crushed garlic cloves. Allicin exhibits anti-parasitic activity against major human intestinal parasites such as *Entamoeba histolytica*, *Ascaris lumbricoides* and *Giardia lamblia* (Kalyesa et al., 1975). *Entamoeba histolytica*, the human intestinal protozoan parasite, is very sensitive to allicin, as only 30 µg/ml of allicin totally inhibits the growth of amoeba cultures (Mirelman et al., 1987). Moreover, researchers have found that at lower concentrations (5 µg/ml), allicin inhibited 90% the virulence of trophozoites of *E. histolytica* as determined by their inability to destroy mono-layers of tissue-cultured mammalian cells *in vitro* (Ankri et al., 1997).

Role of garlic against multi-drug resistant bacteria

Garlic is active against microorganisms that are resistant to antibiotics and the combination of garlic extracts with antibiotics leads to partial and total synergism (Didry et al., 1992). The emergence of multi-drug resistant strains of Gram negative (*Pseudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, *Salmonella* species, etc) and Gram positive (*Staphylococcus*, *Enterococcus*, *Streptococcus* species, etc) bacteria is troubling for human and

animals. The emergence of epidemic methicillin resistant *Staphylococcus aureus* (MRSA) resistant to mupirocin has led many authors to suggest that the use of mupirocin should be controlled more strictly, especially as there is a lack of alternative agents. Consequently, garlic is an alternative agent for the treatment of MRSA and in a great demand (Sharma et al., 2005).

Role of garlic against multi-drug resistant tuberculosis (MDR-TB)

Scientific evidence from randomized clinical trials supports the use of garlic and enhances access for MDR-TB infected people, through the public health system. Its use can allow an effective MDR-TB management, due to its affordability and the absence of toxic effects (Catia et al., 2011). In view of the increased incidence of MDR-TB, the research of new anti-tubercular drugs based on affordable and more effective treatments has already begun. Studies on innovative alternative plant extracts of medicinal values need to be emphasized, as plants are an important source of new antimicrobial agents, with little toxicity, able to replace drugs to which *Mycobacterium* resistance has occurred (Amin et al., 2009).

As garlic is concerned, the *in vitro* tests undertaken about the inhibitory effect on MDR-TB are at an advanced stage whereas few researches *in vivo* have been conducted. The concentration of garlic extract required was in the range of 1.34 to 3.35 mg/ml suggesting that there is only a slight variation in the susceptibility of the strains to allicin (Delaha and Garagusi, 1985). The anti-tuberculosis activity *in vivo* of garlic oil preparation was demonstrated in a study of guinea pigs which were given an intra-peritoneal dose of 0.5 mg/kg. However, when garlic oil was used, a reduced causative process was noted in the organs involved, indicating that garlic oil administration causes less marked lesions in the viscera of the animals inoculated with tubercle bacilli (Jain, 1998). The high potential of garlic extract was revealed to inhibit the growth of *Mycobacterium tuberculosis* H₃₇R_v and *M. tuberculosis* TRC-C1193, susceptible and resistant to isoniazid (first-line anti-tuberculosis medication), respectively. The minimum inhibitory concentration (MIC) of garlic was between 80 and 160 µg/ml for the susceptible strain and 100 and 200 µg/ml for the resistant strain. In addition, water extract of garlic was proven to inhibit the incorporation of ¹⁴C glycine into the whole cells, indicating that the primary mechanism of action is by inhibition of protein synthesis (Ratnakar and Murthy, 1996).

An interesting *in vitro* test about the anti-tubercular activity of garlic was performed in Nigeria using disc diffusion method and compared with standard antibiotics. The anti-tubercular activity of garlic on multiple-drug resistant *Mycobacterium* was investigated among Nigerian HIV-infected-persons and it exhibited maximal activity against all isolates even at reduced concentrations. Only

two of the standard anti-tubercular antibiotics used, streptomycin and rifampicin, showed significant activity against isolates tested (Dibua, 2010).

Antioxidant

Whole garlic and aged garlic extract exhibit direct antioxidant effects and enhance the serum levels of two antioxidant enzymes, catalase and glutathione peroxidase (Prasad et al., 1995). Garlic extract, allicin is efficiently scavenged exogenously generated hydroxyl radicals in a dose dependent fashion, but their effectiveness was reduced about 10% by heating to 100°C for 20 min. Other garlic constituents, such as S-allyl cysteine, also confirmed significant antioxidant effects. The sulfur compounds found in fresh garlic appear to be nearly 1000 times more potent as antioxidants than crude, aged garlic extract. Garlic (both the homogenate of 10% in physiological saline solution and its supernatant) was able to reduce the radicals present in cigarette smoke (Torok et al., 1994).

Drug toxicities and pharmacokinetics

Glutathione is a compound necessary for liver to facilitate detoxification of substances. It has been hypothesized that garlic organo-sulfur compounds may be able to prevent glutathione depletion. Patients who experience increasing in reactive oxygen induced stress on liver function may be protected by garlic ingestion (Sabayan et al., 2006). It was found in *E. coli* cultures that aged garlic extract, S-allyl cysteine, diallyl sulfide and diallyl disulfide do not interfere with the antibiotic activity of gentamycin but may improve gentamycin-induced nephrotoxicity (Maldonado et al., 2005). Aged garlic has also been shown to reverse oxidant effects of nicotine toxicity in rat studies. More researches are required in the future garlic may be a unique choice to help minimize the toxic effects of therapeutic drugs (Sener et al., 2005).

Reduces stress

Among the many uses of garlic, it appears to have the fortunate capacity for protecting against the negative effects of stress that affects the autonomic nervous and neuroendocrine system. Rats that were trained with endurance exercises to physical fatigue enjoyed improved parameters of aerobic glucose metabolism, attenuated oxidative stress, and vasodilations, when given garlic at a dosage of 2.86 g/kg for 30 min before exercise (Morihara et al., 2006). In rats exposed to psychologically stressful situations, aged garlic extracts significantly prevented the decreases in spleen weight seen in control animals. Additionally, the garlic significantly prevented the reduction of hemolytic plaque forming cells in spleen cells.

Moreover, garlic was able to block the lipopolysaccharide induced immune cytokine and plasma corticosterone and catecholamine changes following cold water immersion stress (Nance et al., 2006). Aged garlic extract is also effective to prevent adrenal hypertrophy, hyperglycemia and elevation of corticosterone in hyperglycemic mice induced by immobilization stress. Given the extreme chronic stress many people now face in their daily life, garlic may prove useful to counter the negative impact of this stress on human physiology (Kasuga et al., 1999).

Adverse effects of garlic

The main adverse effect commonly associated with garlic intake is breath odor, especially when raw forms of the herb are used. Nausea and vomiting are other major adverse effects and care should be taken in consuming high quantities. Although an entire bulb produces little juice, it is potent and can act as a strong emetic, even in small quantities. Although garlic generally poses little in terms of safety issues, there are isolated cases of topical garlic burns (Friedman et al., 2006) and anaphylaxis (Yin and Li, 2007). Rare garlic allergy has been attributed to the protein allinase, which has induced immunoglobulin E (IgE) mediated hypersensitivity responses from skin prick testing (Kao et al., 2004). As a result, the literature has generally cautioned against using garlic while using anticoagulant therapy. There is a reported case of spontaneous spinal or epidural hematoma in an 87 years old man, with associated platelet dysfunction related to excessive garlic ingestion (Saw et al., 2006).

CONCLUSION

Garlic, from crushed to capsules, is consumed throughout the world. This review paper demonstrated some of the benefits of garlic for its potential uses in preventing and curing different diseases, and acting as antioxidant for many radicals. Fresh and powdered garlic are popular for food flavor and should continue to be used. Today, with the ever-growing resistant organisms, taking of garlic extract remains a powerful antimicrobial agent. Clearly more studies are needed to refine the use and improvement of the efficacy of this important medicinal plant.

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Full Length Research Paper

Antifungal activity of some species of marine sponges (class: Demospongiae) of the palk bay, southeast coast of India

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Nine species of sponges (Orders: Keratosida, Hadromerida, Haplosclerida and Poecilosclerida) of the class Demospongiae were tested for antifungal activity. In general, only trace and moderate activity was observed against the fungal pathogens tested. However, the encrusting Keratosida sponges depicted strong antifungal activity than the other sponges. *In vitro* antifungal activity of sponge extracts was determined against six species of pathogenic fungi (*Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Penicillium* sp., *Candida albicans*, *Cryptococcus* sp.). Stock cultures of fungi were maintained in Czapek Dox agar. On the surface of the medium, discs inoculated with the extract (20 µl/6 mm disc) were placed. The inhibition zone was measured after 72 h of incubation and the antifungal activity has been expressed as inhibition zone in mm. In the present investigation, four solvents viz; ethanol, methanol, acetone and chloroform were used to obtain crude extracts from sponges. Of these, only chloroform and ethanol extracts showed activity against the fungi. There was higher activity against *Cryptococcus* sp. (10 mm) in the crude extracts of chloroform and minimum activity against *A. fumigatus* (5 mm) in the crude extract of ethanol. From the present study, it could be understood that the sponges might be used for the extraction of useful drugs that have antifungal activity against the important human pathogenic fungal species. Besides, the result of the present study is providing baseline data for the future researches in this line of work and is also throwing more light on the use of sponges by the pharmaceutical technologies for the extraction of useful drugs.

Key words: Demospongiae, solvents, antifungal activity, Palk Bay.

INTRODUCTION

Incidence of fungal infection is emerging worldwide, and despite treatment, mortality remains high (Dannaoui et al., 2003). Many studies have shown an increasing frequency of systemic infections within the last decades in advanced human immunodeficiency virus infected patients and other patients with deficient immune systems (Thiebaut, 2002). Currently, very few antifungal agents are available and their use may be limited by dangerous side effects (Lorthoraly et al., 1999; Andriole, 1999). Furthermore, with the emergence of new triazol-

resistant strains of fungi, new compounds must still be screened in search of fungicidal effect, with a broad spectrum of activity and without side effects. In the marine environment, sponges (Porifera) are one of the richest sources of both biologically active secondary metabolites and chemical diversity (Kijjoo and Sawangwong, 2004; Proksch et al., 2003). These natural products may play a role in warding off predators, and perhaps they also repel fouling organisms. Marine sponges are also a well known source of a unique class

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Table 1. List of marine sponges tested for antifungal activity.

| S/No | Order | Family | Species |
|------|----------------|-----------------|---|
| 1 | | Aplysillidae | <i>Psammaplysilla purpurea</i> (Carter) |
| 2 | Keratosida | Spongiidae | <i>Spongia officinalis</i> Linnaeus var. <i>ceylonensis</i> Dendy |
| 3 | | Spongiidae | <i>Hyattella cribriformis</i> (Hyatt) |
| 4 | | Dysideidae | <i>Dysidea fragilis</i> (Mantagu) |
| 5 | Hadromerida | Spirastrellidae | <i>Spirastrella inconstans</i> var. <i>digitata</i> (Dendy) |
| 6 | | Spirastrellidae | <i>Spirastrella inconstans</i> (Dendy) |
| 7 | Haplosclerida | Haliclonidae | <i>Haliclona tenuiramosa</i> (Burton) |
| 8 | | Callyspongiidae | <i>Callyspongia diffusa</i> (Ridely) |
| 9 | Pocelosclerida | Psammascidae | <i>Desmapsamma anchorata</i> (Carter) |

of metabolites collectively known as C21 bisfuranoterpenes (Fontana et al., 1996; Rochfort et al., 1996; Capon et al., 2001). Hence, the present study was undertaken to investigate the antifungal properties of nine species of sponges (Table 1).

MATERIALS AND METHODS

Collection and preparation of samples

Nine species of marine sponges (class: Demospongiae) were collected from the shallow waters of the Palk Bay region (Lat. 9° 17' N, Long. 79° 17' E) at Gandhi Nagar, southeast coast of India. These samples were rinsed with sterile seawater to remove associated debris and salt. The specimens were weighed (5 g) and preserved separately in ethanol, methanol, acetone and chloroform (1:2) and brought to the laboratory. Afterwards, these specimens were soaked in the above mentioned solvents respectively for 48 h. The extracts were later obtained from the soaked samples by grinding, using pestle and mortar and filtering through Whatman No.1 filter paper. The filterates were centrifuged at 3,000 rpm. The solvent was evaporated under reduced pressure using desiccator. After the evaporation of the solvents, the extracts became brown, yellow, black and pink in colour and were thick; the residue was weighed and dissolved in the same solvents for antifungal testing. Six strains of human pathogenic fungi were obtained from Raja Muthiah Medical College, Annamalai University, India. The characteristics and symptoms of test fungi are summarized in Table 2.

Antifungal susceptibility assays

In vitro antifungal activity of sponge extracts was determined against six species of pathogenic fungi. Stock cultures of fungi were maintained in Czapek Dox agar. Inoculum for *Candida albicans* was prepared by spread plating of 0.2 ml of 24 h old cultures grown on Czapek Dox broth. For *Aspergillus flavus*, *Aspergillus fumigatus* and *Aspergillus niger*, well-drained spores were distributed uniformly on the surface of the agar plates with the help of a sterile cotton swab. Other fungal strains (*Cryptococcus* sp. and *Penicillium* sp.) were inoculated by taking a piece of fungal colony using a sterile cotton swab and gently swabbed on the surface uniformly. Agar was used as the medium for antifungal assay. On the surface

of the medium, discs inoculated with the extract (20 µl/6 mm disc) were placed. The inhibition zone was measured after 72 h of incubation and the antifungal activity has been expressed as inhibition zone in mm (Sithrangabopathy, 2003).

RESULTS AND DISCUSSION

The results of antifungal activity of sponges are shown in Figure 1A to D. In the present study in general, only trace and moderate activity of sponges was observed against the human pathogenic fungi tested. Four species of the order Keratosida (*Psammaplysilla purpurea*, *Spongia officinalis* var. *ceylonensis*, *Hyattella cribriformis* and *Dysidea fragilis*) exhibited antifungal activities. In the other Orders, Poecilosclerida, Haplosclerida and Hadromerida, (*Commelina diffusa*, *Stenalia inconstans*, *Stenalia inconstans* var. *digitata*, *Haliclona tenuiramosa* and *Desmapsamma anchorata*) did not exhibit any antifungal activity. Further, only ethanol and chloroform sponge extracts showed antifungal activity while others (extracts of methanol and acetone) did not affect the growth of the test fungi.

The metabolites extracted from the species of *Hyposmocoma communis* showed activity only against *C. albicans* and *A. fumigatus* (Rifai et al., 2004). The sponges examined in the present investigation come under the Orders: Keratosida, Poecilosclerida, Haplosclerida and Hadromerida and only a few members of these orders have been reported to possess a broad spectrum of biological activities (Amade et al., 1982). Though studies (Lazarua and Anita Mary, 2000) reveal that the chemical composition varies considerably from family to family in the Keratosida sponges, many 'wonder' compounds have been isolated from *Spongia officinalis*, *Psammaplysilla purpurea*, *Dysidea fragilis* and *Dendrilla* sp. Thus, Keratose sponges appear to be good sources than the other orders of the Phylum: Porifera in possessing 'wonder drug' potentials. In the present

Table 2. List of fungal pathogens used for assay and their characteristics.

| S/No | Fungal pathogen | Disease | Symptom |
|------|---|----------------|--|
| 1 | <i>Aspergillus niger</i> Van Tieghem | Aspergillosis | Infection in blood vessels |
| 2 | <i>Aspergillus flavus</i> (Raper and Fennell) | Liver cancer | Anomalous growth of liver cells |
| 3 | <i>Aspergillus fumigatus</i> (Fresenius) | Fungus ball | Lung problem |
| 4 | <i>Penicillium</i> sp. | Penicilliosis | Opportunistic in human infection and opportunistic in HIV infected persons |
| 5 | <i>Candida albicans</i> (C.P.Robin) Berkhout | Candidiasis | Superficial infection in skin, mouth and throat and genital lesions |
| 6 | <i>Cryptococcus</i> sp. | Cryptococcosis | Pneumonitis and cryptococcal meningitis |

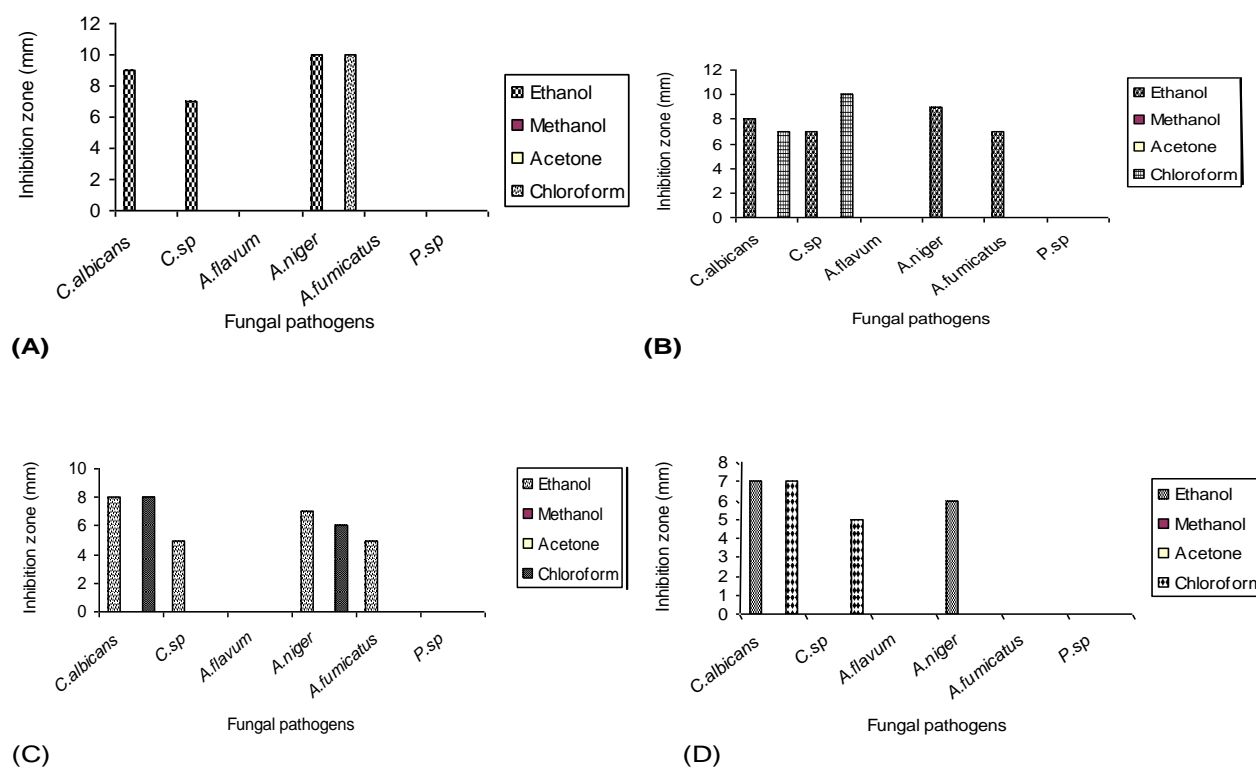


Figure 1. Antifungal activity of some species of marine sponges collected from the Palk Bay. A. Antifungal activity of *S. officinalis* B. Antifungal activity of *P. purpurea* (C) Antifungal activity of *H. cribriformis* (D) Antifungal activity of *D. fragilis*

investigation, it was found that all the sponge species of the Order, Keratosida possessed good activity against *C. albicans*, *A. niger*, *A. fumigatus* and *A. flavus*. This could be attributed to the fact that the Keratosida sponges have elasticity, more water retention capacity and more bioactive substances than the other sponge orders (Lazarus and Anita, 2000).

In an earlier study (Amade et al., 1987), out of the 7 species of Brittany sponges, only two (*D. fragilis* and *Phakellia ventilabrum*) showed slight inhibition on some bacteria and fungi. However, in the present study, *D. fragilis* showed the inhibition against *C. albicans*, *Cryptococcus* sp. *A. fumigatus* and *A. niger*. Antimicrobial activities of *S. officinalis*, *Crambe crambe* and *Ircinia fasciculata* have also been studied (Amade et al., 1987; Burkholder and Ruetzler, 1969; Uriz et al., 1992). In this regard, present study is significant in that the antifungal activity of *S. officinalis* var. *ceylonensis* was higher than that of the earlier reports.

Ethanollic extracts of 19 species of sponges collected from Polynesia were tested against bacteria and fungi. Among these, 8 had no activity, 4 had very weak activity and 7 showed significant activity against bacteria and fungi (Amade et al., 1982). In the present study, out of the ethanolic extracts of 9 species of sponges collected from the Palk Bay region, only 4 species (*S. officinalis* var. *ceylonensis*, *P. purpurea*, *D. fragilis* and *H. cribriformis*) extracts were slightly active against fungi and 5 species (*H. tenuiramosa*, *D. anchorata*, *S. inconstans* and *S. inconstans* var. *digitata*) extracts showed no fungal inhibition. Thus, the antimicrobial activity of sponges may vary from species to species as determined by the biochemical and physiological synthesis of antimicrobial compounds. When the chemical defense and anti-fouling activity were analysed, strong antimicrobial activity was found in the dichloromethane extract of *Ircinia spinosula* (against marine fungi and bacteria) and the ethanol extract of *Ircinia oros* (against diatoms) (Tsoukatou et al., 2002). Such studies including the present one may thus be useful in the prevention and/or control of biofilm formation of microbes.

Though petroleum ether, chloroform and methanol extracts of the sponge *Tethya* sp. were tested, only the petroleum ether extract was very active against mosquito larvae. But the petroleum ether extract showed lesser haemolytic activity whereas the chloroform extract showed maximum lytic activity, indicating the presence of toxicity in the chloroform extract (Madhavi and Sujala, 1998). In the present study, in the chloroform extracts activity was noticed only against *C. albicans*, *A. niger* and *Cryptococcus* sp. But the acetone extracts of the 9 species of sponges had no activity against all the fungal species tested. Of the different species of sponges, only 4 species viz *P. purpurea*, *S. officinalis* var. *ceylonensis*, *H. cribriformis* and *D. fragilis* exhibited inhibitory activity against the pathogenic fungi viz *C. albicans*, *A. niger*, *Cryptococcus* sp. *A. fumigatus* and *A. flavus* and 5

species namely *S. inconstans* var. *digitata*, *S. inconstans*, *C. diffusa*, *D. anchorata* and *H. tenuiramosa* showed no activity against all the fungal pathogens tested in the present investigation.

Thus, it can be inferred that the fungi are more resistant to the sponge extracts. This could be attributed to the fact that the cell walls of the fungi are composed of chitin, a nitrogen containing polysaccharide. The hard cover of the crabs and exoskeletons of arthropods are also composed of this substance chitin, which is relatively resistant, including for microbial decomposition (Ronald, 1997).

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Full Length Research Paper

***Helicobacter pylori* sero-prevalence in different liver diseases**

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This study was carried out to investigate the seroprevalence of *Helicobacter pylori* infection in patients with different liver diseases and determine the association and correlation between the seroprevalence of *H. pylori* infection and the liver diseases. The presence of a *H. pylori* antigen was investigated in serum samples from 274 individuals with liver diseases as well as 120 healthy individuals. *H. pylori* antigen was detected using enzyme-linked immunosorbent assay (ELISA) and Western blot based on specific anti-*H. pylori* antibody. The result was analyzed using the chi-square test. *H. pylori* was detected in sera samples of 31.7% (20/63) ($X^2 = 3.7$) of non-cirrhotic, 50% (11/22) ($X^2 = 3.9$) of cirrhotic and 56.1% (106/189) ($X^2 = 5.2$) of hepatocellular carcinoma (HCC) individuals, compared to 8.4% (10/120) of healthy individuals. The levels of *H. pylori* antigen were significantly higher ($p < 0.05$) in sera of different stages of liver diseases compared by healthy individuals. We found a good correlation between *H. pylori* antigen levels and the severity of the liver diseases (Pathology) ($r = 0.368$, $p < 0.001$). Also, there is a correlation between age and *H. pylori* antigen levels ($r = 0.25$, $p < 0.001$). *H. pylori* infection is correlated with occurrence and development of different stages of liver diseases.

Key words: Liver diseases, *Helicobacter pylori*.

INTRODUCTION

In African countries, there is a high prevalence of *Helicobacter pylori* infection (Lindkvist et al., 1996). *H. pylori* is a Gram-negative spiral organism which colonizes the gastric mucosa causing chronic gastritis (Yang et al., 2003; Dawsey et al., 2002; Peterson et al., 1991). Genetic research has identified polymorphisms of *H. pylori* virulence factors and the host which could play a role in the clinical outcome of the infection (peptic ulcer or gastric cancer) (Buzás, 2012). *H. pylori* are successful colonizers of the human gastric mucosa. Colonization increases the risk of peptic ulcer disease and adenocarcinoma. However, potential benefits of

H. pylori colonization include protection against early-onset asthma and against gastrointestinal infections (Kienesberger et al., 2012). *H. pylori* was classified as a group 1 carcinogen for gastric cancer by the International Agency for Research on Cancer (1994).

It was found that between two to 20 percent of people infected with *H. pylori* will develop ulcers (Kusters et al., 2006). Some evidence also links *H. pylori* infection to gastric cancer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and perhaps pancreatic cancer and cardiovascular disease (Kusters et al., 2006). Also, smoking is identified as a modifiable risk factor for *H.*

pylori infection (Bastos et al., 2013)

Histology investigation of endoscopic biopsies showed the golden standard in the diagnosis of *H. pylori* related gastritis (El-Zimaity et al., 1996). However, endoscopy is an invasive procedure that requires general anesthesia (Bourke et al., 2005). Attallah (2004) developed a sensitive and specific noninvasive immunoassay based on the detection of an *H. pylori* circulating antigen (HpCA) in sera of *H. pylori*-infected individuals (Abdel fattah et al., 2004). Concerning hepatic fibrosis (non-cirrhotic) is a common sequel to diverse liver injuries (Lun-Gen et al., 2003), fibrosis could be a cause of functional hepatic failure (Ahn et al., 2002). Progression of non-cirrhotic liver with the development of cirrhosis is a feature of almost all chronic liver diseases (De Ledinghen et al., 2004). This progression may continually cause hepatocellular carcinoma (HCC) (Giannell et al., 2003). HCC is the fourth cause of death due to cancer worldwide (Poon and Borys, 2011).

A striking finding indicated by Ward et al. (1994) was that a bacterial infection of the liver in healthy A/JCr male mice was capable of inducing a strong inflammatory change in the parenchyma (that is, hepatitis) leading to hepatocellular carcinoma. This bacterial pathogen was demonstrated to belong to *Helicobacter* genus. *H. pylori* infection is also very common in subjects suffering from liver cirrhosis (Goo et al., 2009; Ponzetto et al., 2000), but its prevalence has never been reported in HCC patients.

In this study, we show *H. pylori* sero-prevalence in different liver diseases compared with control subjects. Also, we want to find a correlation between levels of *H. pylori* antigen and the severity of the liver diseases (Pathology).

MATERIALS AND METHODS

A total 274 patients suffered from different liver diseases were recruited in this study (mean age = 50.9±10.8 years), beside 120 healthy individuals (mean age = 32.4 ± 6 years). All patients were taken from Gastroenterology Department, Mansoura University. Patients were classified according to histology into three groups.

1. First group: Non-cirrhotic group included 63 patients (mean age = 45.7±9.5).
2. Second group : Cirrhotic group included 22 patients (mean age = 49.5±9.6).
3. Third group : HCC group included 189 patients (mean age = 52.8±10.9).

ELISA for *H. pylori* circulating antigen (HpCA) in serum

Each well of polystyrene microtiter plates was coated with 50 µl of a tested human serum sample diluted in carbonate-bicarbonate buffer (pH 9.6). The plates were incubated overnight at room temperature

and washed three times with 0.05% (vol/vol) PBS-T20 (pH 7.2), and then free active sites were blocked with 0.2% (wt/vol) nonfat milk in carbonate-bicarbonate buffer. After washing of the plates, 50 µl of the specific antisera/well was added to the 58-kDa antigen diluted 1:100 in PBS-T20, and the mixture was incubated at 37°C for 2 h. After the plates were washed, 50 µl of anti-rabbit IgG alkaline phosphatase conjugate (Sigma)/well diluted in 0.2% (wt/vol) nonfat milk in PBS-T20 was added, and the mixture was incubated at 37°C for 1 h. The amount of coupled conjugate was determined by incubation with 50 µl of p-nitrophenyl phosphate substrate (Sigma)/well for 30 min at 37°C. The reaction was stopped by using 3 M NaOH, and absorbance was read at 405 nm. The cutoff level of the ELISA, above or below which the tested sample is considered positive or negative, was calculated as the mean ELISA optical densities (range, 0.135 to 0.377) of a group of 24 serum samples from noninfected healthy individuals ± 3 standard deviations [i.e., 0.257 ± (3 × 0.047) = 0.398]. The mean absorbance value of a group of 32 *H. pylori*-positive individuals was 0.751 (range, 0.411 to 1.250).

Statistical methods

Results were expressed as mean ± SD and were analyzed by using the Student's t test, and ANOVA tests, as appropriate. Correlation between different parameters was performed using Pearson's correlation test. P ≤ 0.05 was considered to be significant. All statistical procedures were performed using SPSS software, version 11 for Windows.

RESULTS

A total of 274 hepatic patients and 120 control subjects were recruited into this study. The demographic and clinical details of these subjects are shown in Table 1. The group of patients is classified into, a non-cirrhotic patients (n = 63), cirrhotic patients (n = 22) and hepatocellular carcinoma (n = 189). Detection of *H. pylori* circulating antigen (HpCA) in human serum by a novel ELISA was developed by Attallah (2004) for the diagnosis of *H. pylori* infection. Investigation of the seroprevalence of *H. pylori* showed that (Table 1):

1. There were significant differences in the *H. pylori* Antigen levels between the control and study groups of different stages of liver diseases (< 0.05).
2. The levels of *H. pylori* antigen were significantly higher in sera of non-cirrhotic group (p = 0.008), cirrhotic group (p = 0.003) and HCC group (p = 0.001) compared to healthy individuals, where, *H. pylori* antigen levels in control group was 0.23 ± 0.07, compared to non-cirrhotic group (0.29 ± 0.16), cirrhotic group (0.34 ± 0.18) and HCC group (0.35 ± 0.15).

However, there was a good correlation between *H. pylori* antigen levels and the severity of the liver diseases (Pathology) (r = 0.368, p < 0.001). Also, there was a

Table 1. Clinical and *Helicobacter pylori* serological parameters in patients and control subjects.

| Parameter | Controls (n=120) | Patients | | | | | | | |
|---------------------------|---------------------|---------------|---------|-----------|---------|-----------|---------|--------------|---------|
| | | Non-cirrhotic | | Cirrhotic | | HCC | | All patients | |
| | | (n=63) | p-value | (n=22) | p-value | (n=189) | p-value | (n=274) | p-value |
| Age (years) | 32.4±6 | 45.7±9.5 | 0.001 | 49.5±9.6 | 0.001 | 52.8±10.9 | 0.001 | 50.9±10.8 | 0.001 |
| <i>H. pylori</i> Ag level | 0.23±0.07 | 0.29±0.16 | 0.008 | 0.34±0.18 | 0.003 | 0.35±0.15 | 0.001 | 0.34±0.16 | 0.001 |

Data shown are mean ± SD. p-values are for comparing data between controls and different patient groups.

Table 2. Positivity of *H. pylori* antigen in different liver diseases.

| <i>H. pylori</i> Ag | Patholgy | | | |
|---------------------|----------|---------------|-----------|-----|
| | Normal | Non-Cirrhotic | Cirrhotic | HCC |
| +ve | 10 | 20 | 11 | 106 |
| -ve | 110 | 43 | 11 | 83 |
| Total number | 120 | 63 | 22 | 189 |
| Positivity (%) | 8.4 | 31.7 | 50 | 56 |
| Association | - | 3.7 | 3.9 | 5.2 |

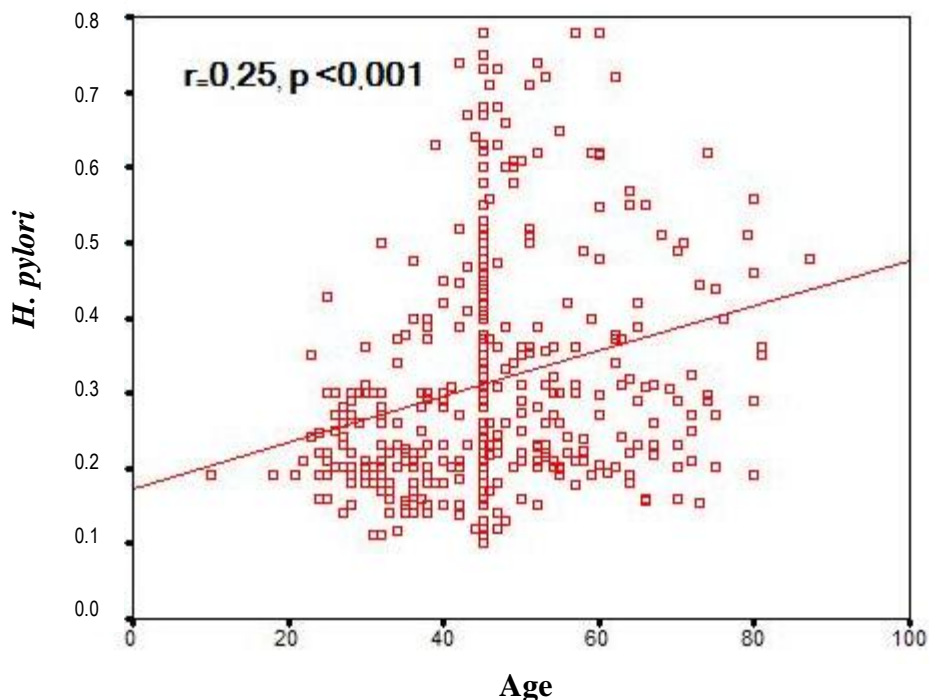


Figure 1. Correlation between age and *H. pylori* antigen levels.

correlation between age and *H. pylori* antigen levels ($r = 0.25, p < 0.001$) as shown as in Figures 1 and 2. *H. pylori* seropositivity was more prevalent among patients with

HCC (106/189, 56.1%) than in controls (10/120, 8.4%) ($p < 0.05$) ($X^2 = 5.2$). While *H. pylori* seropositivity among patients with cirrhosis (11/22, 50%) than in controls ($p <$

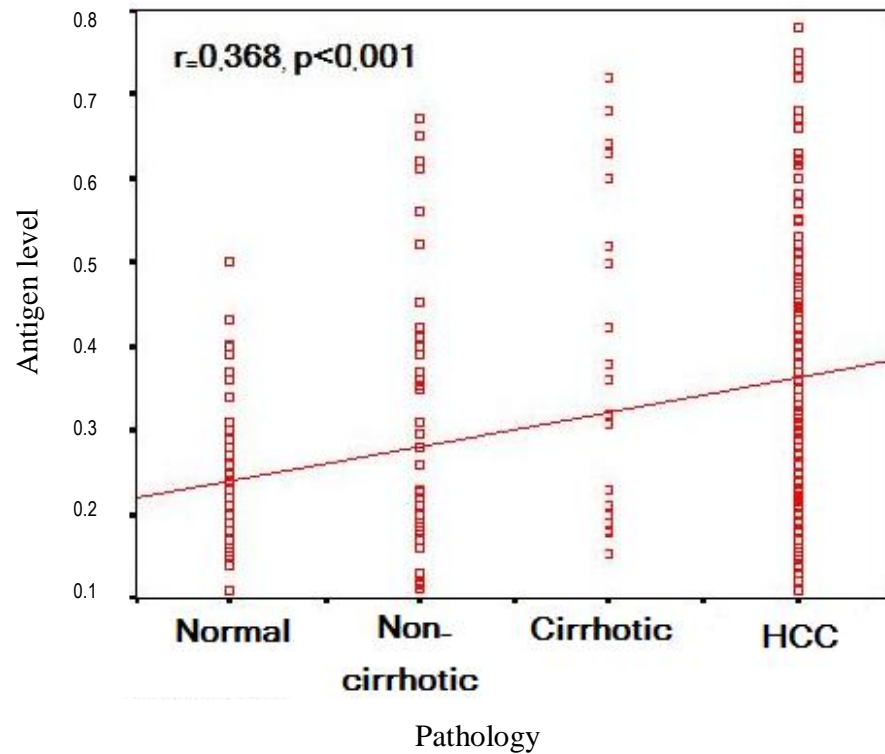


Figure 2. Correlation between Pathology and *H. pylori* antigen levels.

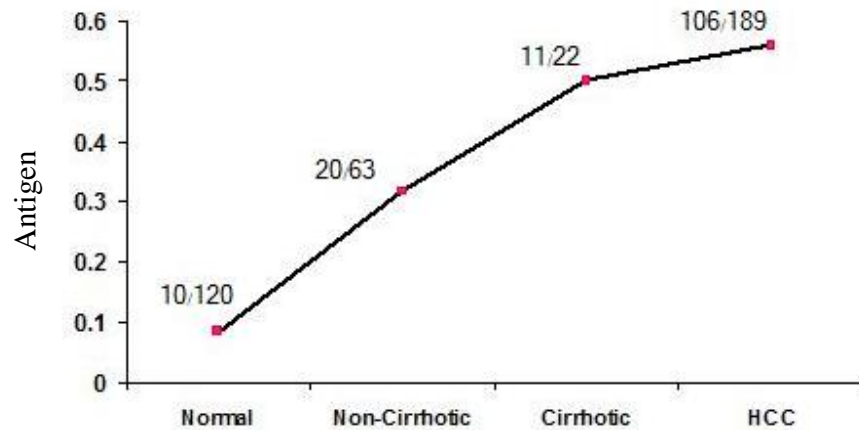


Figure 3. Prevalence of *H. pylori* antigen.

0.05) ($X^2 = 3.9$). Also, seropositivity was (20/63, 31.7%) among non-cirrhotic patients compared by controls ($p < 0.05$) ($X^2 = 3.7$) (Table 2 and Figure 3).

DISCUSSION

H. pylori infection is a chronic infection (Nicola et al.,

2003). In most instances, it is acquired during childhood, and is often associated with low socio-economic class (Mendall et al., 1992; Queiroz et al., 2012). The presence of this bacterium has been strongly established as the main cause of several gastroduodenal diseases, including peptic ulcer disease (Hopkins et al., 1996), gastric carcinoma, and gastric MALT lymphoma

(Wotherspoon et al., 1993). A very high prevalence of *H. pylori* infection in patients with cirrhosis of the liver has been mentioned in several reports: a higher prevalence of *H. pylori* infection was noted by Siringo et al. (1997) and Spinzi et al. (2001), in cirrhotics as compared with blood donors ($p < 0.0005$), in two North Italian towns.

The prevalence of *H. pylori* infection in patients with liver disease needed to be studied on the basis of clinical and experimental considerations (Marilena et al., 2004). From a clinical point of view, the medical history of cirrhotic patients was punctuated by frequent and recurrent hospitalisations due to high rate of complications. Among the most relevant of them, peptic ulcer and upper GI hemorrhage were of peculiar relevance, being life-threatening for the patient and of high cost for Health Care Services, requiring both emergency care and subsequent long hospital stay (Rosina et al., 1996). An important association was observed between recurrent abdominal pain and *H. pylori* infection in some populations (Abolfazl et al., 2013).

From an experimental point of view, infection of healthy A/JCr male mice with *H. hepaticus* could result in chronic hepatitis and liver cancer in a short time (Ward et al., 1994). Since this report, several other *Helicobacter* species have been subsequently found in the liver and biliary tract of cats and dogs suffering from hepatitis and hepatocellular carcinoma (Andersen, 2001). Kirk et al. (1980) demonstrated a frequency 33% of peptic ulcer in patients with chronic liver disease. In an Italian multi-centre study, between 12 and 20% of cirrhotic patients were demonstrated to bear gastric or duodenal ulcer with a high prevalence in the gastric site (Gottardello et al., 1991).

Since peptic ulcer is related to the presence of *H. pylori* infection in non-cirrhotic patients, it is logical to suppose a role for the same bacterium also in subjects with cirrhosis. To search for the presence of the bacterium and to be cured of it, stems from the rationale is used to prevent the development of peptic ulcer and its complications in cirrhotics, too, as we usually do in non-cirrhotic patients. From an epidemiological point of view, several data indicated that only a proportion of patients infected by hepatitis C virus (HCV) develops liver cirrhosis (Guadagnino et al., 1997), and among these only a minority ultimately succumbed to liver cancer, and also that "classical" prognostic features do not explain all the variations of the disease (Wiese et al., 2000). These observations suggested that other factors besides the viral pathogen could concur in generating HCC.

The experimental demonstrated by Ward et al. (1994) showed that *H. hepaticus* causes hepatitis and HCC in male A/JCr mice. A number of *Helicobacter* sp. have been isolated from the liver of cats and dogs with hepatitis (Fox et al., 1996) and from the human biliary tract and gallbladder. In western countries, HCC arises

almost invariably on the background of cirrhosis representing a long-term complication of the disease, after decades of continuing inflammation (Tomiyama et al., 2013; Fox et al., 1998).

One "new" possible mechanism capable of inducing pro-inflammatory cytokines and lymphoid proliferation might indeed be liver or biliary tract infection by bacteria belonging to *Helicobacter* genus. A few papers in the last years reported the finding of genomic sequences belonging to *Helicobacter* spp. in the liver of patients with HCC. We have shown sequences of *Helicobacter* spp. in 23 of 25 human livers with cirrhosis and HCC (Ponzetto et al., 2000).

Avenaud et al. (2000) confirmed these data by demonstrating genomic sequences of *Helicobacter* spp. In eight of liver specimens from patients with HCC and the sequenced polymerase chain reaction (PCR) products confirmed *H. pylori* and *Helicobacter felis* (Nicola et al., 2003). Moreover, Agha-Amiri et al. (1998) found that seven out of 20 patients with HCC, genomic sequences of a bacterium belong to the RNA superfamily VI (Campylobacter, Helicobacter, Arcobacter) with highest homology to *Arcobacter* spp. The role of *Helicobacter* spp. in the evolution of cirrhosis and HCC in humans is unknown, but a potential mechanism has been reported by Taylor et al. (1995), who described a new liver-specific toxin produced by several *Helicobacter* spp.

Conclusion

We found that infection is correlated and associated with occurrence and development of different stages of liver diseases, where the seroprevalence of *H. pylori* in subjects with cirrhosis of the liver and HCC is much more frequent than in controls.

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Full Length Research Paper

Molecular evaluation of antibiotic resistance prevalence in *Pseudomonas aeruginosa* isolated from cockroaches in Southwest Iran

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***Pseudomonas aeruginosa* can cause infection in the hospitals. This microorganism is ubiquitous within the environment and is particularly isolated from moist areas such as water and soil. The aim of this study was to determine the hospital cockroaches as the main factor for antibiotic-Resistant *P. aeruginosa* infections transmission and also to determine antibiogram pattern of *P. aeruginosa*. Also, the pattern of antibiotic resistance showed that a total 14 (11.2%) of 125 samples were contaminated with *P. aeruginosa* isolated from cockroaches in hospitals using molecular sieve of polymerase chain reaction (PCR). The overall susceptible of isolated *P. aeruginosa* strains to antimicrobial agents showed that Amikacin 14 (100%), Ciprofloxacin 14 (100%), Gentamycin 14 (100%), have more susceptibility, respectively. The prevalence of *P. aeruginosa* in cockroaches' hospitals is high and as a potential factor in transmission of *P. aeruginosa*.**

Key words: *Pseudomonas aeruginosa*, antibiotic resistance, cockroaches.

INTRODUCTION

Pseudomonas aeruginosa play an important role in hospital intensive care units, causing a wide spectrum of nosocomial infections (Strom and Lory, 1986; Gomila et al., 2006). *P. aeruginosa* is an aerobic, nonsporulating Gram-negative, motile bacterium (Römling et al., 1994). *P. aeruginosa* causes infection in immune depressed subjects or in those with faulty homeostasis mechanisms (Struelens et al., 1993). *P. aeruginosa* potential pathogens have been isolated from cockroaches collected from hospitals and have proven that cockroaches carry a large flora of pathogenic bacteria (Babalola et al., 2007). The source of the pathogenic versatility of *P. aeruginosa* is undoubtedly its unique genome. Sequencing of this genome was achieved in 2000 (Canduela et al., 2006). For the year 1991 to 1992, *Pseudomonas* species were

the most commonly identified etiologic agents causing dermatitis, conjunctivitis, or otitis in humans following recreational water exposures where water pH or free residual chlorine levels failed to meet Centers for Disease Control and Prevention (CDC) guidelines for public spas and hot tubs (Malathi et al., 2006; Tsuchizaki et al., 2006). *P. aeruginosa* can be a constituent of the bacterial flora of the intestines of laboratory rodents, especially mice. *P. aeruginosa* strains are resistant to β -lactams, aminoglycosides, and quinolones (Buckingham-Meyer et al., 2007; Willcox et al., 2008).

P. aeruginosa is among the most feared pathogens associated with nosocomial infection, especially among mechanically ventilated patients (Trautmann et al., 2005). *P. aeruginosa* is the most common pathogen responsible

Table 1. TEM, SHV, and CTX-M primers of *P. aeruginosa*.

| Gene | Sequence | PCR product (bp) |
|-------|---|------------------|
| Tem | Tem-F: 5'-TCCGCTCATGAGACAATAACC-3' Tem-R: 3'-ATAATACCGCACCACATAGCAG-5' | 296 |
| Ctx-M | Ctx-F: 5'-TCTTCCAGAATAAGGAATCCC-3' Ctx-R: 3'-CCGTTTCCGCTATTACAAAC-5' | 909 |
| Shv | Shv-F: 5'-TACCATGAGCGATAACAGCG-3' Shv-R: 3'-GATTTGCTGATTTGCTCGG-5' | 450 |

for both acute respiratory infections in ventilated or immunocompromised patients and chronic respiratory infections in cystic fibrosis patients (Johnson et al., 2007; Rello et al., 1997). Flagella and pili, the motile surface appendages of *P. aeruginosa* are responsible for bacteria motility and progression towards epithelial contact (Mahenthalingam et al., 1996; Rello et al., 1996). Upon cell contact, the type III secretion system, a major virulence determinant, is activated. Four effectors proteins are known: ExoY, ExoS, ExoT, and ExoU and all participate, at varying levels, in the cytotoxicity of *P. aeruginosa* leading to invasion and dissemination of *P. aeruginosa* (Morfin-Otero et al., 2009). The aim of this research was to determine the prevalence *P. aeruginosa* in cockroaches from hospital in Chaharmahal VA Bakhtiar, Iran using polymerase chain reaction (PCR). The study also tried to specify the pattern of antibiotic resistance in summer of 2011.

MATERIALS AND METHODS

Sample collection

This empirical study was done on 125 cockroaches collected from 6 hospitals located in Chaharmahal Va Bakhtiari province (Hajar and Ayatollah Kashani Hospitals in Shahrekord city, Shohada Hospitak in Farsan city, Imam Javad in Naghan city, Imam Reza in Lordagan city, and Valiasr in Boroujen city). The collection of samples was done using manual and sticky trap methods from hospital kitchens. Then, samples were transmitted to the laboratory of Biotechnology Research Center using separate sterile tube to prevent any contamination mixing of the samples. *Klebsiella pneumoniae* ATCC 700603 (Genekam Biotechnology AG, Germany) and *Escherichia coli* ATCC 25922 was used as positive and negative control, respectively.

Picking up the cultivation of susceptibility

Antimicrobial susceptibility profiles were determined by the dilution method on Mueller-Hinton agar, according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Knowle et al., 1992). The antimicrobial agents tested included trimethoprim, sulfamethoxazol, ceftiazidime, ceftriaxon, imipeneme, tobramycin, amikacin, ciprofloxacin and gentamicin. CLSI breakpoints were used for minimum inhibitory concentration (MIC) interpretation (Empel et

al., 2007). The results were interpreted after 24 h of incubation at 37°C, as sensitive, intermediately sensitive, and resistant according to the zone of diameter around each antibiotic disk.

PCR assay

In order to confirm the presence of *P. aeruginosa* TEM, SHV, and CTX-M genes, PCR test was performed. The primers used for genes amplification are as shown in Table 1.

Amplification reaction was carried out in a total volume of 25 µl, consisting of 1 µM of each primers, 2 mM MgCl₂, 200 µM dNTP, 5 µl of 10X PCR buffer, 1 U of Taq DNA polymerase (Fermentas, Germany) and 1 µg of template DNA. Thermal PCR conditions consisted of 5 min at 95°C and then 30 cycles initial temperature of 94°C, temperature of 58 and 72°C connector at each end for 1 min and final extension was for 5 min at 72°C. The amplified products were analyzed in 1.5% agarose gel. Electrode buffer was TBE (Tris-base 10.8 g 89 mM, Boric acid 5.5 g 2 mM, EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH8.0) combined all components in sufficient H₂O and stir to dissolve). Gels were stained with ethidium bromide. Aliquots of 10 µl of PCR products were applied to the gel. Constant voltage of 80 for 20 min was used for products separation. After electrophoresis images were obtained in UVItec documentation systems (UK).

Statistical analysis

The numbers of cockroaches presenting airsacculitis and the prevalence of re-isolation of *P. aeruginosa* from the swap were analyzed by the chi-square test using the SPSS17 (SPSS Inc. Chicago, IL, USA) software. The probability level for significance was $p \leq 0.05$.

RESULTS

The quality of extracted DNA from samples was examined by electrophoretic analysis through a 1% agarose gel. *P. aeruginosa* was recovered from 14 cockroaches of 125 (11.2%). The percentage of resistant isolates was as follows: 14.28% to ceftriaxon, 13.3% to ceftazidim, 26.66% to sulfamethoxazol and percentage of susceptible isolates was as follows: 100% to tobramycin, imipenem, gentamycin, ciprofloxacin and amikacin. Also, antibiotic resistance pattern is as shown in Table 2.

The TEM gene of *P. aeruginosa* was success fully

Table 2. Antibiotic resistance pattern of *P. aeruginosa*.

| Antimicrobial Agent | Resister | Half Resister | Stark |
|------------------------------------|----------|---------------|-------|
| Trimethoprim sulfamethoxazol (sxt) | 26.66 | 21.42 | 46.66 |
| Amikacin (AN ₃₀) | 0 | 0 | 100 |
| Ciprofloxacin (CP ₅) | 0 | 0 | 100 |
| Gentamycin (GM ₁₀) | 0 | 0 | 100 |
| Ceftriaxon (CRO ₃₀) | 14.28 | 57.14 | 14.28 |
| Imipeneme (IPM ₁₀) | 0 | 0 | 100 |
| Ceftiazidime (CAZ ₃₀) | 13.3 | 28.57 | 53.33 |
| Topramycin (TOB ₁₀) | 0 | 0 | 100 |

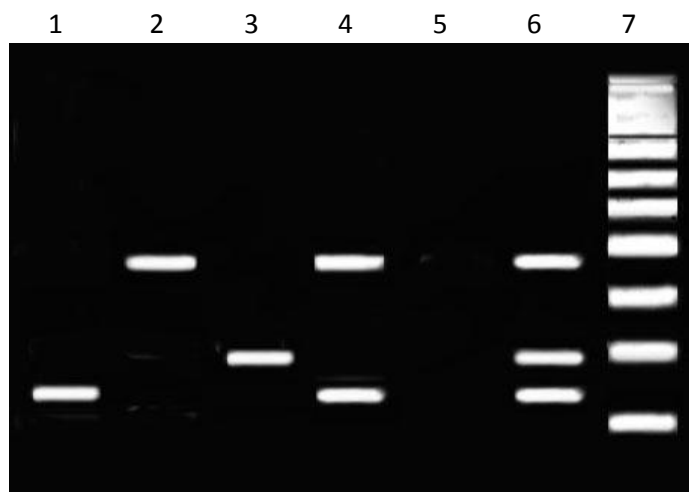


Figure 1. An agarose gel stained with ethidium bromide, for detection of TEM, SHV and CTX-M genes in *P. aeruginosa*. Lines 1, 2 and 3 are positive tests for TEM, CTX-M and SHV, respectively. Line 4 is positive test for TEM and CTX-M. Lines 5 and 6 are negative and positive controls, respectively. Line 7 is 1 kb DNA ladder (Fermentas, Germany).

amplified with the TEM-F and TEM-R primers. Also, agarose gel electrophoresis of the PCR amplified products is as shown in Figure 1. Out of 14 *P. aeruginosa*, 14 samples (100%), 1 sample (7/14%) and 3 samples (21/42%) are positive for TEM, SHV and CTX-M genes by PCR, respectively. The most active agents to treat infection caused by *P. aeruginosa* were amikasin, ciprofloxacin, gentamycin, imipenem and tobramycin.

DISCUSSION

Pseudomonas consists of 5 types that include *Pseudomonas spasya*, *Pseudomonas maltvfyiya*, *Pseudomonas financial*, *Pseudomonas psvdvmalyy* and *P. aeruginosa*. *P. aeruginosa* is one of the most important nosocomial pathogen and is strongly involved in severe and often fatal infections in patients with cystic fibrosis,

burns, ocular diseases, pneumonia, and other immunosuppressive illnesses (5,1). Although *P. aeruginosa* is a nonfermentative aerobe, it can grow under anaerobic conditions using nitrate as an electron receptor. Its ability to survive in a wide range of environmental conditions is partially explained by its versatile nutritional abilities and its ability to resist high concentrations of common antibiotics (Aumeran et al., 2007). *P. aeruginosa* is intrinsically resistant to the most commonly used antibiotics. Antibiotic resistance is achieved through a combination of restricted antibiotic uptake through the outer membrane and a variety of energy-dependent mechanisms (Presteri et al., 2007). Preincubation with antibiotics has been demonstrated to have a number of effects on *P. aeruginosa* including induction of a biofilm form of growth, improved heat and osmotic stress response, changes to hydrophobicity, and reduced bacterial adherence (Panagea et al., 2005).

According to a study in Iranian patients with cystic fibrosis (CF), the antibiograms of the isolates showed 100% sensitivity to imipenem and collistin followed by ciprofloxacin (90.5%), ceftazidime and tobramycin (85.7%), amikacin, piperacillin, gentamycin (62%), and carbenicillin (43%). In other study, MIC determination for amikacin showed a 100% sensitivity as compared to the disk test where 81% sensitivity was observed (Ferrante and Scortichini, 2009; Huson and Bryant, 2006). In other hand, according to Giamarellos-Bourboulis et al. (2006) study, *P. aeruginosa* was resistant to ciprofloxacin (27.1%), ceftazidime (15.7%), cefepime (2.9%), imipenem (67.1%), and piperacilin (14.3%), whereas in our study *P. aeruginosa* was resistant to sulfamethoxazol (26.66%), ceftriaxon (14.28%), ceftiazidime (13.3%), imepenem (0%), amikacin (0%), ciprofloxacin (0%), gentamycin (0%), and topramycin (0%).

Genomic DNA was extracted and the PCR were performed using specific primers for TEM, SHV and CTX-M genes. The results of the resent study showed that TEM gene is present in all of the isolated *P. aeruginosa* and SHV and CTX-M genes present in some isolates.

Conclusion

Conclusively, as stated earlier, this study showed that isolated *P. aeruginosa* of cockroaches from hospital have more resistance to Sulfamethoxazol rate of 26.66%, rather than other studies. Amikacin, ciprofloxacin, gentamcin, and topramycin seem to be the only antimicrobial agent which showed 100% sensitivity and may be used as the drug of choice for treating multidrug resistant *P. aeruginosa* infections. Further, the regular surveillance of hospital associated infections including monitoring antibiotic sensitivity pattern of *P. aeruginosa* and formulation of definite antibiotic policy may be helpful for reducing the incidence of *P. aeruginosa* infection.

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Full Length Research Paper

High prevalence and poor treatment outcome of tuberculosis in North Gondar Zone Prison, Northwest Ethiopia

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The aim of this study was to assess the trend of tuberculosis (TB) prevalence and treatment outcome in a prison system of Northwest Ethiopia. Patients' data on age, sex, TB type, treatment outcome and human immunodeficiency virus (HIV) status was collected from medical records of North Gondar Zone Prison TB Clinic for all patients with TB from 2002 to 2011. The data was analyzed using Statistical Package for Social Sciences (SPSS) version 16. The prevalence of all forms of TB during the ten years ranged from 579 to 2623 per 100,000 populations. The highest treatment success rate, 80% was observed in the year 2002, whereas the lowest treatment success rate, 42% was observed in the year 2004. A total of 114 TB patients were screened for HIV from 2009 to 2011, of which 14 (12.3%) were HIV positive. The prevalence of TB/HIV co-infection ranged from 163 per 100,000 populations in 2009 to 288 per 100,000 populations in 2010. There were high prevalence rates of TB and TB/HIV co-infection among the inmates of North Gondar Zone Prison with poor treatment success rates in comparison to the national figure and world health organization (WHO) target.

Key words: Tuberculosis prevalence, treatment outcome, prison, tuberculosis/human immunodeficiency virus (TB/HIV) co-infection.

INTRODUCTION

Prisons are not mere static venues holding large populations. They represent dynamic communities where at-risk groups congregate in a setting that exacerbates disease and its transmission, including tuberculosis (TB). Prevalence rates of TB in prisons usually exceed the rates in the specific country substantially and can reach up to 50 times higher than national averages (Baussano et al., 2010; WHO, 2007). In line with this, a study from

three major prison settings of Eastern Ethiopia showed a very high prevalence of pulmonary TB, about seven times higher than that of the general population (Abebe et al., 2011).

Routine recording and reporting of the number of TB cases diagnosed and treated, and monitoring of the outcomes of treatment was one of the five elements of TB control emphasized in the directly observed

treatment, short course (DOTS) strategy, and remains one of the core elements of the Stop TB Strategy (WHO, 2001). DOTS as a strategy was introduced to the TB control programme at North Gondar Prison Administration TB clinic in 2002. Nevertheless, achieving the main goals of the DOTS in prisons would be very difficult for prison inmates since they will be freed every time, some of them under treatment for TB. There is a high chance to disappear into the countryside where it is usually difficult to trace and link with the existing health system. Furthermore, even within the prison system, many prisoners would be transferred to other prisons without effective follow up system.

To our knowledge, there was no enough study conducted on trend of TB prevalence and TB treatment outcome in Ethiopian prison inmates. Therefore, this study was conducted to assess the ten years trend of TB prevalence and treatment outcome among prison inmates of North Gondar Zone Prison, Northwest Ethiopia.

MATERIALS AND METHODS

North Gondar Zone Prison Administration is located to the eastern part of the Gondar city which currently accommodates 1754 prison inmates, 1716 men and 38 women. It has got one clinic rendering service to the prison inmates and the prison staff. The clinic also provides TB and HIV diagnostic and treatment services to the inmates and the staff. The medical charts for all the prison inmates who were diagnosed with TB in the prison from 2002 to 2011 were thoroughly reviewed. The medical records of all the 321 TB patients seen in North Gondar Zone Prison were examined. The registration documents reviewed contain basic information, such as patient's age, sex, TB type, treatment outcome and HIV status. Data was analyzed based on TB type (smear-positive pulmonary TB, smear-negative pulmonary TB, extra-pulmonary TB), treatment outcome (cured, completed, defaulted, relapse, death, transferred out), and HIV sero-status.

Definition

According to the standard definitions of the National Tuberculosis and Leprosy Control Program guideline (NTLCP) adopted from WHO (Ministry of Health of Ethiopia 2008), the following clinical case and treatment outcome definitions were used.

Pulmonary TB, smear-positive

A patient with at least two sputum specimens which were positive for acid-fast bacilli (AFB) by microscopy, or a patient with only one sputum specimen which was positive for AFB by microscopy, and chest radiographic abnormalities consistent with active pulmonary TB.

Pulmonary TB, smear-negative

A patient with symptoms suggestive of TB, with at least two sputum specimens which were negative for AFB by microscopy, and with chest radiographic abnormalities consistent with active pulmonary TB (including interstitial or miliary abnormal images), or a patient with two sets of at least two sputum specimens taken at least two

weeks apart, and which were negative for AFB by microscopy, and radiographic abnormalities consistent with pulmonary TB and lack of clinical response to one week of broad spectrum antibiotic therapy.

Extrapulmonary TB (EPTB)

This included TB of organs other than the lungs, such as lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges, etc. Diagnosis of EPTB was based on fine needle aspiration cytology or biochemical analyses of cerebrospinal/pleural/ascitic fluid or histopathological examination or strong clinical evidence consistent with active extrapulmonary TB, followed by a decision of a clinician to treat with a full course of anti-TB chemotherapy. In all the cases of EPTB, sputum examinations and chest radiographs were used to investigate the involvement of lung parenchyma.

Treatment outcome

The treatment outcome was divided into seven categories according to NTLCP guideline. These categories were: cured (finished treatment with negative bacteriology result at the end of treatment), completed treatment (finished treatment, but without bacteriology result at the end of treatment), failure (remaining smear positive at five months despite correct intake of medication), defaulted treatment (patients who interrupted their treatment for two consecutive months or more after registration), died (patients who died from any cause during the course of treatment), transferred out (patients whose treatment results are unknown due to transfer to another health facility) and successfully treated (A patient who was cured or completed treatment).

Treatment success rate (TSR)

It is the sum of the percentages of cure and treatment completed rounded off to the nearest digit (WHO, 2001).

Statistical analysis

Data were entered and analyzed using SPSS version 16. The prevalences of TB and HIV were calculated taking the whole prison population during the respective years. P value less than 0.05 was taken as significant.

Ethical issues

Institutional ethical clearance was obtained from the Institutional Review Board of University of Gondar. Permission from the prison authorities was also obtained before the start of the study. All patients' information was kept strictly confidential.

RESULTS

The number of inmates in North Gondar Prison ranged from 1429 to 2085 during the ten years period with a mean of 1728. During the study period, 321 TB cases were reported and given treatment according to the national guideline. Majority, 311 (96.9%) were male. The mean (\pm standard deviation (SD), range) age of the TB

Table 1. Distribution of TB types (n=321), North Gondar Zone Prison, 2002-2011.

| Year | SP TB [N (%)] | SN TB [N (%)] | EPTB [N (%)] | Total [N (%)] |
|-------|---------------|---------------|--------------|---------------|
| 2002 | 1 (10) | 4 (40) | 5 (50) | 10 (3.1) |
| 2003 | 4 (12.9) | 18 (35.5) | 16 (51.6) | 31 (9.7) |
| 2004 | 6 (17.6) | 11 (55.9) | 9 (26.5) | 34 (10.6) |
| 2005 | 3 (10) | 21 (66.7) | 6 (20) | 30 (9.3) |
| 2006 | 2 (6.4) | 23 (74.2) | 6 (19.4) | 31 (9.7) |
| 2007 | 1 (3.6) | 16 (53.6) | 11 (39.3) | 28 (8.7) |
| 2008 | 7 (15.9) | 22 (50) | 15 (34.1) | 44 (13.7) |
| 2009 | 6 (15.4) | 22 (56.4) | 11 (28.2) | 39 (12.1) |
| 2010 | 4 (14.3) | 17 (60.7) | 7 (25) | 28 (8.7) |
| 2011 | 4 (8.7) | 28 (58.7) | 15 (32.6) | 46 (14.3) |
| Total | 38 (11.8) | 182 (56.7) | 101 (31.5) | 32 (100) |

TB: Tuberculosis; EPTB: extrapulmonary tuberculosis; N: number; SP: smear positive; SN: smear negative.

Table 2. Prevalence of all forms of TB per 100,000 populations in comparison to that of the general population in North Gondar Zone Prison, 2002 to 2011.

| Year | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 |
|---|------|------|------|------|------|------|------|------|------|------|
| Prevalence of TB at the prison per 100,000 population | 579 | 2257 | 1505 | 1736 | 2169 | 1812 | 2557 | 2124 | 1343 | 2623 |
| Prevalence of TB at the general population per 100,000 population | 370 | 520 | 533 | 546 | 641 | 579 | 432 | 406 | 394 | 578 |
| TB in prisons/TB in the general population | 1.6 | 4.3 | 2.8 | 3.2 | 3.4 | 3.1 | 5.9 | 5.2 | 3.4 | 4.5 |

TB: Tuberculosis.

positive inmates was 34 (± 13.9 , 12 to 87) years. Most, 249 (77.6%) were new cases, but there were 4 (1.2%) defaulters and 5 (1.6%) relapses. TB type was categorized as smear positive pulmonary TB in 38 (11.8%), extra pulmonary TB in 101 (31.5%), and smear negative pulmonary TB in 182 (56.7%) (Table 1).

The prevalence of all forms of tuberculosis per 100,000 populations during the ten year period ranged from 579 in 2002 to 2623 in 2011. It was consistently increasing throughout the years and was also higher than the report for the general population (Table 2).

TB treatment outcome was categorized as cured 20 (6.2%), completed 158 (49.5%), defaulted 9 (2.8%), died 10 (3.1%) and transferred out 124 (38.6%). The TSR was calculated for all the ten years. The highest TSR, 80% was observed in 2002 and the lowest TSR, 42% was observed in the year 2004. The prison TSR was lower than the national TSR and WHO target throughout the study period except for the year 2002 (Table 3).

Provider initiated HIV counseling and testing for the TB positive inmates was performed over three years from 2009 to 2011. From the total of 113 screened TB positive inmates, 14 (12.4%) were HIV positive. The prevalence of HIV infection was found to be 3/39 (7.7%), 6/28 (21.4%) and 5/47 (10.6%) in the years 2009, 2010 and 2011, respectively. The prevalence of TB/HIV co-infection was

163, 288 and 285 per 100,000 populations (Table 4).

DISCUSSION

It has been reported that prevalence rates of TB in prisons are usually higher than national averages (Baussano et al., 2010; WHO, 2007). This was reflected in the current study by the high prevalence rate of TB throughout the years ranging from 579/100,000 population in 2002 to 2623/100,000 population in 2011. The high TB prevalence rate observed in this study in the prison setting was also in agreement with the prevalences in the prisons of Eastern Ethiopia which is 1913/100,000 populations (Abebe et al., 2011). However, studies from Zambia, Botswana, Russia and Georgia showed much higher prevalences (Habeenzu et al., 2007; CDC, 2003; Slavuckij et al., 2002; Aerts et al., 2000). On the other hand, lower prevalences were reported from prisons of Asian and European countries, 568/100,000 in Thailand, 259/100,000 in Taiwan, 341/100,000 in Turkey and 215/100,000 in France (Sretrirutchai et al., 2002; Chiang et al., 2002; Hanau-Berçot et al., 2000; Kiter et al., 2003). The low prevalence in these countries could be due to a good TB control strategy in the general population as well as in their

Table 3. Treatment outcome and TSR of the TB patients at North Gondar Zone Prison, 2002 to 2011.

| Year | Treatment outcome | | | | | | TSR at the prison (%) | TSR at the general population (%) |
|-------|-------------------|-------------------|-------------------|--------------|-------------------------|---------------|-----------------------|-----------------------------------|
| | Cured [N (%)] | Completed [N (%)] | Defaulted [N (%)] | Died [N (%)] | Transferred out [N (%)] | Total [N (%)] | | |
| 2002 | 0 (0) | 8 (80) | 0 (0) | 0 (0) | 2 (20) | 10 (3.1) | 80 | 76 |
| 2003 | 3 (7.7) | 16 (41) | 1 (2.6) | 1 (2.6) | 17 (43.6) | 39 (12.2) | 49 | 70 |
| 2004 | 2 (7.7) | 9 (34.6) | 0 (0) | 2 (7.7) | 13 (50) | 26 (8.1) | 42 | 79 |
| 2005 | 2 (6.7) | 13 (43.3) | 0 (0) | 5 (16.7) | 10 (33.3) | 30 (9.4) | 50 | 78 |
| 2006 | 1 (3.2) | 14 (45.2) | 2 (6.5) | 0 (0) | 14 (45.2) | 31 (9.7) | 48 | 84 |
| 2007 | 0 (0) | 13 (46.4) | 0 (0) | 0 (0) | 15 (53.6) | 28 (8.7) | 46 | 84 |
| 2008 | 4 (9.1) | 17 (38.6) | 6 (13.6) | 1 (2.3) | 16 (36.4) | 44 (13.7) | 48 | 84 |
| 2009 | 2 (5.1) | 27 (69.2) | 0 (0) | 0 (0) | 10 (25.6) | 39 (12.1) | 74 | 84 |
| 2010 | 3 (10.7) | 13 (46.4) | 0 (0) | 0 (0) | 12 (42.9) | 28 (8.7) | 57 | 84 |
| 2011 | 3 (6.5) | 28 (60.8) | 0 (0) | 1 (2.2) | 15 (32.6) | 46 (14.3) | 67 | 84 |
| Total | 20 (6.2) | 158 (49.2) | 9 (2.8) | 10 (3.1) | 124 (38.6) | 321 (100) | - | - |

N: Number; TB: tuberculosis; TSR: treatment success rate.

Table 4. Distribution and prevalence of TB/HIV co-infection at North Gondar Zone Prison, 2009-2011.

| Year | HIV status of the TB patients | | Total N | TB/HIV prevalence (per 100,000 populations) |
|-------|-------------------------------|------------------|---------|---|
| | Positive [N (%)] | Negative [N (%)] | | |
| 2009 | 3(7.7) | 36(92.3) | 39 | 163 |
| 2010 | 6(21.4) | 22(78.6) | 28 | 288 |
| 2011 | 5(10.6) | 42(89.4) | 47 | 285 |
| Total | 14(12.3) | 100(87.7) | 114 | - |

HIV: Human immunodeficiency virus; TB: tuberculosis; N: number

prisons. Nevertheless, the high prevalence of TB in the study area could pose problems to the TB control in the general population as TB from prisoners may spread through visitors into the community. Characterization of the associated factors for the high prevalence with a subsequent improvement of the TB control system in the prison could impact on the TB control in the community.

This study also showed that smear positive pulmonary TB was found in only 11.8% of the cases. The reason for this low smear positivity might be due to the poor prison laboratory facilities, inadequate training of laboratory personnel in the prison TB clinic, and by the overall low case detection rate (31 to 38%) in Ethiopia (WHO, 2011). On the other hand, the proportion of smear negative pulmonary TB cases was consistently high throughout the study period (35.7 to 74.2%) and remained the highest as compared to smear positive and EPTB cases over the years (except in 2003 where EPTB was the highest) (Table 1). The large number of smear negative pulmonary TB cases could be due to the poor laboratory facility and high proportion of TB/HIV co-infection. The overall situation obviates the need of strong capacity building in prison health systems and policy commitment towards improving the extremely low smear positivity.

The trend of TSR during the ten years was below the

national TSR and the WHO target (Table 2) (WHO, 2007, 2011, 2004, 2005, 2006, 2008, 2009). While the most effective means of breaking the transmission chain, and thus preventing infection and possible disease in the rest of the community, is to provide appropriate treatment to cure existing cases, the prison's TSR is alarmingly low facilitating transmission and development of drug resistance. The low TSR could be explained, however, by the high transferred out rate of the TB positive inmates to another prison or health institution through the study period. This result, then, emphasizes the necessity of improved DOTS and zonal TB control program in the prison.

Death and default were recorded in five and three of the ten years, respectively. This study also revealed a consistently high transferred out of the prisoners throughout the ten years ranging from 20% in 2002 to 53.6% in 2007. The transfer could be to another prison in the country or after freedom to the nearby health institutions where the prisoners live. Since there are no systems which help to trace and know the final treatment outcome of the transferred out patients, there is a chance they could end up with default, death or treatment failure. If default or treatment failure happened to be the outcome, development and spread of drug resistance to

the community could be the immediate repercussion. On the other hand, as prisoners might face difficulties to return to their home in fear of the victims, they may flee to other areas where they cannot access TB treatment. This could then result into default and subsequently to drug resistance. The result of this study then underscores the importance of studying TB treatment outcome of several prison systems in the region where prisoners are interchanged frequently including the health institutions where the prisoners live after freedom. Devising a mechanism to trace and know the final treatment outcome of the transferred out TB cases could also be of paramount importance in terms of TB control program.

The prevalence of TB/HIV co-infection was found to be high throughout the three years ranging from 163 per 100,000 populations in 2009 to 288 per 100,000 populations in 2011. The prevalence of TB/HIV co-infection in other African prisons ranges from 7700 to 21400 per 100,000 populations which is much higher than the results of this study (Martin et al., 1994; Chaves et al., 1993). Nevertheless, the prevalence rates in all the three years were very high demanding a fair share of attention from all concerned parties.

The major limitations of this study were that the HIV status of the prison inmates treated for TB before September 2009 was not found, since there was no HIV screening service in the prison's TB/HIV clinic.

Conclusion

There was a high prevalence of TB with very low smear positivity and very low TSR in the North Gondar Zone Prison. The high rate of transferred out without subsequent follow up systems, fairly high mortality and TB-HIV co-infections in the prison demand urgent response from all responsible bodies. Hence, TB control programs should give special emphasis towards prison health systems so as to increase case detection rate through periodic active screening for TB and HIV; strengthen DOTS programme to improve the treatment success rate; and establish efficient referral and contact tracing mechanisms for transferred out cases.

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