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Full Length Research Paper

Identification of *Edwardsiella tarda* isolated from duck and virulence genes detection

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Bacteria from 30 duck liver samples were characterized using VITEK System. Two strains showed 99% similarity to *Edwardsiella tarda* (*E. tarda*). A set of seven virulence genes viz. *citC* (citrate lyaseligase), *fimA* (fimbrial operon), *gadB* (glutamate decarboxylase), *katB* (catalase), *mukF* (putative killing factor), *esrB* (TTSS regulator) and *gyrB* (ATPase domain of DNA gyrase) were amplified from obtained duck *E. tarda* and reference fish *E. tarda*. The results obtained show that all seven genes were present in the isolate X1 and reference strain, while only *gyrB*, *citC* and *mukF* genes were present in the isolate X2. The results obtained also indicate that X1, X2, and reference strain were all virulence strains.

Key words: Duck, *E. tarda*, Identification, virulence genes.

INTRODUCTION

Edwardsiella tarda is an intracellular, rod-shaped Gram negative, non-capsulated, motile, facultative anaerobic bacterium that was first isolated from a pond-cultured eel by Hoshina in 1962 (Hoshina, 1962). *E. tarda* is widely distributed in aquatic environments (Pitlik et al., 1987; Wyatt et al., 1979) and is infectious to variety of animals including humans (Clarridge et al.,1980; Janda et al., 1993; Jordan and Hadley, 1969; Mowbray et al., 2003), fish (Mowbray et al., 2003; Amandi et al., 1982; Kusuda et al., 1976; Meyer et al., 1973; Nakatsugawa, 1983; Sae-Oui et al., 1984), amphibians (Kourany et al., 1977; Sharma et al., 1974), reptiles (Kourany et al., 1977; Sechter et al., 1983; Sugita and Deguchi, 1983) and birds (Cook and Tappe, 1985; Winsor et al.,1981). This study was carried out to isolate, identify and characterize *E. tarda* from ducks using various cultural, morphological, and biochemical test.

The basis of pathogenicity depends on the number of virulence factors in *E. tarda*, including the ability to invade epithelial cells (Janda et al., 1991; Ling et al., 2000), resist serum and phagocytemediated killing (Ainsworth and Chen, 1990; Srinivasa, et al., 2001), and production of toxins, such as, haemolysins and dermatotoxins for disseminating infection (Ullah et al., 1983; Hirono et al., 1997). It has been established in *E. tarda* that pathogenic strains have virulence genes absent in nonpathogenic bacteria (Rao et al., 2003). Now, many virulence-related genes in *E. tarda* have been cloned or reported, which include *katB*, *esrB*, *mukF*, *fimA*, *gadB*, *citC*, *pstS*, *pstC*, *ssrB*, *astA*, *isor*, *omp*S2, and *gyrB* (Rao et al., 2003; Rao et al., 2003; Tan et al., 2005; Casiano et al., 2011). In our previous study, we testified that the zebrafish could be used as the animal models for studying the pathogenicity of *E. tarda* (Peng et al., 2011). So in this study, we investigated the prevalence distribution patterns of the seven virulence-associated genes and that of *E. tarda* isolated from duck, and was compared with isolates from diseased fish.

MATERIALS AND METHODS

Reference strains of *E. tarda*

E. tarda strain CD (16S ribosomal RNA gene GenBank accession

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number EF467289) was isolated from a moribund cichlid during an Edwardsiellosis outbreak in a farm in China.

Sample collection

Sterile tryptic soy broth (TSB) (Oxoid Ltd., Basingsoke, Hampshire, England) in sterile glass tubes was carried to the sampling areas and samples were collected from liver of the ducks. The liver samples collected aseptically were transferred immediately into TSB and test tubes containing liver samples were then immediately brought to the bacteriology laboratory, College of Animal Science and Technology, Shandong Agricultural University, China. A total of 30 duck liver samples, including 15 different farms of Xiamen and 15 different farms of Yanzhou, China.

Isolation and characterization of *E. tarda*

Each liver sample was inoculated into freshly prepared TSB, and were incubated at 37°C for 24 h aerobically in bacteriological incubator. Then the culture was plated onto TSB agar and Salmonella shigella agar (SSA, Luqiao, Beijing, China). The single colony of bacteria growth was examined using the incubated tubes. Smears were prepared and fixed by gentle heating for all samples. The fixed smears were stained with Gram's method and examined under microscope at 100x magnifications using immersion oil. In presence of Gram negative rods in the smears, the materials from the tube corresponding to the smears were plated onto Salmonella shigella agar (SSA, Luqiao, Beijing, China). The plates were then incubated at 37°C for 24 h and the plates containing colonies with black pigmentation were selected. Gram's staining test was performed to identify the plates containing *E. tarda* first. Salmonella-Shigella agar culturing was performed from the suspected plates containing Edwardsiella to obtain pure culture and these pure isolates obtained were used for further study.

Biochemical characterization

Isolated organisms with black pigmentation colonies on SSA were selected, and the same strain was maintained on TSA were subjected to the following biochemical tests by VITEK.

Lethal dose (LD_{50}) determination

Zebrafish were anesthetized with tricaine methanesulfonate (MS-222) (Hangzhou Animal Medicine Factory) at a concentration of 195 $\mu g \cdot ml^{-1}$. Then fish were intraperitoneally injected with 1.0×10^1 to 1.0×10^6 CFU/$fish^{-1}$ *E. tarda*, while the control fish were injected with 10 mM phosphate-buffered saline (PBS). Ten fish were used per dose. Mortality was monitored until 1 week post-infection. The experiment was repeated in triplicate. The results were averaged and used to calculate the LD_{50} value, using the method of Reed and Muench (1938).

DNA isolation

Genomic DNAs were extracted from every isolate of *E. tarda* using DNA extraction kit (Takara). Isolated DNA samples were checked for purity and quantified in a ND-1000 (Nano Drop, America). The samples were then resolved on agarose gel (0.8%) with 4 µl of template DNA mixed with 1 µl of loading dye (xylene cyanol + bromophenol blue) and electrophoresed at 120 volts for 70 min. DNA samples showing intact bands were used for polymerase chain reaction (PCR) amplifications.

PCR amplification of virulence genes

The targeted genes of *E. tarda* isolates were *citC* (citrate lyaseligase), *fimA* (fimbrial operon), *gadB* (glutamate decarboxylase), *katB* (catalase), *mukF* (putative killing factor), and *esrB* (TTSS regulator). Specific primers (Table 1) were used to amplify the seven virulence genes in two *E. tarda* isolates. To amplify the genes, 25 µl of reaction mixture was made containing 20 ng of template DNA, 20 pM of primers, 160 µM of dNTP mix, 1.25 U Taq polymerase, 1×Taq buffer, and 0.5 mM $MgCl_2$. Seven genes were amplified individually using the specific primers with 32 cycles of denaturation at 94°C for 1 min, annealing at 55°C for *citC*, 60°C for *fimA*, 57°C for *gadB*, 58°C for *katB*, 55°C for *mukF*, 51.5°C for *gyrB* and 56°C for *esrB* for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. All PCRs were carried out in a Biometra T Gradient thermocycler (Biometra Goettingen, Germany). The PCR product was visualized on 1.5% agarose gels stained with ethidium bromide, and documentation was done using the Gel Doc system (Bio-Rad).

RESULTS

Two samples showed black colonies on SSA after cultural examination (Figure 1). The isolates were Gram negative, short rod shaped organism base on morphological characteristics (Figure 2), and were identified to be *E. tarda* based on biochemical characterization test (Table 2). Positive *E. tarda* were detected from 2 out of 30 samples and both of these two positive samples were from Xiamen. The percentages of positive samples from those two areas were 13.3 and 0%, respectively (Table 3).

Following a challenge infection with *E. tarda*, the fish were monitored daily for 1 week post-challenge. Disease manifestations appeared between day 1 and 7 postchallenge and included reduced activity, anorexia, convulsions and death. Survival differences were observed among the different groups, and the data showed that the nearest LD_{50} values of CD, X1, and X2 strains were 2.69×10^2, 4.87×10^2, and 1.81×10^3 CFU/$fish^{-1}$, respectively.

A set of seven virulence genes were amplified using gene specific primers (Table 1). The results revealed that all seven genes were present in the isolates X1, but three genes in the isolates X2 (Table 4, Figure 3). Amplification of genomic DNA of two *E. tarda* isolates and one reference strain of *E. tarda* for *citC* and *mukF* resulted in the amplification of a fragment of 596 and 357 bp respectively, in all three samples. Among the 3 samples, *E. tarda* strain CD and X1 showed amplification of a 441, 583, 1417, and 311 bp fragment for *fimA*, *gadB*, *katB* and *esrB*, respectively, which were not found in *E. tarda* strain X2.

DISCUSSION

By conducting cultural examination on SSA,

Table 1. Description of the primers used for the amplification of the different virulence-associated genes.

Gene	Primer sequence	Product length (bp)
gadB	F:5'- ATTTGGATTCCCGCTTTGGT-3' R:5'- GCACGACGCCGATGGTGTTC-3'	583
mukF	F:5'- TTGCTGGCTATCGCTACCCT-3' R:5'- AACTCATCGCCGCCCTCTTC-3'	357
citC	F:5'- TTTCCGTTTGTGAATCAGGTC-3' R:5'- AATGTTTCGGCATAGCGTTG-3'	596
fimA	F:5'- CTGTGAGTGGTCAGGCAAGC-3' R:5'- TAACCGTGTTGGCGTAAGAGC-3'	441
esrB	F:5'-TCGTTGAAGATCATGCCTTGC-3' R:5'-TGCTGCGGGCTTTGCTT-3'	311
katB	F:5'-CTTAGCCATCAGCCCTTCC-3' R:5'-GCGAGTGCCGTAGTCCTT-3'	1417
gyrB	F:5'- GCATGGAGACCTTCAGCAAT-3' R:5'-GCGGAGATTTTGCTCTTCTT-3'	415

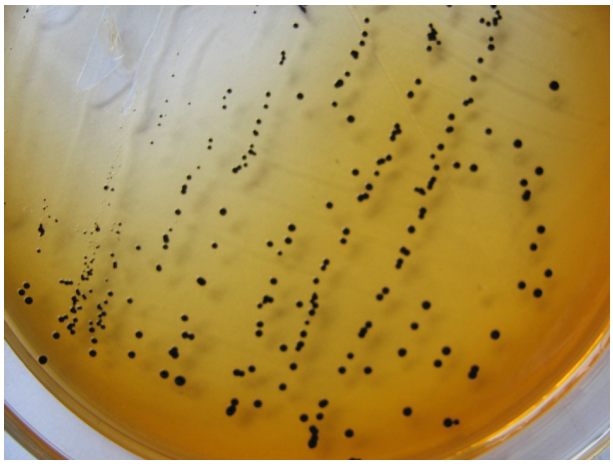

Figure 1. Growth of *E. tarda* on Salmonella shigella agar.

morphological characterization, and biochemical characterization using VITEK test, two strains of *E. tarda* were isolated and identified from duck, which indicated that *E. tarda* was potential pathogenic for duck and other birds, such as, Rockhopper penguins and *Cathartes aura* (Cook and Tappe 1985; Winsor et al.,1981).

Pathogenic bacteria may have virulence genes that are absent in nonpathogenic bacteria, making them virulent. Virulence genes may also be present in both pathogenic and nonpathogenic bacteria but may be functional only in pathogenic ones (Rao et al., 2003). It has been reported that seven genes, namely, *orfA*, *citC*, *fimA*, *gadB*, *katB*, *mukF*, *ssrB*, and *gyrB* were specific to pathogenic *E. tarda* (Rao et al., 2003; Choresca et al., 2011). These genes can therefore be used as biomarkers to perform diagnosis of pathogenic *E. tarda*. Hence, whether these seven genes discussed previously were carried by the *E. tarda* isolated from duck was examined in present study. All seven genes were present in *E. tarda* strains X1 and CD, and only two genes (*citC* and *mukF*) was found in the

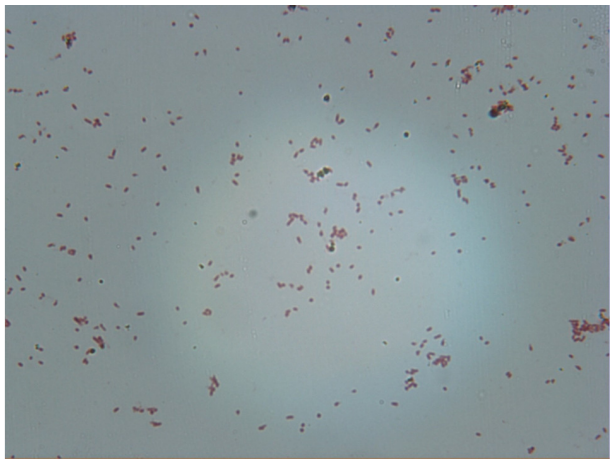

Figure 2. *E. tarda* showing Gram negative small rods arranged singly or pairs (Gram's staining).

Table 2. Biochemical characteristics of isolations from liver samples of duck.

Characteristics	Strains and Response	
	X1	X2
DP-300	+	+
Glucose (oxidative)	+	+
Growth Control	+	+
Acetamide	-	-
Esculin	-	-
Plant Indican	-	-
Urea	-	-
citrate	-	+
Malonate	-	-
TDA	-	-
Polymyyxin B	-	-
Lactose	-	-
Maltase	+	+
Mannitol	-	-
Xylose	-	-
Raffinose	-	-
Sorbitol	-	-
Sucrose	-	-
Inositol	-	-
Adonitol	-	-
p-Coumaric	-	-
H_2S	-	+
ONPG	-	-
Rhamnose	-	-
Arabinose	-	-
Glucose (Fermantative)	+	+
Arginine	-	-
Lysine	+	+
Base control	+	+
Ornithine	-	-

"+" indicates isolates are positive for the characteristic and "-" indicates are negative.

Table 3. Isolation of *E. tarda* from liver samples collected from ducks of Xiamen and Yanzhou.

Name of the place	No. of sample tested	Cultural examination		Biochemical examination		Total positive	
		Positive	Negative	Positive	Negative	No.	%
Xiamen	15	2	13	2	13	2	13.3
Yanzhou	15	0	15	0	15	0	0

Table 4. The different virulence-associated genes found in the isolates.

Isolate	Virulence-associated gene						
	citC	*fimA*	*gadB*	*katB*	*mukF*	*esrB*	*gyrB*
X1	+	+	+	+	+	+	+
X2	+	-	-	-	+	-	+
CD	+	+	+	+	+	+	+

"+" indicates isolates are positive for the different virulence-associated genes and "–" indicates are negative.

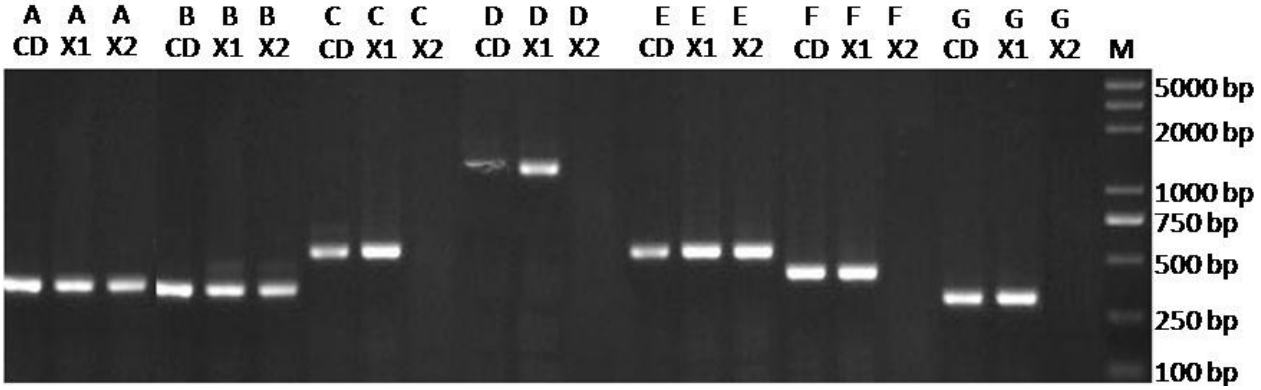

Figure 3. Amplification of the different target genes. A, Amplified fragment (415 bp) of the *gyrB* gene; B, amplified fragment (357 bp) of the *mukF* gene; C, amplified fragment (583 bp) of the *gadB* gene; D, amplified fragment (1417 bp) of the *katB* gene; E, amplified fragment (596 bp) of the *citC* gene; F, amplified fragment (441 bp) of the *fimA* gene; G, amplified fragment (311 bp) of the *esrB* gene; M, DNA marker; CD, X1, and X2, different strains of *E. tarda*.

strain X2, which indicated that *E. tarda* strains X1, X2, and CD, were pathogenic *E. tarda*. Most importantly, the LD_{50} values of CD, X1, and X2, were 2.69×10^2, 4.87×10^2 and 1.81×10^3 CFU/fish^{-1}, respectively. These results indicated that the virulent genes may be correlated with pathogenicity of *E. tarda*, and the more the virulent genes, the more the pathogenicity.

Biocomputational analysis has been carried out to determine the distribution of these genes in other pathogenic bacteria. Most of the 14 virulence genes (*orf20*, *orfA*, *citC*, *fimA*, *gadB*, *katB*, *mukF*, *ssrB*, *astA*, *isor*, *ompS2*, *pstB*, *pstC* and *pstS*) required for *E. tarda* infection have related sequences in other common human pathogens, such as, enterohemorrhagic *Escherichia coli*, *Salmonella enterica serovar* Typhimurium, *Vibrio cholerae*, and others (Rao et al., 2003; Bishai et al., 1994; Elkins et al., 1999; Hensel, 2000; Lucas et al., 2000; Wilson et al., 2000). In this study, the seven tested genes were correlated with pathogenicity of *E. tarda* in zebrafish. Thus, these virulence genes will be useful in determining how pathogenic bacteria interact with the host and cause systemic infections. They may also form the basis for the design of novel therapeutics and common antigens in vaccine development to protect hosts against systemic diseases.

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