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Detection of *Edwardsiella tarda* from fish by the specific amplification of a 450 bp fragment of 16S rRNA gene and its sequencing

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Edwardsiella tarda is a gram-negative, motile, rod shaped bacterium, which causes low chronic mortality in farmed finfishes. In India, *E. tarda* infections occur frequently in fry, fingerlings and adults of many cultured species (Sahoo *et al.* 2000). *E. tarda* were recovered from muscle and kidney samples of live ulcerative *Ophiocephalus punctatus* and biochemically confirmed (Kumar *et al.* 2006). Different serological tests, viz. indirect ELISA, competitive ELISA, dot blot ELISA and serum agglutination tests have been used for rapid and confirmatory identification of *E. tarda* in infected and dead fish (Swain and Nayak 2003). The genetic relationship of *E. tarda* isolates from different habitats in India had been assessed by PCR-RFLP of 16S rDNA (Acharya *et al.* 2007). During recent years this approach has proven its usefulness for identification of bacterial isolates. The objectives of the study were to design, check the sensitivity and specificity of the polymerase chain reaction (PCR) primers based on 16S rRNA gene sequence to *E. tarda* for the rapid detection of the Indian isolates.

Bacterial strains: The bacterial strains used in the study were isolated from visceral organs of diseased Indian major carps and *Ophicephalus* spp. Sixteen different isolates of *E. tarda*, *E. coli*, *Salmonella arizonae*, *Pseudomonas alcaligenes*, *Aeromonas hydrophila, Vibrio cholerae*, *Staphylococcus aureus* and *Flavobacterium* spp. were used in the study.

Construction of specific PCR primers: The 16S rRNA sequences of *E. tarda* were obtained by accessing the nucleotide sequence database, Gene Bank. The data were examined with multiple alignment analysis (CLUSTAL W), conserved region for bacteria was identified, and the specificity was confirmed by BLAST. Specific PCR primers for identification of *E. tarda* were designed from the sequence of 16S rRNA using Laser gene 6 software. Three sets of primers was tested for specificity among different bacterial isolates. The cross-reactivity of *E. tarda* 16S rRNA gene primers were checked by NCBI–BLAST and by genomic DNA amplification of other bacteria, viz. *Escherichia coli, Salmonella arizonae, Pseudomonas alcaligens, Aeromonas hydrophila, Vibrio cholerae, Staphylococcus aureus* and *Flavobacterium* spp. The details of primer sets are given in Table 1.

Preparation of PCR samples: Bacterial pellets were diluted in sterile saline prior to lysis by 10 min of boiling in a water- bath. Bacterial genomic DNA of all *E. tarda* isolates was isolated as per Hiney *et al.* (1992). The nucleic acid preparation was finally suspended in 50 µl of TE buffer. The isolated nucleic acid was quantified and qualified at 260 nm and 260/280 ratios, respectively.

PCR amplification: The PCR amplification was performed using all the 3 sets of primers designed in this study. We used 10 ng of genomic DNA, 50 pmoles of primers, 100 µ

Table 1. Details of primers used for the amplification of the fragments of 16S rRNA of *E. tarda*

Primer	Sequence	Priming site	Product size
ETF6	5'-ACGAGCGGAGGACGAGTAAGT–3'		
ETR6	5'-CTTAACAAACCGCCTGCGTGC–3'	1–500	500 bp
ETF7	5'-TTGGACGTGAAATCACCGGGC–3'		
ETR7	5'-CGCTGGATGTCAAGAGTAGGTA–3'	501–906	406 bp
ETF8	5'-CTGTAGAGATATGGGAGTGCCT–3'		
ETR8	5'-CTCCCGAAGGTTAAGCTAGCTA–3'	911–1360	450 bp

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moles of each dNTP's and 2 mM of $MgCl_2$. Samples were subjected to 35 cycles of amplification (94 °C for 2 min, 64 °C for 1 min and 72 °C for 3 min on a Master cycler). Ten µl of the reaction mixture was then analyzed by submarine gel electrophoresis in 1.2% agarose.

Cloning and sequencing of 450 bp fragment of E. tarda 16S rDNA gene: A fragment of 450 bp 16S rRNA gene of *E. tarda* was amplified using ETF8 and ETR8 primers. The DNA band of interest was excised from the gel at position 450 bp, ligated to pCR 2.1 TOPO cloning vector and transformed into DH5α *E. coli* strain. The recombinant clones were confirmed by PCR using ETF8 and ETR8 primers. Two terminal sequences of the cloned genes fragment were determined with the ABI PRISM Big Dye primer cycle sequencing ready reaction kit and ABI 377 DNA auto sequencing machine by using M13 sequence primers.

Sensitivity of the PCR: For determining the sensitivity of the PCR, 10-fold dilutions (8×10^5 to 8 bacteria) were tested. When nucleic acids were used, the sensitivity of the PCR was determined by amplifying 5 µl of 10–fold serial dilutions (20 ng to 2 pg). PCR amplification was performed with a DNA thermal cycler and as described.

Three primer pairs were designed namely ETF6 and ETR6, ETF7 and ETR7 and ETF8 and ETR8 on the basis of the nucleotide sequence of the 16S rRNA downloaded from NCBI and used to amplify target sequences in genomic DNA from 16 isolates of *E. tarda*. Primer pairs ETF8 and ETR8 yielded a product of the expected 450 bp size for *E. tarda*. Primer pairs ETF6 and ETR6, and ETF7 and ETR7 yielded a product of the expected 500 bp and 406 bp size for *E. tarda*, respectively. But amplification of the expected product size was observed for the other bacteria, viz. *Escherichia coli, Salmonella arizonae, Pseudomonas alcaligenes, Aeromonas hydrophila, Vibrio cholerae, Staphylococcus aureus* and *Flavobacterium* spp., when the primer pairs ETF6 and ETR6 and ETF7 and ETR7 were used. The primer pair ETF8 and ETR8 specifically amplified only *E. tarda*. All strains of *E. tarda* isolated in our laboratory were confirmed and a PCR amplification product of the expected length (450 bp) was obtained when ETF8 and ETR8 were used. Baird *et al.* (2003) were able to describe PCR primers based on *Edwardsiella* small subunit RNA gene for differentiating *E. tarda* from human source (Biotype1) and *E. tarda* from fish (Biotype2). Although the PCR amplification procedure was performed according to the manufacturer's suggestions, minor modifications, viz. 2 Mm $MgCl_2$ was used. No amplification product was obtained with bacterial cells or isolated nucleic acids from *Escherichia coli, Salmonella arizonae, Pseudomonas alcaligenes, Aeromonas hydrophila, Vibrio cholerae, Staphylococcus aureus* and *Flavobacterium* spp. These results suggest that the ETF8 and ETR8 pair of primers used in this study are specific for PCR amplification of a 450 bp fragment from an *E. tarda*. PCR primers specific for *E. tarda* isolates from Japanese eels have been derived from an anonymous species-specific sequence (Aoki and Hirono 1995) or from the hemolysin gene (Chen and Lai 1998). Acharya *et al.* (2007) found that the molecular methods were superior to discriminate the *E. tarda* habitat wise to conventional typing methods. Panangala *et al.* (2005) identified restriction sites that differ between the 16S–23S intergenic spacer regions sequence of the *E. tarda* and *E. ictaluri,* which would be useful in distinguishing the two species. In this study, we described PCR primers based on *Edwardsiella* 16S rRNA gene for identification of *E. tarda*.

The 450 bp fragment of *E. tarda* was cloned in pCR 2.1 TOPO cloning vector for sequencing. The cloned 450 bp fragment of the 16S rRNA gene of *E. tarda* was sequenced and comparison of this sequence with other 16S rRNA sequences from DDBJ/EMBL/Genbank was made. Comparing the 450 bp fragment of the *E. tarda* with other 16S rRNA of bacteria, 450 bp fragment of the *E. tarda* obtained in this study was 100% similar with 16S rRNA sequences of other *E. tarda* strains in the NCBI database viz., DQ884466, AF015259, AB050833, AB050832, AB050829, AB050828, AB050827, etc.

Isolated DNA from *E. tarda* was serially diluted in saline and used as a template. The minimum amount of purified DNA in the reaction mixture needed to obtain a detectable PCR product was 20 pg. The lower limit of detection of *E. tarda* bacterial cells or isolated DNA by PCR was examined for all the strains of *E. tarda*. A suspension of bacterial cells was diluted and processed. Amplification which resulted in detectable levels of PCR product was achieved when a minimum of 8 CFU/ml of *E. tarda* were lysed, on the basis of an average of five repeated testing of viable cells and PCR assays. A PCR amplification product could not be obtained when a sample with more than 10^6 CFU was used in the assay, probably because of accumulation of soluble cell products inhibitory to PCR.

In the study, we used the PCR technique for the identification of the 16S rRNA genes of *E. tarda* and the specificity of the primers used here is noteworthy, and can reach high sensitivity. The detection limit for 16S rRNA gene from *E. tarda* was 20 pg genomic DNA. The PCR method will have a wide application for detection of the *E. tarda*.

SUMMARY

Species-specific primers were developed to detect the 16S ribosomal RNA gene of *Edwardsiella tarda.* The region of 16S rRNA gene 450 bp was amplified in all 16 isolates of *E. tarda.* Amplification, which resulted in detectable levels of PCR product, was achieved when a minimum of 8 CFU/ml of *E. tarda*. The detection limit for 16S rRNA gene by PCR amplification of genomic DNA was 20 pg. There was no cross reactivity found with *Escherichia coli, Salmonella arizonae, Pseudomonas alcaligenes, Aeromonas hydrophila, Vibrio cholerae, Staphylococcus aureus* and *Flavobacterium* spp.

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