

IDENTIFICATION OF BACTERIAL PATHOGENS INFECTING *PENAEUS MONODON*, TIGER SHRIMP BY 16S rDNA AMPLIFICATION AND SEQUENCING

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Detection of shrimp bacterial pathogens based on 16s rDNA amplification and sequencing was carried out in this study. Bacteria were isolated from infected shrimp and shrimp pond water. 16s rDNA of the bacterial isolates, one each from the infected shrimp and shrimp pond water were amplified by polymerase chain reaction (PCR) using specific primers. The PCR amplified products were sequenced and the sequence information was compared with other sequences in the GenBank of National Centre for Biotechnological information (NCBI) using BLASTn program. Their maximum identity showed that the bacteria isolated from infected shrimp and the water sample from the shrimp ponds were *Vibrio harveyi* and *Vibrio alginolyticus* respectively. 16s rDNA technique is a valuable tool for rapid and accurate detection of bacteria.

Shrimp culture is an industry set for a period of strongly growing demand, and is currently worth around US\$10 billion. Identification of bacteria is essential for the diagnosis of diseases in shrimp. Conventional identification of bacteria involves cultivation of bacteria followed by their biochemical identification. Biochemical identification methods are laborious, time-consuming and at times misleading due to the presence of variants among bacterial species (Fredrickson et al., 2004). There are also many bacterial species that are not cultivable by standard methods. Polymerase Chain

Reaction (PCR) has been proved to be a simple and rapid way to identify bacteria.

16s rDNA sequence is a gene encoding small subunit ribosomal RNA. This gene contains conserved sequences of DNA common to all bacteria and divergent sequence unique to each species of bacteria (Woese, 1987). When a small piece of this sequence is used as a primer in a PCR assay, it acts as a "Universal primer" to non-selectively amplify any bacterial DNA in a sample. Identification of the bacterial DNA by sequencing, and searching for a closed match in the database will explain to which the species belong. In this study, we have attempted to identify bacterial pathogens of shrimp based on 16s rDNA amplification and sequencing. The standardized protocol for identification of bacterial isolates based on this technique is presented.

Samples

Live, infected shrimp juveniles with discoloration and weakness were collected from a shrimp farm in Minjur, Chennai, Tamil Nadu and used for the isolation of bacteria. Water samples collected aseptically from the ponds in which the infected shrimps were reared were also used for the isolation of bacteria.

Isolation of bacteria from samples

Haemolymph from shrimp samples and aliquots of water sample from rearing medium were

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inoculated aseptically in Tryptic Soy broth and incubated overnight at 37°C. Then, a loopful of culture from each of the sample was streaked on tryptic soy agar (TSA) plates to get individual colonies. One bacterial isolate each was selected from infected shrimp and pond water and were coded as SDDL F1 and SDDL F2 respectively. These isolates were used in the study.

DNA extraction from bacterial isolates

Overnight grown young cultures (24h old) of SDDL F1 and SDDL F2 from broth or plate were used for DNA extraction. One ml of the culture medium or a loopful of culture were taken in 1.5ml eppendorf tube, pelleted and washed with sterile distilled water and used for DNA extraction. A commercial DNA extraction Kit (Bangalore Genei Pvt Ltd, Bangalore) was used for the extraction of genomic DNA following the manufacturer's protocol. To each of the sample, 600µl of the DNA extraction buffer was added and incubated at room temperature for 5min. The sample with buffer was boiled in a water bath for 10min. The samples were then centrifuged at 10,000rpm for 10 min. An aliquot of the supernatant was used as the template DNA for PCR amplification.

Polymerase chain reaction (PCR)

PCR was performed in a 50µl volume containing PCR buffer (10mM Tris-HCl [pH8.3], 1.5 mM MgCl₂), 40pmoles of 16SrDNA-specific PCR primers (Weisburg *et al.*, 1991), 200pmoles each of dATP, dGTP, dCTP and dTTP and 1µl of extracted sample DNA (1ng) or 1µl of sterile distilled water (negative control) were used as template DNA for PCR. The above PCR mix was subjected to PCR with an initial denaturation of 94°C for 5 min and 30 cycles of 94°C for 1min; 54°C for 45sec and 72°C for 1min and a final extension of 72°C for 10min in a PCR thermal cycler (Eppendorf, Germany).

Separation of PCR amplified products

The PCR amplified 16s rDNA products were separated on a 1% agarose gel by electrophoresis. Briefly 8µl of the PCR products were mixed with 4µl of gel loading solution (Bangalore Genei Pvt Ltd, Bangalore) and electrophoresed in TAE buffer (0.04M Tris-acetate, 0.001M EDTA with 0.8µg of ethidium bromide/ml) for 1 h at 80V. The products were visualized and documented in a gel documentation unit (Vilber Lourmet, France) and photographed under UV illumination.

Automated sequencing of PCR products

Sequencing of PCR products of 16s rDNA was carried out in a commercial automated sequencer (Applied Biosystems). The sequence information of the bacterial isolates, were compared with the similar sequences that were available in the GenBank of National Centre for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov) using Basic Local Alignment Search Tool (BLAST) program. Based on the maximum identity results, the bacterial isolates SDDL F1 and SDDL F2 were identified and submitted to the GenBank, NCBI using *bankit* program for the benefit of other researchers and the accession numbers were obtained.

PCR amplification of 16s rDNA of bacterial isolates resulted in an amplicon of 1487 bp (Fig 1). Bacterial sequence analysis and comparison showed that the isolate SDDL F1 is *Vibrio harveyi* as it shared a homology of 100% with the *Vibrio harveyi* SDDL F2 isolate was found to be *Vibrio alginolyticus* (Table 1). The species level identification of bacterial isolates, SDDL F1 and SDDL F2 based on the maximum identity (%) and their GenBank accession numbers are presented in Table 2.

Diseases in cultured shrimps causing great production loss are attributed mainly to bacterial pathogens, predominantly *Vibrio* species (De la Pena *et al.*, 2001). Vibriosis is a major problem in shrimp

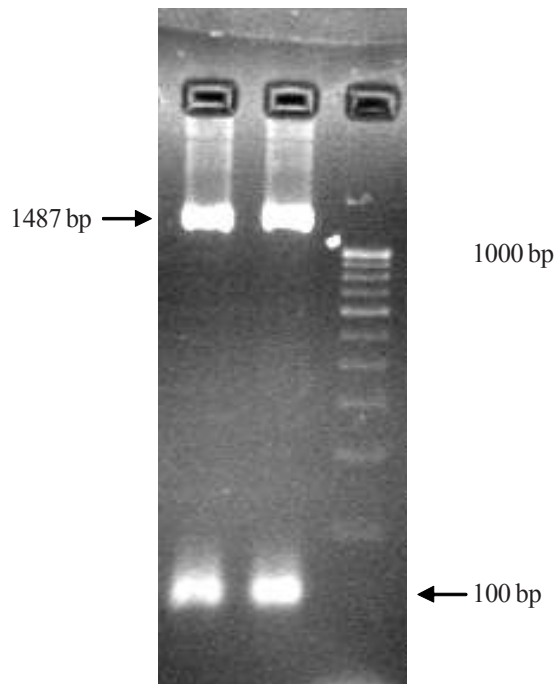
aquaculture (Lightner, 1988; Mohny *et al.*, 1994). *V. harveyi* a luminous species has been reported to cause heavy losses in shrimp hatcheries and farm (Nithimathachoke *et al.*, 1995). Characterization and confirmation of various species of *Vibrio* based on biochemical methods may not be accurate at times, due to the presence of variants. Moreover, conventional methods of bacterial detection are limited by the time and labor involved in analyzing a large number of samples. Hence, molecular tool like PCR has found wide application in specific detection of pathogen with high sensitivity and rapidity. PCR amplification of 16s rDNA sequences enables identification and discrimination of bacteria from complex communities with out cultivation (Drancourt *et al.*, 2000). It is also highly reliable as it provides unambiguous data even for rare isolates, which are difficult to identify with the phenotypic identification schemes. Hence, this technique is being increasingly used for the identification of isolates that cannot be identified by conventional methods as in the case of non-cultivable bacterial species. The potential of sequencing and comparison of 16s rDNA in the identification and discrimination of bacterial species has been well documented by various researchers (Kolbert *et al.*, 1999 and Vela *et al.*, 2002). Identification of bacterial fish pathogen such as *V. anguillarum*, *Aeromonas salmonicida*, *Flexibacter maritimus* have been documented by Hiney and Smith. (1988). Dorsch *et al.* (1992) have shown the close relationships of *V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. proteolyticus*, *V. parahaemolyticus* and *V. nitriengens* by analyzing conserved and variable sequence in this gene by 16s rDNA. In the present study, sequence analysis and homology have shown that the bacterial isolates from infected shrimp and the pond water belong to *V. harveyi* and *V. alginolyticus* with the maximum nucleotide identity of 100%. This confirms with the earlier reports as *Vibrio harveyi* has been shown to be a dominant species among *Vibrio* isolated from moribund shrimp (Huang, 1989; Lavilla-Pitago, 1988) and *V. alginolyticus* has been reported from the tank

water of healthy and diseased penaeid shrimps (Vandenberghe *et al.*, 1999). The results of this study shows that 16s rDNA amplification helps in the rapid and accurate identification of bacterial pathogens which is important for earlier and effective disease management.

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Lanes: 1 and 2 - PCR amplicons of 16S rDNA gene of the bacterial isolates SDDL1 and SDDL2. M- 100 bp molecular weight DNA ladder.

Table 1
Homology search results for 16s rDNA of SDDL F1 and SDDL F2

Accession	Description	Maximum identity
EU082833.1	<i>Vibrio</i> sp. D13 16S ribosomal RNA gene, partial sequence	100%
CP000790.1	<i>Vibrio harveyi</i> ATCC BAA-1116 chromosome II, complete sequence	100%
CP000789.1	<i>Vibrio harveyi</i> ATCC BAA-1116 chromosome I, complete sequence	100%
EU076595.1	<i>Vibrio harveyi</i> strain SDDL VH-1 16S ribosomal RNA gene, partial sequence	100%
AM422800.1	<i>Vibrio harveyi</i> partial 16S rRNA gene, strain 04101	100%
EF635306.1	<i>Vibrio harveyi</i> isolate HQ050227-1 16S ribosomal RNA gene, partial sequence	100%
DQ530297.1	<i>Vibrio harveyi</i> strain UCR7 16S ribosomal RNA gene, partial sequence	100%
DQ530295.1	<i>Vibrio harveyi</i> strain UCR4 16S ribosomal RNA gene, partial sequence	100%
AY264923.1	<i>Vibrio harveyi</i> strain ACMM 642 16S ribosomal RNA gene, partial sequence	100%
AY264921.1	<i>Vibrio harveyi</i> strain 47666-1 16S ribosomal RNA gene, partial sequence	100%
EU069459.1	<i>Vibrio alginolyticus</i> strain SDDL VA-1 16S ribosomal RNA gene, partial sequence	100%
X56576.1	<i>V. alginolyticus</i> 16S ribosomal RNA	100%
DQ173157.1	<i>Vibrio alginolyticus</i> strain zouA 16S ribosomal RNA gene, partial sequence	99%
AY332566.1	<i>Vibrio alginolyticus</i> strain EcGS021001 16S ribosomal RNA gene, partial sequence	99%
EF542800.1	<i>Vibrio alginolyticus</i> strain YJ06167B 16S ribosomal RNA gene, partial sequence	99%
DQ079634.1	<i>Vibrio alginolyticus</i> 16S ribosomal RNA gene, partial sequence	99%
X74691.1	<i>V. alginolyticus</i> (CIP 70.65) gene for 16S ribosomal RNA	99%
DQ995519.1	<i>Vibrio alginolyticus</i> A3G-2 16S ribosomal RNA gene, partial sequence	99%
DQ173157.1	<i>Vibrio alginolyticus</i> strain zouA 16S ribosomal RNA gene, partial sequence	99%
DQ269211.1	<i>Vibrio alginolyticus</i> strain SR1 16S ribosomal RNA gene, partial sequence	99%

Table 2
The species level identification of SDDL F1 and SDDL F2 and their GenBank Accession numbers

Isolate	Identified bacterial sp.	Maximum identity with the sequences in the GenBank, NCBI	Accession No.
SDDL F1	<i>Vibrio harveyi</i>	100%	EU447444
SDDL F2	<i>Vibrio alginolyticus</i>	100%	EU447445