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EVOLUTIONARY IMPORTANCE OF *TOXR* GENE OF *VIBRIO CHOLERAE* ISOLATED FROM SHRIMP INFECTED WITH WHITE SPOT SYNDROME

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ABSTRACT
Shrimp aquaculture has now blooming in Asian countries particularly in India. A variety of bacterial as well as viral pathogens has caused mass mortalities in shrimp production and cause larger economic losses. Majority of shrimp farming has faced white spot syndrome (WSS) disease which leads to death of shrimp in fewer than 3-10 days. The present investigation was aimed to demonstrate the evolutionary pattern of *toxR* gene in *Vibrio cholerae* isolated from shrimp infected with WSS. Homogenized carapaces in PBS were used as inoculums for this study. Genus level identification of the isolate was done by biochemical assay while the particular species was identified by molecular techniques such as gene sequencing and phylogenetic analysis. Colonies grown on TCBS confirmed that the isolated pathogen was *Vibrio* sp. and the phylogenetic tree revealed that the isolated pathogen was *V. cholerae*. Significant divergent was observed in the evolutionary pattern of *toxR* gene of isolated *V. cholerae* that paved a way for exploring novel antibacterial compounds which would improve the shrimp farming in near future.

KEYWORDS: Shrimp, BWSS, *Vibrio cholerae* and *toxR* gene.

INTRODUCTION

Brackish water shrimp culture especially the *Penaeus monodon* called as "Black Tiger Shrimp" is increasing in recent years (Pushparajan, & Soundarapandian, 2010). The rapid expansion of shrimp farming provides room for emergence of various pathogens thereby resulting in serious economic losses to commercial shrimp aquaculture. White spot syndrome (WSS) is one such highly contagious viral disease that accounts for 100% cumulative mortality rate with shorter incubation period of about 3-10 days (Lightner, 1996; Pradeep *et al.,* 2012, Hossain *et al.,* 2015). Epidemics of WSSV have been reported in India since 1994. In 2007, major outbreak of WSSV has been recorded which blown up to 300,000 metric tons of shrimps with 100% mortality (Karunasagar & Ababouch, 2012). It is noteworthy to identify the group of infectious agents involved in the pathogenesis of WSS so that newer antimicrobials or antivirals can be effectively tested upon those pathogens. Till date, it is reported that at least five different viruses have been concerned with the WSSV. Interestingly, *B. subtilis* and *V. cholerae* from white spotted shrimps have been isolated and known as 'bacterial white spot syndrome' (BWSS) (Wang *et al*., 2009).The aim of this present investigation was to set up method based on biochemical tests for identification of the pathogenic *Vibrio cholerae* isolated from white spotted shrimps.

MATERIALS AND METHODS

Collection of shrimp

Shrimps with prominent external white spots were collected from aquaculture farms situated at Thiruvasaladi village located in Chidambaram Taluk, Cuddalore district, at regular intervals during 2016-2017. The specimens were brought to laboratory under cold chain condition and stored at 4°C until further processes.

Isolation and identification phase

Carapaces were peeled from infected shrimp of which one gram was homogenized in sterile PBSat ratio 1:9 and serially diluted from 10-1upto 10-10. About 0.1ml from each dilution was plated on the surface of thiosulphatecitrate bile salt sucrose agar (TCBS; HiMedia, Mumbai, India) (Wang *et al*., 2009). After incubation for 24-48 hrs, the isolate was identified as *Vibrio* sp., based on the morphological characteristics laid down in Bergey's Manual of Systematic Bacteriology (Buchanan & Gibbons *et al.,* 1974). Pure culture was obtained by repeated sub-culture on TCBS and the stock was stored at 10% glycerol at 4°C.

Detection of exoenzyme and catecholate siderophore production

*Vibrio*sp. thus obtained was subjected for analysis of extracellular enzymes using plate assays for amylase & DNase (Jefferies *et al.,* 1957), elastinase (Kessler *et al.,* 1997), gelatinase (Frazier, 1926), lipase (Rhodes, 1959), proteolysis test and other characterization such as hemolytic assay (Swift *et al.,* 1999), slime production (Freeman *et al.*,1989), suicide phenomenon (Namdar & Bottone, 1988), surface hydrophobicity (Rosenberg & Kjelleberg,1986), biofilm formation (O'Toole & R. Kolter,1998), Autoagglutination and precipitation after boiling (Janda *et al.,* 1987) were also assessed by following the standard protocols. Siderophores are produced under iron limiting conditions; Fiss glucose minimal media was employed as per the formulation of Paul and Dubey, 2015 (Paul & Dubey, 2015) supplemented with Low Iron concentration (Fe SO4 – 139 µg/l) and High Iron concentration (Fe SO_4 – 5.56 mg/l). Culture taken in 0.5ml volume was inoculated and

incubated for 24hrs at 28°C. The amount of catecholatetype of siderophore production was estimated by Arnow's assay (Arnow, 1937) using nitrite-molybdate reagent. The end product of pinkish red color solution was read at 500nm in UV-VIS Double beam spectrophotometer (Systronics India Ltd., Ahmedabad, Gujarat).

DNA isolation, PCR amplification and gene sequencing

Genomic DNA from *Vibrio sp.,* was extracted according to Manufacturers instruction (BioBeeTech Pvt. Ltd., Karnataka, India). The quantization of extracted DNA was estimated by 260/280 ratio using spectrophotometer while the integrity was visualized on 1% agarose gel with the help of UV Transilluminator (Biotech R&D Laboratories, Tamil Nadu, India). Universal primer (*forward primer*- 5' GAGTTTGATCMTG and *reverse primer*- 5' AGAGTTTGATCMT) targeted for 16s rRNA have been employed for the molecular level identification of *Vibrio* isolates.16s rRNA and Virulence gene (*toxR*) specific amplification program were conducted as follows:94°C for 1 min,30 cycles, at 94°C for 30 sec, 50°Cfor 30 sec and 72°C for 1min with a final extension step of 72°C for 10 min. Primers used for the implication of *toxR* fragment of 560bp were: *forward primer*- 5' ATACGAGTGGTTGCTGTCATG and *reverse primer*- 5' GTCTTCTGACGCAA TCGTTG. For all PCR experiments, gradient Master cycler (Ependorf Scientific Inc, Westbury, NY, USA) was used with a reaction mixture comprising of 5µl of isolated DNA, 1.5µl of Forward Primer and Reverse Primer, 12µl of TaqMasterMix (Taq DNA polymerase is supplied in 2×Taq buffer, 0.4mM- dNTPs, 3.2mM -MgCl2, and 0.02%-bromophenol blue) and 5µl of deionized water. The resulting amplified PCR products were visualized on 0.8%agarose gel electrophoresis with Marker DNA using 1×TAE Buffer. The resulting DNA patterns were examined under gel documentation and the photographs were documented (Bio-Rad, USA).

Purification and sequencing of PCR product

Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore, US). Single-pass sequencing was performed on each template using 16s rRNA universal primers. Sequencing reactions were performed using a ABI PRISM®BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems, US). The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water, subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems, US) andthe resulting chromatogram was documented.

Multiple sequence alignment and phylogenetic analysis

The obtained 16s rRNA sequence was subjected to nucleotide blast using NCBI blast similarity search tool. The program MUSCLE 3.7 rendered the multiple sequence alignments for closely related gene sequences (Edgar, 2004).The resulting aligned sequences were trimmed using the program Gblocks 0.91b for eliminating poorly aligned positions and divergent regions (Talavera & Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis having HKY85 as substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while be in gone order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al.*, 2008).

RESULTS AND DISCUSSION

In shrimp farming, Asia now stands ahead of other countries both in terms of volume and value contributing 65% of world production, especially by India, china and Japan. Shrimp aquaculture in India have been started in a big way during 1991-1994 and at present, shown tremendous potential of growth in the maritime states including Tamil Nadu, Andra Pradesh, West Bengal and Orissa. In Tamilnadu, Chidambaram, Nellore and Balasore are considered as the epicenter of shrimp farming (Mishra & Shekhar, 2005) and hence the Cuddalore District was selected for the present investigation. The survey revealed the occurrence of bacterial white spot disease in shrimp based on external symptoms, epithelial tissue pigmentation and as white spots were observed in gill. Those shrimps were further used to isolate the pathogen.

Biochemical characterization of *Vibrio* sp.

Basic morphological and biochemical characteristics of isolated bacteria were tabulated in Table 1. The isolate grown from 10-2 dilution have produced yellowish colonies on TCBS plates (selective media for *Vibrio sp*.,) as shown in figure 1. In this study, *in vitro* assays were also performed based on biochemical characters of the *Vibrio* isolates along with virulence factors such as hydrolytic potentials of various extracellular products and surface characteristics which were studied to determine the relationship between these factors and the virulence established. Figure 2 depicted the multiple enzymatic profiles contributing to pathogen's virulence factor in isolated *Vibrio sp.,* including production of amylase, elastinase, protease, gelatinase, Lipase and DNase. The findings (Mejdi *et al.*, 2011) were in accordance with the present outcome who detected enzymatic activity in Vibrios isolated from marine fish. Basically, microbial infection in shrimp involves three basic steps (1) microbe has to enter into the host, then (2)secretes siderophores to chelate iron from the host and (3) eventually damages the host by means of extracellular products such as hemolysins and proteases [Lee *et al*, 1997a]. Besides, the extracellular products have been considered to be vital determinants of virulence in *Vibrio* sp., *Vibrio* sp. isolated in the present study showed hemolytic activity, able to produce catecholate type siderophores, self-pelleting (SP+) in nature and exhibit precipitation even after boiling (PAB+). Earlier study (Soto Rodríguez *et al.,* 2003a, Soto Rodríguez *et al.,* 2003b) recorded that rate of mortality is proportionate with that of isolates capable of producing hemolysins, proteases and siderophores. Quorum sensing system controls biofilm formation and virulence factor expression [McDougald *et al.,* 2003, McDougald *et al.,* 2001]. Recent evidence suggested that in *V. parahaemolyticus*, biofilm formation is positively correlated with surface hydrophobicity (Mizan *et al.,* 2016). In this context, the present study aimed to evaluate the biofilm formation of isolated pathogen with respect to cell surface hydrophobicity when exposed to xylene. It

was also confirmed that *Vibrio sp.,* isolate showed high surface hydrophobicity with xylene which was concominant with the findings [Niderman-Meyer *et al.,* 2010].

TABLE 1: Basic morphological and biochemical characteristic features of *Vibrio sp.*

Tests	Observations
Shape	Curved Rod
Gram Staining	- Ve
Spore Staining	Non Spore forming
Motility	Motile
Capsule	Non Capsulated
Flagella	Flagellated
Indole	+Ve
MR	- Ve
VP	Variable
Citrate	+ Ve
H2S production	- Ve
Urease production	- Ve
Gelatin hydrolysis	+ Ve
Nitrate reduction	+ Ve
Oxidase test	+ Ve
Glucose	+ Ve
Lactose	Variable
DNase	+ Ve
Sucrose	+ Ve
Catalase	- Ve
Casein	- Ve
Starch	- Ve
Fructose	- Ve
Dextrose	- Ve
Galactose	+ Ve
Xylose	- Ve

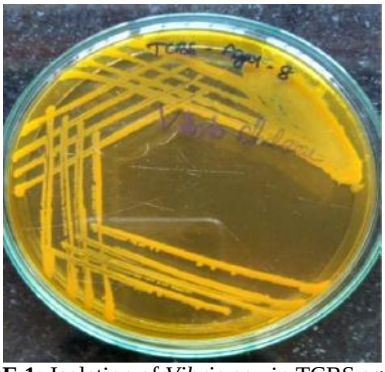

FIGURE 1: Isolation of *Vibrio* sp., in TCBS agar plate

*Yellow colored colonies grown on TCBS agar plate (selective media) is the unique characteristic feature of *Vibrio* sp., which is used for the genus level confirmation of isolated bacteria.

PCR for *V. cholerae* identification and its phylogenetic analysis

The results of overall molecular level analysis were shown in Figure 3 (I-III). Species level identification of *V. cholerae* (accession number-MF772495) was done by sequencing the 16s rRNA and the phylogenetic tree showed branching with Chinese isolates (accession number- KJ725363.1: bootstrap value of 100). The virulence factor of *V. cholerae* was modulated by *toxR* gene which was amplified as 980kb PCR product (figure 3- I). Further phylogenetic analysis revealed that there is a significant divergent ($p< 0.002$) was observed in the evolutionary pattern of *toxR* gene (bootstrap value of 99) amplified from the *V. cholerae* isolated from shrimp infected with WSS (Figure 3- III).Mutation rate analysis has to be performed for obtained *toxR* gene sequence in near future to predict the actual significant modifications.

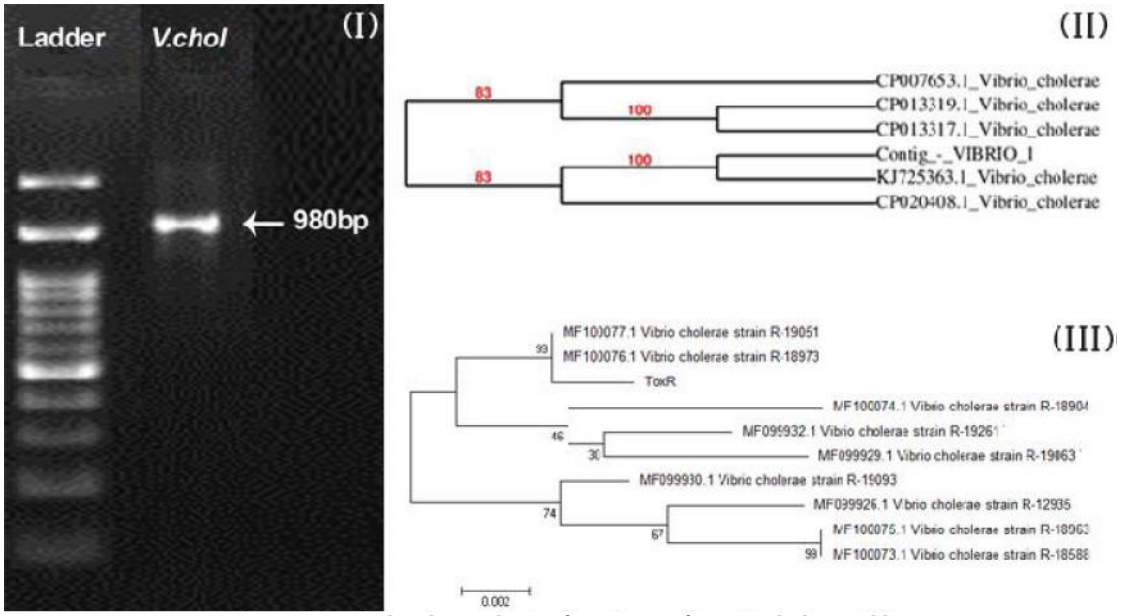

FIGURE 3: Molecular analysis of *toxR* gene from *V. cholerae* 1kb

Conventional PCR was performed with gene specific primer to amplify *toxR* gene - 980bp (I) in length and a phylogenetic tree was constructed for both *Vibrio* sp. level identification (II) and to reveal the evolutionary modifications ($p< 0.002$) (III).

CONCLUSION

Overall, the results of our study showed the existence of genetically diverse *V. cholera* isolated from shrimp farm located at Chidambaram district, Tamil nadu. Additionally, it is important to elucidate the antibiotic susceptibility patterns, level of toxR protein expression and the efficacy of virulence factor in *in vivo* condition for this isolated *V. cholerae* is yet another interesting finding, which requires further study. Therefore, maintaining regular surveillance and performing genetic characterization of *V. cholerae* strains in the shrimp aquaculture should contribute to the early detection and prevention of WSS outbreaks.

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