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VIBRIO HARVEYI INFECTION IN *MUD* CRABS (*SCYLLA TRANQUEBARICA*) INFECTED WITH WHITE SPOT SYNDROME VIRUS

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Abstract

An investigation on the bacteria associated with white spot syndrome virus (WSSV) infection in mud crabs, *Scylla tranquebarica* was carried out. Luminescent bacteria were recovered from the haemolymph and hepatopancreas samples of 15 of the 19 mud crabs affected by WSSV, obtained from the crab fattening farms in Tamil Nadu and Andhra Pradesh. All the 15 bacteria were phenotypically identified as *Vibrio harveyi*, however only nine of these isolates were confirmed as *V. harveyi* based on 16S rRNA gene targeted PCR. Further these nine *V. harveyi* isolates were subjected to BOX-PCR analysis, and the phylogenetic analysis of the isolates based on the banding pattern in BOX-PCR indicated high degree of genetic heterogenecity among these bacteria. All the nine isolates produced hemolysins, protease and phospholipase. A majority of *V. harveyi* isolates were found to be resistant to amoxicillin, ciprofloxacin, oxytetracycline, nitrofurantoin, streptomycin, chloromphenicol, nalidixic acid and norfloxacin. The study has revealed for the first time, that multiple antibiotic resistant *V. harveyi* are important bacteria associated with WSSV infected mud crabs.

Keywords: BOX-PCR, mud crab, *Scylla tranquebarica, Vibrio harveyi,* WSSV

INTRODUCTION

Mud crab farming is a recent activity and is practiced in Bangladesh, China, Indonesia, Malaysia, Singapore, Taiwan, Philippines and Vietnam. In India, crab farming is being mainly carried out in West Bengal, Orissa, Andhra Pradesh, Tamilnadu, Kerala, Karnataka, Goa, Maharashtra and Andaman islands. The mud crabs belonging to the genus *Scylla* are large portunids with high commercial value in terms of domestic markets and export by virtue of their delicacy. In spite of intensive research on several aspects of aquaculture of this species for the past two decades [1], little attention has been given to the causes of disease and mortalities of cultured crab populations [2]. The diseases of mud crabs (*Scylla* spp) have been reviewed recently [3]. Although as many as 30 viruses have been documented to cause infections in crabs [4], most publications on viral infections in mud crab diseases pertain to WSSV. Natural WSSV infections have been reported in wild-caught and farmed mud crabs of various life stages in many countries of Asiatic region [5, 6]. Recently, an icosahedral 150 nm virus causing a disease characterised by muscle necrosis and a reovirus designated as mud crab reovirus (MCRV) with signs of 'sleeping disease' associated with high mortality in cultured mud crabs, *S. serrata* was reported from China [7]. A number of bacterial diseases such as shell disease, filamentous bacterial disease, luminescent bacterial disease etc. have also been reported in mud crabs [8]. It is possible that the intensification of mud crab culture is likely to result in increased occurrence of diseases, as experienced with shrimp farming, with white spot syndrome virus being an important agent [9]. The foregoing overview of diseases of mud crabs indicates that they suffer morbidity and mortality due to viral and bacterial infections affecting productions. In the objective of this study was to understand the kinds and the role of bacteria in the morbidity of mud crabs farmed in the South Indian states of Andhra Pradesh and Tamil Nadu, affected by white spot syndrome virus.

MATERIALS AND METHODS

Isolation of *Vibrio harveyi*:

Nineteen moribund mud crabs *(Scylla tranquebarica)* infected with white spot syndrome virus (WSSV) were collected from different culture farms in Tamil Nadu and Andhra Pradesh of culture duration ranging from 90-170 days with history of chronic mortalities. Crabs were dissected out aseptically and hepatopancreas and haemolymph samples were inoculated on to Zobell's marine Agar (ZMA) (Himedia, India) and thiosulphate citrate bile salt sucrose (TCBS) (Himedia, India) agar plates and incubated at 30°C for 24 h. Bacterial isolates were identified using a battery of phenotypic tests such as salt tolerance, aminoacid decarboxylation, fermentation of sugars, susceptibility to vibriostatic agent O/129 (2,4-diamino-6, 7-diisopropyl-pteridine) etc. [10, 11].

Confirmation of *Vibrio harveyi* isolates by PCR:

Chromosomal DNA from pure cultures of *V. harveyi* was extracted according to the method described earlier [12]. Confirmation of *V harveyi* isolates was carried out by gene specific PCR as described by Oakey et al. (2003) [13]. PCR was carried out with the 16S rDNA gene specific primers VH-1 (5'AAC GAG TTA TCT GAA CCT TC 3') and VH- 2 (5'GCA GCT ATT AAC TAC ACT ACC 3') as the forward and reverse primers, which have been reported to be highly specific. The PCR reaction was carried out in 25 µl reaction mix containing 2.5µl of 10x PCR reaction buffer, 3mM magnesium chloride, 10pmol each primer, 200 µM each dNTPs, 1 U of Taq polymerase and 50-100ng template DNA. The volume of reaction mix was made-up to 25 µl with addition of MilliQ-water. The PCR reaction was carried out using Master cycler gradient thermocycler (Eppendorf, Germany), and the thermo- cycling conditions included denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for one min, 65°C for one min and 72 °C for one min, and a final extension at 72°C for 5 min.

Molecular typing of *Vibrio harveyi* by BOX PCR:

BOX-PCR was carried out to assess the genetic relationship of *V. harveyi* isolates recovered from mudcrabs from various farms as described earlier using a single oligonucleotide primer (5'-CTACGGCAAGG CGACGCTGACG-3') [14]. BOX PCR was performed in a 25 µl reaction volume, containing 2.5µl of 10x PCR reaction buffer, 2 mM $MgCl_2$, 20 pmol primer, 125 µM each dNTPs, 2 U of *Taq* polymerase and 50 ng of template DNA. The thermo-cycling conditions included denaturation at 95°C for 7 min, followed by 30 cycles of 94 °C for one min, 53°C for one min, 65°C for 8 min and a final 16 min extension at 65°C. Phylogenetic analysis of the bacterial isolates based on the fingerprints produced on agarose gel after BOX-PCR was carried out by using finger printing software version 2 (Bio Rad, USA).

Virulence traits of *V. harveyi* isolates:

Ability to produce haemolysins, phospholipases and proteases has been recognized as virulence mechanism of *V. harveyi* [15] and hence, the isolates obtained in this study were tested for the production of these traits *in vitro*. Haemolytic activity of *V. harveyi* was tested on blood agar base (Himedia,India) supplemented with 5% sheep blood and 1% sodium chloride. Bacterial isolates were streaked onto the medium, incubated at 30°C, and the production of haemolysis was observed after 24 hours of incubation. Protease, phospholipase and lipase activity of *V. harveyi* were determined according to the procedures described previously [16]. Bacteria were streaked onto nutrient agar plates supplemented with 1% casein, 1% egg yolk and 1% Tween 80 respectively and incubated for 24 hours at 30°C. Presence of lytic halo zone around the colony was considered as positive for the production of protease, phospholipase and lipase respectively. Eight different antibiotics were tested in the present study to evaluate the antibiotic sensitivity pattern of *V. harveyi* isolates by disk diffusion test according protocols described previously [17].

RESULTS AND DISCUSSION

Luminescent bacteria were isolated on ZMA and TCBS from the haemolymph and hepatopancreas samples of 15 WSSV infected (confirmed by PCR [18], data not shown) cultured mud crabs (*S. tranquebarica*), inclusive of four out of six from Andhra Pradesh and eleven of the thirteen samples from Tamil Nadu. All the 15 isolates were motile, fermented glucose, oxidase positive, arginine dehydrolase negative, lysine decarboxylase and ornithine decarboxylase positive and were sensitive to 150 µg of vibriostatic agent, O/129. Based on these biochemical characteristics, along with their ability to produce luminescence, the isolates were designated to belong to *V. harveyi* [11]. Luminescent bacteria (LB) are ubiquitous and abundant in the marine environment including the surface and gut of marine animals and aquaculture ecosystems (19, 20), and *V. harveyi* has been recognized as a significant pathogen of marine vertebrates and invertebrates [15]. However so far, it is yet unclear whether *Vibrio* spp. are opportunistic or primary pathogens in crabs. Several studies on *V. harveyi* as the causative bacterium of vibriosis in different aquatic animals such as penaeid shrimp, finfish, oysters etc have been documented [21-23].

Vibrio spp can act as primary pathogens of shrimp in pond waters with decrease in the diversity of the *Vibrio* community associated with a dominance of few potentially virulent *Vibrio* species prior to outbreaks of vibriosis [24, 25] and primary disease caused by highly virulent strains has also been reported [24]. Most outbreaks of shrimp vibriosis occur either in combination with physical stress factors or following primary infections with other pathogens [26, 27]. WSSV - Vibrio co-infections in shrimp ponds and a cumulative effect on mortality of shrimp by dual infection under experimental conditions have been documented [28]. It has been indicated that a primary WSSV infection may weaken shrimp, increasing their susceptibility to bacterial infections [26]. *V. harveyi* were the predominant species

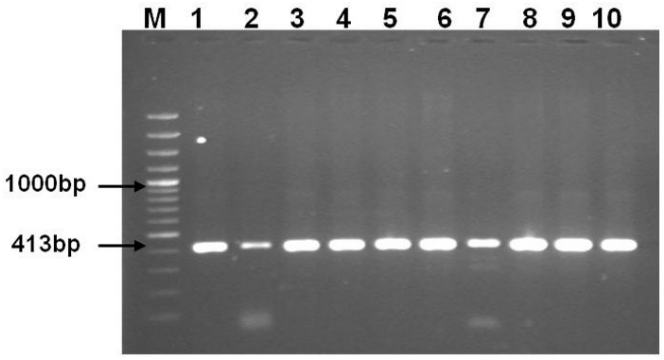

Figure 1. Confirmation of *V. harveyi* isolates by 16S rRNA gene specific PCR: Lane M: 100bp DNA ladder (Fermentas) and Lane 1: *V. harveyi* ATCC 25919, Lanes: Vh1-9

recovered from 15 WSSV infected 19 mud crab samples processed. Mud crabs suffered chronic mortalities due to such dual infection in the farms with a cumulative mortality ranging from 60-75%, and the farmers resorted to harvesting the remaining stock. To our best of knowledge, this is the first report on the association of *V. harveyi* in mud crabs, S*cylla tranquebarica* affected with WSSV, leading to chronic mortalities.

The identification of vibrios is labour-intensive, requiring many biochemical and/or physiological tests [29]. Taxonomically, *V. harveyi* is closely related to *V. campbellii* and *V. alginolyticus* [30]. Complex identification keys using large numbers of phenotypic characteristics have been proposed [31, 32]. Use of such large number of tests is not feasible for routine diagnostic laboratories because of the time and costs involved. In aquaculture, the differentiation becomes a concern as all these species are natural inhabitants of marine waters. Hence, in the present study, 16Sr RNA gene targeted PCR was used for confirmation of *V. harveyi* among the luminescent bacterial isolates recovered from crabs. Nine isolates which produced 413 bp amplification products (Fig.1) by this 16S rRNA gene specific PCR were confirmed as *V. harveyi*.

Molecular typing of nine *V. harveyi* isolates by BOX-PCR generated four to eight bands ranging in size from 300 to 2000bp. BOX-PCR analysis by UPGMA Dice coefficient method showed two major clusters at about 35 percent hierarchical level (fig 2). The first cluster grouped four isolates including the reference strain (ATCC 25919) along with the two isolates from Kovalam (South Chennai) and one isolate from Kakinada (Andhra Pradesh). The second cluster comprised of six isolates, one from Kakinada (Andhra Pradesh) and five from Tamilnadu (Marakkanam and Tuticorin).

We observed that the antibiogram of *V. harveyi* showed mixed patterns of resistance to the eight antibiotics tested in this study. All the isolates were resistant to amoxicillin. Out of the nine isolates of *V. harveyi*, eight were resistant to all tested antibiotics such as ciprofloxacin,

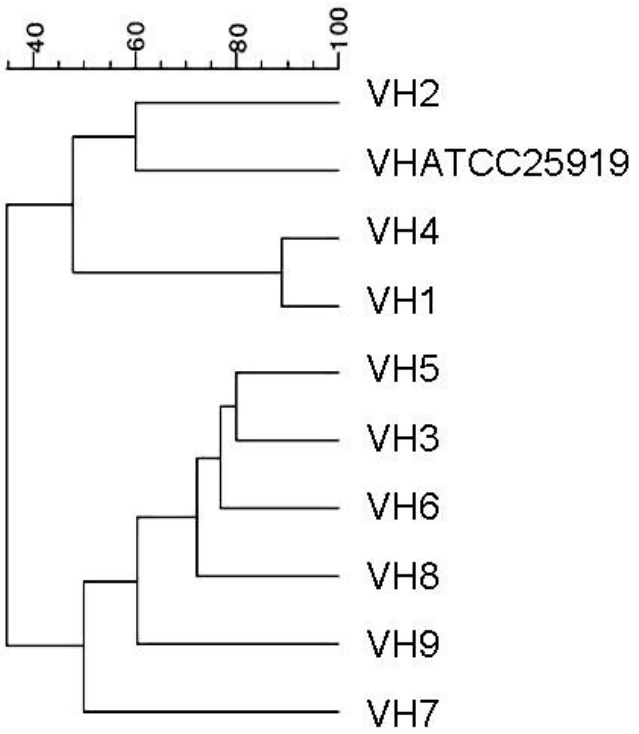

Figure 2. Phylogenetic analysis of *V. harveyi* isolates based on Box PCR fingerprints

oxytetracycline and nitrofurantoin, streptomycin, norfloxacin, chloromphenicol and seven isolates were resistant to nalidixic acid. Resistance of shrimp pathogenic bacteria to commonly used antibiotics such as erythromycin, kanamycin, pencicillin G and streptomycin has been reported earlier [35].

All the isolates were found to be actively hemolytic and also produced protease and phospholipases. Virulence of *V. harveyi* has been reported to be associated with their ability to produce extra cellular products (ECPs) such as proteases, phospholipases, hemolysins or cytotoxins [15]. The production of virulence factors varies with the isolates and has been proposed to be governed by mobile genetic elements and bacteriophages [34]. The present study has revealed that luminescent multiple antibiotic resistant *V. harveyi* is an important secondary bacterial pathogen in mud crabs affected primarily by white spot disease, resulting in the morbidity and mortality of mud crabs.

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