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Research Paper

Molecular variations in *Vibrio alginolyticus* and *V. harveyi* in shrimp-farming systems upon stress

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Abstract

A study was performed to investigate the genomic variations in the shrimp farm isolates of *Vibrio alginolyticus* and *V. harveyi* when the isolates were subjected to environmental stress. Samples of shrimps, water and sediment were collected from Southern Indian coastal shrimp farms. Vibrio isolates were biochemically identified and confirmed using 16S rDNA and *gyrB* gene specific PCR. The bacterial strains were genotyped by PCR fingerprinting using GTG(5) and IS (Insertion Sequence) primers. Seven strains each of *V. alginolyticus* and *V. harveyi* were subjected to 10 passages through trypticase soya broth (TSB), which contained different NaCl concentrations (3, 6 and 8%) and trypticase soya agar (TSA). *V. alginolyticus* was also passaged through TSB with a 12% NaCl concentration. PCR fingerprinting, which was performed on the strains that were passaged through different salt concentrations, confirmed that *V. alginolyticus* and *V. harveyi* could affect the genomic variations, depending on the environmental conditions of the culture. The study highlights the complex genotypic variations that occur in *Vibrio* strains of tropical aquatic environment because of varied environmental conditions, which result in genetic divergence and/or probable convergence. Such genetic divergence and/or convergence can lead to the organismal adaptive variation, which results in their ability to cause a productive infection in aquatic organisms or generation of new strains.

Key words: *Vibrio harveyi*, *Vibrio alginolyticus*, molecular variation, shrimp, *Penaeus monodon*.

Introduction

Luminescent *V. harveyi* has been reported to cause serious infections in shrimp farming systems and can lead to a 100% mortality rate and, consequently, huge economic losses (Lavilla-Pitogo *et al.*, 1990; Lavilla-Pitogo and de la Pena, 1998; Leano *et al.*, 1998). *Harveyi* clade is now recognized as one of the 14 clades in the *Vibrio* genus (Sawabe *et al.*, 2007), which consists of *V. harveyi*, *V. campbellii*, *V. rotiferianus*, *V. parahaemolyticus*, *V. alginolyticus*, *V. natriegens* and *V. mytili*, all of which share a high level of phenotypic and genotypic homology (Cano-Gomez *et al.*, 2009). There are both virulent and avirulent strains in *Vibrio harveyi*; luminescent strains are virulent in almost all cases, although exceptions have been reported (Defoirdt *et al.*, 2007). The expression of bioluminescence in *V. harveyi* is co-regulated with the production of toxin-A; hence, this bioluminescence expression is considered a virulence factor (Manefield *et al.*, 2000). However, different factors, such as adhesion factors, extracellular polysaccharides and biofilm formation, lytic enzymes, siderophores, type III secretion systems and bacteriophages, appear to induce the virulence in *V. harveyi* (Ruwandeepika *et al.*, 2012)

Pathogenicity and genomic variations have been found among the *V. harveyi* and *V. alginolyticus* isolates that were obtained from shrimp-farming systems (Hernandez and Olmos, 2004; George *et al.*, 2005; Satendrakumar *et al.*, 2007). Several processes such as horizontal gene transfer, prophage integration, super integrons, generation of pathogenicity islands via integration of plasmids, phages, or conjugative transposons into specific target genes

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have been suggested to generate pathogenic vibrios from the environmental isolates (Thompson *et al.*, 2004).

Vibrios are dominant in the ocean and shrimp-farming environment because of their versatility in metabolic activity and their ability to serve as nitrifiers, as demanded by the environmental parameters (Grimes *et al.*, 1984; Thangarani, 2001; Urakawa and Rivera, 2006). Starvation studies have indicated that *V. alginolyticus* and *V. parahaemolyticus* can have phenotypic alterations as a survival strategy (Abdallah *et al.*, 2009). It was also observed that the changed osmolarity of the culture medium could change the outer membrane protein patterns of both *V. alginolyticus* (Xu *et al.*, 2005) and *V. parahaemolyticus* (Xu *et al.*, 2004). Therefore, the current study was performed to investigate whether genomic variability would develop in two important *Vibrio* strains of the shrimp farming systems viz. *V. alginolyticus* and *V. harveyi* under the effect of changed environmental culture conditions.

Materials and Methods

Samples of farmed shrimp, brood stock, larvae, gut and intestine of loose-shell-affected shrimps, shrimp farm water and sediment and hatchery water were collected from two Southeast Indian States, Tamilnadu and Andhra Pradesh, brought to the laboratory in ice and processed within 12-24 h. The shrimp samples were tested for the presence of white spot syndrome virus (WSSV) using diagnostic PCR with the standard procedures (Lo *et al.*, 1996).

Seven strains of each *V. alginolyticus* and *V. harveyi* were isolated and biochemically identified (George *et al.*, 2005; Satendrakumar *et al.*, 2007) from the collected samples and were confirmed using *gyrB* gene specific PCR (Thaithongnum *et al.*, 2006) and 16SrDNA analysis (Oakey *et al.*, 2003) (Table 1). To study the genomic variations of the species under different culture environmental conditions, young cultures of the isolates, which were grown in trypticase soya broth, were further inoculated into a battery of culture tubes with media that contained various salt concentrations. All isolates were inoculated into a normal maintenance medium (TSA) with 1% salt concentration. In addition, the *V. harveyi* strains were inoculated into three sets of sterile trypticase soya broth (TSB) (Hi Media, Mumbai, India) at 3, 6 and 8% sodium chloride w/v, whereas the *V. alginolyticus* strains were inoculated into four sets of 3, 6, 8 and 12% salt concentrations. Each of these cultures was subcultured at a 24-h interval to another sterile culture medium of the identical composition. Then, each isolate was passaged for 10 days, and the DNA of the isolates was extracted at the initial 0 passage and at the end of 10 passages. For the DNA extraction, the isolates at 0 passage and at the end of 10 passages were grown in a sodium-chloride-supplemented Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl, pH 7.5) at 32 °C for 16-20 h, and the cells were harvested and washed twice in physiological saline. The chromosomal DNA extraction was performed using standard phenol-chloroform and ethanol precipitation (Sambrook *et al.*, 1989). The extracted DNA was dissolved in sterile deionized water (Biocel, Millipore, Molsheim, France) and used for the PCR analysis. PCR fingerprinting of the isolates were performed using IS-PCR (George *et al.*, 2005) and GTG (5) PCR (Gomez-Gil *et al.*, 2004).

For IS-PCR, amplification reactions were performed in 50 μL with 1.5 unit of Taq polymerase, 20 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μm dNTP (Genei, Bangalore, India). The reaction mixture was incubated at 94 °C for 2 min, followed by 35 cycles at 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min with a final extension at 72 °C for 20 min in a Mastercycler (Eppendorf, Hamburg, Germany). Fingerprinting by (GTG) 5 PCR included denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 3 min, 92 °C for 30 s, 40 °C for 1 min and 65 °C for 8 min, with a final extension of 65 °C for 8 min. The amplification products were visualized in 1.2% agarose gels (Genei), which was stained with ethidium bromide. The dendrograms were analyzed using the unweighted pair group method with mathematic averages (UPGMA) and the Dice coefficient cluster analysis with the UVI bandmap software in a gel documentation system (UVI Tec, Cambridge, UK).

Table 1 - Details of the shrimp (*Penaeus monodon*) -farm-associated bacterial strains in the experiment.

V. harveyi	Source	*V. alginolyticus*	Source
DS134	Uninfected shrimp intestine	DS29	Uninfected shrimp intestine
DS149	Water from WSSV infected pond	DS199	Soil from black spot infected pond
DS158	Soil from WSSV infected pond	DS200	Soil from reservoir pond of the black spot infected farm
DS165	Uninfected shrimp hepatopancreas	DS246	Soil from inlet area
DS184	Water with luminescence	DS263	Soil from *Zoothamnium* sp. infected pond
DS218	Lesions of black spot infected shrimp	DS334	Water from WSSV affected pond
DS260	Soil from *Zoothamnium* sp. infected pond	DS350	Loose shell affected shrimp intestine

Results

The details of different isolates that were obtained from various sources are provided in Table 1. The isolates that were identified as *V. alginolyticus* were Gram-negative, motile fermentative rods. These rods produced enzymes (catalase, oxidase, gelatinase, lysine and ornithine decarboxylase), acid from sucrose, indole from tryptophan, acetoin from glucose and swarming colonies on agar plates. These isolates showed no growth in the medium with 0% NaCl but grew well at 8% NaCl, which corresponds to the description of *V. alginolyticus* (Alsina and Blanch 1994a, b). However, the *V. harveyi* isolates were different because they were luminescent; they produced no acetoin from glucose and appeared as green or yellow non-swarming colonies in the TCBS agar plates.

The *V. alginolyticus* and *V. harveyi* isolates were further identified by the characteristic amplification of the 16S rDNA, and *V. harveyi* was positive for the *gyrB* gene. The isolates were fingerprinted using IS and GTG PCR, which generated characteristic fingerprint patterns for both species before the experiment at 0 passage. Although the seven *V. alginolyticus* could be differentiated into 4 genogroups in GTG PCR, the seven *V. harveyi* strains formed 3 genogroups during the GTG PCR fingerprint analysis (Figure 1a and 1b).

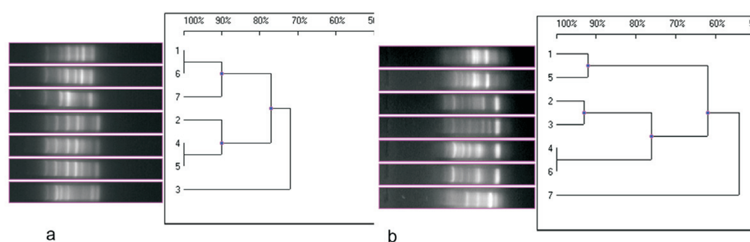

Figure 1 - Fingerprint patterns generated using GTG PCR amplification of the genomic DNA in 1.2% agarose gel (a) *V. harveyi* strains at 0 passage; lane 1- DS134, lane 2- DS149, lane 3- DS158, lane 4- DS165, lane 5- DS184, lane 6- DS218, lane 7- DS260. (b) *V. alginolyticus* strains at 0 passage. Lane1- DS29, lane 2- DS199, lane 3- DS200, lane 4-DS246, lane 5- DS263, lane 6- DS334, lane 7- DS350.

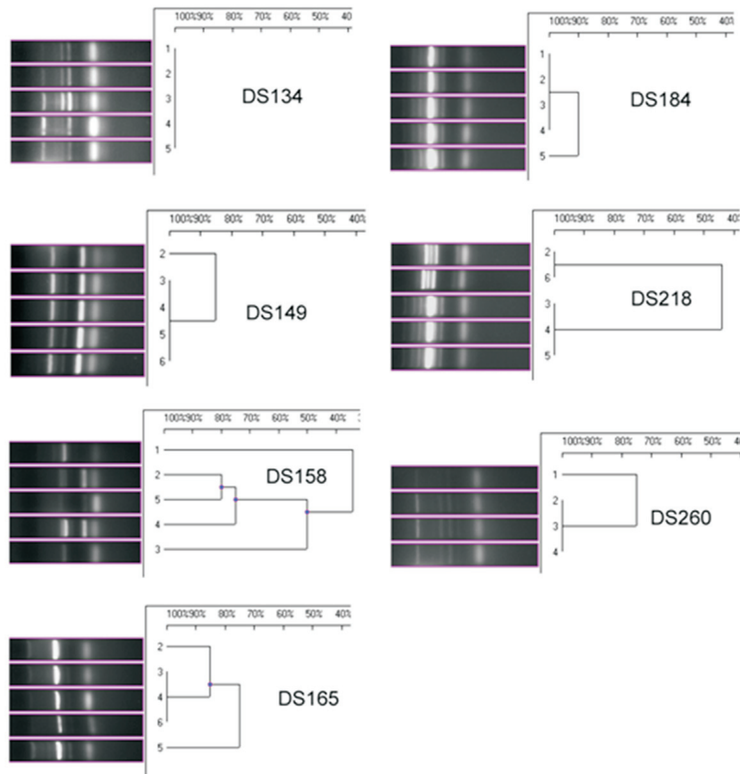

Figure 2 - Fingerprint patterns generated using IS PCR amplification of the genomic DNA of *V. harveyi* with primers, which were targeted at insertion sequences in 1.2% agarose gel. The number of strains is indicated in each gel. Lane 1- 0 passage, lane 2 to 4- after 10 passages in 3, 6 and 8% NaCl containing medium, respectively, lane 5- after 10 passages in TSA for strains DS134, DS158, DS184 and DS260. Lane 2- 0 passage, lane 3 to 5- after 10 passages in 3, 6 and 8% NaCl containing medium, respectively, lane 6- after 10 passages in TSA for strains DS149, DS165 and DS218.

Following 10 passages in different salt concentrations, among the *V. harveyi* strains, three strains (DS 134, DS 184 and DS 149) showed no change in the fingerprint pattern (more than 80% homology) according to the IS PCR analysis (Figure 2). Two isolates DS218 and DS260 exhibited similar types of fingerprint grouping under both IS and GTG PCR analyses (Figures 2 and 3). Among the *V. alginolyticus* strains, the IS and GTG PCR analyses showed a divergence of fingerprint patterns (at less than 80% homology) for all isolates except DS199, which had a single type of fingerprint pattern (Figures 4 and 5).

Discussion

The vibrio species continues to be a serious pathogen in shrimp-farming systems, including hatcheries. Because of the ubiquity of the species in the marine and brackish water environments found in the tropical belt, population control is the best management practice in coastal aquaculture systems compared with attempting to avoid the pathogen. Furthermore, many vibrio species are beneficial to the cultured shrimps, whereas others act as opportunistic pathogens. There are instances of potentiation of these opportunistic pathogens, which cause mass mortalities of shrimp juveniles and adults. Because these vibrios can act as nitrifiers, they often compete with other heterotrophic bacteria in nitrogen-rich environment and increase the population size, which causes the production of quorum sensing signals and secretion of toxins and biofilms. Although the exact trigger to this conversion is not clear, several factors have been implicated in the generation of virulent strains of vibrios in tropical aquaculture systems (Thompson *et al.*, 2004). Because it has been reported that environmental stresses such as starvation and changes in osmolarity can alter the phenotypic characteristics in *V. harveyi* and *V. alginolyticus* (Xu *et al.*, 2004, 2005; Abdallah *et al.*, 2009), the current study was performed to investigate whether the changed environmental stress could lead to any genomic change in the two vibrio species, which could be detected using fingerprinting PCR techniques such as IS PCR and GTG PCR.

The study demonstrated complex genotypic variations that occur in *Vibrio* strains of tropical aquatic environment because of varied environmental conditions; these genotypic variations result in genetic divergence and/or probable convergence, which are necessitated by adaptive requirements for a successful survival strategy. Previous

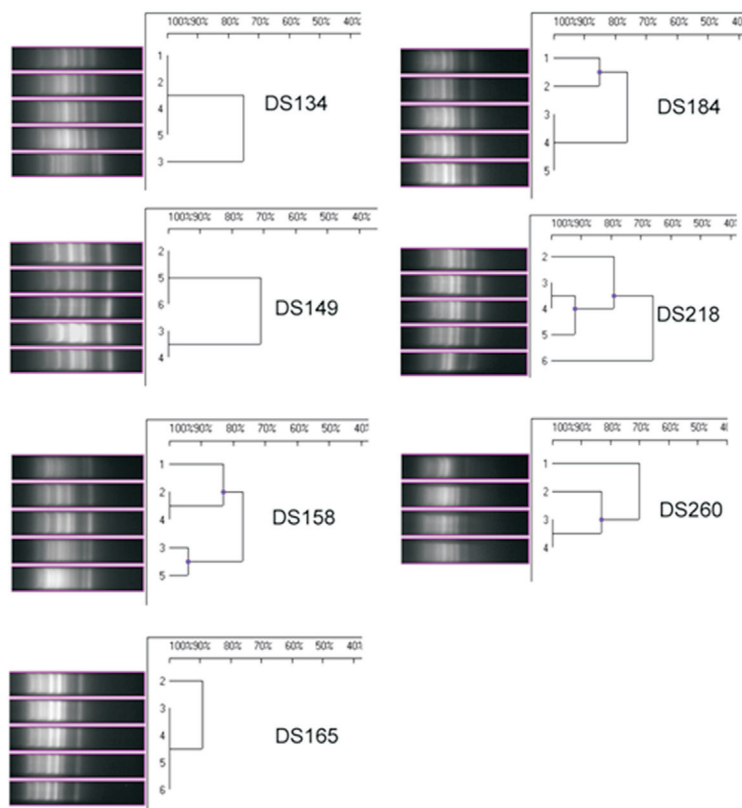

Figure 3 - Fingerprint patterns generated using GTG (5) PCR amplification of the genomic DNA of *V. harveyi* in 1.2% agarose gel. The number of strains is indicated in each gel. Lane 1- 0 passage, lane 2 to 4- after 10 passages in 3, 6 and 8% NaCl containing medium, respectively, lane 5- after 10 passages in TSA for strains DS134, DS158, DS184 and DS260. Lane 2- 0 passage, lane 3 to 5- after 10 passages in 3, 6 and 8% NaCl containing medium, respectively, lane 6- after 10 passages in TSA for strains DS149, DS165 and DS218.

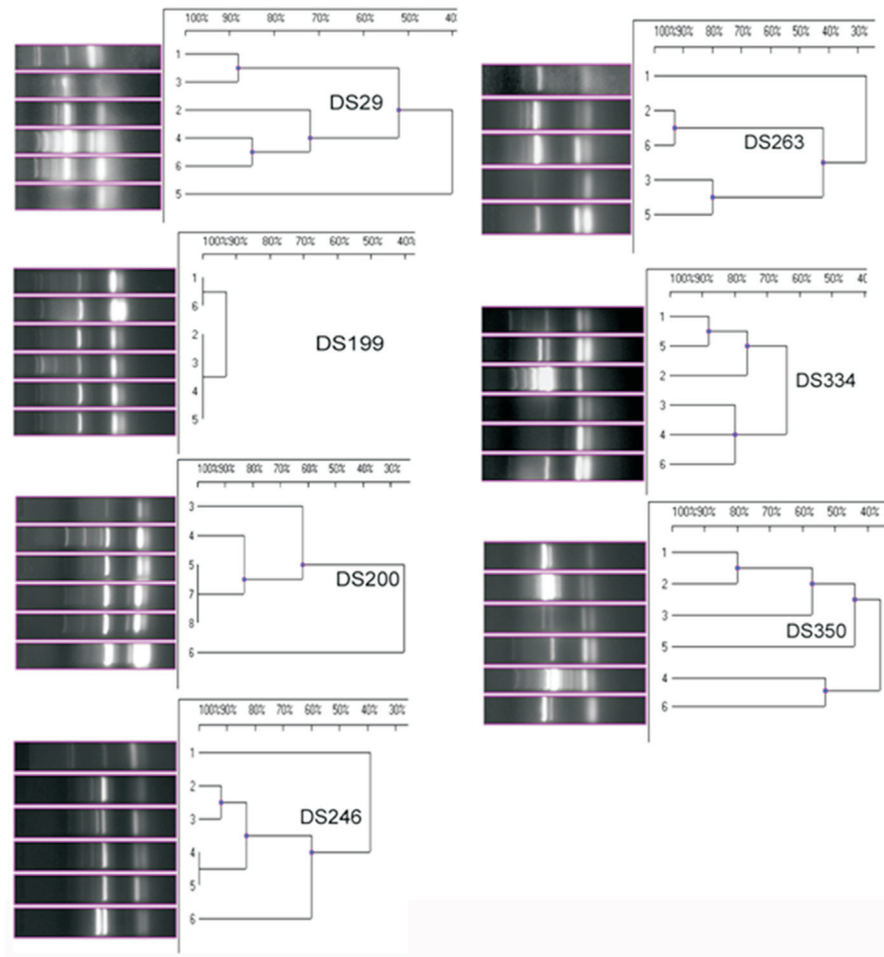

Figure 4 - Fingerprint patterns generated using IS PCR amplification of the genomic DNA of *V. alginolyticus* with primers, which were targeted at the insertion sequences in 1.2% agarose gel. The number of strains is indicated in each gel. Lane 1- 0 passage, lane 2 to 5- after 10 passages in 3, 6, 8 and 12% NaCl containing medium, respectively, lane 6- after 10 passages in TSA for strains DS29, DS199, DS246, DS263, DS334 and DS350. For strain 200, the order starts from 3- 8, respectively.

reports have indicated that the environmental and clinical *V. cholerae* strains have similar genomic organization and that pathogenic strains may arise from nontoxigenic strains in the aquatic environment (Chakraborty *et al.*, 2000; Brazil *et al.*, 2002) via multiple horizontal gene transfers (Heidelberg *et al.*, 2000).

The results of the current study indicate that the insertion sequences are notably suitable to determine the strain variation (Chandler, 1998) and play a remarkably important role in the genomic alterations because of their translocating property and functional ability to affect mutation/genetic variation (Syvanen, 1998). Source-independent genogrouping of both *V. harveyi* and *V. alginolyticus* strains was discernable in the current study. Among *V. harveyi*, two strains DS149 and DS158, which originated from WSSV-infected pond, were differentiated into two genogroups. Similarly, two strains that originated from uninfected shrimp intestine (DS134) and isolated from black-spot-infected shrimp lesion (DS218) from two different locations belonged to a single genogroup, with 100% homogeneity in GTG PCR fingerprinting. Among the *V. alginolyticus* strains, DS246 and DS334 belonged to a single genogroup, although they originated from WSSV-infected pond water and inlet area soil. Similarly, the strains from two distinctly different sources, such as uninfected shrimp intestine (DS29) and soil from *Zoothamnium* sp.-infected pond (DS263), were grouped into a single genogroup. The current study found that the *V. harveyi* and *V. alginolyticus* strains experienced stress-induced genomic alterations regardless of their source of origin. Changed environmental conditions such as starvation have been reported to cause adaptive mutation involving IS movement (Hall, 1988). The changes noted in the present study, which involved possible stress conditions induced by long-term passaging in high-salt (NaCl) medium and laboratory conditions, would have generated the mutation involving IS elements. A similar situation might also occur for the organisms that were growing *in vivo*, where the bacterial isolates would experience a changed propagating en-

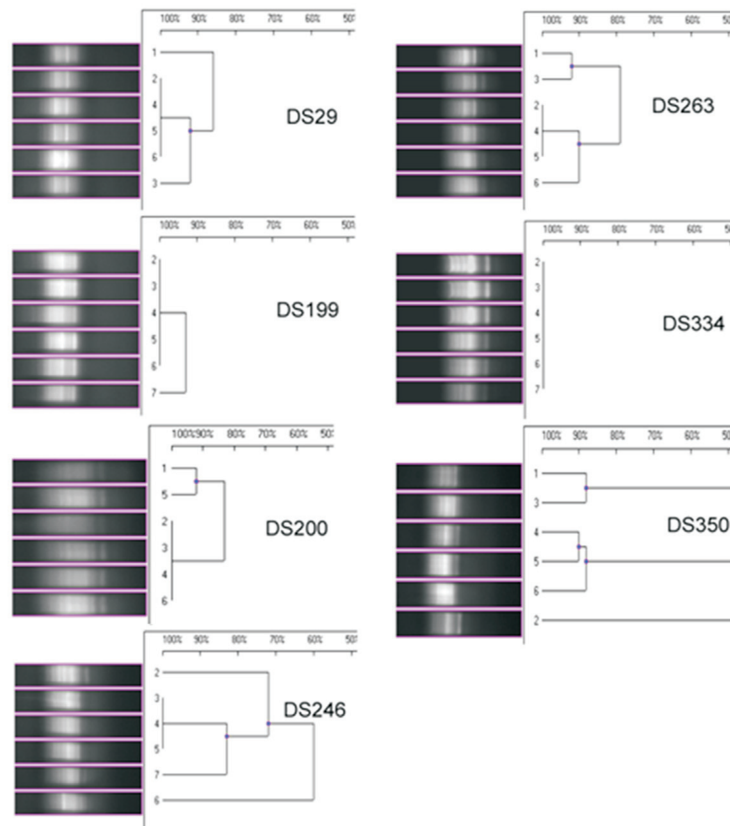

Figure 5 - Fingerprint patterns generated using GTG (5) PCR amplification of the genomic DNA of *V. alginolyticus* in 1.2% agarose gel. The number of strains is indicated in each gel. Lane 1- 0 passage, lane 2 to 5- after 10 passages in 3, 6, 8 and 12% NaCl containing medium, respectively, lane 6- after 10 passages in TSA for strains DS29, DS200, DS263 and DS350. Lane 2- 0 passage, lane 3 to 6- after 10 passages in 3, 6, 8 and 12% NaCl containing medium, respectively, lane 7- after 10 passages in TSA for strains DS199, DS246 and DS334.

vironment, which altered the fingerprint pattern from the original isolates. Similarly, such a change might occur in the isolates from animals that experienced a few cycles of passaging through the laboratory medium before they were fingerprinted using IS PCR. The *V. vulnificus* strains were reported to convert between distinct phenotypes of encapsulated opaque and nonencapsulated translucent forms; in addition, they switched from these variants to a rugose form under environmentally challenging conditions of low temperature (Grau *et al.*, 2005). The rugose variant, which was produced at temperatures below 37 °C and could form prodigious biofilms, has been found to have an important role in aiding the survival of the species at cooler temperatures in its natural marine environment, in nutritionally deficient conditions or otherwise unfavorable conditions. Nevertheless, the rugose variant potentiates the pathogenicity characteristics within the human host. The present study shows the genotypic changes that can occur in *V. harveyi* and *V. alginolyticus* strains that are subjected to altered propagating conditions. The role of IS sequences in revealing such changes has been reported in cases of enteric bacteria such as *E. coli* (Syvanen, 1998).

Our results demonstrate the genetic instability of two important and cosmopolitan *Vibrio* species of shrimp-farming systems. In a similar study, where *V. alginolyticus* and *V. parahaemolyticus* were maintained in low nutrient conditions, Abdallah *et al.* (2009) found that the adaptive response speed of vibrios to starvation was variable, and *V. alginolyticus* more quickly modified its extra chromosomal genetic content compared with *V. parahaemolyticus* to adapt to the changed environmental conditions. In the present study, changes were also visible in the genomic characteristics of *V. alginolyticus* and *V. harveyi* when they were fingerprinted using GTG (5) and IS PCR techniques. These techniques show that the genomic plasticity of the vibrios in the environment is probably a survival strategy that can also result in the phenotypic alterations in the protein expression (Xu *et al.*, 2004, 2005).

The findings of the present study is significant in the current pandemic scenario in the shrimp-farming sector, where the Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) significantly decreases shrimp production worldwide, which leads to huge economic losses to many countries. AHPND has been reported to be caused by a specific strain of *V. parahaemolyticus* (Tran *et al.*, 2013), which was not hith-

erto reported from shrimp aquaculture within the last two decades.

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