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Comparative analysis of RAPD, RFLP and PFGE for the Characterization of Pathogenic *Vibrio parahaemolyticus* Isolated from Seafood and Coastal Environment

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Abstract

Vibrio parahaemolyticus is ubiquitous in coastal marine environment. Certain strains of *V. parahaemolyticus* are pathogenic to humans and harbors either or both thermostable direct hemolysin (*tdh*) gene and *tdh* related hemolysin (*trh*) gene. Pathogenic *V. parahaemolyticus* isolates from the aquatic environment from Mumbai, India were characterized with respect to their virulence genotypes by employing random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE). Out of 140 samples, twenty five samples harboured virulent *V. parahaemolyticus*. Pandemic group specific PCR assays *viz*., GSVP, GSOLD, GSOK, ORF8 and PGS PCR showed differential performances which revealed difficulties in the confirmation of pandemic clone from environmental samples. Results from the molecular characterization employing RAPD and RFLP indicated that the PGS PCR positive isolates (2) showed identical banding pattern in RFLP analysis, whereas GS:OK positive isolates (4) showed similar RAPD banding pattern with 80% similarity among 11 pathogenic strains studied. However, in whole genome comparison by PFGE using *notI* restriction enzyme, these isolates were scattered in different groups at 40% similarity level. This study suggests that the genetically diverse pathogenic strains of *V. parahaemolyticus* with characteristic of pandemic clones are present in the coastal environment.

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Introduction

Among the human pathogenic groups of bacteria, *Vibrio parahaemolyticus* has emerged as one of the major concerns in seafood safety due its global food poisoning incident (Martinez-Urtaza et al., 2004). Pathogenic *V. parahemolyticus* in seafood are represented by those harboring either or both thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes (Xu et al., 2016). Since 1990s, the increase in enteric infections caused by *V. parahaemolyticus* have been associated with the emergence of a new pandemic clonal group comprising of genetically comparable serotypes, the prominent among them being the serotype O3:K6 (Matsumoto et al., 2000). The presence of such strains of *V. parahaemolyticus* in fishery products is definitely a deep concern on safety and acceptability of the fishery product.

Several molecular typing methods have been developed which examine the genetic relatedness of isolates by studying their molecular composition, homology and presence or absence of specific genes (Wong et al., 2000). RAPD PCR uses short oligomers to determine the genetic relatedness among the species as well as among the strains of the same species. Grouping of 80 strains of *V. parahaemolyticus* from different environments such as tropical estuary, shrimp farm and sea foods along the south west coast of India indicated that there was no biogeographical effect on the distribution of RAPD profiles in *V. parahaemolyticus* populations (Silvester et al., 2015). RAPD profiling of *V. parahaemolyticus* serotypes O3:K6 and serovariants of O3:K6 such as

O4:K68 and O1: KUT (untypable) isolated from different sources revealed that they exhibited a similar genetic fingerprint (Gil et al., 2007). RFLP analysis of virulence associated genes has been used as a valuable tool for typing of pathogenic as well as the pandemic strains of *V. parahaemolyticus* (Marshall et al., 1999). Elola-Lopez et al. (2015) used PCR-RFLP analyses of multivalent adhesion molecule 7 (*mam-7* gene) to differentiate clinical and environmental *V. parahaemolyticus* strains. In recent years, pulsed field gel electrophoresis (PFGE) is being widely used in epidemiological studies due to the highly discriminating power as compared to other typing methods (Parsons et al., 2007). Suffredini et al. (2011) examined the intra-species variability and genetic relationships among the environmental isolates of *V. parahaemolyticus* collected from different European countries such as Spain, Italy, Portugal and United Kingdom during 1999 to 2009. Chowdhury et al. (2000) reported that a close genetic relationship exists between O3:K6 and O4:K68 strains from India and Thailand showing 78-91% similarity as revealed by AP-PCR, ribotyping and PFGE. The rapid spread of pathogenic strains of *V. parahaemolyticus* needs global monitoring of the risk posed by this pathogen to ensure the safety of seafoods (Kang et al., 2016).

In the present study, an attempt has been made to ascertain the genotypes of pathogenic isolates of *V. parahaemolyticus* in seafood and aquatic environment by employing molecular typing methods such as RFLP, RAPD, and PFGE.

Materials and Methods

A total of 140 samples from north west coast of Mumbai region during the period 2014-2015, comprising of water (45), fish (40), shellfish (32) and sediment (23) were screened for the presence of *V. parahaemolyticus*. The isolation and identification was carried out as described in our previous study (Ayyappan et al., 2018). Molecular characterization for the presence of *tlh*, *tdh* and *trh* genes were carried out as per Bej et al. (1999). Reference strains used were *V. parahemolyticus* strains AQ4037 (tdh^-, trh^+) and O3:K6 (tdh^+, trh^- and pandemic group-specific (PGS) PCR assays (NICED, Kolkata).

Molecular confirmation of pandemic *V. parahaemolyticus* isolates was carried out by PCR targeting different pandemic group specific markers such as *toxRS/New* (GSVP) (Matsumoto et al., 2000); GS: OK (Okura et al., 2003); *toxRS/old* (GS OLD) (Okura et al., 2003); PGS PCR (Okura et al., 2004) and ORF8 PCR (Myers et al., 2003).

PCR-RFLP in virulent isolates of *V. parahaemolyticus* was done as per the procedure of Elola-Lopez et al. (2015). Initially, PCR targeting *mam-7* gene (2619 bp) was carried out. The reaction mixture was prepared with final reaction volume of 30 µl containing 1X amplification buffer with 2.5 mM $MgCl_2$, 1.5 mM solution of four dNTPs (pH 8.0),10 pM forward primer (*mam*-7F-CGTATGTGCCTGATGTTAAGAGGA) and reverse primer (*mam*-7R- AAGGGCTTAGGAATTGGCGTT), 1U Taq DNA polymerase and 50-75 ng DNA template. The amplification was carried out with 1 cycle of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 30 sec), annealing (65°C for 1 min 30 sec) and extension (72°C for 2 min) with a final extension at 72°C for 10 min. PCR amplified products were further digested with *hindIII* restriction enzyme to release appropriate restriction fragments. Digestion was carried out overnight by using 10 unit of *hindIII* in a final volume of 20 µl containing 0.3 -0.5 µg PCR product The PCR products were analyzed by electrophoresis at 75 voltage with 1.5% agarose gel (Himedia, India) and photographed using gel documentation system (Bio-Rad Gel documentation System, USA).

To determine the genetic relatedness among the isolates, RAPD was carried out by employing oligonucleotide primer (P1) (Maluping et al., 2005). The reaction mixture consisted of 1X amplification buffer with 2.5 mM $MgCl_2$, 10 mM NTPs (pH8.0), 10 pM 10 mer primer, 1U Taq DNA polymerase, 100 ng DNA template. The amplification conditions used was initial denaturation at 95ºC for 4 min followed by 45 cycles of denaturation at 95ºC for one minute, annealing at 36ºC for one minute and extension at 72ºC for 2 min with final extension at 95ºC for 7 min. The PCR products were analyzed by electrophoresis at 75 voltage with 1.5% agarose gel (Himedia, India) and photographed using gel documentation system (Bio-Rad Gel documentation System, USA). The gel photographs was subjected to analysis by using bionumerics 7.6 (Applied Maths, New York) software to determine the level of similarities between the isolates. Dendrograms were calculated by the unweighted pair group method by arithmetic averaging (UPGMA) algorithm using Sorensen-Dice similarity coefficient with 1% tolerance level.

PFGE has been carried out as per pulse net procedure for performing PFGE of *V. cholerae* and *V. parahaemolyticus* (Parsons et al., 2007). Briefly, the bacterial cell suspension [0.8 to 1.0 (OD_{610})] and 1% agarose (1:1) were mixed in microcentrifuge tubes containing 20 µl proteinase K (20 mg ml^{-1}) and transferred to disposable plug molds (Amersham Biosciences, USA) for agarose plugs. Cell lysis was performed by transferring the plugs into the lysis buffer containing 10% SDS and kept for 2 h in a shaker water bath (54-55°C). After lysis, the plugs were washed four-five times initially with preheated sterile ultrapure water and later with TE buffer for 10-15 min in shaker water bath (54-55°C). The plugs were cut in to 2-2.5 mm wide slice and transferred to a tube containing diluted restriction buffer (1:10) for pre-restriction incubation at room temperature for 10-15 min. After preincubation, restriction digestion was carried out using *Notl* restriction enzyme. The electrophoresis was performed in CHEFF MAPPER pulsed electrophoresis system (Bio-rad Laboratories, USA). Gel images were captured by gel documentation system (Bio-Rad Gel documentation System, USA). PFGE gel photographs were analysed using Bionumerics 7.6 (Applied Maths, New York) software to determine the level of similarities between the PFGE patterns. The dendrogram was constructed using UPGMA (unweighted pair group mean analysis) based on Sorensen-Dice similarity coefficient with 1% tolerance level.

Results and Discussion

Pathogenic strains of *V. parahaemolyticus* carrying virulence genes such as *tdh* and/or *trh* genes are responsible for food poisoning illness in humans and seafood may act as a source of this bacterium (Ayyappan et al., 2018). Although the prevalence and the characterization of pathogenic strains from clinical sources have been well studied, information available is scant regarding their presence in the seafood and coastal environments.

Twenty-five samples out of 140 samples analyzed from different seafood and coastal environments of North west coast of Mumbai, harbored pathogenic *V. parahemolyticus.* A total of 688 bacterial isolates were confirmed as *V. parahaemolyticus* by biochemical and PCR targeting *tlh* gene (Table 1). Out of 688 isolates, 38 and 3 isolates were positive for *trh* and *tdh* gene respectively. Among the 41 isolates, none of the isolates were positive for *toxRS/new* (GSVP) and *toxRS/old* (GS OLD) sequence of the pandemic strain. Four isolates were positive for GS:OK primer which specifically amplified the area within the *toxRS/New* sequence region by producing the gene amplicon of 651 bp. Two isolates yielded an amplicon of 235 bp with PGS PCR. One isolate showed positive result with ORF8 PCR. The virulent characteristics of the isolates for molecular typing methods (RAPD, RFLP and PFGE) are depicted in Table 2.

In general, an isolate possessing both *tdh* and *toxRS/new* can be considered as a pandemic strain (Okura et al., 2003). *tdh*-negative GS PCR-positive O3:K6 serovars have been isolated from Japanese coastal environment (Hara-Kudo et al., 2003). Recent studies from India have examined the pandemicity of pathogenic *V. parahaemolyticus* isolates from marine environment (Anjay et al., 2016; Pal & Das, 2014; Parthasarathy et al., 2016). However, in this study, none of the isolate showed positive for any of the group specific PCRs tested.

Table 1. Samples screened for the presence of virulent *V. parahaemolyticus* by biochemical and molecular methods

*Sample	Samples positive for *V. parahaemolyticus* (%)	No. of confirmed isolates of *V. parahaemolyticus* by biochemical tests and *tlh* gene by PCR	No. of Isolates harboring *trh* gene	No. of isolates harboring *tdh* gene
Fish (40)	100	143	24	3
Shell fish (32)	100	223	-	-
Sediment (23)	100	132	5	-
Water (45)	100	190	9	-
Total (140)		688	38	3

***-** value in parentheses indicates the total number of samples screened for the presence of virulent *V. parahaemolyticus*

Table 2. Virulent characteristics of isolates selected for molecular typing analysis

Isolates name	*trh*	*tdh*	PGS	GS OK	*orf8*
VP1	-	+	-	+	-
VP2	-	+	-	-	-
VP3	-	+	-	-	-
VP4	-	-	-	-	-
VP5	+	-	-	-	-
VP6	+	-	-	+	-
VP7	+	-	-	+	-
VP8	+	-	+	-	-
VP9	+	-	-		+
VP10	+	-	-	+	-
VP11	+	-	+	-	-

(-): Negative; (+): Positive

PCR results with *tdh* positive and GS:OK positive isolate (VP1) were in agreement with the characteristics of pandemic strains as reported by Matsumoto et al. (2000). There are reports of *tdh* negative O3:K6 strains lacking ORF8, but positive with *toxRS/new* gene (Osawa et al., 2002, Okura et al., 2003). However, in this study, *V. parahaemolyticus* isolate showing *tdh* negative, ORF8 positive lacking *toxRS/new* gene (VP9) suggests that the gene might have been acquired from the bacteriophge f237 (Chan et al., 2002). The *tdh* negative GS-PCR positive O3:K6 strain has been reported from Japan which differed from the *tdh* positive O3:K6 strain in PFGE analysis (Chowdhury et al., 2000). Similarly this study observed three isolates with *tdh* negative GS: OK positive (VP6, VP7 and VP10). Okura et al. (2003) reported the lack of specific genetic markers exclusively associated with pandemic clones. Bhuiyan et al. (2002) reported that neither *toxRS/new* nor ORF8 was sufficient for the identification of the pandemic strains. Parthasarathy et al. (2016) demonstrated PCR yielding an amplicon of 235-bp pandemic group-specific sequence based on an arbitrarily primed PCR fragment. Using this PCR, considerable percentage (11.9%) of shellfish in the coastal areas of eastern India were found harboring pathogenic (tdh^+) and pandemic (6.5%) *V. parahaemo-lyticus.* In this study also it was observed that two isolates harbored group-specific sequence of 235 bp in their genome, revealing the presence of isolates possessing pandemic characteristics in the coastal environments.

All eleven isolates from different seafood and coastal environments harbored *mam*-7 gene (Fig. 1). Further, RFLP anlaysis with *hindIII* restriction enzyme indicated similar RFLP pattern in two PGS positive isolates when compared with reference O3:K6 strain showing a high degree of relatedness among them (Fig. 2). However, other isolates showed different digestion pattern.

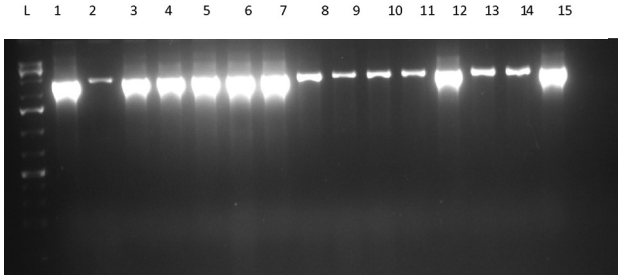

Fig. 1. PCR amplification of *mam 7* gene of *V. parahaemolyticus*

Lane L: GeneRuler 100 plus bp (Fermentas, USA); Lane1: Reference strain *V. parahaemolyticus* O3:K6; Lane 2: Reference strain *V. parahaemolyticus* AQ 4037; Lane 3: VP1; Lane 4: VP9; Lane 5: VP6; Lane 6: VP7; Lane 7: VP11; Lane 8: VP5; Lane 9: VP4; Lane 10: VP2; Lane 11: VP3; Lane 12: VP1; Lane 13: VP12; Lane 14: VP13; Lane 15: VP8

Mam-7 gene is considered to be highly variable among *V. parahaemolyticus* strains, due to the conservation of nucleotide sequences flanking the virulence genes such as *tdh* and *trh* genes (Elola-Lopez et al., 2015). In this study, RFLP analysis revealed identical nucleotide sequence of *mam-*

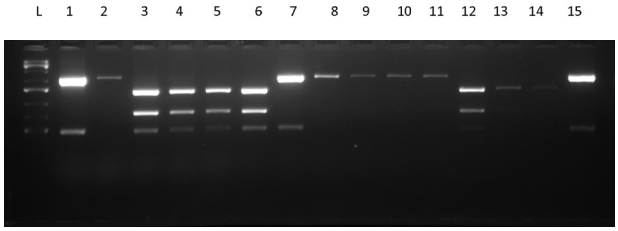

Fig. 2. RFLP digestion pattern of *mam 7* gene of *V. parahaemolyticus*

Lane L: GeneRuler 100 plus bp (Fermentas, USA); Lane1: Reference strain *V. parahaemolyticus* O3:K6; Lane 2: Reference strain *V. parahaemolyticus* AQ 4037; Lane 3: VP1; Lane 4: VP9; Lane 5: VP6; Lane 6: VP7; Lane 7: VP11; Lane 8: VP5; Lane 9: VP4; Lane 10: VP2; Lane 11: VP3; Lane 12: VP1; Lane 13: VP12; Lane 14: VP13; Lane 15: VP8

7gene in PGS PCR positive isolates suggesting that RFLP is useful in the pathotyping of *mam*-7 gene in virulent *V. parahaemolyticus* isolates. Elola-Lopez et al. (2015) reported use of RFLP analysis of virulence gene (*mam*-7) for the discrimination of pathogenic and nonpathogenic *V. parahaemolyticus*. However, RFLP analysis lacks discriminatory power as it selects only certain portion of DNA for PCR amplification and subsequent digestion.

RAPD profiling revealed three distinct groups (Group A, Group B and Group C) with a total of eight distinct patterns at 80% similarity level (Fig. 3). Isolates included in group A were AQ4037, VP5, VP4, VP2, VP3, and VP9. The group B comprised of only PGS-PCR positive isolates (VP8 and VP11) and these exhibited identical patterns. GS: OK positive isolates (VP7, VP6, VP10 and VP1) formed a separate group designated as group C. RAPD results suggested that GS: OK positive isolates are genetically related to each other when compared with pandemic strain O3:K6.

RAPD typing method uses short 10-mer primers to determine the genetic relatedness among the species as well as among the strains of same species (Silvester et al., 2015). *V. parahaemolyticus* strains have been successfully distinguished by wide range of RAPD PCR primers such as P1, P2, P3, P4, P5, and P6 (Maluping et al., 2005). Of these various primers used, P1 has been shown more reproducibility and discriminatory power to differentiate the pandemic strains of *V. parahaemolyticus* especially the old and new 03:K6 isolates (Matsumoto et al., 2000; Okuda et al., 1997a; Okuda et al., 1997b). In the present study, RAPD profiling clearly differentiated the isolates into three groups with 80% similarity. However, four GS:OK positive isolates closely related to the reference strain of pandemic O3:K6 appeared in same group designated as group C. Silvester et al. (2015) reported RAPD fingerprinting revealed high intra specific genomic diversity among *V. parahaemolyticus* isolated from tropical estuary, shrimp farm and seafoods from south west coast of India. Islam et al. (2004) reported that the virulent strain of *V. parahaemolyticus* isolated from aquatic environment samples from Bangladesh possess pandemic group specific genetic markers and showed identical RAPD banding pattern with pandemic O3: K6 strains.

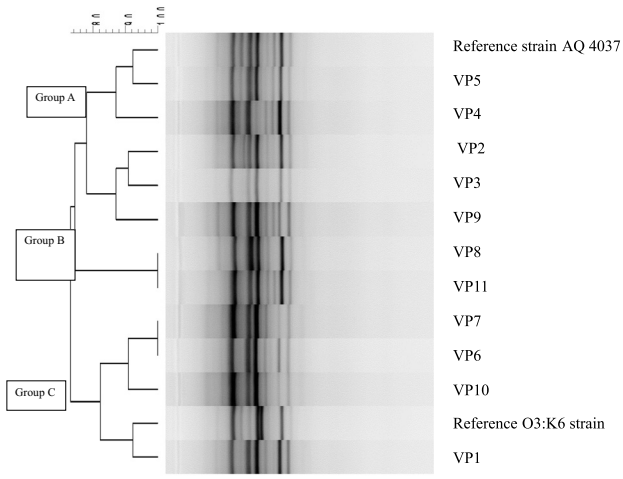

Fig. 3. Dendrogram and RAPD amplification patterns of *V. parahaemolyticus* isolates using primer 1

Differences in the estimation of genetic similarity between RFLP and RAPD clearly indicated the nucleotide sequence differences exist among the virulent *V. parahaemolyticus* isolates. In addition to the results obtained from the RAPD and RFLP typing methods, we further typed all the virulent *V. parahaemolyticus* isolates using whole genome comparison by PFGE. The variation of banding pattern was noted among the isolates in PFGE profiling of *V. parahaemolyticus*. Software analysis revealed the presence of four different groups (group A, group B, group C and group D) with seven distinct genotypes (genotypes A, B, C, D1, D2, E1, F1, F2, F3, F4, G1 and G2) at 40% similarity level suggesting that they are genetically diverse (Fig. 4). The Group A consisted of reference strain AQ4037, and VP3. Group B consisted of VP7, VP6, VP1, VP11 and VP2. Group C consisted of VP10, VP9, and VP8. Group D consisted of VP5 and VP4.

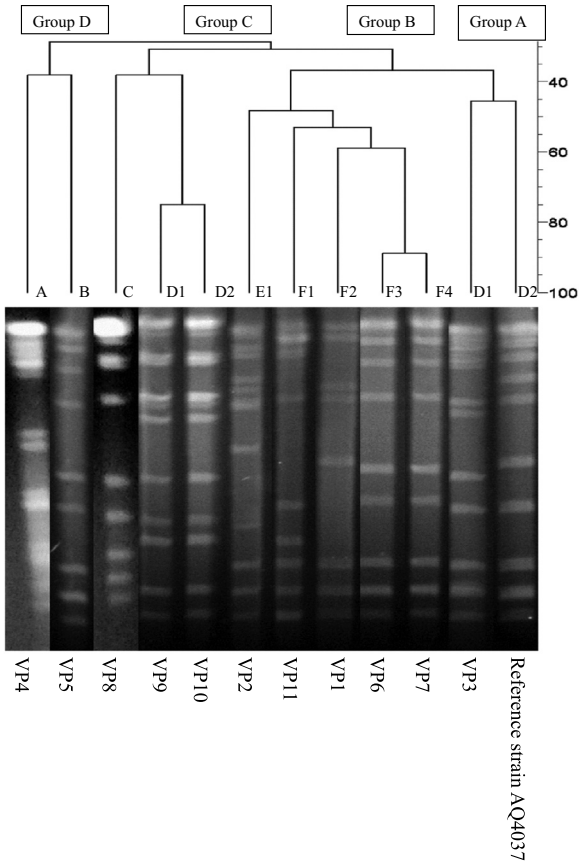

Fig. 4. Dendrogram and PFGE banding pattern of *notI* digested chromosomal DNA fragments of virulent *V. parahaemolyticus* isolates

In the present study, the banding pattern of reference strain of *V. parahaemolyticus* O3:K6 available in the public domain was used for the comparison. The PFGE pattern of all O3:K6 strains isolated from different geographical locations were identical (Chowdhary et al., 2000) and were similar with other pandemic groups such as O4:K68, O1:KUT. Suffredini et al. (2011) examined the intraspecies variability and genetic relationships among environmental isolates of *V. parahaemolyticus* from different European countries and they reported that *trh* positive strains appeared in more than one cluster. Hence, from the molecular typing methods, it was concluded that, the genetically diverse pathogenic strains of *Vibrio parahaemolyticus* with characteristic of pandemic clones are present in the coastal environment. Further, in the present study the banding pattern of the isolates were similar with the banding pattern of non-pandemic strains of various serotypes.

In conclusion, difficulties exist in the confirmation of pandemic clone due to differential performances of pandemic group-specific primers and lack of genetic markers exclusively associated with pandemic clones. RAPD and RFLP primer have less discriminatory power for identifying the genetic relationship among the virulent isolates of *V. parahaemolyticus*. Simultaneous use of whole genome comparison by PFGE gave clear reflection of genetic relationship among the isolates and proved to be the best in the typing of *V. parahaemolyticus* isolates. Seafood and environmental samples may harbor pathogenic strains of *V. parahaemolyticus* which may not fit into the classical definition of pandemic clones, but possess characteristics of pandemic clones. The pathogenic significance of such strains remains to be elucidated. It is therefore imperative to routinely monitor seafood and the coastal environment for the presence of pathogenic *V. parahaemolyticus* and implement proper measures for the control and prevention of *Vibrio*-related diseases.

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