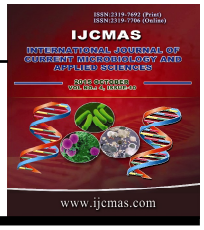
Int.J.Curr.Microbiol.App.Sci (2015) 4(10): 816-828

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 816-828**
http://www.ijcmas.com



Original Research Article

Use of Randomly Amplified Polymorphic DNA Analysis to Differentiate *Vibrio anguillarum* and *Vibrio parahaemolyticus*

B. B. Behura[1], B.R. Samantray[3], S. K. Samal[3], S.C. Mukherjee[2] and B. K. Das[3]*

[1]Balikuda College, Balikuda, Jagatsinghpur-754108, India
[2]ICAR-Central Institute of Fisheries Education, Mumbai, India
[3]ICAR-Central Institute of Freshwater Aquaculture, Kaushalyaganga, Bhubaneswar, 751002, Odisha, India
**Corresponding author*

Keywords

DNA fingerprinting, RAPD-PCR, *Vibrio parahaemolyticus*, *Vibrio anguillarum*

A B S T R A C T

From 20 isolates representating one strain of *Vibrio parahaemolyticus* and 25 isolates representating one strain of *Vibrio anguillarum* isolated from *Macrobrachium malcolmsonii* and *Penaeus monodon* were selected for the genotyping properties using RAPD-PCR. The isolates were isolated from different organs as haepatopancreas, external surface, pleopod and telson from prawn and confirmed on thiosulphate-citrate bilesalt-sucrose agar with supplemented with 2% NaCl. Both the isolates of *Vibrio* produced β-haemolysis and were virulent. For the RAPD study, 10 decamer random primers e.g. OPA, OPB, OPC, OPE, OPF, OPG, OPH, OPJ, OPK and OPY were used for screening and primers producing three or more than three fingerprints such as OPC 1, OPG 14, OPY 9, OPA 4, OPB 18, OPE 15, OPK 20, OPF 11 and OPJ 20 with genomic DNA of *Vibrio* species were selected. The number of amplified fragments varied from 4-9 with molecular weight 0.525-5.67 Kb. The molecular weight of amplified fragments of *Vibrio parahaemolyticus* and *Vibrio anguillarum* with primers OPH 19 and OPG 14 ranged from 0.58-1.99 Kb. The genetic similarity and diversity between the two species was 0.383 and 0.617 respectively. RAPD appeared to be a reliable and fast technique for discriminating between the two species of *Vibrio* on the basis of their form of isolation and therefore, represents a powerful tool for epidemiological studies of these prawn pathogens.

Introduction

Intraspecific characterization of pathogenic bacteria for epidemiological purposes can be achieved by several procedures. Phenotypic characterization widely used in the past, takes into account only few characters and has proven to be non-reliable. In the past couple of decades, the development of molecular techniques that allow the comparison of strains by directly looking at their genomes has overcome the shortcomings of analyzing phenotypic properties. The *Vibrios* are now been associated with many fish and prawn infections (Austin and Austin, 1987). *Vibrio*

anguillarum is a marine gram-negative bacterium that causes disease in approximately 50 different fish species (Austin and Austin, 1999). In vibriosis caused by the organism, the diseased animals show the symptoms of melanosis, abdominal swelling and ascitic fluid accumulation in the peritoneal cavity. The organisms are also associated with raw seas foods. So the V*ibrionaceae* that include pathogens of human, fish, crustacea and bivalve mollusks is one of the limiting factors in the coastal aqua production and exports. Vibriosis is an economically important disease of fish, marine invertebrates (particularly penaeid shrimps), and large marine mammals and is responsible for high mortality rates in aquaculture worldwide. Some *Vibrio* species are also responsible for zoonoses, whereas others are relatively nonpathogenic. Particular attention is now being focused on Vibriosis caused by infection with *Vibrio anguillarum* has been responsible for numerous epizootics among cultured and feral population of fish throughout the world (Hasten and Holt, 1972; McCarty *et al.,* 1974; Novotny, 1975). Characterization of chromosomal DNA allows typing of most bacterial strains. At species level, the genetic relationship of *V. anguillarum* and *V. ordaii* has been examined for 16S rRNA sequence analysis (Rehnstam *et al.,* 1989; Dorsch *et al.,* 1992; Kita-Tsukamoto *et al.,* 1993) as well as with DNA fingerprinting (Tsai *et al.,* 1990; Martinez *et al.,* 1994).

Vibrio parahaemolyticus is a marine bacterium, and some strains can cause gastroenteritis in human through the consumption of contaminated seafood. Microorganisms have traditionally been identified by comparison of their morphologies and through biochemical assays. Such screening is time consuming especially when a large number of isolates is involved and also the methods are not sensitive enough to identify different strains belonging to same species. But DNA fingerprinting techniques such as restriction fragment length polymorphisms (RFLP) and random amplification of polymorphic DNA (RAPD), provide alternate approaches to distinguish genotypic variants (Willams *et al.,* 1990; Myhr *et al.,* 1991; Marhual and Das, 2009; Marhual *et al.,* 2009; Marhual *et al.,* 2012). In epidemiology, random amplification of ploymorphic DNA (RAPD) assays is appropriate for screening large panel of strains (Hilton *et al.,* 1996).

Vibrio parahaemolyticus is a major food-borne pathogen that causes worldwide health problems. Prevention of *V. parahaemolyticus* contamination of foods, especially contamination with serotype O3:K6, which has been associated with recent outbreaks (Bag *et al.,* 1999) is an important public health concern.

So there is the need of characterization of several *Vibrio* species. Bacterial identification is a growing field of interest within microbiology. The differentiation of closely related strains by comparing RAPD patterns has been introduced in the field within the few years (Welsh and McClelland, 1990; Williams *et al.,* 1990; Welsh *et al.,* 1992; Marhual *et al.,* 2014). This technique is based on the use of a single arbitrary primer of low stringency in the polymerase chain reaction to amplify segments of the genome. The resulting fragments indicate the polymorphism of DNA. RAPD technique so far has been perhaps the most convenient method to reveal the intraspecies DNA polymorphism. In the present study, we have used RAPD-PCR to differentiate *V. anguillarum* and *V. parahaemolyticus* and to calculate the genetic similarity and genetic diversity within the species.

Material and Methods

Sample collection, isolation and identification

A total of 20 samples each from *Macrobrachium malcolmsonii* from Central Institute of Freshwater Aquaculture, prawn hatchery and *Penaeus monodon* from Phalcon Marine Exports, Bhubaneswar, India were collected. The different organs as hepatopancreas, external surface, pleopods and telson from prawn were collected and grown in tryptone soya broth added 2% NaCl (Himedia, India). The samples were streaked on thiosulphate-citrate-bile salt-sucrose agar (Himedia, India) and incubated at 37^0C for 24h. The biochemical test was done according to Mac Fadden, 1976 and West and Colwell, 1984. From 20 isolates representing one strain of *Vibrio parahaemolyticus* and 25 isolates representing one strain of *Vibrio anguillarum* were selected for RAPD fingerprinting study. After identification *V. parahaemolyticus* and *V. anguillarum* were maintained on tryptone soya agar. The haemolytic tests were performed according to (Miyamoto *et al.*, 1969) to know the virulence of the isolates.

DNA isolation

For isolation of genomic DNA, (Williams *et al.*, 1990) with slight modification was followed: *V. anguillarum* and *V. parahaemolyticus* species were grown at 37^oC for 24 hours in 20 ml of tryptone soya broth added 1.5% NaCl (Hi Media, India). A volume of 1.5 ml culture was transferred to microcentrifuge tube and centrifuged at 13,000 g for 5 min. The supernatant fluid was discarded and a further 1.5 ml of culture added to the pellet, centrifuged again, the supernatant fluid was discarded and the combined pellets resuspended in 367 µl of Tris- EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 8.0), 30 µl of 10% SDS (BDH) and 5 µl proteinase K (20mg/ml, BDH). The suspension was thoroughly mixed and incubated at 37^oC for 2 hours (Williams *et al.*, 1990). The DNA sample was then extracted with phenol: chloroform: isoamyl alcohol (25:24:1), the aqueous phase recovered and precipitate with $1/10^{th}$ volume of 3M sodium acetate (SRL, pH 5.2) with double volume of isopropanol. After precipitation, the samples were centrifuged at 13,000 g for 10 min at 4^0C. Then the pellet was washed with 70% alcohol twice and finally dissolved with 40 µl of Tris-EDTA buffer and stored at 4^0C. The absorbance of the DNA solution was read at 260 and 280 nm in UV-spectrophotometer (Biorad, SmartSpec 3000) to determine the concentration and purity. The genomic DNA samples was also examined on an 0.8% (w/v) agarose gel to check that, the genomic DNA had not become excessively fragmented and stored at -20^0C for further use.

RAPD-PCR amplification

RAPD-PCR was used to determine the relatedness of the two strains of *V. anguillarum* and *V. parahaemolyticus*. Reaction mixtures (25 µl) were made consisting of 2.5µl 10X PCR buffer, 100 µM each dNTPs (dATP, dGTP, dCTP and dTTP), 5 pMol primer, 2.5mM $MgCl_2$, 25ng of genomic DNA and 1 unit of Taq DNA polymerase, made up to 25 µl with sterile double distilled water, amplifications were performed for 35 cycles (MJ Research, Inc.) at 94^0C for 45 sec (denaturation), 36^0C for 45 sec (annealing), 72^0C for 1min 30 sec (extension) with initial denaturation at 94^0C for 4 min. A final elongation step at 72^0C for 7 min was included. The PCR amplification products were visualized by running 10 µl of the reaction mixture on 1.2% (w/v) agarose

gel and detected by staining with ethidium bromide (BDH). After staining, the gels were visualized under UV transilluminator and photographed in gel doc system (UVI Soft, Inc.). DNA ladder (1Kb) (MBI, Fermentas) was used as molecular mass marker. In each experiment a control reaction was set up containing no template DNA, so that any contaminating DNA present in the reaction would be amplified and detected in the agarose gel.

Primers

Each of the 20 OPA, OPB, OPC, OPE, OPF, OPG, OPH, OPJ, OPK and OPY decamer primers obtained from operon technologies in the form of kit were assessed with two local strains of *V. anguillarum* and *V. parahaemolyticus.* Those primers which produced clear and reproducible bands (at least three and above but identical tests) were selected for genomic studies. These primers had a G+C content of 60 to 70% and that they have no self- complementary ends. The primers amplified are OPA-4 (5'-AATCGGGCTG-3'), OPB-18 (5'-CCACAGCAGT-3'), OPC-1 (5'-TTCGAGCCAG-3'), OPE-15 (5'-ACGCACAACC-3'), OPF-11 (5'-TTGGTACCCC-3'), OPG-14 (5'-GGATGAGACC-3'), OPH-19 (5'-CTGACCAGCC-3'), OPJ-20 (5'-AAGCGGCCTC-3'), OPK-20 (5'-GTGTCGCGAG-3') and OPY-9 (5'-AGCAGCGCAC-3').

Evaluation of RAPD performance

Molecular weight determination

The molecular weight was determined using the software UVI soft comparing it with known molecular weight marker (1Kb ladder, MBI Fermentas, SM 0313).

Data analysis

Comparing the RAPD patterns between different species, images of the gels were captured by gel documentation system (UVI soft) and data analysis was performed using one-way ANOVA. For this pairwise comparison between all the samples were calculated manually using the following equation.

$S_{AB} = 2 N_{AB} / (N_A + N_B)$

Where S_{AB} =Shared DNA band between two species A and B/ Genetic Similarity between A and B

N_{AB} =Number of DNA bands shared in common between species A and B

N_A and N_B= Total number of bands possessed by the species A and B respectively.

Results and Discussion

V. parahaemolyticus and *V. anguillarim* isolated from different body parts viz. hepatopancreas, external surface, pleopod and telson of marine prawn *P. monodon* and freshwater prawn *M. malcolmsonii* (Table 1). The two strains were found to produce β-haemolysis around colonies on blood agar.

The molecular weight of the amplified DNA fingerprinting of randomly selected primers of *V. parahaemolyticus* and *V. anguillarum* are shown in table 2. The molecular weight of all the selected primers ranged from 0.525-5.67 Kb. Figure 1 shows the DNA fingerprints of *V. parahaemolyticus* and *V. anguillarum* against OPH 19 and OPG 14; in which the mol. Wt. ranged from 1.99-.580 Kb and 1.56-0.590 Kb. The mol. Wt of the amplified product with primers OPY 9 and OPA 4 ranged from 0.525-3.96 Kb (Fig. 2

and 3). Figures 4, 5 and 6 show fingerprinting patterns with primers OPC 1, OPB 18, OPE 15, OPK 20, OPF 11 and OPJ 20. The banding pattern of *V. parahaemolyticus* and *V. anguillarum* shows similarity between them. The genetic similarity and diversity between the two species was 0.383 and 0.617 respectively. The probability factor and variance between the two groups of the above species was calculated to be 405.116 and 0.028906 respectively (Table 4) by using one-way ANOVA.

The highest numbers of amplified fragments were seen in *V. anguillarum* with primer OPE-15 (9 bands); followed by OPC 01, OPY 09 and OPF 11. Whereas the minimum number of PCR amplified fragments was observed in *V. anguillarum* and *V. parahaemolyticus* (4 fragments) amplified with OPG 14 and OPB 18. The amplified fragments of *V. parahaemolyticus* and *V. anguillarum* with OPH 19 showed 4-5 fragments varying from 0.590 to 1.99 Kb. In OPG 14 the amplified fragments ranged from 0.717 to 1.26 Kb; which showed there is close similarity in the banding patterns. In all the primers chosen for the study, showed that the fragments amplified using *V. parahaemolyticus* and *V. anguillarum* ranged between 0.5 to 1.9 Kb were species specific and unique.

There was little similarity and difference in the amplified fragments of DNA of *V. parahaemolyticus* and *V. anguillarum* with the selected amplified primers. By using OPH 19 primer *V. parahaemolyticus* produced two clear and distinct bands i.e. 1.99 Kb and 1.73 Kb. Then using OPG 14 primer; both the species produced same amplified fragments of 0.59-1.26 Kb. Amplified fragment of size 1.30 Kb and 3.12 Kb in *V. anguillarum* with primer OPY 9 and OPA 4 is selective one, which can be used to differentiate the above two species. Amplified DNA fragments of 4.49 Kb and 5.04 Kb molecular weight in *V. parahaemolyticus* using OPC 1 and OPB 18; whereas absent in *V. anguillarum* amplified with the same. The amplified fragments of 2.98 Kb and 2.45 Kb as seen in *V. anguillarum* amplified with OPE 15 and OPK 20, whereas the above two fragments were not amplified in *V. parahaemolyticus.* The molecular weight as 3.3 Kb and 2.05 Kb amplified fragments in *V. anguillarum* is distinguishable as compared to *V. parahaemolyticus*. The above fragments amplified by the selected primers can be used in the epidemiological fields to distinguish between the two virulent species of *Vibrio* i.e. *V. anguillarum* and *V. parahaemolyticus.*

Table.1 Species source from which isolations were made

Sl. No.	Bacteria code	Bacteria	Organ of isolation	Source of isolation	Area of isolation
1	Va	*V. anguillarum*	External surface Hepatopancreas Pleopod Telson	*P. monodon*	Falcon Marine Exports, Bhubaneswar
2	Vp	*V. parahaemolyticus*	Hepatopancreas Pleopod Telson External surface	*M. malcolmsonii*	CIFA*, prawn hatchery

* Central Institute of Freshwater Aquaculture

Table.2 Molecular weight of amplified bands of *V. parahaemolyticus* and *V. anguillarum* using selected primers

Molecular weight in Kb																			
OPH-19		OPG-14		OPY-9		OPA-4		OPC-1		OPB-18		OPE-15		OPK-20		OPF-11		OPJ-20	
Vp	Va	Vp	Va	Vp	Va	Vp	Va	Vp	Va	Vp	Va	Vp	Va	Vp	Va	Vp	Va	Vp	Va
1.99 1.73 1.46 1.26 0.580	1.56 1.40 0.665 0.590	1.26 0.886 0.748 0.663	3.15 1.13 0.890 0.717	1.15 1.09 1.02 0.992 0.639 0.535	1.30 1.09 0.981 0.718 0.594 0.525	3.96 2.14 1.47 1.33 1.14	5.67 3.12 2.72 1.78 1.03	4.49 2.00 1.80 1.66 0.892	2.82 2.07 1.80 1.07 0.892 0.670 0.650	5.04 1.86 1.76 1.13	4.00 1.85 1.77 1.60 0.975	2.73 2.45 2.08 1.78 1.05 0.857	2.98 2.41 1.94 1.74 1.30 1.19 1.01 0.990 0.845	4.26 1.92 1.73 1.61 0.991	3.73 2.45 1.96 1.52 1.14 0.932	3.91 2.25 2.00 1.60 1.37	3.57 3.13 2.67 1.76 1.47 1.33 1.22	4.98 2.05 1.75 1.46 1.31	1.77 1.50 1.37 1.23

N.B: Vp-*Vibrio parahaemolyticus*, Va- *Vibrio anguillarum*

Table.3 Summary of the genetic similarity and genetic diversity values between the 2 species of *Vibrio* obtained from ten decamer primers of random sequence

Combinations using OPC, OPY, OPJ, OPA, OPB, OPE, OPF, OPK, OPH and OPG series of primers	Genetic similarity (S_{AB})	Genetic diversity (1- S_{AB})
V. parahaemolyticus- V. anguillarum	0.383	0.617

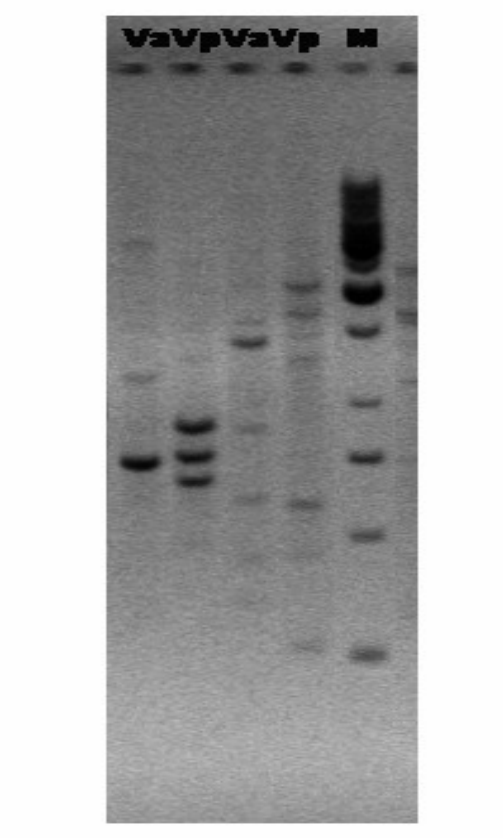


Figure.1 RAPD profile generated by PCR using *V. parahaemolyticus* and *V. anguillarum* with OPH-19 and OPG-14 primers

(R-L) Lanes: 1- 1 Kb ladder , 2- OPH-19, 3- OPG-14, 4- OPH-19, 5- OPG-14

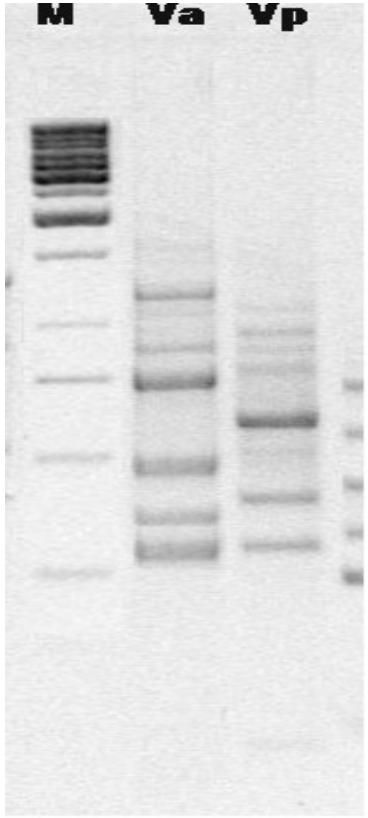


Figure.2 RAPD profile generated by PCR using *V. anguillarum* and *V. parahaemolyticus* with OPY- 9 primer

(L-R) Lanes: 1- 1 Kb ladder, (2-3) - OPY- 9

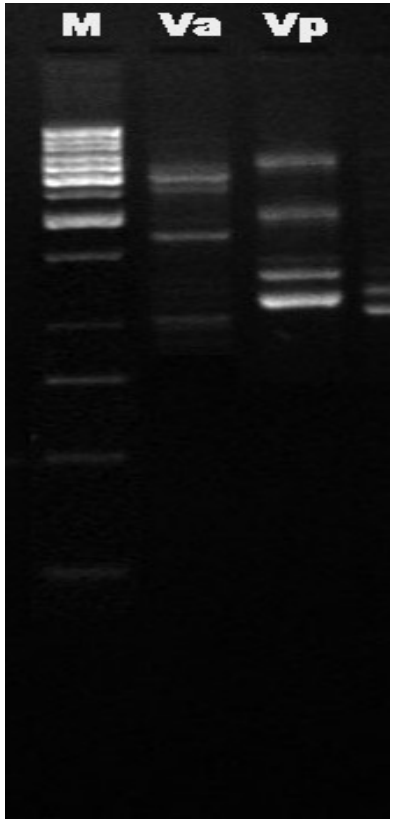


Figure.3 RAPD profile generated by PCR using *V. anguillarum* and *V. parahaemolyticus* with OPA- 4 primer

(L-R) Lanes: 1- 1 Kb ladder, (2-3) - OPA- 4

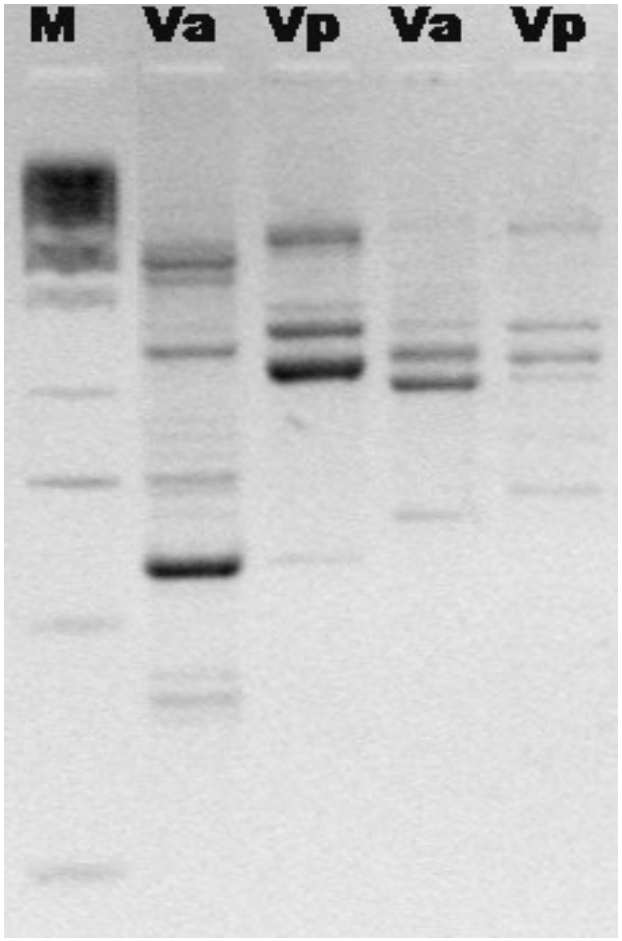


Figure.4 RAPD profile generated by PCR using *V. anguillarum* and *V. parahaemolyticus* with OPC-1 and OPB-18 primers

(L-R) Lanes: 1- 1 Kb ladder, 2-OPC-1, 3- OPB-18, 4-OPC-1, 5-OPB-18

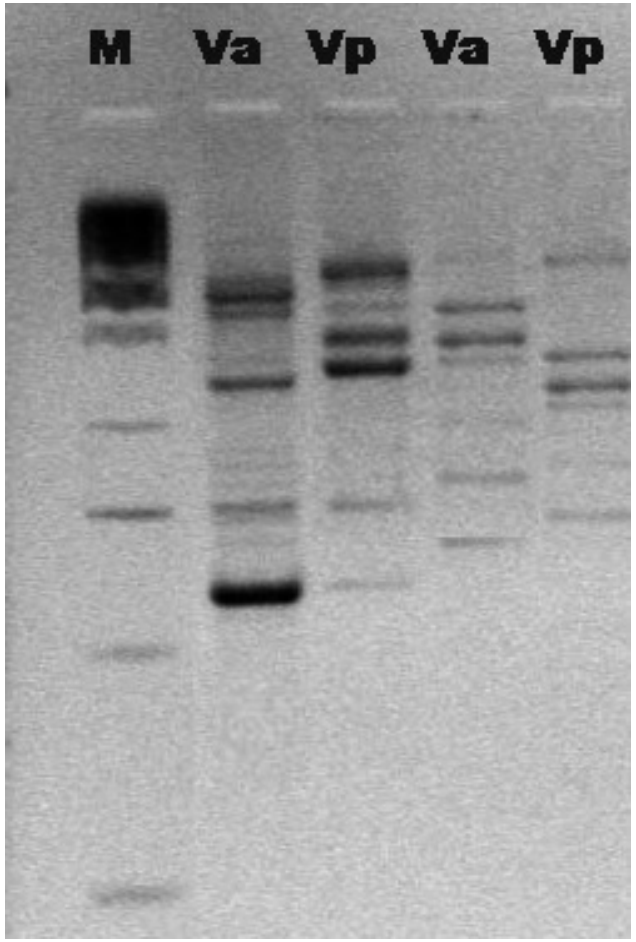


Figure.5 RAPD profile generated by PCR using *V. anguillarum* and *V. parahaemolyticus* with OPE-15 and OPK-20 primers

(L-R) Lanes: 1- 1 Kb ladder , 2-OPE-15, 3-OPK-20, 4-OPE-15, 5-OPK-20

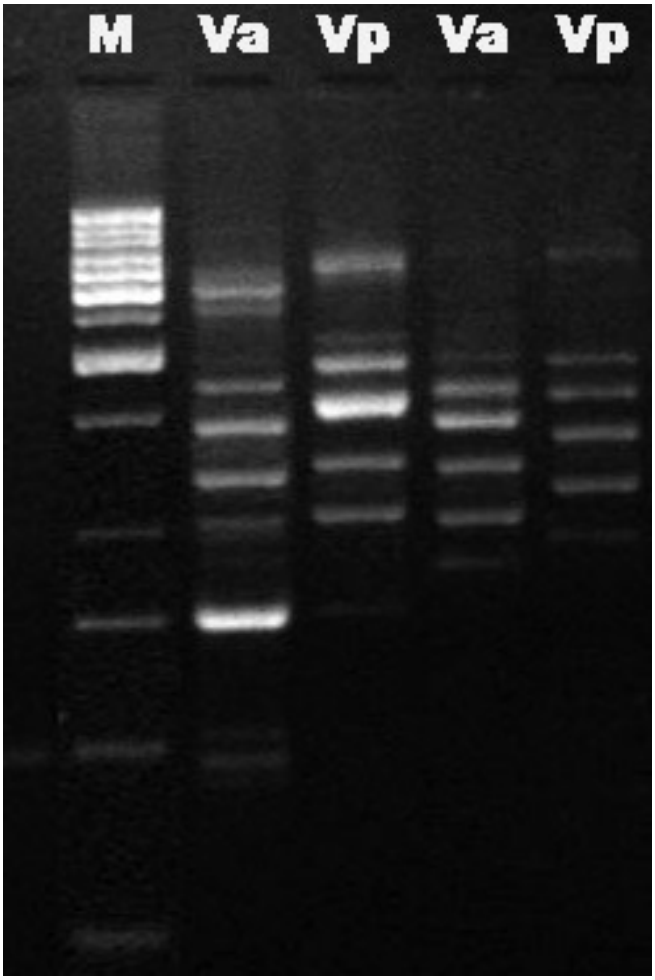


Figure.6 RAPD profile generated by PCR using *V. anguillarum* and *V. parahaemolyticus* with OPF-11 and OPJ-20 primers

(L-R) Lanes: 1- 1 Kb ladder, 2-OPF-11, 3-OPJ-20, 4-OPF-11, 5-OPJ-20

Table.4 One way ANOVA using genetic similarity between *V. parahaemolyticus* and *V. anguillarum* from figure 1–6

Source of variance	SS	df	MS	F	P	Fcrit	Variance
Between Group	130.9184	1	130.9184	28.47	405116	8.2854	0.028906
Within Group	82.76016	18	4.597787				

The genetic relationship among the *V. parahaemolyticus* and *V. anguillarum* species tested was determined by the RAPD-PCR analysis with primers OPH-19 to OPJ-20 respectively. The primers and amplification conditions used in the study allowed generation of 4 to 9 weaker or stronger DNA bands in each of *V. parahaemolyticus* and *V. anguillarum* species.

The different species of prawns were infected by most potential pathogenic strains of Vibrios as *V. parahaemolyticus* and *V. anguillarum*. In the study area, in which seven of 20 samples were positive for the pathogen. The haemolytic test, associated with one of the haemolysin produced by *V. parahaemolyticus* and *V. anguillarum*; that produces a β-type haemolysin on blood agar media. Haemolytic blood agar medium has been considered as an important virulence factor as well as a marker for virulence strains (Miyamoto *et al.,* 1969). *V. parahaemolyticus* and *V. anguillarum* isolated from clinical specimens demonstrates this haemolytic activity (Miyamoto *et al.,* 1969; Lee and Pan, 1993). The data obtained from present study indicate that both of the species were haemolytic positive.

RAPD analysis is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequences (Welsh and Mc Clellnd, 1990), and therefore, it is useful for scanning the entire genome to look for differences between strains. Interestingly, the DNA profiles of *V. parahaemolyticus* and *V. anguillarum* strains isolated were highly divergent.

The technique described here can be readily implemented to identify for quickly diagnosis of species or strains of *Vibrio*. After an initial isolation and characterization by plating of presumably contaminated samples from fishes, reliable results confirming the presence or absence of the pathogenic *Vibrio* can be again confirmed by RAPD-PCR.

In conclusion, our data showed that there is genetic similarity between the *Vibrio* species isolated from shellfish. *V. parahaemolyticus* and *V. anguillarum* were enteric pathogen and have been reported as sources of disease outbreaks in Taiwan, Japan and other coastal regions (Joseph *et al.,* 1982; Cook and Rupple, 1989; Chiou *et al.,* 1991; Janda *et al.,* 1998).

The RAPD and RFLP techniques had been tested earlier, with a cross-section of bacterial families and species including our most recent research findings on the genetic fingerprinting of *Vibrio* species (Tamplin *et al.,* 1996). Recently nucleic acid hybridization has been introduced for the rapid identification of pathogenic bacteria (Festl *et al.,* 1986; Haun *et al.,* 1987; Jiang *et al.,* 1987; Romaniuk *et al.,* 1987). RAPD-PCR has been shown in recent years to be useful for clarifying a number of bacterial species (Williams *et al.,* 1990; Medies *et al.,* 1995) but our results indicate that under the conditions employed in this study, RAPD-PCR appropriate for developing molecular tools for *Vibrio* species. However this method can be applied to the identification of individual strains or to the subtyping because of its high sensitivity. Also, RAPD-PCR may be used as a diagnostic tool in tracing the source of infections associated with the consumption of seafood, because results can be obtained less than 24 h after sampling.

The result obtained with the RAPD analysis showed that this molecular method was sensitive enough to reveal inter and intra specific genetic differences among *V.*

parahaemolyticus and *V. anguillarum.* More ever, the RAPD–PCR is easier to perform and more sensitive than other molecular based techniques (Wang *et al.,* 1993). DNA fingerprinting has been successfully used for clonal descrimination of strains of *Vibrio* species (Versalovic *et al.,* 1991; Rivera *et al.,* 1995). In summary, the results of this work showed that molecular characterization of *V. anguillarum* and *V. parahaemolyticus* isolates using the RAPD polymorphism analysis is a quick and convenient method to differentiate Vibrios of same and different serogroups. The possibility of using the RAPD; for molecular differentiation of *V. anguillarum* and *V. parahaemolyticus* isolates could provide a better knowledge of their genetic relatedness and clonal origin.

The possibility of using the RAPD; for molecular differentiation of *V. anguillarum* and *V. parahaemolyticus*: RAPD analysis is based on the amplification of random DNA segments with a single primer of arbitrary nucleotide sequences (Welsh and Mc Clelland, 1990); and therefore it is useful for scanning the entire genome to look for differences between strains. Genotyping systems including PFGE and RAPD analysis may prove to be useful tools for epidemiological studies on *V. anguillarum* and *V. parahaemolyticus* isolated from shrimps. As expected, the arbitrary primers used in this study enabled even closely related strains within the same *Vibrio* species to be differentiated. RAPD analysis performed in this study, using primers OPC-1, OPB-18, OPE-15 and OPK-20; revealed intraspecific variations among isolates of *V. anguillarum* and *V. parahaemolyticus*. Both RAPD primers used differentiated the *Vibrio* isolates by producing four to nine DNA fingerprints respectively. These RAPD profiles identify could be also defined the genetic relatedness of the isolates. The RAPD patterns obtained by *V. anguillarum* with primer OPE-15 generated more DNA amplified bands than those obtained with primer OPJ-20 and OPG-14. The DNA fingerprint obtained by *V. parahaemolyticus* with primer OPY-9 and OPE-15 generated more amplified bands than those obtained with primer OPG-14 and OPB-18. It is clearly evident that the random primers OPC 1, OPH 19, OPY 9, OPA 4, OPB 18 and OPJ 20 could be used for genetic differentiation of *V. parahaemolyticus* and *V. anguillarum*. In conclusion, RAPD analysis is a better method for discriminating between prawn isolates *V. anguillarum* and *V. parahaemolyticus*.

Acknowledgements

Acknowledgement is due to Director, ICAR-CIFA, and Bhubaneswar for providing all necessary facilities for conducting experimental trials.

Reference

Austin, B., Austin, D.A. 1987. Bacterial fish pathogens: disease in farmed and wild fish. John Wiley and Sons, Chichester.

Austin, B., Austin, D.A. 1999. Bacterial fish pathogens: diseases of farmed and wild fish. Springer Verlag, Berlin, Germany.

Bag, P.K., Nandi, S., Bhadra, R.K., Ramamurthy, T., Bhattacharya, S.K., Nishibuchi, M., Hamabata, T., Yamasaki, S., Takeda, Y., Nair, G.B. 1999. Clonal diversity among recently emerged strains of *Vibrio parahaemolyticus* O3:K6 associated with pandemic spread. *J. Clin. Microbiol.*, 37: 2354–2357.

Chiou, A., Chen, L.H., Chen, S.K. 1991. Foodborne illness in Taiwan, 1981-989. *Food Aust.,* 43: 70–71.

Cook, D.W., Rupple, A.D. 1989. Indicator

bacteria and *Vibrionaceae* multiplication in post-harvest shellstock oysters. *J. Food Prot.,* 52: 343–349.

Dorsch, M., lane, D., Stackeband, E. 1992. Towards the phylogeny of the genus *Vibrio* based on 16s rRNA sequences. *Int. J. Syst. Bacteriol.,* 42: 58–63.

Festl, H., Ludwig, W., Schleifer, K.H. 1986. DNA hybridization probe for the *Pseudomonas fluorescens* group. *Environ. Microbiol.,* 52: 1190–1194.

Hastein, T., Holt, G. 1972. The occurrence of *Vibrio* disease in wild Norwegian fish. *J. Fish Biol.,* 4: 33–37.

Haun, G., Gobel, U. 1987. Oligonucleotide probe, genus-, species-., subspecific identification of sentatives of the genus *Proteus*. *FEMS Microbiol. Lett.,* Pp. 187–193.

Hilton, A.C., Banks, J.G., Penn, C.W. 1996. Random amplification of polymorphic DNA (RAPD) of Salmonella: strain differentiation and characterization of amplified sequences. *J. Appl. Microbiol.,* 81: 575–584.

Janda, J.M., Powers, C., Bryant, R.G., Abbott, S.L. 1998. Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* species. *Clin. Microbiol. Rev.,* 1: 245–267.

Jiang, X., Estes, M.K., Metcalf, T.G. 1987. Detection of hepatitis A virus by hybridization with single-stranded probes. *Appl. Environ. Microbiol.,* 54: 2487–2495.

Joseph, S.W., Colwell, R.R., Kaper, J.B. 1982. *Vibrio parahaemolyticus* and related *Vibrios*. *Crit. Rev. Microbiol.,* 10: 77–124.

Kita-Tsukamoto, K., Oyaizu, H., Nanba, K., Simidu, U. 1993. Phylogenetic relationship of marine bacteria, mainly members of the familiar\e Vibrionaceae, determined on the basis of 16S rRNA sequences. *Int. J. Syst. Bacteriol.,* 43: 8–19.

Lee C., Pan, S.F. 1993. Rapid and specific detection of the thermostable direct haemolysin gene in *Vibrio parahaemolyticus* by the polymerase chain reaction. *J. Gen. Microbiol.,* 139: 3225–3231.

Mac Fadden, J.F. 1976. Biochemical tests for the identification of medical bacteria. Willims and Wilkens Co. Baltimore. 310 Pp.

Marhual, N.P., Das, B.K. 2009. Use of random amplified polymorphic DNA and restricted fragment polymorphism for typing of *Vibrio alginolyticus* isolated from black tiger shrimp *Penaeus monodon*. *J. Pure Appl. Microbiol.,* 3(1): 75–82.

Marhual, N.P., Das, B.K., Sahu, S., Nayak, K.K. 2014. Genetic differentiation of field isolates of *Vibrio alginolyticus* and *V. parahaemolyticus* by using RAPD fingerprinting pattern. *J. Aqua. Tropics,* 29(3and4): 71–78.

Marhual, N.P., Das, B.K., Samal, S.K. 2012. Characterization of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* isolated from *Penaeus monodon*: Antibacterial Resistance, plasmid profiles and random amplification of polymorphic DNA analysis. *Afr. J. Microbiol. Res.,* 6(20): 4270–4276.

Marhual, N.P., Samal, S.K., Das, B.K. 2009. Random amplified polymorphic DNA-PCR typing of *Vibrio parahaemolyticus* isolated from black tiger shrimp *Penaeus monodon. E-planet.* 7(1): 1-6.

Martinez, I., Espelid, S., Johanson, A., Welsh, J., McClelland, M. 1994. Fast identification of species and strains of *Vibrio* by amplification of polymorphic DNA. *J. Fish Dis.,* 17: 297–302.

McCarty, D.H., Stevenson, J.P., Roberts, M.S. 1974. Vibriosis in rainbow trout. *J. Wildlife Dis.,* 10: 2–7.

Medies, N.S., Akopyants., Berg, D.E. 1995. Arbitrary primer PCR DNA

fingerprinting of *Escherichia coli* O157:H7 strains by using templates from boiea cultures. *J. Clin. Microbioiol.,* 33: 1534–1536.

Miyamoto, Y., Obara, Y., Akiyama, S., Takizawa, K., Yamai, S. 1969. In vitro hemolytic characteristics of *Vibrio parahaemolyticus*: its close correlation with human pathogenecity. *J. Bacteriol.,* 100: 1142–1149.

Myhr, E., Larsen, J.L., Lillehaug, A., Gudding, R., Heum, M., Hastein, T. 1991. Characterisation of *Vibrio anguillarum* and closely related species isolated from farmed fish in Norway. *Appl. Environ. Microbiol.,* 57: 2750–2757.

Novotny, A.J. 1975. Netpen culture of pacific salmon marine waters. *Marine Fish Review* 37, 36-47.

Rehnstam, A.S., Nordqrist, A., Wolf-Watz, H., Hagstrom, A. 1989. Identification of *Vibrio anguillarum* in fish by using partial 16S rRNA sequences and specific 16S rRNA oligonucleotide probe. *Appl. Environ. Microbiol.,* 55: 1907–1910.

Rivera, I.G., Chowdhury, M.A.R., Huq, A., Jacob, D., Martius, M.T., Colwell, R.R. 1995. Enterobacterial repetitive intergenic consensus sequences and the PCR to generate fingerprints of genomic DNAs from *Vibrio cholerae* O, O139 and non-O1 strains. *Appl. Environ. Microbiol.,* 61: 2898–2904.

Romaniuk, P.J., Trust, T.J. 1987. Identification of pylobacter species by southern hybridization of genomes using an oligonucleotide probe for 16S rRNA genes. *Microbiol. Lett.,* Pp. 331–335.

Tamplin, M.L., Jackson, J.K., Buchrieser, C., Murphee, R.L., Portier, K.M., Gangar, V., Miller, L.G., Kaspar, C.W. 1996. Pu;se-field gel electrophoresis and ribotye profiles of clinical and environmental *Vibrio vulnificus* isolates. *Appl. Environ. Microbiol.,* 62: 3572–3580.

Tsai, H.J., Yeh, M.S., Song, Y.L. 1990. Characterization of *Vibrio* species by using genomic DNA fingerprinting techniques. *Fish Pathol.,* 25: 201–206.

Versalovic, J., Koeuth, T., Lupski, J.R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genome. *Nucleic Acids Res.,* 19: 6823–6831.

Wang, G., Whittam, T.S., Berg, D.E. 1993. RAPD (arbitrary primer) PCR is more sensitive then multilocus enzyme electrophoresis for distinguishing related bacterial strains. *Nucleic Acid Res.,* 21: 5930–5933.

Welsh J., Pretzman C., Postic D., Saint Girons I., Baranton G., McClelland, M. 1992. Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phylum groups. *Int. J. Syst. Bacteriol.,* 42: 370–377.

Welsh, J., McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acid Res.,* 18: 7213–7218.

West, P.A., Colwell, R.R. 1984. Identification of *Vibrionaceae*: an overview. In: Colwell, R.R. Ed., Vibrios in the environment. New York, USA, John Wiley. Pp. 205–363.

Williams, J., Kubelik, G.K., Livak, A.R., Rafalski, K.J., Tingley, T. 1990. DNA polymorphisms amplified by arbitrary primers as genetic markers. *Nucleic Acids Res.,* 18: 6531–6535.