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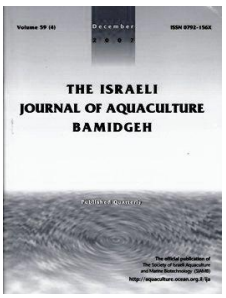

RAPD-PCR and Outer Membrane Protein Characterization of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* Isolated from Diseased shrimp

Nilima Priyadarshini Marhual, Basanta Kumar Das, Jyotirmayee Pradhan, Priyabrata Swain, Bibudhendra Kumar Mishra, Ambekar Eswar Eknath

Fish Health Management Division, Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, Bhubaneswar 2, Orissa, India

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Abstract

Vibrio alginolyticus and *V. parahaemolyticus* were characterized by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and profiling of outer membrane proteins (OMP) to the genetic level to enable easier identification of these microorganisms from diseased shrimp (*Penaeus monodon*) and adopt control measures. Of 20 tested Operon primers (OPC), only OPC5 and OPC18 were used for PCR amlification of 70 isolates representing three strains of each species. OPC5 amplified 1-8 bands with sizes ranging 0.42-2.95 Kb in *V. alginolyticus*, 5-10 fragments with sizes ranging 0.449-3.55 Kb in *V. parahaemolyticus*, and one unique common band of 1.34 Kb in both species. When OPC18 was used, one dark band (2.4 Kb) was common to all *V. alginolyticus* strains and another dark band (1.77 Kb) was common to two strains and the *V. alginolyticus* ATCC reference strain. One protein band (37.63 kDa) was common to both *V. alginolyticus* and *V. parahaemolyticus* and could be used as a diagnostic OMP marker for both species. The simpler and more rapid dot-ELISA and indirect ELISA methods confirmed identification of these pathogenic bacteria.

* Corresponding author. Tel.: +91-943-7080411, fax: +91-674-2465407, e-mail: basantadas@yahoo.com

Introduction

Vibrio are pathogenic to aquatic animals and can cause serious infections on farms (Durborow, 1999) amongst fish and prawns (Austin and Austin, 1993). The genus *Vibrio* of the family Vibrionaceae contains more than 50 species and its taxonomy is continuously being updated due to the addition of new species detected by molecular taxonomic techniques (Tantillo et al., 2004). Since the aquatic environment is a reservoir for these microorganisms, detailed studies on the pathogenic potential of environmental *Vibrio* will contribute to understanding the virulence properties of these bacteria (Baffone et al., 2005). Vibriosis, an economically important disease of fish, marine invertebrates (particularly penaeid shrimps), and large marine mammals, is responsible for high mortality rates in aquaculture worldwide (Sparagano, 2002). *Vibrio alginolyticus* and *V. parahaemolyticus* are primary pathogens that cause vibriosis in all stages of shrimps (Manilal et al., 2010).

Genotypic characterization of bacteria is a growing field (Busse et al., 1996). Intraspecific characterization of pathogenic bacteria for epidemiological purposes can be achieved by several procedures. Widely-used phenotypic characterization takes into account only a few characters and has proven non-reliable (Arias et al., 1997). Molecular techniques allow comparison of strains through genomes, overcoming the shortcomings of phenotypic properties. The differentiation of closely related strains by random amplified polymorphic DNA (RAPD; Welsh and McClelland, 1990; Williams et al., 1990) is based on the use of a single arbitrary primer of low stringency in the polymerase chain reaction (PCR) to amplify segments of a genome. The resulting fragments indicate the polymorphism of the DNA. This technique is the most convenient for revealing intraspecies DNA polymorphism (Brandt et al., 2001). In the present study, we used RAPD-PCR to differentiate between *V. alginolyticus* and *V. parahaemolyticus* and to calculate the genetic similarity and genetic diversity within the species.

Outer membrane proteins (OMP) are thought to relate to antigenic determinants in gram-negative bacteria (Zorrilla et al., 2003; Akayli et al., 2008). Due to their exposed epitopes on the cell surface, OMP are highly immunogenic components of bacteria. It would be interesting to understand the biological function of OMP and their immunogenicity characteristics in the immune response. OMP profiling is a valuable method for typing and differentiating between bacterial fish pathogens. Immunogenicity of OMP antigens has been studied by indirect enzyme-linked immunosorbent assay (ELISA) and dot-ELISA, which are simpler and more rapid for detecting specific immune responses.

Sensitive, specific, and rapid methods for detecting and identifying pathogenic microorganisms are needed to control bacterial infections in intensive fish culture. RAPD and OMP profiling can be used to compare microorganisms at the inter and intraspecies levels with high discrimination (Welsh and McClelland, 1990). This study aimed at characterizing *V. alginolyticus* and *V. parahaemolyticus* by proteomics to the genetic level so that future identification of these microorganisms will be easier and subsequent control measures can be adopted to prevent this disease.

Materials and Methods

Isolation and identification of bacteria. Bacteria were isolated from diseased *Penaeus monodon*, collected from private farms in the Puri, Balasore, and Cuttack Districts of Orissa, that showed clinical signs of vibriosis such as whitish musculatures and reddening of the telson. Isolated bacteria were screened on thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia) medium. The biochemical, antigenic, and molecular properties of 70 isolates representing six strains (three of each species) were examined and compared to two reference strains (ATCC 17749 and ATCC 17802).

Biochemical analysis. Test cultures were grown on tryptic soya broth (TSB) supplemented with 1.5% NaCl for 24 h at 37°C and inoculated into test media for biochemical testing according to Mac Fadden (1976) and West and Colwell (1984).

RAPD-PCR assay. Genomic DNA was extracted using the MB505 HipurATM Bacterial and Yeast Genomic DNA purification Spin Kit. Bacteria were inoculated into nutrient broth

(NB) supplemented with 1.5% NaCl and incubated at 37°C for 24 h. Bacterial cultures (1.5 ml) were centrifuged at 10,000 rpm for 5 min. The pellet was resuspended in 180 µl lysis solution AL and 20 µl RNase A solution followed by incubation for 2 min at room temperature. 20 µl of Proteinase K (20 mg/ml) was added and incubated for 30 min at 55°C. After that, 200 µl lysis solution C1 was mixed and incubated at 55°C for 10 min, followed by addition of 95-100% ethanol (200 µl). The entire contents of the tube was loaded into a binding column and centrifuged at 10,000 rpm for 1 min. The column was placed in a new 2-ml collection tube. 500 µl Prewash solution (PWB) was added to the column and centrifuged at 10,000 rpm for 1 min, then 500 µl wash solution was added to the column and centrifuged at 10,000 rpm for 1 min. The column was placed in a new 2-ml collection tube, followed by addition of 200 µl elution buffer (ET), and centrifuged for 2 min at 10,000 rpm to elute genomic DNA which was stored at -20°C.

RAPD-PCR was conducted according to Marhual and Das (2009) and Marhual et al. (2009). Two randomly designed 10-mer oligonucleotide primers - OPC5 (GATGACCGCC) and OPC18 (TGAGTGGGTG) - were used for amplification. DNA samples were diluted to a concentration of 25 ng/µl before PCR reaction and mixed with 2.5 µl 10X PCR buffer (Bangalore Genei Pvt. Ltd.), 2 µl deoxynucloside triphosphate (dNTP) mixture (Bangalore Genei Pvt. Ltd.), 5 pmol primer (Operon Technologies, Inc., Alameda, USA), and 1U Taq DNA polymerase (Banglore Genei Pvt. Ltd.). The final volume was adjusted to 25 µl with sterilized double distilled water. PCR reactions were run in a thermo cycler (MJ Research) using the following program: one cycle of initial denaturation at 94°C for 4 min followed by 45 cycles of 45 s at 94°C for denaturing, 45 s at 36°C for annealing, and 1.3 min at 72°C for extension. Cycling was concluded with a final extension at 72°C for 7 min and reaction products were stored at 4°C until further analysis. RAPD-PCR products were electrophoresed in 1.2% agarose gel in TBE buffer at 100 V for 90 min (Sambrook et al. 1989), stained with ethidium bromide and photographed by gel documentation system.

Analysis of DNA fingerprinting patterns. The molecular weights of the RAPD products were determined using the software, Alpha Innotech. RAPD analysis was performed using Gene Profiler rev 10.98 (Scanlytic, Fairfax, VA) and treeCon software (Van de Peer and De Watcher, 1994). For the phylogenic study, data were transformed to estimate distances (Nei and Li, 1979). The unweighted pairgroup method using arithmetic averages (UPGMA) was used for cluster analysis (Sokal and Michener, 1958).

Extraction of OMP. OMP were extracted following the methodology of Austin and Rodgers (1981) with little modification.The bacterial isolates were cultured in brain heart infusion broth (BHIB) supplemented with 1.5% NaCl after incubation at 37°C for 24 h. The cultures were centrifuged at 8000 *g* for 45 min. The pellet was washed in 20 mM tris buffer (pH 7.2) and resuspended in the buffer containing 10 mM ethylenediaminetetraacetic acid (EDTA). The cell suspensions were subjected to sonication in a sonicator (Artek Sonic Dismembrator Model 150) at 50 hz for about 10 min to disrupt the cell wall. After sonication the unbroken cells were sedimented by centrifugation at 8000 *g* and the supernatant was collected. The collected supernatant was ultracentrifuged at 45,000 *g* in an ultracentrifuge (Hitachi Model CP 100x). The pellet was resuspended in Tris buffer containing 0.5% sarcosyl or sodium dodecyl sulphate (SDS) to dissolve the cell membrane then further centrifuged at 45,000 *g* for about 45 min. The obtained OMP was dissolved in Tris buffer and OMP profiles of the six bacteria and two reference strains were analyzed using SDS poly-acrylamide gel electrophoresis (SDS-PAGE) in a Mini-PROTEAN Tetra Cell (Bio-rad). The proteins were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue.

Immunization and preparation of antiserum of OMP. The OMP obtained by centrifugation was used as an unheated sonicated antigen. 75 µl Freund's complete adjuvant (FCA) was injected intramuscularly into the hind legs of rabbits. The animals were given a booster on 14, 28, and 35 days after immunization with the same emulsion dose, but in Freund's incomplete adjuvant (FIA) instead of FCA. Blood was collected from a puncture in the ear vein of the rabbits 42 days after immunization. Serum was collected by centrifugation (1500 *g*) of the clotted blood for 15 min and stored at -20°C until further use.

Indirect enzyme-linked immunosorbent assays of rabbit serum. ELISA was conducted by the method of Swain et al. (2002) with slight modifications using 96-well microtiter polystyrene plates (Nunc, Denmark). The wells were separately coated with 50 ml OMP (1-2 µg/well) diluted overnight in carbonate-bicarbonate buffer (pH 9.6) at 4°C. The plates were washed in phosphate buffer saline (PBS) containing Tween-20 (PBS-T, pH 7.2) and blocked with 100 ml 3% skim milk powder for 2 h at 37°C. The wells were further washed in PBS-T. Rabbit sera raised against OMP was serially two-fold diluted after initial dilution of 1:10 with PBS (pH 7.2) added to homologous antigen-coated wells in duplicate per serum dilution. The plates were incubated at 37°C for 45 min and washed thrice in PBS-T. Anti-rabbit-horseradish peroxidase (HRPO) conjugated goat serum (the second antibody) was added and incubated for 45 min. The wells were then thoroughly washed and 50 ml of the substrate solution (5 mg O-phenylene diamine tetra hydrochloride plus 10 ml of H_2O_2 [38%, v/v] plus 5 ml acetate buffer, pH 5.0) was added. The plates were incubated at 37°C for 5 min in a dark chamber and optical density (OD) was recorded at 450/655 nm in a microplate reader (Bio-Rad, USA). Antibody activity was expressed in terms of OD, after subtracting the value in unimmunized healthy sera.

Dot-ELISA. Nitrocellulose paper strips (NCP) of 5-5 mm^2 were coated with 2 µl OMP as an antigen. The coated strips were dried at room temperature for 3 h then blocked in PBS containing 0.05% Tween 20 (PBS-T) and 5% skim milk powder at 37°C for 30 min. The strips were washed thrice with PBS-T and incubated with primary Ab (1:400) for 1 h at 37°C, then washed several times in PBS-T and incubated with secondary Ab for 30 min. The strips were incubated with anti-bovine-complex (ABC) reagent for 30 min, then transferred to a fresh plate and incubated with 3 amino-9 ethyl carbazole (AEC) reagent for 10 min in the dark. The strips were washed with distilled water, air dried at room temperature, and blots were detected.

Results

Vibrio alginolyticus and *V. parahaemolyticus* showed positive reactions for oxidation fermentation, oxidase test, motility test, hydrolisis of starch and variable reactions to Simmon's citrate test, ornithine decarbozylase test, liquification of gelatin, fermentation of maltose, mannitol, etc. The organism was negative to urease test, lysine decarboxylase test, arginine decarboxylase test, fermentation of arbinose, and salicin.

The RAPD assay using OPC primers was chosen to type three *V. alginolyticus* (V2, V10, V18) and three *V. parahaemolyticus* (V7, V15, V16) strains and their respective ATCC strains. Of 20 tested OPC primers, only OPC5 and OPC18 were used for PCR amplification. Using these primers, PCR amplification successfully differentiated between the eight strains as validated by RAPD (Table 1). The RAPD-PCR profiles of the eight strains showed genetic variation (Fig. 1). Primer OPC5 amplified 1-8 bands ranging 0.42-2.95 Kb in *V. alginolyticus* and 5-10 fragments ranging 0.449-3.55 Kb in *V. parahaemolyticus*. In *V. alginolyticus*, the V10 and ATCC strains had the maximum banding patterns with 8 bands ranging 0.41-2.73 Kb. In all cases, there was one distinct common band with a molecular weight of 1.34 Kb. The V10, V18, and ATCC strains had 7-8 bands. Common dark bands (0.681, 1.34, 2.17, 2.95 Kb) were present in all *V. parahaemolyticus* strains, including the ATCC strain. The maximum number of bands using OPC5 (10) was found in V7 and the minimum number (5) in the ATCC strains. In both species, a common band of 1.34 Kb was noticed using OPC5. Primer OPC18 yielded

Table 1. Number and molecular weight of amplified fragments of *Vibrio alginolyticus* and *V. parahaemolyticus* by RAPD primers OPC5 and OPC18.

Primer	*Sequence (5'-3')*	*No. amplified fragments*								*Size of fragments (Kbp)*							
		A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*
OPC5	GATGACCGCC	1	8	7	10	8	8	8	5	1.343	0.415 -2.167	0.428 -2.167	0.449 -2.951	0.435 -2.951	0.442 -2.951	0.620 -2.731	1.343 -3.551
OPC18	TGAGTGGGTG	2	3	3	6	5	4	7	6	1.769 -2.405	0.752 -2.405	0.719 -2.405	0.334 -2.405	0.334 -2.405	0.719 -2.405	0.553 -3.342	0.326 -2.405

Vibrio alginolyticus: A = V2, B = V10, C = V18, G = ATCC17749; *Vibrio parahaemolyticus*: D = V7, E = V15, F = V16, H = ATCC17802.

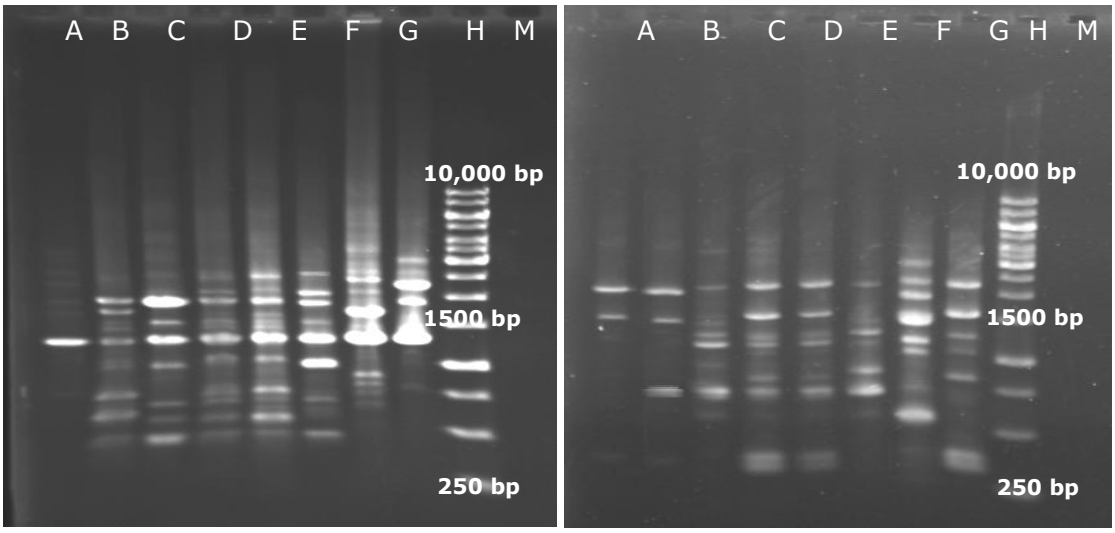

Fig. 1. RAPD band patterns in *Vibrio alginolyticus* (A, B, C, G) and *V. parahaemolyticus* (D, E, F, H) using (a) OPC-5 and (b) OPC-18 random primers; M = 1 Kb DNA ladder.

2-7 bands ranging 0.55-3.3 Kb in *V. alginolyticus* and 4-6 bands ranging 0.33-2.40 Kb in *V. parahaemolyticus*. Using OPC18, a dark band of molecular weight 2.4 Kb was common in all strains of *V. alginolyticus* while a dark band (1.77 Kb) was found in strains V2, V10, and the ATCC strain of *V. alginolyticus*. All strains of *V. parahaemolyticus* produced two unique bands (1.45 and 2.40 Kb) while a band at 1.77 Kb was prominent in V7, V15, and the ATCC strain of *V. parahaemolyticus*. Similarly, a band of 0.72 Kb was found in all *V. parahaemolyticus* strains except the ATCC strain. The percentage of dark to weak bands was lowest in *V. parahaemolyticus* using OPC18. Analysis by PHYLIP correlated the eight *Vibrio* strains according to the combined similarity matrix generated with the two RAPD primers (Fig. 2). The dendrogram shows high diversity; similarity between strains A and D was 60%, followed by strains E and F, and B and G. Inter-species similarity among strains A, D, E, and F was 40% and between these strains and H was 30%. Strains B and G show 58% similarity and strains B, G, and C show 38% similarity.

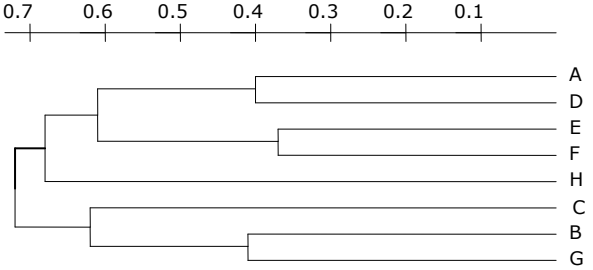

Fig. 2. Dendrogram based on UPGMA generated by PHYLIP showing genetic similarity derived from RAPD profiles of four strains of *Vibrio alginolyticus* (A = V2, B = V10, C = V18, G = ATCC17749) and four strains of *Vibrio parahaemolyticus* (D = V7, E = V15, F = V16, H = ATCC17802).

SDS-PAGE (10%) showed that the OMP structures of the eight strains were heterogeneous. The number of protein bands and molecular mass of corresponding bands varied among strains. The protein bands of OMP ranged 17.24-50.54 kDa (Table 2) in *V. alginolyticus* and 14.75-45.54 kDa in *V. parahaemolyticus*. However the lower molecular weight of 17.24 KD was common in V2, V10, and V18 but not in the ATCC

Table 2. Number and molecular weight of polypeptide bands of OMP in *Vibrio alginolyticus* and *V. parahaemolyticus*.

	Vibrio alginolyticus				*Vibrio parahaemolyticus*			
Sl no	*A*	*B*	*C*	*G*	*D*	*E*	*F*	*H*
Mw in KDa	37.63 34.51 17.24	37.63 27.07 17.24	37.63 31.64 26.15 22.37 17.24 14.75	50.54 37.63 24.39 21.98 19.13	47.98 37.63 31.64	37.63 14.02	37.63 25.25 22.76	45.54 37.63 26.15 23.97
Total no. bands	3	3	6	5	3	2	3	4

A = V2, B = V10, C = V18, D = V7, G = *Vibrio alginolyticus* ATCC17749, E = V15, F = V16, H = *Vibrio parahaemolyticus* ATCC17802.

strain of *V. alginolyticus*. Only one protein band of OMP (37.63 kDa) was common to both species (Fig. 3). In all strains, the protein fraction after profiling ranged 3-7 and the molecular weight 14.75-50.54 kDa. Indirect ELISA assays against OMP antibodies showed a gradual increase of antibody titer as the dilution decreased (Fig. 4). All strains tested positive for dot-ELISA, which was revealed by brown coloration of the nitrocellulose paper strips.

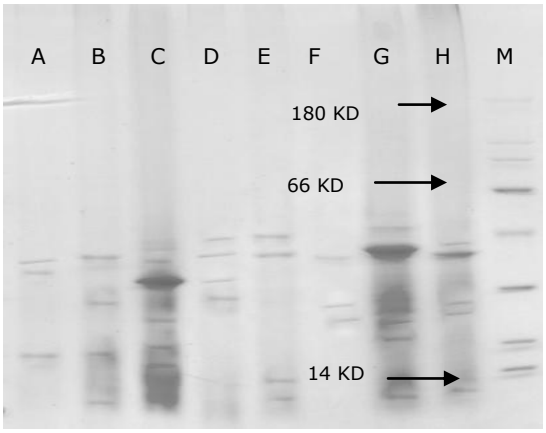

Fig. 3. SDS-PAGE of outer membrane proteins of *Vibrio alginolyticus* (A, B, C, G), *V. parahaemolyticus* (D, E, F, H), and a protein molecular weight marker (M).

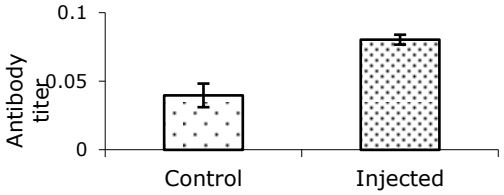

Fig. 4. Optical density (means±SE less titer value of the control group) of specific antibody level at 42 days post-immunization.

Discussion

The identification of genera and species of Vibrionaceae is problematic, mainly because of great variability in diagnostic phenotypic features such as arginine dehydrolase, indole production, and carbon utilization (Akayli et al., 2008). DNA polymorphism in genome fingerprinting generated by RAPD markers can distinguish between strains of almost any organism (Somarny et al., 2002). Molecular techniques for characterization of microorganisms have been widely used for epidemiological studies. In the present study we used two methods, RAPD-PCR and bacteria OMP profiling, to polymorphically differentiate between *V. alginolyticus* and *V. parahaemolyticus*, both of which are prevalent in eastern India.

In comparison with other molecular typing methods, RAPD is faster and less labor intensive. Only a small amount of template DNA is required for amplification reactions and, in some cases, the method better discriminates among bacterial strains (Wang et al., 1993). Two random operon primers, OPC5 and OPC18, used in RAPD-PCR showed genetic variation between the two *Vibrio* species. Cluster analysis based on the similarity matrix grouped all the strains into five clusters indicating that genetically heterogenous groups of *V. alginolyticus* and *V. parahaemolyticus* were prevalent among the diseased shrimps. The present observations are in agreement with previous studies observed in *V. parahaemolyticus* (Najiah et al., 2003), *V. alginolyticus* (Sudheesh et al., 2002) and *V. vulnificus* (Gutacker et al., 2003).

The ability of RAPD-PCR to cluster these eight strains suggests that this technique could be used to differentiate between the two species. Among the OPC5-generated amplicons, two amplicons (2.1 and 1.3 Kb) were shared by all *V. parahaemolyticus* strains, one amplicon (1.34 Kb) was shared by all *V. alginolyticus* strains, and one amplicon (1.3 Kb) was common to both *V. alginolyticus* and *V. parahaemolyticus*. Similarly, OPC18 generated one amplicon (2.4 Kb) that was common to all strains of *V. alginolyticus* and *V. parahaemolyticus*. Though varying genetic profiles have been generated using RAPD analysis (Sudeesh et al., 2002; Akayli et al., 2008), our findings show unique common distinct bands in all strains including the ATCC strains of both species that were not reported by previous workers. These common bands, amplified by OPC5 and OPC18, could be used as diagnostic RAPD markers for epidemiological studies, disease diagnosis, and health management of shrimp. They have further significance because amplicons of common fragments of RAPD-PCR using the OPC18 primer for both *V. alginolyticus* and *V. parahaemolyticus* are useful in genetic amplification and hybridization assays for diagnostic purposes. These highly conserved fragments could be ideal for identifying strains that may be difficult to distinguish phenotypically.

OMP reflect the antigenicity of particular pathogens. OMP profiles of *V. alginolyticus* vary (Zorrilla et al., 2003; Akyali et al., 2008). In our study, OMP profiles of *V. alginolyticus* and *V. parahaemolyticus* also differed. However, unlike other workers, we found a common protein fragment (37.63 kDa) in both *V. alginolyticus* and *V. parahaemolyticus* strains and a protein fragment (17.24 kDa) common to all *V. alginolyticus* strains except the ATCC strain. Though we did not study individual protein fractions, these specific protein fingerprints are unique and can be used as diagnostic OMP markers for both *Vibrio* species. OMP also confirmed identification of the pathogens even when the serum was diluted to 1:100. The antibody raised against one OMP was positive for all strains tested, indicating that the requirements for using OMP antibodies and dot-ELISA are uncomplicated and can be recommended for field use.

In conclusion, RAPD-PCR using OPC5 and OPC 18 primers produced unique distinct amplicons that can be used as diagnostic markers for identifying *V. parahaemolyticus* and *V. alginolyticus*. Further development of a sequence-characterized amplified region would be useful in epidemiological studies and disease diagnosis. To develop effective vaccines against these *Vibrio*, more attention should be given to these immunogenic OMP and more studies on other protective antigens of this pathogen should be carried out. Many strains of *V. parahaemolyticus* and *V. alginolyticus* are avirulent and could be used as probiotics.

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