



## Selection of specific cell wall antigen for rapid detection of fish pathogenic *Vibrio parahaemolyticus* by enzyme immunoassay

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### ABSTRACT

An enzyme linked immunosorbant assay (ELISA) was developed, using polyclonal antibodies against a specific cell surface protein of *Vibrio parahaemolyticus*, for rapid detection of the organism. Nine virulent strains and one type strain of *V. parahaemolyticus*, one strain each of *Vibrio vulnificus* and *Vibrio alginolyticus* were used for the study. Cell surface proteins were extracted from all the strains and were analysed by SDS PAGE. One distinct band with molecular weight of 34 kDa, abundant in all the *V. parahaemolyticus* strains and lacks in other *Vibrio* species, was selected as cell wall antigen for immunisation. Polyclonal antibodies were raised against the selected 34 kDa protein of *V. parahaemolyticus* after preparative electrophoresis. An indirect plate ELISA was developed using this antiserum for detection of crude cell surface protein as well as whole cells of *V. parahaemolyticus*. All the 9 virulent strains and one type strain of *V. parahaemolyticus* tested, produced positive results, using the ELISA technique. To assess the specificity of the polyclonal serum, cross reaction studies with other *Vibrio* species such as *V. vulnificus* and *V. alginolyticus* were conducted by indirect plate ELISA. The results have clearly shown that antibodies directed against 34 kDa cell surface protein can be used for specific detection of fish pathogenic *V. parahaemolyticus*.

Keywords: Enzyme linked immunosorbant assay (ELISA), Fish pathogen, Polyclonal antiserum, *Vibrio parahaemolyticus*

### Introduction

Mariculture has rapidly expanded in the recent decades in many Asian countries with better economic return over investment. However, fish diseases of bacterial origin have been one of the most important factors leading to economic loss since the beginning of mariculture (Kumar *et al.*, 2007; Sobhana, 2009). Vibriosis is one of the major bacterial diseases that affect marine finfish, with the major etiological agents being *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio parahaemolyticus* (Harikrishnan *et al.*, 2010; Krupesha Sharma *et al.*, 2012). Species under the genus *Vibrio* are Gram - negative bacteria found in fresh, estuarine and marine ecosystems and exist as part of normal microbiota or as primary or secondary pathogens (Austin and Austin, 2007). *V. parahaemolyticus* is one of the major *Vibrio* species isolated from vibriosis infected marine fishes (Sudheesh and Xu, 2001; Liao *et al.*, 2004; Harikrishnan *et al.*, 2011). This *Vibrio* species has been reported to cause diseases in cultured groupers in Thailand (Danayadol, 1999), Malaysia (Najiah *et al.*, 2003), China (Li *et al.*, 2010) and Vietnam (Do *et al.*, 2008; Nguyen and Nguyen, 2008) resulting in high mortalities and economic losses.

For diagnosis of bacterial diseases, conventional methods of isolation and identification like biochemical methods are most commonly used, but these are time consuming and laborious. The use of nucleic acid probes and PCR for detection of microbial pathogens are gaining importance, but these need sophisticated equipment and are not suitable for large scale screening of samples (Tyagi *et al.*, 2009; Rosec *et al.*, 2009). Development of rapid and sensitive immunodiagnostic techniques for important bacterial pathogens, based on polyclonal/monoclonal antibodies, can help in timely diagnosis of diseases and to adopt more scientific health management measures (Chi *et al.*, 2007; Diasda and Tambourgi, 2010).

Among the different methods, which may be used for rapid detection of pathogenic bacteria, immunological techniques are promising, because of their sensitivity and rapidity (Qian *et al.*, 2008; Thuy *et al.*, 2013). However it is necessary, to select appropriate antigens for developing immunoassays, in order to avoid cross reactions. For Gram negative bacteria, the selection of appropriate antigen is somewhat easier, as different proteins from the outer membranes are known to be common antigens for a genus or a species (Fofstra and Dankert, 1979; Inoue *et al.*, 1995; Ningqiu *et al.*, 2008). The outer membranes of

Gram negative bacteria contain several distinct proteins, with molecular weights in the range of 30 to 40 kDa (Manning *et al.*, 1982; Li *et al.*, 2010). These proteins are sometimes species-specific and have high antigenicity (Kabir, 1986; Seltman and Holst, 2002; Mao *et al.*, 2007). Therefore, antibodies against these specific proteins can be explored as tools for rapid identification of these bacteria (Swain and Nayak, 2003).

A great deal of interest has been developed towards exploring the possibility of evolving enzyme immunoassays, based on specific cell surface proteins, for detection of bacterial pathogens (Bhunia and Johnson, 1992; Chen and Chang, 1996; Swain *et al.*, 2001; Tully *et al.*, 2006). Keeping this in view, the present study was undertaken to develop an indirect enzyme linked immunosorbent assay, using polyclonal rabbit antiserum raised against a specific cell wall antigen (34 kDa) for detection of fish pathogen, *V. parahaemolyticus*.

## Materials and methods

### Test strains

Nine fish pathogenic strains of *V. parahaemolyticus* (VP1; VP2; VP3; VP4; VP5; VP6; VP7; VP8 and VP9); one strain each of *V. vulnificus* (VV) and *V. alginolyticus* (VA), originally isolated from vibriosis affected groupers (*Epinephelus malabaricus* and *Epinephelus coioides*) from different disease incidences, were used for the study. The bacterial strains were isolated from diseased groupers from the experimental tanks, broodstock facilities at Marine Hatchery of the Central Marine Fisheries Research Institute (CMFRI) at Cochin Fisheries Harbour, Kochi and at the Mandapam Regional Centre of CMFRI at Mandapam. All the strains used were tested for virulence in juveniles of *E. malabaricus*. One type strain of *V. parahaemolyticus* (MTCC 451), procured from Institute for microbial technology (IMTECH), Chandigarh, India was also used.

### Phenotypic and biochemical characteristics of test strains

All the test strains used were reconfirmed for their phenotypic and biochemical characteristics. The strains were characterised based on their growth on selective media [Thiosulfate citrate bile salts sucrose (TCBS) agar, Difco], colony characteristics, Gram staining, motility and a series of biochemical tests such as amino acid decarboxylase test (Mac Faddin, 1980), sugar fermentation test (West and Colwell, 1984), growth in salt tryptone broth to study halophilism, growth at 43°C, cytochrome oxidase test (Holding and Collee, 1971), nitrate reduction test (Crosby, 1967), catalase test, methyl red and Voges Proskauer test (Lee *et al.*, 1979), indole production, triple sugar iron agar (TSI) and Kligler iron agar (KIA)

reactions, oxidation/fermentation (O/F) test, urease test, gelatinase test (Smith and Goodner, 1958), citrate utilisation test, O-nitrophenyl  $\beta$ -D galactopyranosidase, ONPG ( $\beta$ -galactosidase) test (Paik, 1980) and sensitivity to 0/129 discs. All media used for the tests contained 2.5% NaCl (W/V). The isolates were then identified to species level according to Bauman and Schubert (1984).

### Kanagawa reaction to test haemolytic activity ( $\beta$ - haemolysis)

Kanagawa reaction was performed on Wagatsuma agar (Wagatsuma, 1967). Haemolytic activity of the bacterial strains were tested by spot inoculating the strains onto each freshly prepared and dried Wagatsuma agar containing a suspension of mammalian/fish red blood cells. The plates were incubated for 18 $\pm$ 2 h at 37°C and were observed for presence of zone of haemolysis around the colony.

### Extraction and purification of cell surface proteins (CSP)

Cell surface proteins were extracted from all the 10 strains of *V. parahaemolyticus*, including the type strain, MTCC 451 and one strain each of *V. vulnificus* (VV) and *V. alginolyticus* (VA). Extraction of outer membrane proteins from the above mentioned test strains was carried out following the method described by Chen and Chang (1996) with modifications. Briefly, the organisms grown overnight on nutrient agar (NA) slants were harvested in 2 ml phosphate buffered saline (PBS, pH 7.2) and inoculated in tryptone soya broth (TSB) for 18-24 h at room temperature. The cells were pelleted by centrifugation at 5,000 g for 20 min at 4°C and then washed three times with PBS (pH 7.2). The resultant pellet was suspended in 10 ml PBS containing 1% sodium dodecyl sulphate (SDS) and heated in a water bath maintained at 70°C for 2 h with occasional shaking. The bacterial cells were centrifuged at 10,000 g for 20 min at 4°C in a refrigerated centrifuge. The supernatant containing SDS extracted cell surface proteins was collected and stored at -20°C, till further use. The crude cell surface extracts were dialysed against PBS (pH 7.2) using dialysis tubing (Sigma) having a molecular weight cut off (MWCO) >12 kDa. The protein content of the crude cell surface proteins was determined following dialysis, from each test strain using the method of Lowry *et al.* (1951).

### SDS PAGE analysis of crude cell surface protein

The extracted cell surface proteins were analysed by SDS PAGE on 12.5% (w/v) acrylamide resolving gel and 4% stacking gel (acrylamide 30% and bisacrylamide 8%) containing 0.1% SDS as described by Laemmli (1970) using a compact dual mini vertical slab gel electrophoresis unit (Scie-Plas, UK). The samples were mixed with

2x Laemmli sample buffer in a proportion of 1:2 and subsequently heated at 95°C for 5 min before loading in to the wells. Approximately 200 µg of proteins were loaded to each lane of the gels. Standard molecular weight markers (Genei, Bangalore) were run parallel along with sample proteins, to determine the relative molecular weights of the polypeptides. After electrophoretic run, the protein bands were visualised by staining with Coomassie Brilliant blue-R250.

#### *Antiserum against purified cell surface proteins*

The 34 kDa cell surface antigen of *V. parahaemolyticus* (VP6) was purified by preparative SDS PAGE on 3 mm thick gels. After the electrophoretic run, protein bands were rapidly visualised by staining with 4 M sodium acetate solution for 60 to 80 min with shaking (Higgins and Dahmus, 1979). The protein bands got visualised as clear bands on the milky white background. After appropriate visualisation, the band corresponding to molecular weight, 34 kDa was excised with a scalpel and immediately washed thrice (2 min each) in deionised water with shaking.

The polyacrylamide gel strip containing the 34 kDa protein (approximately 5 mg) was cut in to small pieces and then crushed in to fine particles using a micro-pestle in an eppendorf vial. The fine gel particles were suspended in PBS (2-3 ml) and emulsified with an equal volume of Freund's incomplete adjuvant (FIA). Two milliliters of the emulsified antigen was injected subcutaneously at 4 to 6 dorsal sites on the back of a New Zealand white rabbit (average weight of 2 kg). Three booster doses were given at 3 weeks intervals in a similar way. Ten days following the 3<sup>rd</sup> booster, blood was collected by ear vein puncture using sterile 26-gauge needle and syringe. Blood was allowed to clot and serum was separated by centrifugation at 3000 g for 10 min. Control/negative serum was also raised by injecting a New Zealand white rabbit (average weight of 2 kg) with 1 ml PBS emulsified with an equal quantity of FIA. The sera were de complemented at 56°C for 30 min in a water bath and stored at -20°C until further use.

#### *Enzyme immunoassay (Indirect plate ELISA)*

An indirect plate ELISA test was developed to detect the cell surface proteins and heat killed whole cells of *V. parahaemolyticus*, using antiserum raised against the specific 34 kDa outer membrane protein. The ELISA used for the detection was as per the methods described by Cheng and Chang (1996) with slight modifications.

The ELISA plates (Griener, Germany) were coated with 100 µl per well of coating buffer (carbonate bicarbonate buffer, pH 9.6) containing cell surface protein

extracted from VP6 as an antigen at 10, 20, 40, 60, 80 and 100 µg ml<sup>-1</sup>. The plates were incubated overnight at 4°C and subsequently washed thrice with PBS-T (PBS containing 0.05% Tween 20, pH 7.4). The unsaturated sites of the plates were blocked by adding 100 µl per well of 1% BSA in PBS-T and incubated at 37°C for 1 h. The plates were washed thrice with PBS-T. Dilutions ranging from 1:100 to 1:6400 of the hyperimmune antiserum raised in rabbit against 34 kDa protein were added to the wells (100 µl per well) in duplicate set. Similar dilutions of negative serum were also added. The plates were incubated at 37°C for 2 h and then washed thrice with PBS-T. Subsequently, goat anti-rabbit horseradish peroxidase (HRPO) conjugate (Genei, Bangalore), diluted with PBS-T in the ratio 1:2000 was added (100 µl). The plates were incubated at 37°C for 1 h and then washed thrice with PBS-T. Subsequently 100 µl of 3, 3', 5, 5' tetramethyl benzidine (TMB) substrate (Genei, Bangalore) solution was added and then incubated for 10 min in the dark. The reaction was stopped by adding 100 µl per well of 2N H<sub>2</sub>SO<sub>4</sub> and colour development was measured at 450 nm in a microplate reader (Cary 50, Varian). The positive to negative ratios of more than 2 was considered positive.

The suitability of the indirect plate ELISA was also studied for the detection of whole cells of *V. parahaemolyticus* by standardising the indirect ELISA separately using various concentrations (10<sup>3</sup> to 10<sup>9</sup> cells ml<sup>-1</sup>) of heat killed (80°C for 10 min) cells of *V. parahaemolyticus* as antigen for coating the plates (100 µl per well).

#### *Cross reaction studies*

The polyclonal antiserum against 34 kDa protein was tested for cross reaction with *V. alginolyticus* and *V. vulnificus* strains by indirect plate ELISA. Heat killed (80°C for 10 min) cells of these strains were suspended in coating buffer (carbonate bicarbonate buffer, pH 9.6) at a cell density of 10<sup>6</sup> cells ml<sup>-1</sup> and indirect plate ELISA was performed, as described earlier, using antisera raised against 34 kDa cell surface protein of *V. parahaemolyticus* at a dilution of 1: 3200.

## **Results**

All the 9 strains and one type strain of *V. parahaemolyticus* (MTCC 451) gave typical reactions as per Bauman and Schubert (1984). They were Gram negative motile short rods, oxidase and catalase positive, producing green colonies on TCBS agar. In addition, they were resistant to the vibriostatic agent 0/129 at 10 mg and sensitive at 150 mg concentration. All the strains showed growth in tryptone broth, supplemented with 3, 6 and 8% (w/v) NaCl and did not grow at 0 and 10% (w/v) NaCl. All the strains of *V. parahaemolyticus* were negative for arginine while positive for lysine and ornithine

decarboxylation. They were fermentative for arabinose, dextrose and mannitol without gas production, while non-fermentative for sucrose, cellobiose, inositol, salicine and lactose. The strains were positive for gelatinase, urease and nitrate reduction tests and negative for ONPG test. All the test strains and the MTCC reference strain of *V. parahaemolyticus* gave negative reaction for haemolytic activity on Wagatsuma agar, indicating that they were all Kanagawa negative.

The electrophoretic profiles obtained after the SDS PAGE analysis of cell surface protein patterns of test strains showed several distinct protein bands with molecular weight in the range of 30 to 97 kDa (Fig. 1a, lanes 2, 3, 4, 6 and Fig. 1b, lanes 1 to 6). Outer membrane protein pattern of all the *V. parahaemolyticus* test strains were identical, with 5 to 6 major bands having molecular weights of 34 kDa, 36 kDa, 37 kDa, 55 kDa, 75 kDa and 93 kDa. The outer membrane protein pattern of the type strain of *V. parahaemolyticus* (MTCC 451) were

and specificity, the 34 kDa protein was selected as antigen for immunisation to develop polyclonal antiserum.

The extraction of cell surface antigens with 1% SDS was effective in solubilising the cell surface proteins and highly reproducible, showing similar banding patterns whenever repeated. The protein bands got visualised as clear bands on the milky white background (Fig. 2) in the preparative SDS PAGE gel, by staining with 4 M sodium acetate solution. Antiserum was raised against cell surface protein having molecular weight of 34 kDa, which was found to be common in all *V. parahaemolyticus* isolates and not present in other vibrios. Immunisation of rabbits was accomplished by inoculation of protein along with FIA, after excising the specific molecular weight bands directly from preparative SDS PAGE gels. This method of immunisation, using proteins in PAGE, was found to be effective for production of antisera and after 3 boosters good titer was obtained.

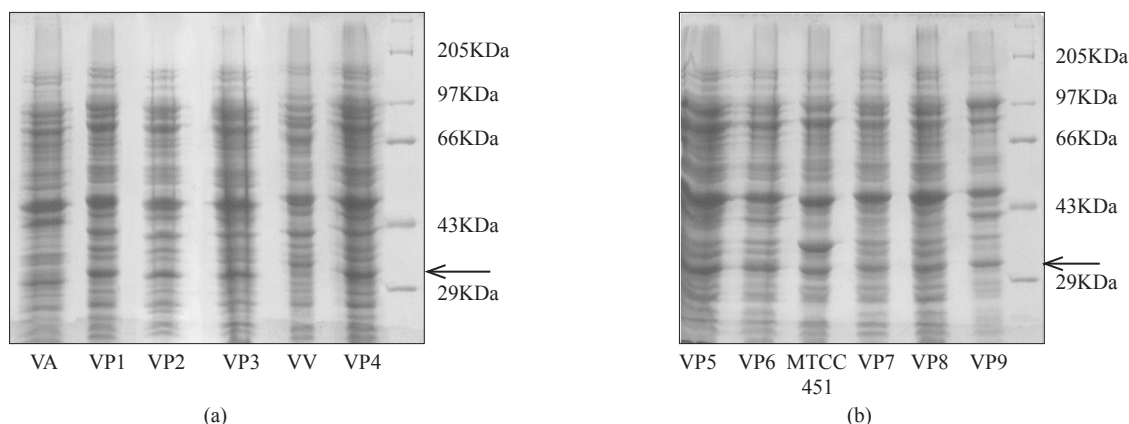


Fig. 1. SDS PAGE profile of the cell surface protein of the test strains stained by Coomassie Brilliant blue-R250 1a: VP1, VP2, VP3 and VP4 - *V. parahaemolyticus* test strains; VA - *V. alginolyticus*; VV - *V. vulnificus*; 1b: VP5, VP6, VP7, VP8 and VP9 - *V. parahaemolyticus* test strains; MTCC 451 - *V. parahaemolyticus* type strain (Arrow - 34 kDa protein band)

also quite similar, but with the 37 kDa band being very prominent (Fig. 1b, lane 3), which was not so prominent in other 9 strains of *V. parahaemolyticus*. The outer membrane proteins of the two other closely related *Vibrio* species viz., *V. alginolyticus* and *V. vulnificus* are also shown in Fig. 1a (lanes 1 and 5 respectively). However, the patterns were quite different from those of *V. parahaemolyticus*. The major protein band of 55 kDa was found to be common in all the three *Vibrio* species i.e., *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*.

One distinct band (Fig. 1a, indicated with arrows) with apparent molecular weight of 34 kDa was abundant in all the *V. parahaemolyticus* strains and were not observed in the other two *Vibrio* species analysed. Due to high concentration

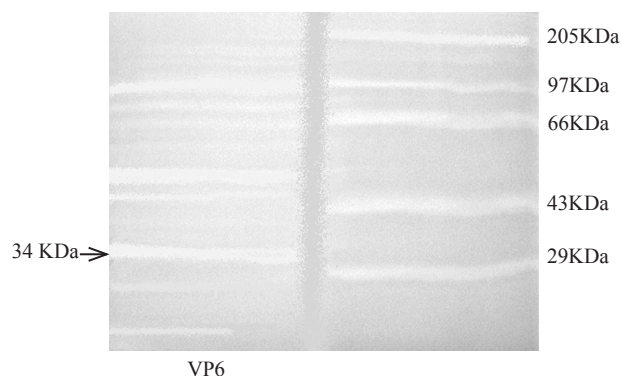


Fig. 2. Preparative SDS PAGE profile of the cell surface protein of *V. parahaemolyticus* (VP6) stained with 4 M sodium acetate (Arrow - 34 kDa protein band of VP6)

ELISA titer (OD values at 450 nm) for different concentrations of crude cell surface proteins of *V. parahaemolyticus* at various dilutions of the antiserum raised against the 34 kDa cell surface protein are given in Table 1. The positive to negative ratios of more than 2 were only considered. Accordingly, even the lowest concentration of 10 µg cell surface protein used for testing, gave positive reaction at a maximum dilution of 1: 800 of the anti-34 kDa protein serum.

Results of the indirect plate ELISA for detection of heat killed whole cells of *V. parahaemolyticus* at various dilutions of the anti-34 kDa rabbit antiserum are presented in Table 2. The minimum detection limit for heat killed whole cells of *V. parahaemolyticus* was estimated as  $10^6$  cells  $ml^{-1}$  (Table 2; Fig. 3) using the anti 34 kDa antiserum at a maximum dilution of 1: 3200. The cross reaction studies revealed that polyclonal rabbit antiserum directed against 34 kDa cell surface protein was specific for *V. parahaemolyticus* detection. The antiserum gave strong

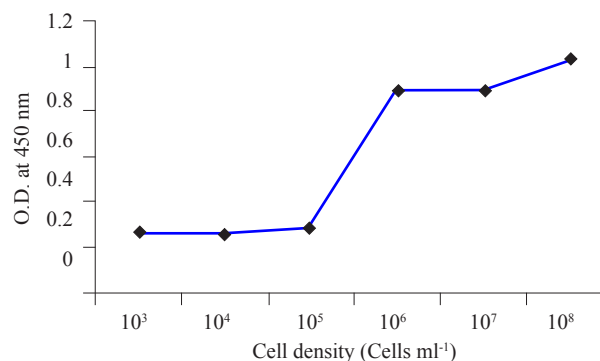


Fig. 3. ELISA titre for different concentrations of heat killed whole cells of *V. parahaemolyticus* by indirect plate ELISA using the antiserum raised against the 34kDa protein at 1:3200 dilution

positive reaction with all the ten *V. parahaemolyticus* strains, while it gave negative reaction with other *Vibrio* species tested viz., *V. vulnificus* and *V. alginolyticus* (Table 2).

Table 1. ELISA titres (OD values at 450 nm) for different concentrations of crude cell surface proteins of *V. parahaemolyticus* at various dilutions of the antiserum raised against the 34 KDa cell surface protein

Antiserum dilution	Antigen concentration (µg)					
	100	80	60	40	20	10
1:100	0.983	0.903	0.677	0.957	0.991	0.957
1:200	0.629	0.629	0.565	0.699	0.807	0.969
1:400	0.513	0.493	0.450	0.507	0.501	0.570
1:800	0.389	0.380	0.383	0.416	0.460	0.431
1:1600	0.381	0.348	0.323	0.329	0.371	0.326
1:3200	0.362	0.314	0.312	0.303	0.341	0.331
1:6400	0.266	0.253	0.241	0.257	0.232	0.236
Control	0.227	0.199	0.174	0.212	0.174	0.194

Table 2. ELISA titres (OD values at 450 nm) for different concentrations of heat killed whole cells of *V. parahaemolyticus* at various dilutions of the antiserum raised against the 34 KDa cell surface protein. ELISA titres for heat killed whole cells of *V. vulnificus* and *V. alginolyticus* at a cell density of  $10^6$  cells  $ml^{-1}$  at antiserum dilution of 1: 3200 are also presented

Antiserum dilution	Antigen concentration (cells $ml^{-1}$ )					
	$10^8$	$10^7$	$10^6$	$10^5$	$10^4$	$10^3$
<i>V. parahaemolyticus</i>						
1:100	1.988	1.699	1.699	0.593	0.580	0.561
1:200	1.923	1.606	1.606	0.458	0.450	0.508
1:400	1.765	1.473	1.473	0.427	0.351	0.415
1:800	1.599	1.238	1.238	0.359	0.309	0.328
1:1600	1.177	1.026	1.026	0.305	0.268	0.284
1:3200	1.023	0.891	0.891	0.284	0.256	0.265
1:6400	0.693	0.512	0.338	0.275	0.202	0.228
<i>V. vulnificus</i>						
1: 3200	-	-	0.411	-	-	-
<i>V. alginolyticus</i>						
1: 3200	-	-	0.331	-	-	-
Control	0.231	0.282	0.282	0.233	0.192	0.200

## Discussion

The specificity and to some extent the sensitivity of any immunoassay depends on the quality of the antibodies used in the reagents (Swain *et al.*, 2003; Thuy *et al.* 2013). Cross reactions due to related antigens can occur, and these can limit the usefulness of the test. Therefore it is necessary to select appropriate antigens for developing immunoassays in order to avoid cross reactions (Swain *et al.*, 2001; Tully *et al.*, 2006).

The phenotypic variability of isolates from the environment and from fish/shellfish makes it difficult to distinguish between *V. parahaemolyticus* and other members of this genus, particularly *V. alginolyticus*, *V. harveyi* and *V. mimicus* accurately by means of biochemical tests (Wachsmuth *et al.*, 1980; Croci *et al.*, 2007). One of the major difficulties in biochemical identification of *V. parahaemolyticus* is the variability in some of the activities, such as fermentation of sugars like sucrose, arabinose and cellobiose (Karunasagar *et al.*, 1997; Tyagi *et al.*, 2009). However, in the present study, only the strains giving typical reactions as per Bauman and Schubert (1984) were considered.

All the test strains and the MTCC reference strain of *V. parahaemolyticus* used for the studies were Kanagawa negative and tested positive for urease production. Most human pathogenic strains of *V. parahaemolyticus* produce a major virulence factor, the thermostable direct hemolysin (tdh) and are designated as Kanagawa phenomenon (KP<sup>+</sup>) positive (Johnson, 2009). Another virulence factor, the tdh-related hemolysin (trh) is generally associated with the Kanagawa phenomenon negative (KP<sup>-</sup>) strains or with urease positive strains of *V. parahaemolyticus* (Okuda *et al.*, 1997; Kumar *et al.*, 2011). Though most of the environmental strains of *V. parahaemolyticus* are typically not human pathogens, they cause disease in fish and shellfish (DePaola *et al.*, 2003; Nordstrom and DePaola., 2003; Martinez-Urtaza *et al.*, 2006).

Extraction with 1% SDS was very effective in solubilising the cell surface proteins. Though the procedure involved heating at 70°C, the bacterial cells remained intact in the procedure. This indicated that only cell surface proteins and not the cytoplasmic proteins were extracted. The extraction of cell-envelope bound protein with SDS was found to be an effective method by previous workers (Chang *et al.*, 1994; Chen and Chang, 1996).

The major protein band of 55 kDa was found to be common in all the three *Vibrio* species *i.e.*, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*. The outer membrane protein pattern of all the *V. parahaemolyticus* test strains were identical, with 5 to 6 major bands. The 34 kDa protein band which was very

prominent in all the *V. parahaemolyticus* test strains was not present in *V. alginolyticus* and *V. vulnificus*. Hence, this cell surface protein was selected as species-specific antigen for *V. parahaemolyticus*.

In the present study, the growth conditions and extraction procedures used were identical for all the test strains and all the *V. parahaemolyticus* strains gave similar banding patterns, except for the type strain (MTCC 451) for which the 37 kDa protein band was very distinct as compared to the same of *V. parahaemolyticus* isolates from fish. Chang *et al.* (1994) identified 3 to 4 major bands having molecular weight in the range 30 kDa to 40 kDa in *V. parahaemolyticus* isolated from seafood. They found that two distinct bands with apparent molecular weights of 36 kDa and 34 kDa were abundant in all the *V. parahaemolyticus* strains. They used these two proteins collectively for immunising rabbits to raise antisera and developed a latex agglutination test for rapid identification of food borne *V. parahaemolyticus*. In the present study too, the 36 kDa protein was recorded in all the *V. parahaemolyticus* strains tested, but it was not as prominent as the 34 kDa protein. Moreover, the 36 kDa protein band was also present in the other species of *Vibrio* tested *viz.*, *V. alginolyticus* and *V. vulnificus*.

The 34 kDa cell surface protein of *V. parahaemolyticus* was purified by preparative SDS PAGE and visualised by staining with sodium acetate (Higgins and Dahmus, 1979). This is a rapid and sensitive method for the detection of protein bands in SDS PAGE gels that is suitable for the recovery of individual polypeptides on a preparative scale. The process is reversible and allows for the complete recovery of unmodified protein from preparative gels. The visualisation is rapid, requiring between 60 and 80 min for 3 mm gels and sensitive, detecting as little protein as 0.1 µg mm<sup>-3</sup>. In this method high concentrations of sodium acetate (4M) is used to precipitate SDS, not bound to protein. In such gels protein bands are transparent within a white background and are effectively viewed against a dark background. After appropriate visualisation, the band corresponding to the molecular weight 34 kDa was excised with a scalpel and immediately washed thrice in deionised water and used as antigen for immunising rabbit to develop polyclonal antiserum (Sachan and Agarwal, 2002).

Immunisation of rabbits was accomplished by inoculation of protein along with FIA after excising 34 kDa band directly from preparative SDS PAGE gels. This method of immunisation using proteins in PAGE was found to be effective for production of antisera and good titer was obtained after 3 boosters. The extraction of cell surface proteins from *V. parahaemolyticus* with SDS and the excision of the desired bands directly from SDS gels

are convenient ways to obtain highly purified antigens for immunisation purposes. The procedure eliminates the step of eluting proteins from polyacrylamide gels, thus avoiding further loss of the desired proteins. In addition, polyacrylamide gel can act as an adjuvant (Weintraub and Raymond, 1963) by slowly releasing antigens from the gel. Though animals can produce antibodies against polyacrylamide (Weintraub and Raymond, 1963) this does not cause problems of cross reaction, since the assay systems normally do not contain polyacrylamide. By using this simple technique, antigen purification and animal immunisation could be done within a period of 2 to 3 days. This methodology was also successfully applied to prepare specific antibodies against *Staphylococcus aureus* (Chang and Ding, 1992); *V. parahaemolyticus* (Chang *et al.*, 1994; Chen and Chang, 1996) and *Listeria monocytogenes* (Chen and Cheng, 1996).

ELISA techniques are widely used for detection of pathogenic *Vibrio* species in aquaculture (Robertson *et al.*, 1998; De Pinto *et al.*, 2012). In the indirect plate ELISA test developed for rapid detection of fish pathogenic *V. parahaemolyticus* using the anti-34 kDa protein serum, even the lowest concentration (10 µg) of the cell surface protein tested, gave positive reaction. The minimum detection limit for heat killed whole cells of *V. parahaemolyticus* was estimated as 10<sup>6</sup> cells ml<sup>-1</sup> using the anti-34 kDa antiserum at a maximum dilution of 1: 3200. Sensitivity of the indirect ELISA enabled to understand cross reaction between closely related OMP proteins unlike agglutination test (Kumar *et al.*, 2010). These findings are in agreement with other ELISA systems, where optimum reaction at a cell concentration of 10<sup>6</sup> cells ml<sup>-1</sup> was achieved (Sachan *et al.*, 2002). The cross reaction studies revealed that polyclonal rabbit antiserum directed against 34 kDa cell surface protein was specific for *V. parahaemolyticus* detection. The antiserum gave strong positive reaction with all the ten *V. parahaemolyticus* strains, while it gave negative reaction with other *Vibrio* species tested *viz.*, *V. vulnificus* and *V. alginolyticus* (Table 2).

The use of cell wall antigens to develop immunological methods for the rapid detection of bacteria has also been advocated by other researchers (Gharaibeh Dima *et al.*, 2013). ELISA techniques are widely used immunodiagnostic techniques to detect pathogens in culture systems (Romestand *et al.*, 1993; Kumar *et al.*, 2011) due to its sensitivity. In the latex agglutination test for the rapid identification of *V. parahaemolyticus* (Chang *et al.*, 1994), two bacterial outer membrane proteins, with molecular weights of 36 kDa and 34 kDa were used as antigens for producing polyclonal antibodies. However, in their study, some strains of vibrios belonging to the species *viz.*, *V. alginolyticus*, *V. harveyi* and *V. mimicus* produced false positive results. From the present

study it is obvious that the 36 kDa protein is also present in *V. alginolyticus* and this could be the reason for the cross reaction observed with this particular species using the latex agglutination test.

In the sandwich ELISA developed for rapid detection of *V. parahaemolyticus* in foods (Chen and Chang, 1995), the detection limit for total outer membrane proteins of *V. parahaemolyticus* was 10 ng ml<sup>-1</sup>. Their study indicated that the 36 kDa and 34 kDa outer membrane proteins are common antigens of *V. parahaemolyticus*. They have recommended ELISA technique for rapid screening of *V. parahaemolyticus* in foods which can be finished within 24 h (18 h of growth in enrichment broth and 4 h for ELISA). However, cross reactions were still observed with several other *Vibrio* species *viz.*, *V. tubiashi*, *V. campbelli* and *V. vulnificus*. From the results of the present study, it is evident that the cross reaction with *V. vulnificus* could be due to the sharing of the 36 kDa outer membrane protein.

The results of the present study have clearly shown that the 34 kDa fractionated antigen from the cell surface proteins of *V. parahaemolyticus*, having no apparent cross reactive components with related bacterial antigens, can be exploited for diagnostic purposes. The antiserum raised against the 34 kDa protein gave strong positive reaction with all the ten *V. parahaemolyticus* strains tested and negative reactions against two other common *Vibrio* species associated with vibriosis in groupers *viz.*, *V. vulnificus* and *V. alginolyticus*. The possibility of cross reaction with other closely related vibrios such as *V. harveyi*, *V. mimicus*, and *V. campbelli* needs to be further evaluated. However, the high titre of the antiserum indicates possibility of elimination of any cross reactions with other taxa, while giving positive reactions with the majority of *V. parahaemolyticus* strains. Castro *et al.* (1995) have shown that cross reaction can be minimised using higher dilution of antiserum. The detection technique can be made more sensitive by developing immunocapture (sandwich) ELISA using antibodies against crude cell surface proteins/killed whole cells as capture antibodies and the anti-34 kDa antiserum as tracing antibody. There is also scope for developing dot ELISA technique using the anti-34 kDa antiserum for rapid diagnosis of *V. parahaemolyticus* without the need for sophisticated equipment.

The findings from the present investigation may be used as baseline information for designing rapid, sensitive and cost-effective immunodiagnostic kits for important fish bacterial pathogens, which can help in timely diagnosis of diseases and in adopting more scientific health management measures in aquaculture. Compared with the conventional identification procedures, requiring

a battery of physiological and morphological tests, the proposed identification method is simple and time saving.

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