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SPECIFIC IDENTIFICATION OF VIBRIO PARAHAEMOLYTICUS EMPLOYING MONOCLONAL ANTIBODY BASED IMMUNOASSAY

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ABSTRACT

The present study was carried out to achieve a simple, specific and cost effective detection system for *Vibrio parahaemolyticus*. The Outer Membrane Proteins (OMPs) of *V. parahaemolyticus* were extracted by treating the cell pellet with N- lauroyl sarkosyl and analysed by SDS-PAGE. Female BALB/c mice were immunized with the crude OMP extract and antigenicity was confirmed by subsequent Western blotting with anti-OMP antisera. The mice with higher antibody titre were selected as spleen donors for fusion. OMP based indirect plate and dot ELISA were standardized for screening of hybridoma and four hybridoma cell lines producing monoclonal antibodies (MAbs) were stabilized. Among these, MAb 212 detected a 36 kDa OMP that was present in all *V. parahaemolyticus* strains irrespective of their serotypes. Cross-reactivity of this MAb was observed only with *V. alginolyticus* and *Aeromona*s spp. from different organisms tested, but these species could also be clearly differentiated from each other in Western blot as *V. alginolyticus* had reaction at 38 kDa and *Aeromona*s sp. reacted at 39 kDa protein. These species therefore, appear to share the same MAb 212 epitope located at different antigens. These specific and sensitive assays could detect diverse *V. parahaemolyticus* serovars and may be applicable as a reliable and rapid diagnostic procedure for the identification and direct detection of *V. parahaemolyticus* in seafood samples.

KEY WORDS: *V. parahaemolyticus,* Outer Membrane Protein, Western blot, ELISA.

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INTRODUCTION

Vibrio species are ubiquitous member of the bacterial community in temperate and tropical marine coastal waters. Most of them are recognised as fish and shellfish pathogens[1.] but some are involved in human infections. Vibrio parahaemolyticus has been frequently involved in food borne gastroenteritis illness worldwide[2,3], when raw or undercooked seafood were consumed[4], but occasional wound infections and septicaemia were also reported. The pathogenic strains of the organism are known to carry two haemolysins- Thermostable Direct Haemolysin (TDH) and TDH-Related Haemolysin (TRH)[5]. The V. parahaemolyticus strains were classified into 13 O serotypes and 71 K serotypes[6]. Since 1996, a new serovar O3:K6 and its clonal derivatives are regarded as the predominant serovars responsible for most outbreaks worldwide and are emerging as pathogens of great concern[7,8].V. parahaemolyticus strains are generally detected and identified by following basic conventional methods. The isolation from samples includes a pre-enrichment step in the Alkaline Peptone Water (APW) followed by plating in selective media, Thiosulphate Citrate Bile Salt Sucrose agar (TCBS). The presumptive colonies are further subjected to key biochemical tests for confirmation. But these methods are time consuming and at times show ambiguity in the results. Alternatively, confirmation through the use of biomolecular methods, such as PCR[7,8], fingerprinting methods like ribotyping[9,10] and pulsed field gel electrophoresis (PFGE)[11] has been attempted. PCR and other molecular methods though are specific[12], require highly trained personnel and equipments, and thereby tend to become relatively expensive. So immunological methods are desired for detection and diagnostics, since they are simple and highly sensitive to detect accurately several bacterial species[13,14]. Until recently, few studies have reported the use of antibodies for the identification of V. parahaemolyticus isolates/serovars but were found to be of limited value owing to the occurrence of cross-reactivity with other bacterial species[15,16,17]. The present study was initiated to attempt a crude OMP preparation as a source of antigen for generation of specific monoclonal antibodies for use in detection immunoassay.

MATERIALS AND METHODS

1. Bacterial culture and growth

The standard bacterial strains used in this study are listed in Table 1. All the *Vibrio* species were grown in APW with 3 % NaCl while other bacterial cultures were grown in Brain Heart Infusion (BHI) broth at 37 ^{o}C overnight. DFR strains of *V. parahaemolyticus* and *V. alginolyticus* were isolated in Defence Food Research Laboratory, Mysore, from different seafood samples. All the reagents and chemicals used in this study were procured from Himedia, India, unless mentioned otherwise.

TABLE 1
List of bacterial strains used in this study

Sl No.	Bacterial strain	Source	Dot ELISA	Western blot
1	*Vibrio parahaemolyticus* ATCC 17802	ATCC	+	36 kDa
2	*Vibrio parahaemolyticus* K11555	NICED	+	36 kDa
3	*Vibrio parahaemolyticus* K21886	NICED	+	36 kDa
4	*Vibrio parahaemolyticus* K12262	NICED	+	36 kDa
5	*Vibrio parahaemolyticus* K12262	NICED	+	36 kDa
6	*Vibrio parahaemolyticus* L10847	NICED	+	36 kDa
7	*Vibrio parahaemolyticus* E2270	NICED	+	36 kDa
8	*Vibrio parahaemolyticus* E2253	NICED	+	36 kDa
9	*Vibrio parahaemolyticus* VP1	CF	+	36 kDa
10	*Vibrio parahaemolyticus* VP2	CF	+	36 kDa
11	*Vibrio parahaemolyticus* VP3	CF	+	36 kDa
12	*Vibrio parahaemolyticus* VP4	CF	+	36 kDa
13	*Vibrio parahaemolyticus* AQ4037	CF	+	36 kDa
14	*Vibrio parahaemolyticus* DOH1187	CMFRI	+	36 kDa
15	*Vibrio parahaemolyticus* DOH747	CMFRI	+	36 kDa
16	*Vibrio parahaemolyticus* DOH 742	CMFRI	+	36 kDa
17	*Vibrio parahaemolyticus* VPF12	DFRL	+	36 kDa
18	*Vibrio parahaemolyticus* VPSQ54	DFRL	+	36 kDa
19	*Vibrio cholerae* 2/2001	NICED	-	-
20	*Vibrio vulnificus* CB21	CBT	-	-
21	*Vibrio fluvialis* J20015	NICED	-	-
22	*Vibrio furnissi* NCTC 11218	NCTC	-	-
23	*Vibrio furnissi* ATCC 35016	ATCC	-	-
24	*Vibrio aesturianus* ATCC 35048	ATCC	-	-
25	*Vibrio harveyi* CB13	NICED	-	-
26	*Vibrio alginolyticus* ATCC 17749	ATCC	+	38kDa
27	*Vibrio alginolyticus* VA1	DFRL	+	38 kDa
28	*Vibrio alginolyticus* VA2	DFRL	+	38 kDa
29	*Vibrio metschnikovii*	NICED	-	-
30	*Vibrio fisherii* MTCC 1738	IMTECH	-	-
31	*Vibrio splendidus* CB6	CBT	-	-
32	*Aeromanas hydrophila* ATCC 49190	ATCC	+	39 kDa
33	*Aeromonas hydrophila* ATCC 35654	ATCC	+	39 kDa
34	*Aeromonas culicicola* NCIM 5147	NCIM	+	39 kDa
35	*Aeromonas sobria* MTCC 1608	IMTECH	+	39 kDa
36	*Aeromonas liquefaciens* MTCC 2654	IMTECH	+	39 kDa
37	*Aeromonas salmonicida* MTCC 1945	IMTECH	+	39 kDa
38	*Aeromanas formicans* MTCC 2319	IMTECH	+	39 kDa
39	*Pleisomonas shigelloides* MTCC 1737	IMTECH	-	-
40	*Salmonella typhimurium* ATCC 14028	ATCC	-	-
41	*Salmonella typhi* TF 687	NICED	-	-
42	*Shigella boydii* ATCC 9207	ATCC	-	-
43	*Shigella sonneii* ATCC 25931	ATCC	-	-
44	*Yersinia enterocolitica* ATCC 23715	ATCC	-	-
45	*Escherichia coli* ATCC 10536	ATCC	-	-
46	*Bacillus cereus* ATCC 10876	ATCC	-	-
47	*Listeria monocytogenes* ATCC 15313	ATCC	-	-

ATCC- American Type Culture Collection, NICED-National Institute of Cholera and Enteric Disease (Calcutta), CF-College of Fisheries (Mangalore), IMTECH-Institute for Microbial Technology (Chandigarh), NCTC- National* Collection *of Type Cultures, CBT-Centre for biotechnology (Chennai), NCIM- National Collection of Industrial Microorganisms (Pune), CMFRI-Central Marine Fisheries Research Institute (Cochin), DFRL-Defence Food Research Laboratory (Mysore).

2. Extraction and analysis of crude OMPs

Crude OMPs were prepared from standard *V. parahaemolyticus* strain ATCC 17802 by following the modified protocol as described by Crosa and Hodges[18]. Briefly, the *V. parahaemolyticus* was cultured in 100 ml of APW with 3 % NaCl and the cells were harvested by centrifuging at 5,000 *g* for 30 min at 4 ^{o}C. The cells were washed with Phosphate Buffered Saline (PBS) twice and re suspended in 3 ml of 10 mM tris (hydroxyl methyl) amino methane buffer containing 0.3 % (w/v) NaCl (pH 8.0) and sonicated to disrupt the cell membrane. The debris was removed by centrifuging at 10,000 *g* for 2 min. The supernatant was further transferred to new tubes and centrifuged at 17,000 *g* for 1 hr at 4 ^{o}C. The pellet thus obtained was incubated with 3 % (w/v) sodium lauroyl sarcosinate (sarkosyl) in 10 mM tris buffer overnight at 4 ^{o}C, followed by centrifugation at 17,000 *g* for 1 hr at 4 ^{o}C. The crude OMP extract pellet was

washed twice in sterile PBS and then re suspended in 1 ml of sterile PBS. The concentration of the OMP extract was determined by Lowry's method[19] and was examined in SDS-PAGE on 14 % (wt/vol) polyacrylamide gel.

3. Generation of polyclonal and monoclonal antibodies against crude OMP

The polyclonal antibodies were raised in female BALB/c mice (6-7 week old), by injecting subcutaneously and intramuscularly 50 µg of crude OMP extract emulsified with an equal volume of Freund's complete adjuvant (Sigma, India). This was followed by four booster doses with Freund's incomplete adjuvant (Sigma, India) which was given in weekly intervals. The mice were bled one week after the last immunization and the antiserum was collected after centrifuging the blood clot. OMP specific polyclonal antibodies were detected by using a standard plate ELISA format in which 20 µg of the OMP antigen was coated to the solid phase. When the required titre was obtained, a final dose of 40 µg proteins was injected intraperitoneally.The MAbs was produced by following the method of Kohler and Milstein[20] with minor modifications. Three days after the final immunization, the splenocytes of the mice and Sp2/o-Ag 14 myeloma cell line were mixed at a ratio of 5:1 and fused with 40 % polyethylene glycol. The resulting hybridoma cells were selected and grown in Dulbecco's modified Eagle's medium (Sigma, India) supplemented with L-glutamine hypoxanthine aminopterin thymidine (HAT) and 10 % of fetal bovine serum (Gibco, India).The culture supernatants of actively growing hybridoma cells were screened for MAb production by plate ELISA. The positive hybridoma cells were further subjected to recloning twice by limiting dilution for stabilization of the clones and isotyped by plate ELISA.

4. Western blot analysis

The crude OMP extract was separated by 14 % SDS-PAGE and transferred to nitrocellulose membrane by electroblotting (Biorad laboratories, USA). The membrane was then incubated overnight in PBS with 5 % skimmed milk to saturate protein binding site, followed by incubation with primary antibody (hyperimmune sera or the MAb supernatant) for 1 hr. The membrane was washed 3-4 times with PBST (PBS with 0.05 % tween 20) and re-incubated for another 1 hr with peroxidise-conjugated anti-mouse IgG (Sigma, India), diluted 1:1,000, followed by 3-4 washes with PBST. Enzyme reaction was detected by immersing the blot in PBS containing 0.3 % diaminobenzidine tetrachloride (DAB) (Sigma, India) and 0.03 % H_2O_2 at 37 ^oC. The reaction was stopped by washing with distilled water.

5. Specificity of monoclonal antibodies

The Specificity of MAb was tested by dot ELISA and Western blotting across various Gram positive and Gram negative bacterial strains mentioned in Table1. It was also validated against the *V. parahaemolyticus* strains isolated from sea food samples. For dot ELISA, 1 ml of each bacterial strain which were grown overnight in BHI were centrifuged at 10,000 *g* for 3 min. The pellet thus obtained was resuspended in carbonate-bicarbonate buffer (pH 9.6). 5ul was deposited onto nitrocellulose membrane and was allowed to air dry for 10 min. Dot blot membranes were blocked with 5 % skimmed milk powder solution in PBS. After blocking, the samples were incubated with MAb supernatant for 1 h at 37 °C. To detect the MAb, the membrane was immersed in a 1:1,000 dilution of peroxidase-conjugated anti-mouse IgG. The enzyme reaction was detected using the substrate DAB in PBS and was stopped by soaking the blots in distilled water.

6. Isotyping of murine immunoglobulins

Typing of MAbs was done by sandwich-ELISA using class and sub-class specific antimouse immunoglobulins using mouse type sub-isotyping kit (Sigma, India), following the manufacturer's recommendations.

RESULTS

1. Extraction and analysis of crude OMPs

The OMPs which were extracted by ultrasonication and centrifugation were separated in 14 % SDS PAGE. Seven

prominent (molecular weights ranging from 18 kDa to 54 kDa approximately) and several minor OMP bands were seen after the polyacrylamide gel was viewed according to standard procedures by staining with Coomassie brilliant blue G250 (Sigma, India) as depicted in figure 1.

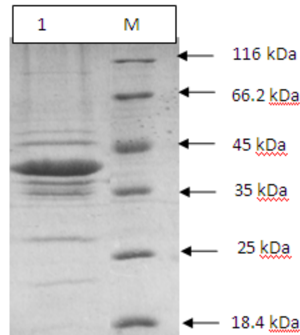

Figure 1
SDS PAGE analysis of Vibrio parahaemolyticus ATCC 17802 crude OMP extract.

2. Generation of polyclonal and monoclonal antibodies against crude OMP

Following immunization with OMP, a titre of 1: 24,000 was observed in plate ELISA. This polyclonal antiserum when tested onto the crude OMPs which were electroblotted onto nitrocellulose membrane, showed reactivity against 4 major bands of proteins of size 52, 36, 22 and 21 kDa (figure 2) .

Figure 2
Reactivity of polyclonal anti sera with Vibrio parahaemolyticus ATCC 17802 OMP extract. The Pageruler prestain protein ladder SM0671 (MBI Fermentas, India, was used as marker.

By murine hybridoma technique, 4 MAbs were obtained which was designated clone 104, 202, 201 and 212. All these clones showed high reactivity in plate ELISA and in Western blot it showed reaction with the 36 kDa protein.

3. Specificity of MAb

To test for the conservation of antigenic epitopes recognized by the MAbs, the antibodies were tested against a wide range of Gram-negative and Gram-positive bacteria, by both dot ELISA and Western immunoblot technique. MAb 212 was found to display cross-reactivity with only *V. alginolyticus* and *Aeromonas* sp. with reaction against *V. alginolyticus* at 38 kDa and with

Aeromonas sp. at 39 kDa. All *V. parahaemolyticus* strains had reactions at 36 kDa protein with this MAb (figure 3&4). The other 3 MAbs showed cross-reactivity with other bacterial strains tested.

4. Isotyping of murine immunoglobulins

The clones were designated as 212, 201, 202, and 104. Among the four clones, 212 was IgG_1, 201 and 104 were IgM and 202 was IgG_{2b} in nature.

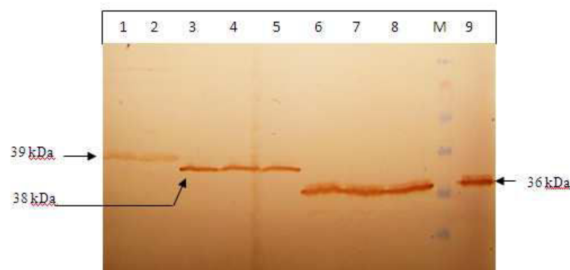

Figure 3
Evaluation of reactivity of MAb 212 with strains of Vibrio parahaemolyticus, Vibrio alginolyticus and Aeromonas species by Western blot

1. A. hydrophila ATCC 49140, 2. A. sobria MTCC 1608, 3. V. alginolyticus VA1, 4. V. alginolyticus VA2, 5. V. alginolyticus ATCC 17749, 6. V. parahaemolyticus VPSQ54, 7. V. parahaemolyticus VP1, 8. V. parahaemolyticus K21886, M. Pageruler prestain protein ladder SM0671 (MBI Fermentas, India), 9. V. parahaemolyticus ATCC 17802 OMP extract.

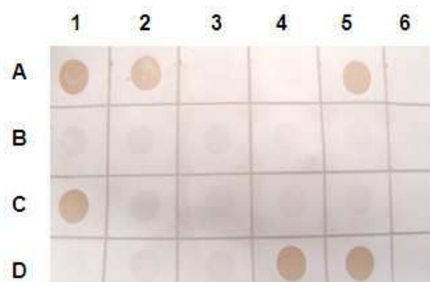

Figure 4
Evaluation of reactivity of MAb 212 with Vibrio species and other bacterial species by dot ELISA

A-1. V. parahaemolyticus ATCC 17802 OMP extract (Positive control), 2. V. parahaemolyticus K21886, 3. V. cholera 2/2001, 4. V. vulnificus CB21, 5. V. alginolyticus ATCC 17749, 6. V. furnissi ATCC 35016, B-1. V. fluvialis J20015, 2. V. fisherii MTCC 1738, 3. V. metschnikovii, 4. V. splendidus CB6, 5. V. aesturianus ATCC 35048, 6. V. harveyi, C-1. A. hydrophila ATCC 49140, 2. Pleisomonas shigelloides MTCC 1737, 3. Salmonella typhi TF 687, 4. Shigella sonneii ATCC 25931, 5. Yersinia enterocolitica ATCC 23715, 6. Bacillus cereus ATCC 10876, D- 1. Escherichia coli ATCC 10536, 2. Listeria monocytogenes ATCC 15313, 3. Shigella boydii ATCC 9207, 4. V. parahaemolyticus VPSQ54, 5. V. alginolyticus VA1, 6. Negative control.

DISCUSSION

The outer membranes of Vibrios contain a number of distinct proteins which play major role in infection and pathogenicity like in all Gram-negative bacteria[21] and have recently become more attractive for the development of vaccine candidates [22,23]. In Vibrios, some of these proteins may be species-specific and have high antigenicity[24,25,26] and some like 25 kDa OMP is common to all *Vibrio* sp.[27]. Therefore, polyclonal antibody raised against the crude antigenic preparation is unlikely to clearly delineate the Vibrios at species level. This problem is overcome to an extent by the use of MAbs where epitope response could be distinct despite similar structural components present among OMPs. MAb based immunological detection methods were developed for specific identification of V. cholerae[28] and V. vulnificus[29]. Recombinant OMPs such as OmpW, OmpV, OmpK, OmpU and TolC were also used to analyze the immunogenicity and development of vaccines[30]. Recently, a polyclonal antibody based flow cytometry was reported for detection of environmental V. parahaemolyticus, but it showed significant cross-reactivity between V. harveyi and V. alginolyticus[31].The crude OMP preparation of V. parahaemolyticus used in the present work contained a number of antigenic proteins in the size range of 18 kDa to 54 kDa, among which, 4 predominant OMP bands of the sizes 21 kDa, 22 kDa, 36 kDa and 52 kDa displayed strong positive reaction in Western blot analysis. The four stabilized MAbs generated against the mixture of OMPs were found to have reaction only to the 36 kDa protein thereby reflecting the highly immunodominant nature of this OMP in V. parahaemolyticus. This apparently immunodominant antigen too had many cross-reactive epitopes as was evident from the reactions observed in 3 out of 4 MAbs. Only clone 212 had restricted cross-reactivity shared among the closely related species, namely, V. alginolyticus and Aeromonas sp. Interestingly, the shared epitope of this MAb resided onto very different antigens in these 3 species and this provided a useful application of this MAb in distinctly identifying all the 3 species. Moreover, all the 7 Aeromonas sp. tested had the epitope for clone 212 in the same 39 kDa protein. Thus, dot ELISA developed in this study based on clone 212 proved to be useful in detecting V. parahaemolyticus, V. alginolyticus and the group of Aeromonas sp. Applying this MAb, it was still possible to selectively identify V. parahaemolyticus when the sample was initially inoculated into a selective media like, TCBS agar where V. alginolyticus developed into a clear yellow sucrose fermenting colonies and the growth of Aeromonas sp. is either inhibited or may produce yellow colonies occasionally. This step could be overlooked if the Western blot analysis is employed.

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REFERENCES

1. Gopal S, Otta SK, Kumar S, Karunasagar I, Nishibuchi M and Karunasagar I, The occurrence of *Vibrio* species in tropical shrimp culture environments; implications for food safety, Int J Food Microbiol, 102: 151–159, (2005).
2. Joseph SW, Colwell RP and Kaper JB, *Vibrio parahaemolyticus* and related halophilic vibrios, Crit Rev Microbiol, 10: 77-124, (1982).
3. Shekar M, karunasagar I and karunasagar I, *In silico* identification and comparison of surface binding pockets of *Vibrio*

parahaemolyticus thermostable direct hemolysin proteins, Int J Pharm Bio Sci, 3: 280 – 288, (2012).

4. Venkitanaranan KS and Doyle MP, Food-borne infections and infestations. In Berdanier, C.D (eds.). Handbook of Nutrition and Food.p.1135-1161.CRC Press, New York, (2001).
5. Shirai H, Ito H, Hirayama T, Nakamoto Y, Nakabayashi N, Kumagai K, Takeda Y and Nishibuchi M, Molecular epidemiologic evidence for association of thermostable direct hemolysin (TDH) and TDH-related hemolysin of *Vibrio parahaemolyticus* with gastroenteritis, Infect Immun*,* 58: 3568–3573, (1990).
6. Iguchi T, Kondo S and Hisatsune K, *Vibrio parahaemolyticus* O serotypes from O1 to O13 all produce R-type lipopolysaccharide: SDS PAGE and compositional sugar analysis, FEMS Microbiol Lett, 130: 287– 292, (1995).
7. Okuda J, Ishibashi M, Hayakawa E, Nishino T, Takeda Y and Mukhopadhyay AK, Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travellers arriving in Japan, J Clin Microbiol, 35: 3150–3155, (1997).
8. Matsumoto C, Okuda J, Ishibashi M, Iwanaga M, Garg P and Rammamurthy T, Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and toxRS sequence analyses, J Clin Microbiol, 38: 578–585, (2000).
9. 9Bag PK, Nandi S, Bhadra RK, Ramamurthy T, Bhattacharya SK and Nishibuchi M, Clonal diversity among recently emerged strains of *Vibrio parahaemolyticus* O3:K6 associated with pandemic spread, J Clin Microbiol, 37: 2354–2357, (1999).
10 DePaola A, Ulaszek J, Kaysner, CA, Tenge BJ, Nordstrom JL and Wells J, Molecular, serological and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food and clinical sources in North America and Asia, Appl Environ Microbiol*,* 69: 3999–4005, (2003).
11. Marshall S, Clark CG, Wang G, Mulvey M, Kelly MT and Johnson WM, Comparison of molecular methods for typing *Vibrio parahaemolyticus*, J Clin Microbiol*,* 37: 2473–2478, (1999).
12. Bej AK, Patterson DP, Brasher CW, Vickery MC, Jones DD and Kaysner CA, Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh*, *tdh* and *trh*, J Microbiol Methods*,* 36: 215–225, (1999).
13. Hegarty JP, Dowd MT and Baker KH, Occurrence of *Helicobacter pylori* in surface water in the United States. J Appl Microbiol, 87: 697–701, (1999).
14. Rengpipat S, Pusiririt S and Rukpratanporn S, Differentiating between isolates of *Vibrio vulnificus* with monoclonal antibodies, J Microbiol Methods*,* 75: 398–404, (2008).
15. Chen D, Hanna P, Altmann K, Smith A, Moon P and Hammond LS, Development of monoclonal antibodies that identify *Vibrio* species commonly isolated from infections of humans, fish, and shellfish, Appl Environ Microbiol*,* 58: 3694–3700, (1992).
16. Chen HC and Chang TC, Rapid detection of *Vibrio parahaemolyticus* in oysters by immunofluorescence microscopy, Int J Food Microbiol*,* 29: 311–319, (1996).
17. Datta S, Janes ME and Simonson JG, Immuno magnetic separation and coagglutination of *Vibrio parahaemolyticus* with anti-flagellar protein monoclonal antibody, Clin Vaccine Immunol*,* 15: 1541–1546, (2008).
18. Crosa JH and Hodges LL, Outer membrane proteins induced under conditions of iron limitation in the marine fish pathogen *Vibrio anguillarum*, Infect Immun*,* 31: 223–227, (1981).
19. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent, J Biol Chem, 193: 265-275, (1951).
20. Kohler G and Milstein C, Continuous cultures of fused cells secreting antibody of predefined specificity, Nature, 256: 495-497, (1975).

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21. Tsolis RM, Comparative genome analysis of the alpha proteobacteria: relationships between plant and animal pathogens and host specificity, PNAS, 99: 12503–12505, (2002).
22. Ding XZ, Smallridge RC, Galloway RJ and Kiang JG, Rapid assay of HSF1 and HSF2 gene expression by RT-PCR. Mol cell biochem, 158: 189-192, (1996).
23. Pizza M, Scarlato V and Masignani V, Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing, Science, 287: 1816–1820, (2000).
24. Kabir S, Composition and immunochemical properties of outer membrane proteins of *Vibrio cholerae*, J Bacteriol, 144: 382-389, (1980).
25. Kabir S, Composition and immunochemical properties of the cell surface proteins of *Vibrio cholerae,* J Gen Microbiol, 132: 2235-2242, (1986).
26. Koga T and Kawata T, Isolation and characterization of the outer membrane from *Vibrio parahaemolyticus*, J Gen Microbiol, 129: 3185-3196, (1983).
27. Manning PA and Haynes DR, A common-immunogenic *Vibrio* outer membrane protein, FEMS Microbiol Letters, 24: 297-302, (1984).
28. Colwell RR, Hasan JAK, Hug A, Loomis L, Siebeling RJ, Torres M, Galvez S and Islam S, Development and evaluation of a rapid, simple, sensitive, monoclonal antibody-based co-agglutination test for direct detection of *Vibrio cholerae* O1, FEMS Microbiol Lett, 97: 215–220, (1992).
29. Tamplin M L, Martin AL, Ruple AD, Cook DW and Kaspar CW, Enzyme immunoassay for identification of *Vibrio vulnificus* in seawater, sediment, and oysters. Appl Environ Microbiol, 57: 1235-1240, (1991).
30. Mao Z, Yu L, You Z, Wei Y and Liu Y, Cloning, expression and immunogenicity analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003, Fish Shellfish Immunol, 23: 567-575, (2007).
31. Manti A, Falcioni T, Campana R, Sisti D, Rocchi M, Medina V, Dominici S, Papa S and Baffone W, Detection of environmental *Vibrio parahaemolyticus* using a polyclonal antibody by flow cytometry, Environ Microbiol Reports, 2: 158–165, (2010).