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Development of monoclonal antibody based sandwich ELISA for the rapid detection of pathogenic *Vibrio parahaemolyticus* in seafood

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ABSTRACT

Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are considered important virulence factors of *Vibrio parahaemolyticus* and strains producing either of these or both are considered pathogenic. In this study, we generated monoclonal antibodies (mAbs) against purified TRH recombinant protein of pathogenic *V. parahaemolyticus*. Sandwich enzyme-linked immunosorbent assays (ELISA) using the hybridoma clone 4B10 showed higher sensitivity of detection compared to other clones. Using mAb 4B10 based sandwich ELISA, we could detect pathogenic *V. parahaemolyticus* in 41.18% (14 out of 34) of the seafood samples analyzed. PCR targeting the *toxR* gene showed the presence of *V. parahaemolyticus* in 64.7% (22 out of 34) seafood samples. Further, PCR targeting the virulence genes showed that 6 seafood samples harboured the *tdh* gene while 9 harboured the *trh* gene indicating the presence of pathogenic *V. parahaemolyticus*. Our results show that mAb 4B10 sandwich ELISA developed in this study could be used as a rapid method for screening seafood samples for the presence of pathogenic *V. parahaemolyticus*.

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1. Introduction

Vibrio parahaemolyticus is a Gram negative, halophilic bacterium, autochthonous to water and sediments of marine and estuarine ecosystem. The incidence of gastroenteritis due to *V. parahaemolyticus* was first reported in Japan during the 1950s (Fujino et al., 1951, 1953) and since then, cases have been reported from several parts of the world. The pathogenesis of this organism has been associated with thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) coded by the *tdh* and *trh* genes respectively (Honda et al., 1987a, 1987b; Honda and Iida, 1993). Therefore, the strains bearing *tdh* and/or *trh* genes are considered as pathogenic (Nishibuchi and Kaper, 1995).

The establishment of an effective control programme for vibrios in seafood necessitates reliable, accurate and sensitive methods to assess the presence of pathogenic vibrios in seafood. Conventional culture based techniques are slow, laborious and often require several days. Moreover, these assays, may fail to detect strains of bacteria which are present in the samples at very low levels (Aono et al., 1997). β-hemolysis on high salt blood agar, Wagatsuma agar is used to detect *V. parahaemolyticus* producing TDH, but there is no phenotypic test for the detection of TRH. PCR has been employed for the detection of *V. parahaemolyticus* from both clinical and environmental samples. These methods target the genes encoding virulence determinants and also species specific markers that include *tdh*, *trh*, *tlh* and *toxR* (Taniguchi et al., 1985; Tada et al., 1992; Karunasagar et al., 1996; Bej et al., 1999; Kim et al., 1999; Dileep et al., 2003). Though the PCR is more sensitive than conventional culture methods, inability of PCR to discriminate between live and dead bacteria due to the persistence of DNA after cell death is considered as a major drawback (Hayashi et al., 2006). Hence specific detection of pathogenic *V. parahaemolyticus* is of utmost importance from the public health and seafood industry point of view.

Monoclonal antibody based tests are considered as powerful tools for the identification of microorganisms from various sources. The first report on the production of monoclonal antibody against pathogenic *V. parahaemolyticus* (TDH and TRH) was made by Honda et al. (1989, 1990) wherein they found that the developed enzyme-linked immunosorbent assay (ELISA) was highly sensitive for the identification of TDH and TRH of pathogenic *V. parahaemolyticus* from clinical samples. In this study, we report, the production of monoclonal antibodies (mAbs) against TRH of *V. parahaemolyticus* and their application in ELISA to detect pathogenic *V. parahaemolyticus* (*tdh* and/or *trh*) from seafood enrichment cultures.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study are listed in Table 1. All isolates used in this study were revived from stock culture preserved

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Table 1
List of bacterial strains used in this study.

Name of organism	Absorbance values of mAb based sandwich ELISA (mean OD ± SD)		
	1B10	4B10	4C12
Recombinant TRH	0.814 ± 0.233	1.137 ± 0.054	0.701 ± 0.100
Vibrio parahaemolyticus (*trh*$^+$) (n = 21)	0.440 ± 0.184	0.718 ± 0.348	0.503 ± 0.162
V. parahaemolyticus (*tdh*$^+$) (n = 03)	0.355 ± 0.053	0.684 ± 0.391	0.310 ± 0.007
V. parahaemolyticus (*tdh*$^+$and *trh*$^+$) (n = 06)	0.444 ± 0.163	0.665 ± 0.227	0.412 ± 0.252
V. parahaemolyticus (non-pathogenic) (n = 05)	–	–	–
V. cholerae (n = 05)	–	–	–
V. harveyi (n = 03)	–	–	–
V. mimicus (n = 02)	–	–	–
V. alginolytiucs (n = 03)	–	–	–
V. anguillarum (n = 02)	–	–	–
V. damsella (n = 02)	–	–	–
Aeromonas hydrophila (n = 02)	–	–	–
A. veronii biovar veronii (n = 03)	–	–	–
Escherichia coli (n = 05)	–	–	–
Salmonella (n = 06)	–	–	–

Note: Positive–negative threshold (negative index) was calculated using the formula, negative OD × 2. More than 2 times that of a negative control considered as positive. The OD of non-pathogenic *V. parahaemolyticus* and other bacterial strains was below the threshold value 0.280 (ranging from 0.120 to 0.264).
"–" means negative for pathogenic *V. parahaemolyticus* (below the threshold value of 0.280).

at −80 °C (Sanyo, Japan) in 30% glycerol broth at Department of Fishery Microbiology, College of Fisheries, Mangalore. These strains were grown in 2 mL of Luria–Bertani broth (HiMedia, India) at 37 °C overnight with shaking.

2.2. Cloning and expression of the trh gene

The *trh* gene was amplified from the *V. parahaemolyticus* using primers (5′–3′) trh5-TTGCTATTGGTTTCAATATT and trh6-AATTTGTGA-CATACATTCAT. The amplified PCR products were purified using QIAquick PCR purification kit (Qiagen, USA). Purified products were analyzed by 1.5% agarose gel electrophoresis. Purified PCR product was ligated to pQE30 UA vector (Qiagen, USA) and transformed into SG13009 *E. coli* cells. Recombinant clones were confirmed by PCR using gene specific primers. TRH expression in recombinant clones was done by inducing with a final concentration of 1 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) and expression of recombinant protein was studied by 15% SDS gel electrophoresis. Recombinant protein was purified by Ni-NTA (Qiagen, USA) affinity chromatography, and concentration was estimated by Lowry's method (Lowry et al., 1951).

2.3. Development of monoclonal antibodies against TRH of V. parahaemolyticus

Initially BALB/c mice (6 weeks old) were immunized with 50 μg of TRH recombinant protein in complete Freund's adjuvant (CFA) and three subsequent doses were injected with incomplete Freund's adjuvant (IFA) at 14 day intervals. Antibody titres were determined by plate ELISA. Final booster dose was delivered by injecting the mice intraperitoneally with 100 μL (100 μg) TRH without adjuvant. Hybridoma clones were developed by using the procedure of Kohler and Milstein (1975) with minor modifications. Sensitized spleen cells were fused with mouse myeloma cell line, Sp2/0 using polyethylene glycol after three days from the final booster dose. Hybridoma cells were cloned by limiting dilution and positive clones were selected by plate ELISA. The class of immunoglobulin was determined by using mouse monoclonal antibody Isotyping Reagents (Sigma, USA).

2.4. Characterization of monoclonal antibodies

The generated mAbs were tested for its specificity using all bacterial strains mentioned in Table 1 by plate ELISA. Western blotting was performed by the procedure of Towbin et al. (1979) using supernatants from positive clones for the reactivity with recombinant TRH protein.

2.5. Production of monoclonal antibodies

Positive hybridoma clones were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (Sera Lab Ltd., UK), Antibiotic Antimycotic solution (100×) 1 mL/100 mL (Sigma, USA), Gentamicin (40 mg/mL) 40 μL/100 mL and 2 mM L-glutamine (Sigma, USA). Cells were allowed to grow to form a monolayer in a 37 °C incubator supplemented with 5% CO_2. Antibody containing culture supernatants were collected from the hybridoma culture flasks and were centrifuged at 1000 × *g* for 10 min to remove the cell debris. mAbs were purified using CBindD™ L resin (Sigma, USA) column and the purified antibodies were stored at −20 °C.

2.6. Monoclonal antibody based sandwich ELISA for the detection of pathogenic V. parahaemolyticus from spiked seafood homogenate

All bacterial strains included in this study were used for the evaluation of mAbs. 100 μL of each overnight grown bacterial culture was spiked into 10 g of bacteria free seafood homogenate. Each spiked seafood homogenate was inoculated into 90 mL of alkaline peptone water (APW). Uninoculated sterile seafood homogenate served as a negative control. The enrichment broths were incubated at 37 °C for 16 h and 1.5 mL aliquots of the enriched broth were centrifuged (Heraeus, Germany) at 10,000 × *g* for 10 min and the supernatant was used for ELISA.

2.7. Determination of the sensitivity of sandwich ELISA and PCR

Two strains of *V. parahaemolyticus* namely *tdh* positive O3:K6 obtained from the National Institute of Cholerae and Enteric Diseases (NICED), Kolkata (kindly provided by Dr. T. Ramamurthy) and *trh* positive AQ4037 obtained from ATCC were used in this study for determining and comparing the sensitivity of mAb based sandwich ELISA and PCR. Finfish (*Stolephorus indicus*) confirmed to be negative for *V. parahaemolyticus* was used in this study for preparing the homogenates. 90 mL of APW was added to 10 g of finfish homogenate in a conical flask that was spiked with varying concentrations of pathogenic *V. parahaemolyticus* to give a final count ranging from 10^0 to 10^6 cells/mL. To unspiked finfish homogenate, 90 mL of APW and 1 mL of Luria–Bertani (HiMedia, India) broth was added and served as negative control. All the conical flasks were incubated at 37 °C for 16 h followed by centrifugation at 10,000 × *g* for 10 min. The resulting supernatants were tested by sandwich ELISA. In parallel, DNA lysate for PCR was prepared by taking aliquots of 1.5 mL of seafood enrichment broths from each flasks and centrifuged at low speed (800 × *g* for 10 min) (Heraeus, Germany) to sediment the meat particles and the supernatant was centrifuged further at 10,000 × *g* for 10 min to pellet the bacteria. Pellets were suspended in 100 μL of 1× Tris–EDTA buffer (pH 8.0) and crude DNA was extracted from pellet by heating at 95 °C for 10 min followed by cooling in ice. Aliquots of 2 μL of DNA lysate were used as template DNA for PCR assay.

2.8. Sandwich ELISA for the detection of pathogenic V. parahaemolyticus in seafood homogenate

Polystyrene ELISA plates (Greiner Bio-One, Germany) were coated with 100 μL of polyclonal rabbit anti-TRH immunoglobulin (Raghunath

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