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RECOMBINANT OUTER MEMBRANE PROTEIN, OMP26La OF *VIBRIO ANGUILLARUM* IS AN EFFECTIVE VACCINE CANDIDATE

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Abstract: Asian sea bass (*Lates calcarifer*) is an economically important cultivable fish species for aquaculture. *Vibrio anguillarum* is one of the major causative agents of classical Vibrios in sea bass. Serotypes of O2a and O2b are the main causative *Vibrio anguillarum* species in sea bass. Fish vaccination is a useful strategy for several disease problems in aquaculture. Though several studies have shown that different vaccine formulations against *V.anguillarum*, currently there is. no effective vaccine available against this important bacterial pathogen. The present study explored identification of immunogenic antigens of *V.anguillarum* to control Vibriosis. The major immunogenic protein antigens of *V. anguillarum viz*., Outer Membrane Protein (OMPs), Extra-Cellular membrane Proteins (ECPs) and Lipopolysaccharide(LPS) were purified and checked for the immune response. Outer membrane proteins of bacteria are considered ideal candidates for vaccine development. OMP26La gene from pathogenic *V.anguillarum* was cloned and expressed. Specificity was cross checked by western blotting. OMPs were purified from pathogenic *V. anguillarum* and separated by 2D gel electrophoresis analysis and confirmed by immuno-blotting. The challenge experiment studies carried-out with recombinant protein OMP26la showed survival rate of 81% against controls (34%) with Relative Percentage Survival (RPS) of 70.3%. The study indicated OMPs can be potential immunogens for vaccination against vibriosis caused by *V.anguillarum*.

Key words: *Lates calcarifer,* 2D gel electrophoresis, Immuno-blotting, Immunogen, Vaccination

INTRODUCTION

Vibrio anguillarum a gram-negative, curved rod shaped marine bacterium of the class *Gammaproteobacteri* is causative agent of systemic vibriosis is important fish pathogen infecting many commercial farmed fish species. Vibriosis disease characterized by hemorrhagic septicemia is often fatal disease results in huge economic losses in finfish aquaculture world wide (Rucker, 1958; Ross *et al*., 1968; Mahnken, 1975; Austin and Austin, 1999; Evelyn, 1996). Up to 23 serotypes, designated O1–O23, are recognized in the European system(Pedersen *et al*., 1999), with O1 and O2 being the main ones implicated in disease (Toranzo *et al*., 1997).The use of antibiotics to combat diseases led to antibiotic resistance and is a major concern due to the risk of transfer of antibiotic resistance to human pathogens through fish treatment (Karunasagar *et al*., 1994).

Vaccination is a viable strategy in the prophylaxis of infectious diseases plays important role in large scale commercial fish farming(Sommerset *et al*., 2005). The vaccine preparations based on inactivation of the pathogen have certain limitations., enabled by means of Through recombinant DNA technology, it has become evident the display of heterologous proteins on the surface of bacterial cells and their applications in vaccine development (Zhu *et al*., 2006). It has been proved that surface expression of antigen in vaccines can elicit superior immune protection (Isoda *et al*., 2007). However till date few recombinant vaccines are commercially available for use in aquaculture (Leong *et al*., 1997). The outer membrane proteins of gram-negative pathogenic bacteria play important role in the bacterial pathogenicity and hosts interaction. They are considered as good vaccine candidates since they have protective antigenicity, as the outer membrane components are easily recognized as foreign substances by immunological defense systems of the hosts, inhibit bacterial colonization in hosts, and induce specific cell-mediated immunity and antibodies (Maiti *et al*., 2009; Seltman and

Holst, 2002; Bricknell *et al*., 1999). In the present study the immunogenic efficacy of the expressed recombinant Omp26La protein for control of *V. anguillarum* was explored.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Fish samples were collected from Muttukadu,Chennai (Tamil nadu) and Kakadwip sundarbans area of West Bengal. Bacterial samples were isolated from the infected Asian sea bass tissue, kidney, liver and intestine. Water samples from the sea bass tank was also taken for bacterial isolation. The tissue samples were removed, homogenized in sterile phosphate-buffered saline (PBS) using a homogenizer. Then serially diluted 10 fold in saline solution and dropped onto Thiosulphate citrate bile salts (TCBS) containing 1.5% Nacl . *V. anguillarum* strain isolated during 2009–2010 from diseased seabass (*Lates calcarifer*) and reference strain *V. anguillarum* NB10 (serotype O1) were used in this present study. Milton *et al*. (1992). The isolates were maintained at 80°C in nutrient broth containing 20% glycerol. Identification of the bacterial strains was done by morphological, cultural, physiological and biochemical characteristics tests.The biochemical characterization of the bacterium was done based on their ability to grow on the TCBS, sensitivity to O129, facultative fermentation without gas production, positive for L-arginine dihydrolase reaction, negative for ornithine decarboxylase, production of Catalase, Indole, Mannitol and sucrose (Pacha and Kiehn, 1969).

Molecular characterization of Bacteria

Species-specific PCR detection of *Vibrio anguillarum* using the amiB (N-acetylmuramoyl-L-alanine amidase). The Sensitive and specific PCR primer designed to target amiB gene for the detection of *V. anguillarum* were used. Forward primer :-(5'-ACATCATCCATTTGTTAC-3') and Reverse primer :- (5'-CCTTATCA CTATCCAAATTG-3') amplified product length was 429 bp .

16s rDNA sequences analysis of Bacteria

The rRNA gene is the most conserved DNA in the cells. Portion of the rDNA sequences from distantly related organisms are remarkably similar. So, for this reasons, the gene that encodes the rRNA (rDNA) have been used for bacterial identification. The polymerase chain reaction was carried by using the 16s rDNA primer fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RP2 (5'-ACGGCTACCTTGTTACGACTT-3'). The amplified product length is 1450bp .

Extraction of OM membrane proteins (OMPs)

OMPs was extracted following the method described by Filip *et al*. (1973) using sarcosyl L (N-laurylsarcosine) (Sigma, USA) with minor modifications. Briefly *Vibrio anguillarum* cells form overnight were collected by centrifugation and washed with Tris-Hcl (pH 8) containing 0.3% Nacl. The cells were disrupted by sonication. The suspension was centrifuged to remove the intact cells. The cell fragments were sedimented by centrifugation. The outer membrane protein fractions were isolated by sodium lauryl sacrosinate dissolved in Tris-Hcl (pH 8) containing 0.3% Nacl. Elecrophoresis of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4% stacking gel and 12% Separating gel being used. Gels were stained with Coomassie brilliant blue G-250.

2D gel electrophoresis, MALDI-TOF/TOF analysis and immunoproteomics

2D gel electrophoresis: 2DE was performed as in Li *et al*. (2007) with the following modifications. Briefly, OM protein extracts containing 200 ìg of proteins were dissolved in lysis solution and then rehydrate a pH 3–10 linear IPG strip (11 cm length, Bio-Rad). Isoelectric focusing was performed at 200 V for 10 min, 300 V for 15 min and 400 V for 6 h with linear pH gradient from low pH at the anode (10 mM H_3PO_4) to high pH at the cathode (20 mM NaOH). After the equalized by 2% (W/V) DTT and 2.5% (W/V) IAA (6M urea, 20% glycerol, 2% SDS) (Sigma) for 30min, the proteins resolved on IEF gel were applied to second dimension to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) that contained 12% acrylamide (from 30% T, 2.66% C). The analytical gels were stained with Coomassie brilliant blue G-250.

Immunoblotting

The proteins separated in the 1D and 2D PAGE gels were transferred onto membrane (PVDF, bio rad). The membranes were blocked with 5% defatted milk in PBS for 2 hours, then incubated overnight at 4ºC with antibody conjugated with HRP diluted 1: 1000. Antigen-antibody reactions were observed after detection of peroxidase activity by H_2O_2 and diaminobenzidine (Sigma). The OMP immune responsive proteins from 2D immunoblot analysed by MALDI-TOF LC- MS/MS

MALDI- TOF LC-MS/MS

2D resolved protein spots were excised from the gel, washed successively with 5mMNH4HCO3, pH 8.0 and 50% acetonitrile in 25 mM NH4HCO3, pH 8.0. After a final wash with triple distilled water, dehydration in a vacuum dryer was performed. "In gel," trypsin digestion was performed for 5 h at 37 ºC in 5 ìl of trypsin solutions (10 ng/ìl trypsin in 5 mM NH_4HCO_3, pH 8.0/5% acetonitrile) per sample, depending on the gel volume and protein amount.

Outer membrane protein Omp26la expression

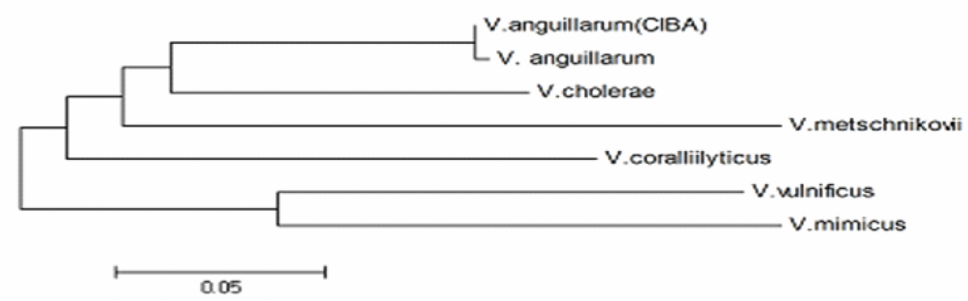
The outer membrane protein OMP26la was amplified from the DNA sample of serotype O1 with the primer having the restriction enzyme site (forward with Eco R1) and (reverse primer with Ava1) the amplified product of 778bp was cloned into PET32a and then transformed into bacterial strain DH5á and protein was expressed in expression strain BL21. The protein expression was induced with IPTG for 3 h at 37 °C after the optimization of expression conditions.Bacterial cells were harvested by centrifugation, then washed and resuspended. The cell suspensions were disrupted by sonication in an ice bath and centrifuged. The supernatants containing the recombinant proteins were subsequently purified by affinity chromatography on Ni-NTA Super flow resin (Qiagen) according to the manufacturer's instructions. Concentrations of the recombinant proteins were determined by the Bradford method. The purified proteins were stored at "80 °C before use. Western analysis was done as described previously (Wang *et al*., 2002) using a rabbit polyclonal antiserum raised against OMPs of *V. anguillarum*.

Challenge experiments and Immunization of sea bass

Challenge experiments with recombinant expressed OMP 26La was taken for the immunization studies. Sea bass animals of 10g were taken for immunization studies. Recombinant expressed *Vibrio anguillarum* OMP26la protein of 5µg in 100µl of PBS was injected for 10g animal. Serum was collected after two boosters dosage. ELISA titer was carried for the hyper immunized serum sample.

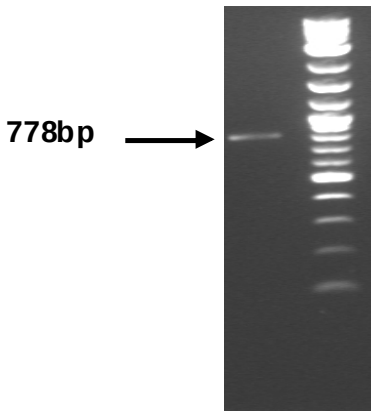
RESULTS AND DISCUSSION

Vibriosis is the most common bacterial disease affecting brackish water fish. Vibriosis has been a major problem for the aquaculture industry throughout the world (Actis *et al*., 1999; Austin and Austin, 1999) resulting in severe economic losses because of high mortality rates *Vibrios* has been associated to environmental stress such as high temperature, salinity or high stocking density. The influences of temperature, salinity and starvation on growth, motility and chemotactic response of V. anguillarum have been determined (Larsen 1984; Larsen *et al*., 2004).The most commonly encountered fish pathogenic *Vibrio* species is *V. anguillarum (Ellis,* 1988). One *Vibrio anguillarum* isolate obtained on screening of 60 samples from CIBA research station in Kakadwip and Muttukadu was characterized and detected by cultural, biochemical tests. Species specific PCR detection of *Vibrio anguillarum* using the specific PCR primer designed to target amiB gene (N-acetylmuramoyl-L-alanine amidase) of *Vibrio anguillarum* yielded 429bp PCR product. Our findings were in accordance with that of Hong and his colleagues (2007). 16s rDNA sequencing and phylogentic analysis (1450bp), using FD1 and RP2 universal 16s primers showed homology to sequences of *V. anguillarum* isolates available in public domain. *V. anguillarum* is hardly distinguishable from other related species due to high biochemical similarities and the existence of variable reactions within each species. Therefore a molecular based identification is necessary to validate the biochemical detection (Pasoz *et al*., 1993).



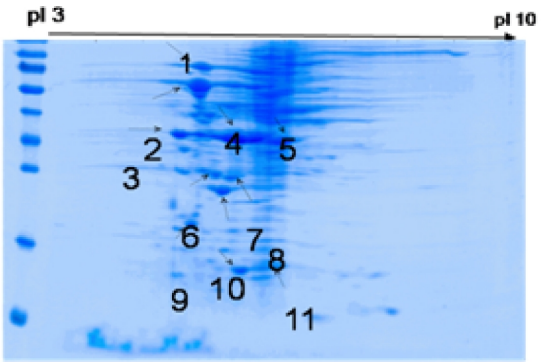
Outer membrane protein Omp26la expression

Outer membrane proteins (OMPs) are likely to play a critical role in the adaptation to changes of environmental conditions. The possible roles of the major OMP of *V. anguillarum* are bile resistance and stimulation of biofilm formation (Wang *et al*., 2002, 2003).The outer membrane protein OMP26la amplified from the DNA sample of serotype O1 with the primer having the restriction enzyme site (forward with Eco R1) and (reverse primer with Ava1) yielded amplified product of 778bp which was cloned into PET32a and then transformed into bacterial strain DH5α and protein was expressed in expression strain BL 21.



PCR amplified fragment (778bp) of OMP26La

The outer membrane proteins of gram-negative pathogenic bacteria are good vaccine candidates since they have important role in the interaction with hosts in the bacterial pathogenicity during adherence, uptake of nutrients from the host and eliminating host-defense mechanisms. Also they have protective antigenicity, because the components of the outer membrane are easily recognized as foreign substances by immunological defense systems of the hosts (Maiti *et al*., 2009; Seltman and Holst, 2002). The OMPs can induce specific bactericidal antibodies,inhibiting bacterial colonization on hosts and inducing cell-mediated immunity (Bricknell *et al*., 1999). Outer Membrane proteins extracted and subjected to 2D gel electrophoresis and Immunoblotting. OMPs 26la is one of the eight immunogenic proteins identified from *V. anguillarum* by 2D gel separation and immunoblotting. OMP261a (28KDa), is one out of five majorly expressed outer membrane proteins that were seperated by gel filtration chromatography. The challenge experiment studies with recombinant protein OMP26La showed the survival rate of 70.3%. After fourth week of immunization experiment the serum samples collected to determine the immune response by ELISA and western blotting.



2D gel of OMPs

Omp26La is a porin-like OMP first cloned by Suzuki *et al*. (1998). Because of limited research information, the biological function of Omp26La remains unknown. However, according to a BLAST search against NCBI database, Omp26La showed markedly high sequence identity with OmpV from *Vibrio cholerae* (96%), *Vibrio parahaemolyticus* (72%), *Vibrio alginolyticus* (70%) and *Photobacterium damselae* (72%), and were thus considered as an OmpV homologue. Kao *et al* (2009) noted that Omp26La of *V. anguillarum* has 96% sequence similarity with OmpV of *V. cholera* and these are considered homologues. Indeed limited immunogenic informations are available with regard to the Omp26La, though recently it has been demonstrated to be salt-responsive which help *V. anguillarum* to adapt to lower salinities (Kao *et al*., 2009). ELISA results revealed an elevated level of antibody in the serum of vaccinated fish compared to that of control fish. It has been shown that vaccinated fish possess high amounts of antibodies (Mao *et al*., 2007) which are believed to play an important role in protecting fish against bacteria (Gonzalez *et al*., 1988).

In summary, recombinant protein OMP26la vaccination of juvenile sea bass resulted in a high efficacy (70.3% RPS). The high level of protection obtained by vaccination in this study is effective and the study indicated OMPs can be potential immunogens for vaccination against vibriosis caused by *V. anguillarum*.

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