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Characterization of immune response elicited by the recombinant outer membrane protein OmpF of *Aeromonas hydrophila*, a potential vaccine candidate in murine model

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Abstract Porins, the outer membrane proteins of gram negative bacteria, perform vital roles in bacterial survival and virulence, such as nutrient transportation across the membrane as well as adhesion to host cells during infection. The outer membrane proteins, OmpF and OmpC, are part of a two-component regulatory system, essential for the maintenance of solute concentrations in the cytoplasmic milieu of bacteria, and are thus considered vital for bacterial survival. Exposed on the surface of gram-negative bacteria, these channel proteins are highly immunogenic and can thus be exploited as vaccine candidates. In the present study, we have cloned, characterized, and expressed outer membrane protein OmpF of *Aeromonas hydrophila*, a major fish pathogen and also known to cause severe infections in humans. The cloned *ompF* gene of *A. hydrophila* consisting of an open reading frame corresponding to mature OmpF was expressed and purified from the heterologous host, *E. coli*. High level of expression resulted in recovery of ~120 mg/L of the purified rOmpF at shake flask level. Polyclonal antisera raised against the recombinant OmpF showed a very high endpoint titer (>1:80,000) and were able to specifically agglutinate live *A. hydrophila.* Further, anti-OmpF antisera cross-reacted with the cell lysates of various *Aeromonas* isolates, suggesting that anti-rOmpF antibodies can be used to identify different *A. hydrophila* isolates in infected conditions. Antibody isotyping, cytokine ELISA, and ELISPOT assay indicated predominantly Th1 type of immune response. The recombinant OmpF reported in the present study thus has the potential to be used as a vaccine candidate against *A. hydrophila*.

Keywords OmpF · *Aeromonas* · Immune response · Vaccine · Agglutination

Introduction

Aeromonas hydrophila, a motile gram-negative bacillus, is found in water sources, soil, and foodstuff. Along with *A. caviae, A. sobria, and A. salmonicida* [1], the organism belongs to *Aeromonadacea* family. Members of the *Aeromonadacea* family are predominantly motile via a single polar flagellum (except *A. salmonicida*) and produce catalase, oxidase, nitrate reductase, and an array of other exoenzymes. *Aeromonas hydrophila* is a fish pathogen and causes several diseases in fish including gastroenteritis, wounds, respiratory infections, and eye infections. It can also cause human illnesses, including acute gastroenteritis, soft tissue infections, meningitis, hepatobiliary tract infections, pneumonia, empyema, and primary septicemia. Various routes of transmission include the intake of contaminated food, exposure of wounds to pathogen contaminated environments, etc. [2].

As a major fish pathogen, *A. hydrophila* causes large economic losses to the aquaculture industry. The infection spreads very fast, especially in contained fish populations. Various strategies including vaccines and antibiotics have

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been employed to combat *A. hydrophila* infections. Commercially available vaccines consist of avirulent strains and heat killed cells. However, these vaccines have several disadvantages, as the pathogens must be cultured and the inconsistency in preparation methods results in a wide variation in their effectiveness. Therefore, there is a need to develop a recombinant vaccine which can provide protective immunity. Potential candidates for recombinant vaccine development include various virulence factors of *A. hydrophila* such as hemolysin (hlyA), protease (oligopeptidase A), adhesins and several outer-membrane proteins [3]. In addition to these virulence factors, the outer membrane proteins such as porins, which are essential for bacterial cell survival, may also be targeted for vaccine development. As components of the outer membrane, and due to their exposed epitopes [4], these are easily recognized as foreign substances by host immunological defence systems. The role of outer membrane proteins in pathogenesis for many important bacterial pathogens has also been reported [5]. Deletion mutations of *aro omp* have been reported to render enterotoxigenic *E. coli* avirulent [6]. Immunization with outer membrane proteins of various organisms including *A. hydrophila* has been reported to confer protection against the bacteria [7–9].

Porins situated at the outer membrane of gram negative bacteria play a major role in the regulation of bacterial metabolism by regulating the transportation of solutes into the cell [10]. Porins are specific for certain molecules such as sugars and non-specific for the transportation of small solutes. They exist most frequently as trimers and the sequence homology among porins of several species has shown a highly conserved nature [11].

The expression of the major outer membrane proteins of *A. hydrophila*, OmpF and OmpC, is regulated by a two component regulatory system containing *envZ* and *ompR* genes. Bacteria regulate the elevation of these porins in different environmental conditions. For example, OmpF is preferentially expressed during low osmolarity conditions or in the presence of high levels of cAMP, while OmpC is exclusively expressed in media of high osmolarities [12]. This enables the bacteria to survive in different environmental conditions. Synthetic peptides representing certain epitopes of the OmpF of *Pseudomonas aeruginosa* have been reported to confer protection against the bacterium [13]. The role of OmpR-dependent genes in the virulence of a number of bacteria has been demonstrated [14–16]. Combined mutants of OmpF and OmpC rendered the *Salmonella typhimurium* avirulent [17]. *Aeromonas* also infects human beings; however, no extensive immunization studies have been conducted to assess the immunization and protective potential of outer membrane proteins of *A. hydrophila* in a mammalian model.

As a critical constituent of the two-component regulatory system crucial for the survival of *A. hydrophila* in adverse conditions, OmpF is therefore a promising vaccine candidate against *A. hydrophila*. Therefore, the present study was undertaken to express recombinant OmpF of *A. hydrophila* and evaluate its immunogenic potential.

Materials and methods

Materials

The Ni^{+2}-NTA Fast Flow and plasmid DNA mini-prep kit were purchased from Qiagen, Germany. Expression vector pET28a (+) was purchased from Novagen, USA. *Taq* DNA polymerase was obtained from Bangalore Genie, India. DNA modification and restriction enzymes were purchased from New England Biolabs, USA. All other chemicals (analytical grade) used in the study were procured from Sigma-Aldrich Chemical Co., USA, unless otherwise stated. Nitrocellulose membrane (0.45 μM) was purchased from Millipore, USA. Oligonucleotides used in the present study were obtained from Sigma-Aldrich Chemical Co., USA.

Bacterial strains and animals

Aeromonas hydrophila strain EUS112 and other *Aeromonas* strains used in the study are listed in Supplementary Table 1. The characteristics of various *Aeromonas* isolates are given in Supplementary Table 2. *Escherichia coli* DH5α and *E. coli* BL21(λDE3) strains were procured from GIBCO BRL, USA and Novagen, USA, respectively. Female Swiss albino mice, 4–6 weeks ($n = 6$/group) were procured from JNU animal house facility. The experimental animals were maintained on feed and water ad libitum. The usage of animals for the purpose was approved by the Institutional Animal Ethics Committee of the University (IAEC approval # 7/2009).

Cloning of *ompF* gene of *A. hydrophila*

Primers were designed on the basis of the putative *ompF* sequence of *A. hydrophila* strain ATCC 7966 (NCBI Acc. No. CP000462.1). The *ompF gene* amplification was carried out using *A. hydrophila* genomic DNA as template and gene specific forward (5′-GGATCCGTGGTTTATGACAAAGA CGGTACC-3′) and reverse (complementary to 5′-ACGA GTGGACTGTTGCCCTGCAATACAACTTCTAACTCG AG-3′) primers containing *Bam*HI and *Xho*I restriction enzyme sites, respectively. The reaction was performed at the following specified conditions: initial denaturation at 95 °C for 5 min followed by 30 thermal cycles of denaturation at 95 °C for 1 min; annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min. Final extension was carried

out at 72 °C for 7 min. The *BamH*I and *Xho*I digested PCR product was cloned into the pET28a (+) vector digested with the same enzymes and transformed into competent *E. coli* DH5α cells. The transformants were selected on LB agar plates containing 50 μg/ml kanamycin and putative recombinants were confirmed by restriction enzyme digestion of the plasmid DNA and automated DNA sequencing (DNA sequencing facility, University of Delhi, South Campus, New Delhi). The recombinant construct thus made was designated *pETAhompF*.

Expression and purification of the recombinant outer membrane protein OmpF (rOmpF)

Recombinant construct *pETAhompF* was transformed into competent *E. coli* BL21 (λDE3) cells. Primary expression analysis of the rOmpF from *E. coli* BL21 (λDE3) cells harbouring the *pETAhompF* was carried out as described earlier [18]. An inoculum (1 %) from the O/N grown culture of *E. coli* BL21 (λDE3) cells harbouring the recombinant construct *pETAhompF* was inoculated in 5 ml of LB media containing 50 μg/ml kanamycin and grown at 37 °C at 200 rpm. The cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.8 $O.D_{600}$ and grown further for 4 h. Cell lysates prepared from the induced and uninduced cultures were analysed for expression of the recombinant protein on 12 % SDS-PAGE [19].

The induced *E. coli* BL21λ(DE3) cells harbouring the *pETAhompF* were grown as described in the previous section. The cells were harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. Different cellular fractions viz extracellular, cytoplasmic, periplasmic, inclusion bodies and membrane fractions, were prepared as described earlier [18] and analysed on 12 % SDS-PAGE followed by Coomassie brilliant blue staining.

Inclusion bodies from the induced culture of *E. coli* BL21 (λDE3) cells harbouring the *pETAhompF* were prepared as described by Vashishta et al. [20] with minor modifications. Secondary culture (200 ml) of *E. coli* BL21 (λDE3) cells harbouring the *pETAhompF* was induced as described earlier. After 6 h of induction, the cells were harvested at 8,000 rpm for 10 min at 4 °C and resuspended in 10 ml of lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 10 mg/ml lysozyme) followed by sonication (five pulses of 1 s for 40 cycles) till the lysate became clear. The sonicated solution was centrifuged at 13,000 rpm for 20 min at 4 °C. The pellet was washed thrice with PENGU buffer (0.2 M sodium phosphate buffer pH 7.3, 1 mM EDTA, 50 mM NaCl, 5 % glycerol and 1 M urea), followed by three washes with homogenization buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.5 % TritonX-100, 0.1 % sodium-Azide). After a final wash with 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, the pellet was solubilised in solubilisation buffer (6 M guanidinium chloride, 10 mM Tris–HCl, pH 8.0, 500 mM NaCl) for 1 h at 4 °C, followed by centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatant thus obtained represented the solubilised inclusion bodies fraction and was used for purification of the recombinant protein.

The solubilised inclusion bodies were allowed to bind to Ni^{+2}-NTA Sepharose pre-equilibrated with solubilisation buffer for 1 h at 4 °C. The non-specific proteins were removed by washing with ten column volumes (CV) of wash buffer-I (8 M urea, 20 mM Tris–HCl, pH 8.0, 500 mM NaCl). The bound rOmpF was eluted with elution buffer containing 8 M urea, 20 mM Tris–HCl, pH 8.0, 500 mM NaCl and 75 mM imidazole. Eluted fractions (1 ml each) were collected and analysed by 12 % SDS-PAGE. The fractions containing the desired proteins were pooled and dialysed using urea gradient dialysis method. Final dialysis was done against 1 × Phosphate buffer saline (PBS) using 25 kDa cut-off dialysis membrane. The protein concentration was estimated by Lowry's method [21]. The rOmpF was aliquoted in small aliquots and stored at −80 °C until further use.

Western blot analysis

Western blot analysis using anti-His-tag monoclonal antibody or anti-rOmpF antibody was carried out as described earlier [22]. The cell lysates from uninduced, induced, and control *E. coli* BL21 (λDE3) cells were resolved on 12 % SDS-PAGE and transferred onto the nitrocellulose membrane using electrode transfer buffer (25 mM Tris–HCl, pH 8.3, 192 mM glycine, 20 % (v:v) methanol). Non-specific sites were blocked by incubation in 3 % BSA in 1 × PBST (0.15 M PBS, pH 7.3, and 0.2 % Tween 20) at 4 °C O/N followed by three washes with 1 × PBST. The membrane was incubated with the primary antibody (anti-His-tag monoclonal antibody or anti-rOmpF antibody raised in mice at the dilution indicated in the legend) for 1 h at RT followed by three washes with 1 × PBST (10 min each). The membrane was then incubated with alkaline phosphatase conjugated goat anti-mouse secondary antibody (1:10,000 dilution) for 1 h at RT, followed by three 1 × PBST washes. The immunoreactive bands were visualized by the addition of Western blue stabilized substrate solution (Promega, USA). The reaction was stopped by the addition of double distilled water.

Immunization of mice with rOmpF

After collection of pre-immune sera, groups of 6 mice (Swiss albino, female 4–6 weeks) were immunized with different amounts of the rOmpF (diluted in 1 × PBS) emulsified in complete Freund's adjuvant. Boosters in incomplete Freund's adjuvant were given on day 14, day

28, and day 42. The mice were bled a week after each booster, on day 21, day 35 and day 49. Sera were collected by centrifugation at 5,000 rpm for 10 min at 4 °C and were stored in small aliquots at −20 °C until further use.

Enzyme linked immunosorbent assay (ELISA) for determination of antibody titers and antibody isotyping

The rOmpF (500 ng/100 µl) was coated in round bottom 96 well plate (Nunc, USA) and incubated overnight at 4 °C and blocked with 2 % BSA for 2 h at 37 °C. Different dilutions made in 100 µl of 1 × PBS of the anti-rOmpF antisera were added to the wells and incubated for 1 h at 37 °C. This was followed by the addition of alkaline phosphatase conjugated IgG antibody. PNPP substrate (P-nitrophenylphosphate, 1 mg/ml) made in AP buffer (1 mM $MgCl_2$ pH 9.8, 50 mM Na_2CO_3) was used for color development and analysis.

For determination of the type of immune response generated, antibody isotyping of the anti-rOmpF sera was carried out using anti-IgG1, anti-IgG2a, and anti-IgG2b, conjugated with horseradish peroxidase (1:5,000). The color was developed by addition of TMB substrate (3,3′,5,5′-Tetramethylbenzidine, BD biosciences, USA) and the absorbance was measured at 405 nm.

Agglutination assay

The assay was performed to assess the ability of anti-rOmpF antisera to agglutinate live *A. hydrophila* cells. *A. hydrophila* cells and other bacterial strains were inoculated (1 %) in LB from an overnight culture and grown for 5–6 h. For agglutination assay, 5×10^8 cfu of each were taken from the log phase culture and agglutination reaction set was made in 1 × PBS containing 1:200 dilution of the polyclonal sera. Equal numbers of *A. hydrophila* (EUS112) cells in 1 × PBS, with preimmune sera, were included in the study as a control. The reaction mix was incubated for 1 h at 37 °C followed by centrifugation at 5,000 rpm for 10 min. The pellet was resuspended in 1 × PBS. The resuspended cells were uniformly smeared on a clean glass slide and dried. The slide was heat fixed by passing through a flame transiently, and stained with methylene blue (Sigma-Aldrich Chemical Co., USA), followed by washing to remove the excess stain and visualized under microscope (Model Eclipse TE2000S, Nikon, USA).

Lymphocyte proliferation assay

Mice immunized with rOmpF were sacrificed 7 days after the last booster administration. Spleens were removed and splenocytes were isolated. RBCs were lysed using 0.9 % ammonium chloride and cells were washed with complete Dulbecco's modified eagle medium (DMEM, Biological Industries, USA) and were counted using Trypan blue. Splenocytes isolated from immunized and control groups were stimulated in vitro with rOmpF to evaluate lymphocyte proliferation. Splenocytes isolated from unimmunized mice and splenocytes from immunized mice not stimulated with rOmpF in vitro were included as controls. Splenocytes were seeded at a density of 1×10^5 cells/well in 96 well plates and stimulated with rOmpF (15 µg/ml) or Concanavalin A (ConA, 5 µg/ml, included as a positive control). The cells were incubated at 37 °C in a 5 % CO_2 humidified incubator for 24 h, 48 h, and 72 h post stimulation. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium benzidine (MTT) assay as described earlier [23]. MTT (0.5 µg/ml) was added to each well and the plate was further incubated for 2 h at 37 °C in a CO_2 incubator. DMSO was added to dissolve the formazan crystals and the absorbance was measured at 540 nm.

Cytokine ELISA

Splenocytes (1×10^5/100 µl) were treated with rOmpF (15 µg/ml) while ConA (5 µg/ml) was included as a positive control. Cells treated with an equal volume of PBS served as the control. Cells were plated in triplicates for each treatment and for each time point. Supernatant collected at different time intervals (24, 48 and 72 h) post-treatment was stored at −80 °C until further use. The levels of IFN-γ and IL-4 in the supernatant were measured using BD cytokine-ELISA kit (Becton–Dickinson pharmingen) as per the manufacturer's instructions.

Enzyme-linked immunosorbent spot (ELISPOT) assay

The immune response generated by the rOmpF immunization was also assessed by enumerating the IL-4 and IFN-γ secreting splenocytes isolated from the immunized mice using BD pharmingen ELISPOT kit. For this, 100 µl of the purified rat antimouse IL-4 or IFN-γ antibodies were coated in the multiscreen 96-well plate (Millipore, USA) at a concentration of 10 µg/ml and incubated at 4 °C O/N. After washing thrice, the non-specific binding sites in the coated wells were blocked with complete DMEM medium for 2 h at 37 °C. Thereafter, splenocytes (1×10^5/100 µl) from the immunized mice were added to each well, and the cells were restimulated with rOmpF (20 µg/ml) for 72 h at 37 °C in a CO_2 incubator. Unstimulated cells and ConA (5 µg/ml) stimulated cells were used as controls. The plate was further incubated with 2 µg/ml of biotinylated rat mouse IFN-γ and IL-4 antibody after extensive washes, followed by the addition of streptavidin-horse radish peroxidase enzyme conjugate. The spots were developed using

AEC (3-amino-9-ethylcarbazole substrate, Sigma-Aldrich Chemical Co., USA) and the SFU was counted by ELISPOT reader (IMMUNOSPOT, CTL Technologies, USA).

Cross reactivity analysis using slot−blot assay

In order to assess the cross reactivity of anti-rOmpF antisera to different *Aeromonas* isolates, a slot blot assay using cell lysates from different *Aeromonas* isolates together with other bacteria/proteins was performed. For this, the cell lysates (1 µg each) of various *Aeromonas* strains were blotted onto a nitrocellulose membrane using a slot blotter (Cleaver Scientific Ltd, UK). BSA (2 %) made in 1 × PBST was used for blocking the non-specific sites for 1 h at 37 °C. The membrane was washed thrice with washing buffer (0.15 M PBS, pH 7.3, and 0.2 % Tween 20). Mouse anti-rOmpF antisera (1:5,000 made in 1 × PBS) was added to the NC membrane and incubated for 1 h. After washing three times with 1 × PBST for 10 min each, the membrane was incubated for 1 h with anti-mouse IgG conjugated with alkaline phosphatase, followed by three washes with wash buffer. Color was developed by the addition of the NBT-BCIP (nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolyl-phosphate p-toluidine salt) Western blue substrate.

Statistical analysis

The data represent mean and standard deviation (SD) of two independent experiments performed in triplicate. Statistical analysis was performed using Student's *t* test and *p* value <0.05 was considered statistically significant.

Results

Cloning, expression and purification of rOmpF

Amplification of the mature *ompF* gene (*AhompF)* of the *A. hydrophila* strain EUS112 resulted in an amplicon of ∼1 kb. Restriction enzyme analysis of the putative recombinants generated after ligation of the *BamH*I and *Xho*I digested PCR product with the plasmid pET28a+ -digested with the same enzymes resulted in the release of an insert of the expected size, thus confirming the successful cloning of the *AhompF* in pET28a+ . BLASTX analysis of the sequence of the cloned insert (GenBank accession no. HF545837) further confirmed it to be the *ompF* of the gram negative bacteria. The recombinant clone harbouring the *ompF* encoding the mature OmpF under the control of T7 promoter was designated as *pET-AhompF*. The recombinant OmpF from this construct is expected to be of ∼40 kDa, comprising 362 amino acid residues (including 34 residues from the vector that also include the 6 × Histidine tag). Expression analysis of the recombinant Histidine tagged OmpF (rOmpF) in *E. coli* BL21 (λDE3) cells transformed with the *pETAhompF* was performed after induction with 1 mM IPTG. A band at the expected size of ∼40 kDa corresponding to the expressed rOmpF was observed in the induced cells only (Fig. 1a, lane 2). Western blot analysis using anti-His antibodies confirmed the authenticity of the rOmpF as a clear band at the expected position was detected in the induced cell lysates only (Fig. 1b, lane 2). Expression was obtained at IPTG concentrations as low as 0.2 mM (Fig. 1c). Time kinetics of rOmpF expression showed an increase in the

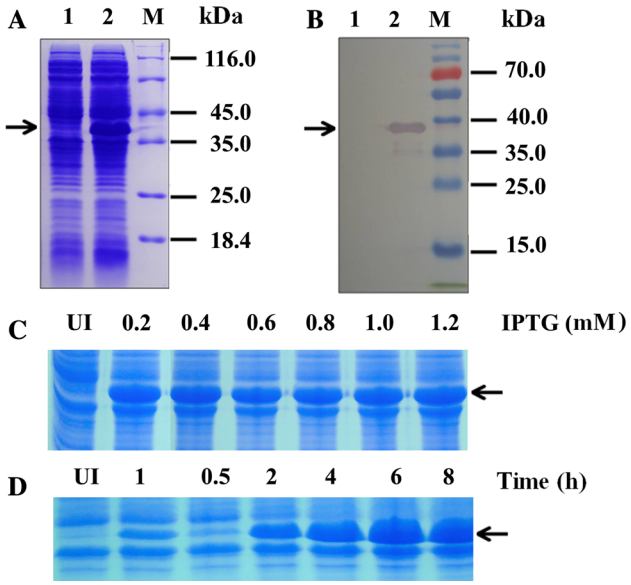


Fig. 1 **a** SDS-PAGE (12 %) analysis of the *E. coli* BL21(λDE3) cells harboring *pETAhompF* for rOmpF expression. *Lanes 1* and *2* show the cell lysates prepared from the uninduced, and induced *E. coli* BL21(λDE3) cells harboring *pETAhompF,* respectively. *M* indicates protein molecular weight (kDa) marker. The *arrow points* to the ∼40 kDa rOmpF expressed only in the induced cell lysate. **b** Western blot analysis of the rOmpF. The authenticity of the expressed product was established by immunoblot analysis using anti-His antibody. *Lanes 1* and *2* depict the uninduced and induced cell lysates of the *E. coli* BL21 (λDE3) cells harboring the pET*AhompF,* respectively. A band of ∼40 kDa (indicated by *arrow*) could be seen in the induced cell lysate only (*lane 2*). **c** Optimization of inducer concentration for rOmpF expression. *E. coli* BL21 (λDE3) cells harboring *pETAhompF* were induced with different concentrations of IPTG (shown on *top* of the panel) for 4 h. Cell lysates (∼50 µg each) were analyzed on 12 % SDS-PAGE. UI refers to the uninduced cell lysates. The *arrow points* to the rOmpF. rOmpF expression could be seen at IPTG concentrations as low as 0.2 mM. **d** Time kinetics of the rOmpF expression. Cell lysates of *E. coli* BL21 (λDE3) cells harboring p*ETAhompF* induced with 1 mM IPTG for different time periods (shown on *top* of the panel) were analyzed by SDS-PAGE (12 %). Maximum expression of rOmpF is observed at 6 h, which remained constant till 8 h. The *arrow points* to the rOmpF

expression of the rOmpF from 1 h onwards, peaking at 6 h (Fig. 1d).

Analysis of various cellular fractions prepared from the induced cell lysates of *E. coli* BL21 (λDE3) harbouring *pETAhompF* indicated that the rOmpF expressed exclusively as inclusion bodies (Fig. 2a, lane 7) and no expression was observed in any of the other fractions. Purification of the rOmpF protein using Ni–NTA affinity chromatography resulted in the elution of the rOmpF in 75 mM imidazole. The protein was purified to near 95–98 % homogeneity as can be seen in Fig. 2b (lane 6). MALDI-TOF–MS analysis of the purified protein further confirmed it to be recombinant OmpF (Supplementary Fig. 1). Approximately 120 mg/L of purified rOmpF could be obtained at shake flask level.

rOmpF immunization results in high antibody titer

Immunization of the Swiss albino mice with different amounts of the purified rOmpF resulted in high antibody titers (end point titers >1: 80,000). In the group immunized with 10 μg of the protein, a slight decrease in the titers was observed after the 2nd booster, and titers increased again after 3rd booster (Fig. 3a). Unlike the aforementioned group, mice immunized with 20 μg of the rOmpF showed a consistent increase in immunoglobulins levels with each booster (Fig. 3b). The anti-rOmpF antisera were highly specific, indicated by the single and sharp immunoreactive band at the expected size of rOmpF observed only in the induced cell lysates of the *E. coli* BL21 (λDE3) cells transformed with *pETAhompF* (Fig. 3, lane 2). No band was detected in the uninduced cell lysates (Fig. 3, lane 1).

The type of immune response generated post-rOmpF immunization is dose dependent

Antibody isotyping (i.e. determination of the levels of different types of immunoglobulins i.e. IgG1, IgG2a, and IgG2b) of the anti-rOmpF antisera elicited different types of immune responses in mice immunized with different amounts of protein. The antisera from mice immunized with 10 μg rOmpF showed IgG1:IgG2a/IgG2b ratios of less than 1 after all three boosters (Fig. 4a) indicating a Th1, or cell mediated immune response. On the other hand, immunization with 20 μg of rOmpF showed the ratio of IgG1: IgG2a/IgG2b to be ≤ 1 after the 1st and 3rd boosters, while it was greater than 1 (~ 1.9) after the 2nd booster, indicating a switch in the immune response from cell mediated to humoral to cell mediated with respective boosters (Fig. 4b).

The rOmpF stimulates proliferation of splenocytes from immunized mice

In vitro stimulation of the splenocytes isolated from rOmpF immunized mice demonstrated a strong antigen response and stimulated cell proliferation (Fig. 5). No stimulation of proliferation was observed in the splenocytes isolated from PBS-immunized control mice. The proliferation index (PI) for rOmpF-stimulated splenocytes (1.7) was significantly greater than that of the control cells (1.3).

Cytokine profile of rOmpF immunized mice

Cytokine ELISA of the culture supernatants of the splenocytes from the rOmpF immunized mice showed very high levels of IFN-γ. The IL-4 levels increased

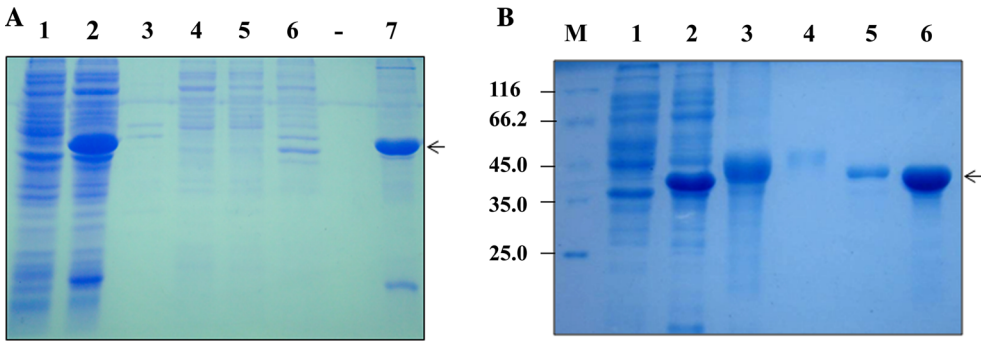


Fig. 2 **a** Localization of expression of the rOmpF. Different cellular fractions of induced *E. coli* BL21 (λDE3) cells harboring the *pETAhompF* were analyzed on SDS-PAGE. *Lanes 1* and *2* contain cell lysates prepared from the uninduced and induced cells, respectively. *Lanes 3–7* indicate extracellular, periplasmic, cytoplasmic, membranous, and inclusion bodies fractions, respectively, prepared from the induced cell lysates. A band corresponding to the expected size of rOmpF can be seen in the inclusion bodies fraction only (*lane 7*, indicated by the *arrow*). **b** Purification of the rOmpF using Ni^{+2} - NTA affinity chromatography. *Lanes 1* and *2* refer to the cell lysates of the uninduced and induced cells, respectively. *Lanes 3–5* indicate the solubilized inclusion bodies, flowthrough, wash fractions. The purified protein eluted with 75 mM imidazole is shown in *lane 6* (indicated by *arrow*). *M* indicates migration of protein molecular weight (kDa)

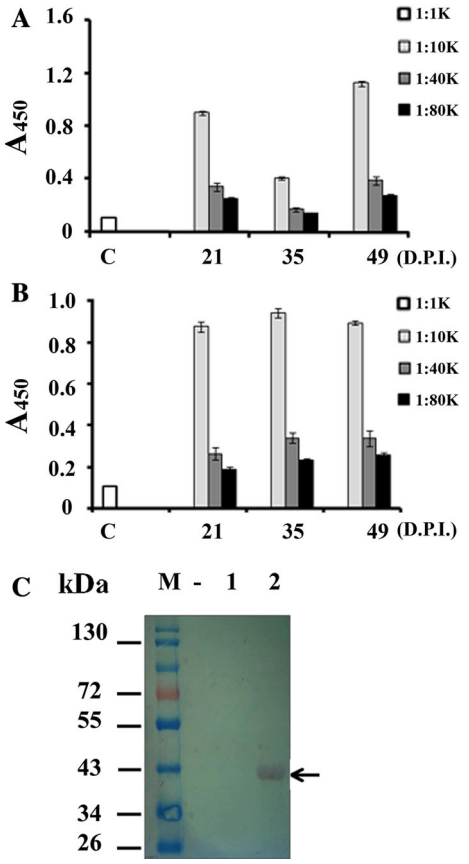


Fig. 3 **a**, **b** Antibody titer determination against the rOmpF. Sera of Swiss albino mice immunized with 10 μg (**a**) and 20 μg (**b**) rOmpF drawn on different days post-immunization (DPI; day 21, 35, and 49) were analyzed for the presence of anti-rOmpF antibodies by ELISA. Antibody titer of anti-rOmpF antibody is found to be >1:80,000. **c** Specificity of the anti-rOmpF sera by immunoblot analysis: the cell lysates of uninduced (*lane 1*) and induced culture (*lane 2*) of *E.coli BL21* (λDE3) cells harbouring *pETAhompF* were transferred on to a nitrocellulose membrane and immunoblotted with the anti-rOmpF antisera (1:10,000). A distinct immunoreactive band (indicated by the *arrow*) visible only in the induced cell lysate (*lane 2*) confirms the anti-rOmpF antisera to be highly specific. *M* indicates protein molecular weight (kDa) markers

significantly after 48 h and reached their maximum levels of 193 pg/ml ($p = 0.013$), with no further increase after 48 h when compared to that of control splenocytes (Fig. 6a). Unlike IL-4, the IFN-γ levels increased significantly from 7,000 pg/ml at 24 h ($p = 0.0004$) to ~15,000 pg/ml at 72 h ($p = 0.0001$) (Fig. 6b).

ELISPOT analysis indicates generation of mixed immune response by rOmpF immunization

Analysis of IFN-γ and IL-4 secreting cell populations in the splenocytes isolated from rOmpF-immunized mice and stimulated with rOmpF in vitro showed a significant increase in the IFN-γ and IL-4 secreting cell population after restimulation with rOmpF in immunized mice when compared to the control. The splenocytes of mice immunized with rOmpF (20 μg) showed a significant increase in the spot forming units (SFUs/10^6 cells) for both IFN-γ ($p = 0.04$) and IL-4 ($p = 0.0008$) secreting cells upon in vitro stimulation with 20 μg/ml rOmpF (Table 1) when compared to splenocytes isolated from control mice. No spots were observed in the unstimulated splenocytes isolated from immunized mice or in rOmpF-treated splenocytes isolated from unimmunized mice.

Agglutination ability of anti-rOmpF antisera

Incubation of *A. hydrophila* (Fig. 7a, d), *E. coli* DH5α (Fig. 7g) and *Staphylococcus aureus* (Fig. 7d) with pre-immune sera did not show any agglutination, whereas incubation of the anti-rOmpF antisera with live *A. hydrophila* agglutinated the bacterial cells efficiently (Fig. 7b, c) and no agglutination was observed with *E. coli* DH5α (Fig. 7h, i) and *Staphylococcus aureus* (Fig. 7k, l), indicating the specificity of the antisera towards *Aeromonas* sp. Further, pre-incubation of the anti-rOmpF antisera with rOmpF prior to addition to *A. hydrophila* (EUS112) cells resulted in loss of agglutination (Fig. 7e, f).

Cross-reactivity of anti-rOmpF antisera with different *A. hydrophila* isolates

Slot blot analysis of lysates of different *A. hydrophila* isolates (Supplementary Table 1) using anti-rOmpF antisera indicated that the antiserum is able to cross react with all the *A. hydrophila* isolates (Fig. 8a). While very intense bands were observed in the rOmpF slot (Slot B14) and in the induced cell lysate of the *E. coli* BL21 (λDE3) cells harbouring *pETAhompF* (Slot C9), no reaction was observed with negative controls such as BSA (Slot C2), control *E. coli* DH5α cell lysate (Slot C11), or with the lysate prepared from Chinese hamster ovary cells (Slot C10).

Discussion

Outer membrane proteins of bacteria are known to be immunogenic and have been reported to confer protective immunity [8, 24, 25]. Both purified recombinant proteins and whole membrane protein fraction of *A. hydrophila* have been evaluated for their immunogenic and vaccine potential against *A. hydrophila* infection in *Labeo rohita* and *Carassius auratus* [8, 26, 27]. Khushiramani et al. [8, 26] evaluated the immunogenic potential of purified recombinant OmpTs and Omp48 proteins of *A. hydrophila*. The anti-OmpTs antisera showed cross reactivity only with *A. hydrophila* and *A. sobria* [8]. On the other hand, the

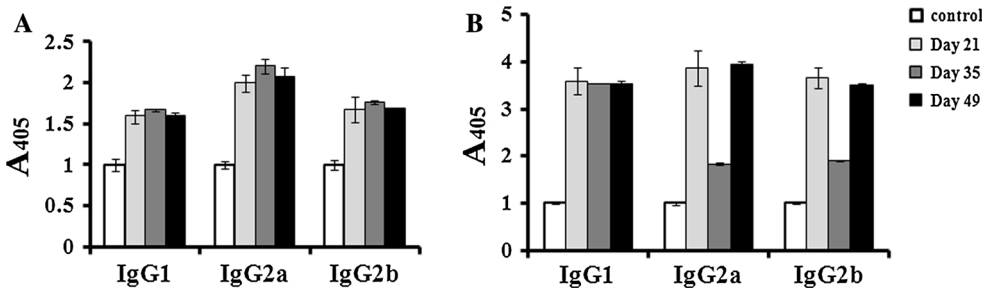


Fig. 4 Antibody isotyping of anti-rOmpF antisera raised in mice. Swiss albino mice were immunized (i.p.) with different amounts of the purified rOmpF in CFA. Sera collected at different time-points after booster injection in IFA were analyzed for the levels of antibody isotypes by using isotype-specific secondary antibodies. *Panels A* and *B* represent ELISA using mouse anti-rOmpF antisera of mice immunized with 10 and 20 μg rOmpF per mouse, respectively

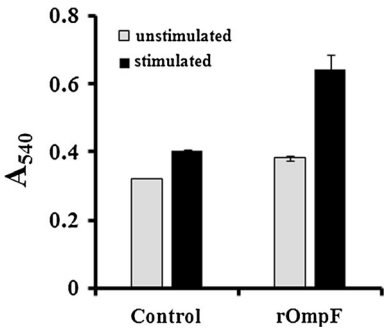


Fig. 5 In vitro stimulation of lymphocytes proliferation by rOmpF. Swiss albino mice were immunized with the rOmpF (20 μg) in CFA, followed by two boosters in IFA on day 14 and 28. Splenocytes (1×10^5 cells/well) were collected a week after the 2nd booster and cultured either in the absence (unstimulated) or in the presence of rOmpF (15 μg/ml, stimulated) for 72 h in a humidified 5 % CO_2 incubator at 37 °C. Lymphocyte proliferation was determined by MTT assay

antisera raised against the Omp48 of *A. hydrophila* could cross react with the whole cell proteins of *A. veroni, Vibrio parahaemolyticus, Edwardsiella tarda*, and *E. coli* [26]. Thanga et al. [27] investigated the vaccine potential of whole cell lysate, membrane fraction, extracellular fraction, and biofilms of *A. hydrophila* in *Carassius auratus,* and reported improved survival of the immunized fish against *A. hydrophila* infection. These investigators used ~300 μg of the membrane fraction for immunization of gold fish weighing ~16.4 ± 1 g, i.e. ~18 μg/g body weight of fish, which is several fold higher than the dose of 1.5 μg/g body weight used by Khushiramani et al. [8, 26]. Significantly higher dose of the preparation used by Thanga et al. [27] was possibly required as a mixture of membrane proteins was used for immunization.

In the present study, we have studied the immunogenic potential of outer membrane protein F of *A. hydrophila* and the modulation of cellular and humoral immunity in a murine model. The rOmpF was expressed in heterologous host (*E. coli*) and purified by affinity chromatography. The exceptionally high expression of the rOmpF (Fig. 1a, lane 2, approximately 62–65 % of total cellular protein) may be due to efficient translation of the coded mRNA by the protein synthetic machinery of the cell [28, 29]. Overexpression of the rOmpF in *E. coli* resulted in the formation of inclusion bodies as has been reported for other outer membrane proteins as well [30]. Refolding of membrane proteins is generally difficult and proteins tend to aggregate. We have been successful in refolding the rOmpF using the urea gradient dialysis method, possibly due to the absence of disulfide bonds in the OmpF. Further, long term storage of the refolded rOmpF did not result in aggregation, indicating that the rOmpF attained a stable conformation upon refolding. Thus, in addition to very high expression, high yields of the refolded rOmpF were achieved suggesting that refolding did not result in major losses of the purified rOmpF.

Antisera generated against the rOmpF was of very high endpoint titers. When compared to the OmpTs of *A. hydrophila* [8], the rOmpF is significantly more immunogenic than the OmpTs of *A. hydrophila* as high end point titers (>1:80,000) were obtained with only 10 μg of the rOmpF. The antisera generated against the rOmpF was able to agglutinate the live *A. hydrophila* in vitro, suggesting that the antisera has neutralizing potential. Agglutination assays have been used for the identification of bacterial strains [31, 32]. Since the anti-rOmpF antibodies specifically agglutinated *A. hydrophila* cells only, these anti-rOmpF antibodies could be used for identification of *Aeromonas*. Loss of agglutination ability of anti-rOmpF antisera by pre-incubation with rOmpF clearly indicates the specificity of interaction between the antibodies present in the anti-rOmpF antisera and OmpF on *A. hydrophila* membrane.

Immunization with lower concentrations of the rOmpF (10 μg) resulted in predominantly a Th1 (cell mediated, type I) type immune response, which changed to a Th2 (humoral, type 2) immune response when the mice were immunized with higher concentration (20 μg) of the rOmpF. Our results are in agreement with previous reports

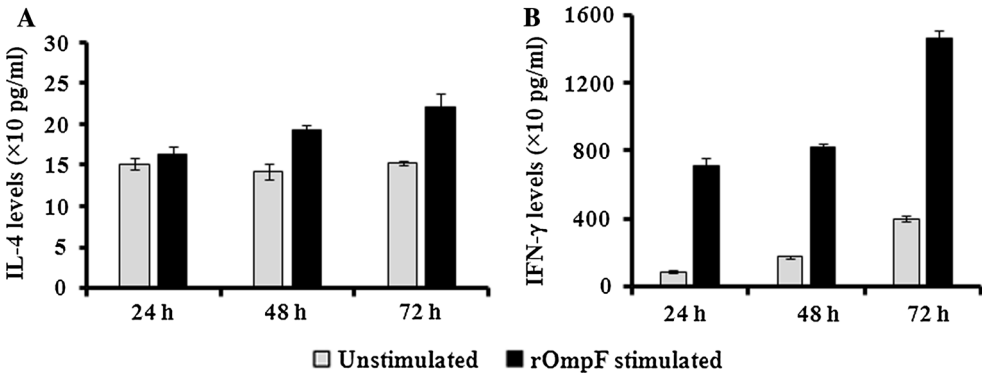


Fig. 6 Analysis of in vitro T cell response by Cytokines ELISA. Splenocytes (1×10^5 cells/well) isolated after 7 days of the administration of the second booster of rOmpF (20 μg/mouse) were stimulated in vitro with 15 μg/ml of rOmpF. Culture supernatants were collected at 24, 48, and 72 h post-stimulation and analyzed by cytokine ELISA for IL-4 (*Panel A*) and IFN-γ (*Panel B*) levels. The levels of both IFN-γ and IL-4 in the culture supernatants of stimulated splenocytes are higher when compared to their respective levels in the culture supernatant of unstimulated control splenocytes

Table 1 In vitro evaluation of T cell response by ELISPOT

Experimental group	IFN-γ SFU/10^6 cells (mean ± SD)	IL-4 SFU/10^6 cells (mean ± SD)
PBS-immunized	No spots	No spots
rOmpF-immunized (unstimulated)	No spots	No spots
rOmpF-immunized (restimulated with rOmpF)	26333 ± 321	91.6 ± 4.5

Swiss albino mice were immunized with 20 μg rOmpF in CFA followed by two boosters in IFA as described earlier. PBS-immunized mice were included as controls. Splenocytes isolated 7 days after administration of the 2nd booster were restimulated with 20 μg/ml rOmpF for 72 h. Different sets of splenocytes were analyzed for IFN-γ and IL-4 secreting cells (BD ELISPOT kits) using ELISPOT reader. The data are represented as SFU (spot forming unit) and expressed as mean ± SD of the experiment, performed in triplicates

by Spellberg and Edwards [33], who demonstrated that the antigen dose is an important determinant of the elicited immune response. Immunization with higher concentrations of the antigen shifts the immune response from Th1 to Th2, protecting the host from the devastating effect of a cytotoxic immune response, which can cause tissue necrosis and liver damage in the process of combating the infection [34].

An increased proliferation of the splenocytes isolated from the rOmpF immunized mice upon in vitro restimulation indicates that the rOmpF immunization is able to generate T-cell memory. Increased levels of both IFN-γ and IL-4 in the culture supernatants of the stimulated splenocytes of the rOmpF-immunized mice suggest a mixed immune response (cell mediated as well as humoral). However, the relatively higher levels of IFN-γ in comparison to IL-4 levels indicate that the rOmpF immunization resulted in a predominantly Th1 immune response. Our results are in accordance with the immunization studies conducted with the OmpF of *Pseudomonas aeruginosa* which also elicited a predominantly Th1 immune response [4]. Mice strains which produced high levels of IFN-γ in response to bacterial infections were able to overcome and clear the microbial infection more effectively [35]. Since vaccines promoting a Th1 immune response have been found to be more protective against chronic *P. aeruginosa* pneumonia [36], *Yersinia* [37], and *Klebsiella* [38], it is expected that an rOmpF immunization resulting in a predominantly Th1 immune response will be able to offer protection against *A. hydrophila* infections. In addition to participating in the cell mediated immune response, Th1 cells are capable of eliciting antibody production by B-cells, enhancing the effectiveness of the immune response [39]. As earlier reports have shown that in animal models, vaccines that generated Th1 or mixed type immune response provided better protection in comparison to those which induced only a Th2 response [40], rOmpF, that generated a predominantly Th1 mixed immune response would likely prove to be a good vaccine candidate.

A desired characteristic of a vaccine candidate is its ability to recognize, and be effective against various strains of a bacterial species. Guan et al. [25] have reported that immunization with a recombinant outer membrane protein conferred protective immunity against two strains of *A. hydrophila*. *Aeromonas hydrophila* is a highly heterogeneous group of bacteria and therefore, it is all the more important that the antisera raised against a potential vaccine candidate is able to interact with as many strains of this bacterium as possible. The conservative nature and surface exposure of the outer membrane protein further makes it attractive as a potential vaccine candidate. The antisera raised against the rOmpF of *A. hydrophila* (EUS112) was able to interact with the whole cells lysates

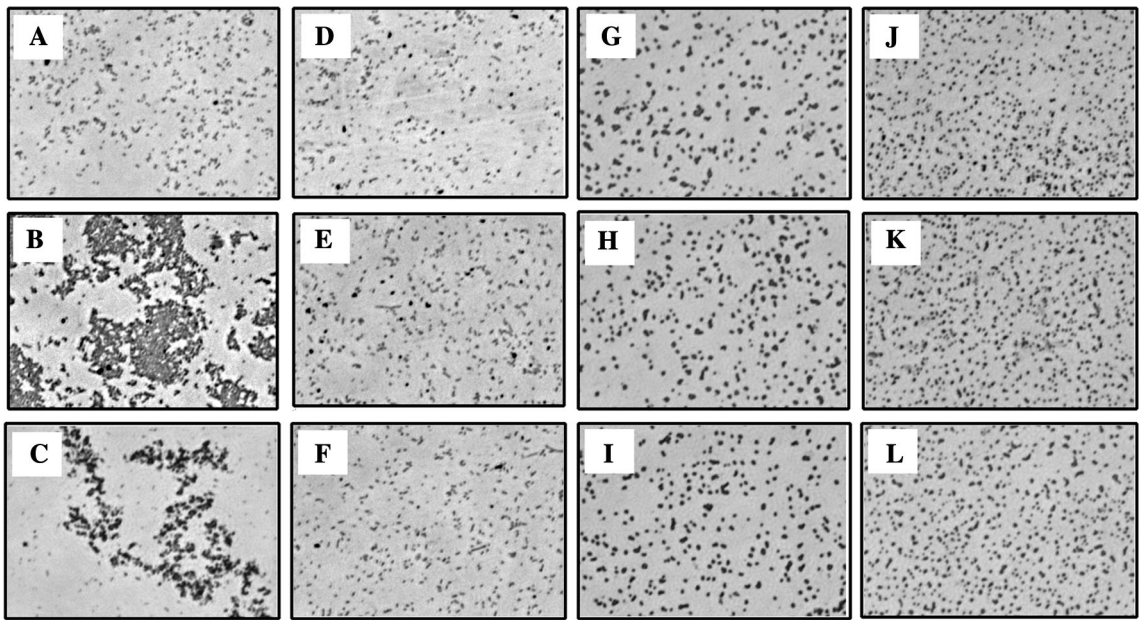
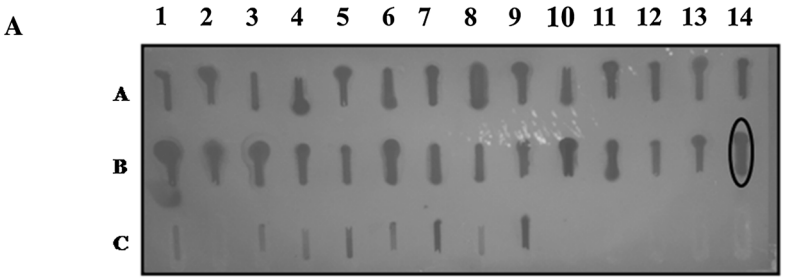


Fig. 7 Agglutination ability of anti-rOmpF antisera. Live *A. hydrophila* (strain EUS112), *E. coli* DH5α *and Staphyococcus aureus* (MTCC, India) cells (5×10^8 CFU each) in 0.5 ml PBS were incubated with either pre-immune serum or anti-rOmpF antisera (1:200 dilution each). **a**, **d** show *A. hydrophila* cells pre-incubated with pre-immune sera whereas **g**,**j** show *E. coli* DH5α *and S. aureus* pre-incubated with pre-immune sera. **b** and **c** show the *A. hydrophila* incubated with anti-rOmpF antisera, whereas **e**, **f** show the *A. hydrophila* treated with anti-rOmpF antisera that was incubated with rOmpF (1.5 μg/μl of neat antisera) for 30 min prior to the addition to the cells. **h**, **i**, **k**, **l** show the *E. coli* DH5α (**h**, **i**) and *S. aureus* (**k**, **l**) incubated with anti-rOmpF antisera. Agglutination is visible only in *A. hydrophila* cells that were incubated with anti-rOmpF antisera. Images are taken at ×40 magnification



B

Slot	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	EUS112	Ah30	Ah31	Ah32	Ah34	Ah35	Ah37	Ah38	Ah39	Ah40	Ah41	Ah42	Ah43	Ah1
B	Ah2	Ah3	Ah4	Ah8	Ah9	Ah10	Ah15	Ah17	646	2654	1944	3249	1608	rOmpF
C	F/HF2	BSA	A/OS5	B/CB4	A/OS2	E/OS2	Ah7	UI	I	CHO-K1	DH-5α	-	-	-

Fig. 8 Cross-reactivity analysis of mouse anti-rOmpF antisera with different *Aeromonas* strains/isolates. Cell lysates (1 μg/12 μl) of various *Aeromonas* strains were blotted on a nitrocellulose membrane and immunoblotted with the anti-rOmpF antibody (1:5,000). Secondary antibody (alkaline phosphatase conjugated anti-IgG antibody) was used at a dilution of 1:10,000 and the color was developed by NBT-BCIP Western blue substrate. *Panel A* shows the slot blot analysis whereas *panel B* shows the details of bacterial strains and lysates spotted at different slots. Purified rOmpF (B14) was included as a positive control whereas BSA (*C2*), CHO-K1 (*C10*) cell lysates, and *E. coli* DH5α cell lysates (*C11*) were included as negative controls. *Spots C8* and *C9* represent cell lysates from the uninduced and induced *E. coli* BL21 (λDE3) cells harboring the *pETAhompF*, respectively. The immunoreactive band was visible in the cell lysate of all the isolates of *Aeromonas* while no band was seen in the negative controls

of a number of *Aeromonas* strains, as indicated by Slot blot analysis, and hence can be used as a potential vaccine against the heterogeneous *Aeromonas spp.*

Thus, the present study reports for the first time, a comprehensive analysis of the immune response generated by the recombinant outer membrane protein F of *A.*

hydrophila in a murine model. The results clearly indicate that the rOmpF of *A. hydrophila* can be used as a potential and effective vaccine candidate against *A. hydrophila.*

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