



Immunogenicity of recombinant outer membrane protein (OmpW) of *Pasteurella multocida* serogroup B:2 in mouse model

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ABSTRACT

Haemorrhagic septicaemia (HS) caused by *Pasteurella multocida* serogroup B:2, in cattle and buffalo especially in tropical regions of Asian and African countries, is known to possess several outer membrane proteins (OMPs) as virulent factors which are being targeted to evaluate their immunogenicity and protective efficacy as candidate antigens for vaccine. In the present study, *ompW* gene encoding for OmpW protein of *P. multocida* serogroup B:2 strain P52, an Indian HS vaccine strain, has been cloned and over-expressed in recombinant *Escherichia coli*. The recombinant OmpW fusion protein (~37 kDa) including histidine tag was purified by affinity chromatography under denaturing condition and confirmed by Western blotting. Further, mice immunized with rOmpW (50µg/dose) along with FCA/FIA resulted in antigen specific IgG antibodies as well as subtypes (IgG₁ and IgG_{2a}). The study indicated the potential possibilities to use the rOmpW antigen in developing subunit vaccine for HS as well as other diseases caused by members of Pasteurellaceae.

Key words: Haemorrhagic septicaemia, Immunogenicity, Mouse model, *Pasteurella multocida*, Recombinant OmpW protein

Haemorrhagic septicaemia [HS], predominantly caused by *Pasteurella multocida* serogroup B:2 strains in Asian tropical countries, is known to affect large ruminants (cattle and buffalo) leading to high morbidity and mortality (Shivachandra *et al.* 2011). Although, several control strategies including use of modified live/killed/subunit vaccines have been tried in the past to prevent the disease outbreak (Verma and Jaiswal 1998, Ahmad *et al.* 2014), none of them has been absolutely effective due to variability in strains and their virulence factors (Biswas *et al.* 2004, Hatfaludi *et al.* 2010). Identification and evaluation of key virulence factors especially surface proteins of *P. multocida* strains is of paramount importance for design and development of efficient control measures including novel vaccine (Wilkie *et al.* 2012).

HS as well as different disease manifestations caused by *P. multocida* strains are presumably driven by various incriminating stress factors (Bisgaard 1993). Comparison of draft genomes of the HS causing strains identified 96 unique genes (Moustafa *et al.* 2015) which need to be characterized in order to attribute their role in virulence and immunity. Nevertheless, in the recent past, several outer

membrane proteins with their distinct roles such as Omp16, Omp87, OmpH, TbpA, HbpA, HemR, HasR, VacJ and many more have been evaluated for their immunogenicity in animal model with varying protective efficacies (Hatfaludi *et al.* 2010, 2012; Kumar *et al.* 2013, Shivachandra *et al.* 2014, 2015, 2017). Unfortunately, bacterial surface proteins involved in physiological modifications and retention of virulence factors/antigens under adversaries have not yet been fully characterized. Recently, a very limited comparative whole genomic and proteomic analysis of selected *P. multocida* strains revealed presence of *ompW* gene encoded OmpW protein (Paustian *et al.* 2001, Boyce *et al.* 2006, Fernández-Rojas *et al.* 2014), which is one among several uncharacterized proteins till date.

OmpW protein encoded by *ompW* gene is a member of ubiquitous small family of OMPs, characterized well in several gram-negative bacterial species (e.g. *Escherichia coli*, *Vibrio cholerae*), and attributed its multifunction role in osmoregulation, anti-phagocytosis and transporter of small molecules (Albrecht *et al.* 2006, Wu *et al.* 2013). Although, well characterized OmpW proteins were utilized in development of novel diagnostics and vaccines against several gram-negative bacterial species such as *E. coli*, *V. cholera*, and *A. hydrophila* (Nandi *et al.* 2000, 2005, Maiti *et al.* 2012), no such studies have been reported with respect to OmpW protein among *P. multocida* strains. Hence, our study reports the immunogenicity potential of recombinant

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OmpW protein of HS causing *P. multocida* in mouse model.

MATERIALS AND METHODS

Bacteria, vector and host cells: *Pasteurella multocida* serogroup B:2 strain P52 (an Indian HS vaccine strain) maintained in the 'Clinical Bacteriology laboratory, ICAR-IVRI, Mukteswar, India, was used. A prokaryotic expression vector (pET32a) and bacterial host cells such as *E. coli* strain Top10F' and *E. coli* BL21-CodonPlus (DE3)-RIPL were used.

Cloning of OmpW gene: Genomic DNA isolated from *P. multocida* B:2 strain P52 as per the standard procedure, was used as template for amplification of *ompW* gene. Initially, the OmpW protein characteristics of *P. multocida* were predicted by proteomics tools available at ExPaSy website.

A primer set targeting *OmpW* gene sequence (Nucleotide region–Nt: 37–55 and Nt: 552) from *P. multocida* F:1 encoding for mature *OmpW* (13A–E₁₈₄ aa) without signal peptide sequence (1 M–A₁₂ aa) and terminal 20 aa (185H–F₂₀₄) was designed based on available gene sequence (NC_002663) (May *et al.* 2001). The sequences of the oligonucleotides with added restriction sites (underlined) for *Bam*HI and *Xho*I at 5' end of forward and reverse primer respectively, were as follows:

OMWNF: 5'-CGCGGATCCGCATTAGTGGCAGGTT-CGG-3' and OMWNR: 5'GTGCTCGAGCTCTACATC-ACCAA-CTTTAA-3'

The target *OmpW* gene was amplified using gene-specific primers and standard PCR conditions described earlier (Chacko *et al.* 2015). Both the PCR product and the pET32a vector were purified by gel extraction, digested with *Bam*HI and *Xho*I, and ligated using T4 DNA ligase to generate clone (pOmpW). Recombinant plasmids were transferred into *E. coli* strain Top10F' and, subsequently, into expression host cells, *E. coli* BL21-CodonPlus (DE3)-RIPL, followed by selection using ampicillin (50 µg/ml) and chloramphenicol (35 µg/ml).

Expression and purification of recombinant OmpW protein: For induction, *E. coli* BL21-CodonPlus (DE3)-RIPL cells were grown in 1 L LB broth in a shaking incubator at 37°C before induction with 1 mM IPTG. After 3h post induction, cells were harvested and cell pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and lysozyme) before lysis by sonication (Sonics, USA) and analysis of cell lysate using 10% SDS-PAGE. For purification of rOmpW under denaturing condition, the insoluble fraction of lysate was solubilized using denaturing buffer (8 M Urea, 50 mM Tris-HCl, pH 7.8, 100 mM NaCl and 10 mM imidazole) and purified by affinity chromatography using Ni-NTA superflow cartridges (Qiagen, USA) as per the standard protocol (Shivachandra *et al.* 2012, 2015).

Immunogenicity of rOmpW in animal model: Swiss albino mice (16; 6–8 week old) reared in pathogen-free environment and maintained at 'Laboratory animal section', ICAR-IVRI, Mukteswar, India, were used. Two groups (O

and C) with 8 mice each were formed. Each mouse in Group 'O' received rOmpW (50 µg/dose) along with equal volume of FCA in a total volume of 100 µl through subcutaneous route. On 21 day, booster dose of antigen was given along with FIA. The animals in control Group 'C' received PBS only. All the animals were fed and watered *ad lib.* and monitored regularly for 56 days. All the laboratory animal experiments were conducted according to the norms of Institutional Animal Ethics Committee (IAEC), ICAR-IVRI, Mukteswar, India.

All the immunized as well as control group mice were bled on 0, 21, 42 dpi and subjected for detection of rOmpW specific IgG as well as subtypes (IgG₁ and IgG_{2a}) in serum by an indirect-ELISA using purified OmpW protein (100 ng/well) as a coating antigen and assay was performed as per the standard protocol described earlier (Kumar *et al.* 2013, Shivachandra *et al.* 2014). For determining the significance of the observations, Student's t-test was carried out using SPSS 16.1 software. P values ≤0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Our initial attempt to understand the presence of *ompW* gene/protein among several *P. multocida* strains by a comparative phylogenetic, multiple sequence alignment and structural features with that of homologues in other bacterial species revealed the presence of evolutionarily highly conserved OmpW protein across members of Pasteurellaceae (Yogisharadhya *et al.* 2019). The characteristics of OmpW protein were predicted by proteomics tools from ExPaSy website. The predicted homology structure of OmpW protein of *P. multocida* revealed a predominant eight strands region (β1 to β8) forming an ellipsoid barrel shape in outer membrane with four external extended loops (L1 to L4) space as well as short turns (T1 to T3) on periplasmic side, which indicated similarity with that of OmpW protein of *E. coli* (Albrecht *et al.* 2006, Hong *et al.* 2006).

Cloning, expression and purification of rOmpW protein: Initial bioinformatics analysis revealed that OmpW protein was encoded by a 615 bp ORF with 40.3% GC. Full length OmpW protein (204 aa, MW ~21.9 kDa) was predicted to contain a signal peptide (1–12 aa) using PSIPRED (Fig. 1 Panel A). OmpW also had a very high antigenic index, and hydrophilicity as shown by PROTEAN (Fig. 1, subpanels a, b, c). The expression construct was designed such that the OmpW region was fused between hexa-histidine tags on both termini. The design of recombinant construct is shown schematically in Fig. 1 Panel 2. The PCR amplified product, ~519 bp was cloned in pET32a vector and recombinant clones were confirmed by colony PCR.

Induction of recombinant cells resulted in over-expression of rOmpW protein which accounted for a total molecular weight of approximately 36.8 kDa (342 aa), of which ~18 kDa (169 aa) was OmpW, and ~18.8 kDa (173 aa) was the coding region of pET32a vector including the two hexa-histidine tags, as appeared on 10% SDS-PAGE

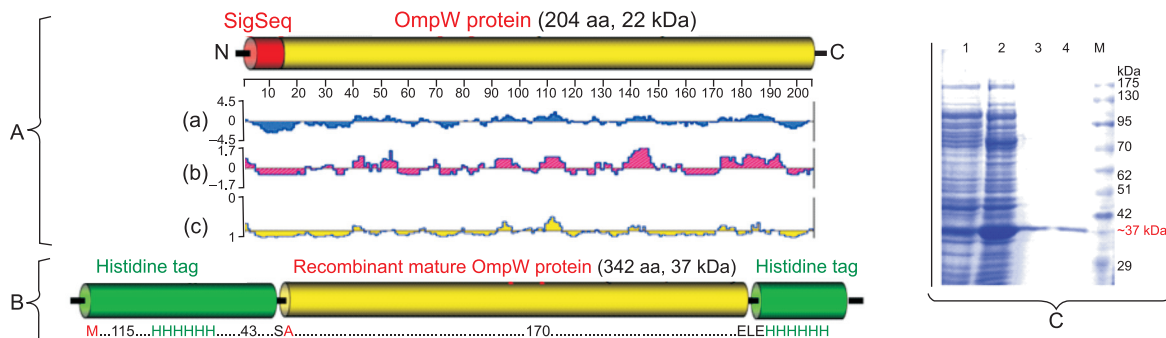


Fig. 1. Schematic of mature OmpW construct, expression, purification and detection. Panel A: Full length OmpW protein; Subpanel: Predicted characteristics of OmpW protein by PROTEAN programme of DNASTAR: (a) hydrophilicity plot as per Kyte-Doolittle, (c) antigenic index as per Jameson-Wolf and (d) surface probability plot as per Emimi; Panel B: Recombinant OmpW protein with N- and C-terminus hexa-histidine tag. The rOmpW fusion protein was found to contain a total of 30 basic, 40 acidic, 129 hydrophobic and 74 polar residues with an isoelectric point of 6.274; Panel C: Analysis of OmpW protein expression and purification on 10% SDS-PAGE; Lanes-M: Protein standard marker; Lane 1: Uninduced *E. coli* cell lysate; Lane 2: Induced *E. coli* cell lysate showing expressed rOmpW; Lane 3 and 4: purified rOmpW protein (~37 kDa) fractions by affinity chromatography. N-, amino terminus; C-, carboxyl terminus; SigSeq, signal sequence; aa, amino acid; kDa, kilo dalton.

(Fig. 1, Panel C, Lane 2). The solubility analysis following cell lysis indicated the partitioning of over-expressed rOmpW into the insoluble fraction of the lysate. Upon purification, the purified rOmpW protein migrated as a single band at ~37 kDa during 10% SDS-PAGE (Fig. 2, Panel C, Lane 3). The rOmpW protein was also detected by an immunoblot assay using polyclonal antibodies against *P. multocida* B:2.

The expression of OmpW protein was found to be influenced by variables such as availability of nutrients (iron, salinity) various environmental (temperature, osmolarity, pH) and stress inducing factors. To note, the expression of OmpW was found to be regulated by iron in case of *E. coli* (Wu *et al.* 2013). A seven-fold down-regulated *ompW* gene expression in *P. multocida* in response to low iron was noted by DNA microarray analysis (Paustian *et al.* 2001). Contrary, *ompW* gene was up-regulated by 2.55 fold in *P. multocida* *crp* mutant strain following transcriptome sequencing analysis (Zhao *et al.* 2016). In correlation to genomic studies, proteomic studies of *in vitro*-grown *P. multocida* revealed presence of OmpW protein in the sarcosine-insoluble membrane fraction (Boyce *et al.* 2006). It implies that the OmpW of *P. multocida* strains could be playing a vital role in iron-related infectious diseases such as HS, etc.

In this report, upon induction of recombinant *E. coli* cells, we observed successful expression of rOmpW fusion protein (Fig. 1, Panel C). As the rOmpW partitioned in to insoluble fraction due to high hydrophobic residues (129), denaturation/renaturation method was employed during affinity chromatography. Previously also, rOmpW was purified from the inclusion bodies under denaturing conditions (Stanczak *et al.* 2012). No oligomeric state of the purified rOmpW observed during SDS-PAGE analysis. Since, earlier studies indicated that rOmpW failed to demonstrate porin-like activity in liposome-swelling data (Nandi *et al.* 2005), we did not perform any functional

characterization studies on monomeric rOmpW of *P. multocida*.

Immunogenicity of rOmpW protein: Following mice immunization trials, the serum antibody titres of rOmpW specific IgG and subtypes (IgG₁ and IgG_{2a}) at different days of post immunization are indicated in Fig. 2. The change in serum antibody titers (total IgG, IgG₁ and IgG_{2a}) of immunized mice showed that there were significant differences between the immunized mice and the control group from 21 to 42 days after immunization ($P < 0.05$). The rOmpW specific IgG antibody as well as subtypes titres showed increasing trend (~8 fold) following booster dose. The ratio of IgG₁/IgG_{2a} was 1.03 and 0.75 in immunized and control mice groups, respectively, at 42 dpi. There was no significance ($P = 0.0605$) between IgG₁ and IgG_{2a} levels

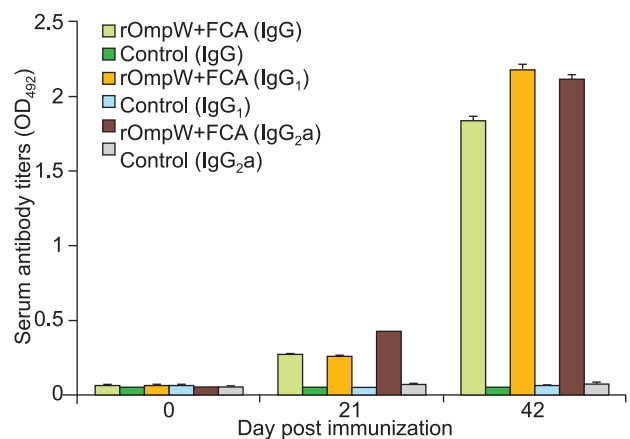


Fig. 2. Immune response and protective efficacy of rOmpW in mouse model. Immune response in rOmpW (50 µg) immunized mice. The bar diagram indicating the rOmpW specific serum antibody titers (IgG, IgG₁ and IgG_{2a}) of sera collected from all the mice at 0, 21 and 42 days post immunization with rOmpW as measured by indirect-ELISA and recorded at OD 492 nm. The absorbance values are means \pm standard error (SE) of eight mice per group.

in 42 dpi in immunized mice group.

Immunogenicity and protective potential of OmpW protein has been well established in several other bacterial species in the past (Nandi *et al.* 2005). In a very recent preliminary analysis, native OmpW protein of different *P. multocida* serogroups was found to be immunogenic as it reacted with hyperimmune sera from chicken during characterization of outer membrane vesicles (OMVs) released into the culture medium (Fernández-Rojas *et al.* 2014). We also noted rise in antigen specific serum IgG antibodies in mice immunized with rOmpW antigen of *P. multocida* indicating immunogenicity potential. Although the cellular immune response was not examined in this study, the serological response ($IgG_{2a}/IgG_1 = 1.03$) suggested both Th-1 and Th-2 response (Fig. 2). The generated antibodies could provide protection in host species following micro-agglutination or immobilization of *Pasteurellae* mediated through binding of antibodies to the OmpW protein and blocking of small molecules transport across the membrane, which is required for survival of bacteria in the host environment. In a fowl cholera infection model, rOmpW (100 µg/chicken) was found to be immunogenic (Hatfaludi *et al.* 2012). Notably, in rabbit model, antisera against OmpW protein was found to provide protection to varying degrees against challenge with live *V. cholerae* cells of both homologous and heterologous serotypes (Das *et al.* 1998). Arguably, rOmpW protein of *P. multocida* could act as an attractive candidate target for development of an alternative recombinant subunit vaccine along with synergistic antigens/adjuvants to provide a cross-protection against diverse infections (HS/Pasteurellosis) caused by capsular (A, B, D, E and F) and somatic serotypes (1–16) of *P. multocida* strains, which need to be evaluated in respective host species following challenge studies.

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