

## Comparative immunogenicity and protective efficacy of different preparations of outer membrane proteins of *Pasteurella multocida* (B:2) in a mouse model

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### ABSTRACT

The present study was undertaken to evaluate the protective efficacy of recombinant outer membrane protein (OmpH) of *Pasteurella multocida* (B:2). The 34 kDa native OmpH protein was purified from OMP-rich extract. Cloning, expression and purification of recombinant OmpH were carried out. Protection studies in mice with sonicated whole cell lysate (Group I), OMP (Group II), native 34 kDa OmpH (Group III) and recombinant OmpH (Group IV), along with a positive (Group V; commercial vaccine) and negative control (Group VI) showed a survivability percentage of 83.33%, 100%, 50%, 63.67% and 83.33% in Groups I-V, respectively, against 100% mortality in Group VI. Serum IgG titres of 30 pooled sera samples from each of the 5 groups, collected at weekly intervals, was assessed using i-ELISA. In Group I, a significant increase was observed in titres on d7, d14 and d21, having mean values (log<sub>10</sub> titre) of 2.58, 3.93 and 4.49 respectively. In Group II, there was a significant increase in antibody response on d7, d21 and d28, having mean values of 3.0, 4.04 and 4.41, respectively. In Group III, there was a significant increase in titres on d7, d21 and d28, with a mean titre of 3.70, 4.23 and 4.67, respectively. In Group IV there was a significant increase in titres on d7 and d14, having titre values of 3.36 and 3.58, respectively. In the positive control, a significant increase in titres was obtained on d7 and d14 with titres of 4.06 and 4.41, respectively. Taking these observations together, it may be concluded that OMP plays an immuno-protective role and possesses strong potential for the development of a candidate subunit vaccine against HS.

**Key words:** *Pasteurella multocida*, OMP, OmpH, recombinant, vaccine

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## Introduction

*Pasteurella multocida* is a Gram negative coccobacillus that causes a wide range of clinical presentations within a broad host range, including pigs, sheep, goats, camels, deer etc. (CHRISTENSEN and BISGAARD, 2006). However it primarily affects water buffaloes and cattle, where it causes hemorrhagic septicaemia (HS), an acute and highly fatal septicaemic disease prevalent in South and South-East Asia and sub-Saharan Africa, caused by specific serotypes of *P. multocida* i.e. B:2 in Asia and E:2 in Africa. In India, it is estimated to cause economic losses of more than 10 million rupees annually (VENKATARAMANAN et al., 2005) thus it is responsible for maximum mortality of livestock in the country, according to the report by the National Animal Diseases Referral Expert System (NADRES) of the Project Directorate on Animal Disease Monitoring and Surveillance (2012). The pathogenesis of the disease is due to complex interactions between host factors and specific bacterial virulence factors, such as: lipopolysaccharides (LPS), capsule, outer membrane proteins (OMP), fimbrial protein and putative haemolysin (BOYCE et al., 2010; CHACHRA et al., 2011; YASIN et al., 2011).

Existing vaccines, such as alum precipitated or oil adjuvanted whole cell bacterin, have certain limitations and the live vaccine based on an antigenically related deer strain of *P. multocida* (serotype B: 3, 4) has been questionable for its safety (MYINT and CARTER, 1990). A modified double emulsion vaccine provided 100% protection under experimental conditions in rabbits (KUMAR et al., 2012). The current focus on developing a newer vaccine has identified membrane antigens as potential immunogens in Gram negative bacteria (ZHOU et al., 2009; BHAT and JAIN, 2010). Likewise, OMP of *P. multocida* have been implicated as protective immunogen, as reviewed by HATFALUDI et al. (2010). The immunogenicity of OMP of *P. multocida* has been demonstrated in chickens (ZHANG et al., 1994; LUO et al., 1997; LUO et al., 1999; STHITMATEE et al., 2008; HERATH et al., 2010), calves (PATI et al., 1996; DABO et al., 1997), rabbits (CONFER et al., 2001; WASNIK et al., 2004), goats (ZAMRI-SAAD et al., 2006) and mice (BASAGOUDANAVAR et al., 2006; LEE et al., 2007; KHARB and SHIV CHARAN, 2010; TAN et al., 2010; KHARB and SHIV CHARAN, 2011; KUMAR et al., 2011).

OmpH is a surface-exposed conserved immunodominant porin that is detected in 100% of bovine isolates, and it has been viewed as a potential vaccine candidate (DABO et al., 2008). It contains a high proportion of antiparallel  $\beta$ -chains, giving it a barrel shape (CHEVALIER et al., 1993; CONFER, 1993). Work has been carried out on the prospect of using OmpH as a subunit vaccine in native and recombinant forms. Its protection has been demonstrated as a subunit or synthetic peptide vaccine against fowl cholera (LUO et al., 1997; LUO et al., 1999; LEE et al., 2007) and atrophic rhinitis (KIM et al., 2006). TAN et al. (2010) obtained high antibody titres against recombinant OmpH, using four local Malaysian strains of *P. multocida* serotype B:2. Additionally, a novel DNA vaccine

encoding OmpH and OmpA has been found to confer resistance against avian *Pasteurella multocida* (GONG et al., 2013).

Therefore, the present study was carried out to evaluate the efficacy of native and recombinant OmpH in providing protection against *P. multocida* serotype B:2.

### Materials and methods

**Animal.** Laboratory-bred male Swiss albino mice (*Mus musculus*, 25-30 g), procured from laboratory animal house facility, IVRI, Mukteswar, were used as the experimental host. They were housed in a climatically controlled room and fed with standard feed and water *ad libitum*. The use of mice was approved by the Institute's Animal Ethical Committee.

**Bacterial strain.** The vaccine strain P<sub>52</sub> of *P. multocida* B:2 was obtained from the Biological Products Division, Indian Veterinary Research India (IVRI), Izatnagar, India and maintained on brain heart infusion agar enriched with 5-10% (v/v) sheep blood by incubating at 37 °C for 18 h.

**Purification of native OmpH protein.** OMP-enriched fractions were extracted employing the standard protocol (CHOI-KIM et al., 1991) as described previously (SINGH et al., 2011). The OMP pellet was resuspended in PBS (pH 7.4), aliquoted and stored at -20 °C. Protein concentration was determined using bovine serum albumin, as standard, employing the method of LOWRY et al. (1951). For purification of OmpH, the band corresponding to 34 kDa was excised from the preparative gel, of 3 mm thickness, and incubated in a rotary shaker at 30 °C overnight in 1 ml elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5). Centrifugation was done at 7,000 g for 10 min to obtain the supernatant as OmpH. The protein was tested for the presence of single band in SDS-PAGE.

**Expression and purification of *P. multocida* recombinant OmpH protein.** In order to generate recombinant OmpH, an open reading frame of OmpH was amplified by PCR using *P. multocida* P<sub>52</sub> genomic DNA as template and the forward 5'-TCAGGATCCCAGCAACAGTTTACAATCAAGA-3' and reverse 5'-CTACCCGGGTTAGAAGTGTACGCGTAAACCA-3' primers with BamHI and SmaI restriction sites. Cloning of the *ompH* gene was undertaken into the pQE32 expression vector (Qiagen, USA) as per SINGH et al. (2009). The positive transformants were sequenced by South Campus, Delhi University (New Delhi, India) and submitted to the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/nucleotide/HM117642>; Accession No. HM117642). The plasmid carrying the *ompH* gene was transformed into *E coli* M15 cells and grown in Luria Bertini (LB) broth to OD<sub>600</sub> of 0.6. The recombinant cells were induced by adding IPTG (Promega) at 0.5 mM, 1 mM and 1.5 mM concentration and were allowed to grow for 1 h, 2 h, 4 h and overnight, to study

expression kinetics. Recombinant proteins were purified by affinity chromatography with nickel-NTA-agarose columns (Qiagen, USA) under denaturing conditions. The purity of the recombinant proteins was assayed by SDS-PAGE. The experiments were conducted as per the protocols described earlier (SINGH et al., 2009).

*Immunization of mice.* For the vaccine study, mice were divided into six groups containing six mice per group. The mice in Groups I-IV were immunized with 50 µg of antigen with Freund's Complete Adjuvant (FCA) on day 0 and with 50 µg of antigen with Freund's Incomplete Adjuvant (FIA) (Banglore Genei, India) on day 14 by the subcutaneous route. Sonicated whole cell lysate, outer membrane proteins, native OmpH and recombinant OmpH served as immunogen for Groups I-IV, respectively. Group V received HS alum precipitated vaccine (Indian Immunologicals, India) and Group VI was given sterile PBS, pH 7.4 as control. Sera samples were collected at weekly intervals starting from day 0 to day 28, for evaluation of antibody response.

*Challenge studies.* Protective ability was assessed by direct challenge studies. On day 28 post immunization all six groups of mice were challenged with 0.1 ml of 100 LD<sub>50</sub> of *P. multocida* P<sub>52</sub> by the intraperitoneal route. The LD<sub>50</sub> of *P. multocida* was calculated by the method of REED and MUENCH (1938). The mortality pattern was recorded in each group and sera were collected on days 5 and 10 post challenge.

*Indirect enzyme linked immunosorbent assay (i-ELISA).* i-ELISA was performed to determine antibody response, as per the method of NATALIA et al. (1992). Briefly, OMP was coated on microtitre plate wells (Maxisorb® 96-well flat bottom ELISA plate, Nunc, Denmark) at a concentration of 1 µg/mL at 4 °C overnight. Unbound sites were blocked with 1% BSA in PBS-Tween 20 (0.05%), pH 7.2. Test sera in a dilution of 1:100 were added in triplicate and reacted with coated antigen for 2 h at 25 °C. The bound antibody was then detected using 1:1000 dilutions of affinity purified goat anti-mouse IgG horse-radish peroxidase (HRPO) conjugate (Bangalore Genei, India). The enzymatic reaction was assayed using orthophenylene diamine (Sigma, USA) and the change in intensity of the colour was determined at A<sub>492 nm</sub> by the ELISA reader (Tecan-sunrise). The antibody titres were calculated by the following formula:

$$\text{Log antibody titre} = X + (A - C / A - B) \times D$$

where X = log dilution of the test sample, with A<sub>492</sub> immediately higher than the cut-off value; A = A<sub>492</sub> of the test sample dilution immediately higher than the cut-off; B = A<sub>492</sub> of the test sample dilution immediately lower than the cut-off; C = Cut-off A<sub>492</sub> (Mean A<sub>492</sub> of the negative samples + 3x S.D.) and D = log dilution factor i.e. 1.

*Statistical analysis.* Antibody responses, as measured by i-ELISA, were analyzed by Student's *t* test to find the statistical significance of the differences between the groups of mice.

## Results

**Purification of 34 kDa OmpH protein.** The protein content in the OMP-rich extract of the P<sub>52</sub> strain was 6.0 mg/ml. The OMP rich preparation of *P. multocida* P<sub>52</sub> revealed the presence of OmpH as a 34 kDa protein band among other major OMPs, eluted as a single polypeptide band when subjected to SDS-PAGE.

**Expression and purification of *P. multocida* recombinant OmpH protein.** The cloning, expression and purification of recombinant OmpH has been previously described (SINGH et al. 2009). PCR amplification of *ompH* gene of *P. multocida* P<sub>52</sub> yielded the expected product of about 1 kb.

**Nucleotide sequence accession number.** The DNA sequence of the *ompH* gene of *Pasteurella multocida* P52 has been deposited in GeneBank under accession number HM117642.

**Challenge studies in mice.** The protective ability of different immunogens in the challenge study showed 100% protection with OMP fraction used as a vaccine. The groups immunized with sonicated whole cell lysate and commercial vaccine showed a survival rate percentage of 83.33%. The recombinant OmpH group showed 63.67% survivability, as compared to the native OmpH group, that showed a 50% survival rate. 100% mortality was recorded in the control group mice.

**Antibody response studies.** The humoral immune response, measured by i-ELISA of sera from mice inoculated with sonicated whole cell lysate, OMP, native 34 kDa OmpH, recombinant OmpH and commercial vaccine, is shown in Fig. 1.

In Group I (Sonicated whole cell lysate), there was a significant increase ( $P < 0.05$ , Student's *t* test) in titres from d0 to d7, d14 and d21, with mean values ( $\log_{10}$  titre) of 2.58, 3.93 and 4.49 respectively, whereas the antibody response increased non-significantly from d21 to d28 post immunization.

In Group II (OMP fraction), there was significant increase ( $P < 0.05$ ) in antibody response from d0 to d7, with titre values of 3.00 on d7, although a non-significant increase in immune response was seen from d7 to d14. Further, there was a significant rise in antibody titres up to d28, with  $\log_{10}$  titre values of 4.04 and 4.41 on d21 and d28 respectively.

In Group III (Native OmpH), there was significant increase ( $P < 0.05$ ) in titres from d0 to d7, with a mean titre of 3.70, while the antibody response increased non-significantly from d7 to d14. Further, there was a significant rise in antibody titres up to d28 with  $\log_{10}$  titre value of 4.23 and 4.67 on d21 and d28, respectively.

In Group IV (Recombinant OmpH), there was a significant increase ( $P < 0.05$ ) in titres from d0 to d7 and d14 with titre values of 3.36 and 3.58, respectively. Antibody response further increased non-significantly until d28 post immunization.

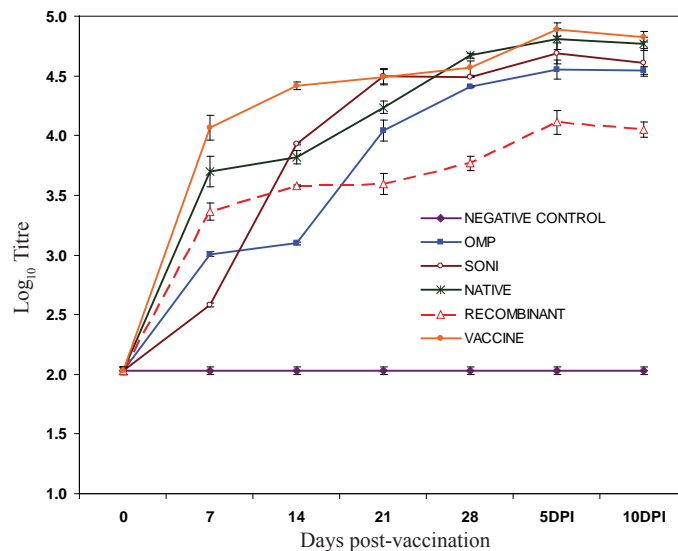


Fig. 1. Humoral immune response measured by i-ELISA of sera from mice inoculated with sonicated whole cell lysate, OMP, native 34 kDa OmpH, recombinant OmpH and commercial vaccine.

In the positive control (Group V), there was a significant increase ( $P < 0.05$ ) in titres from d0 to d7 and d14, with titres of 4.06 and 4.41 respectively, while antibody response further increased non significantly up to d28.

In Group VI (negative control), the value of the  $\log_{10}$  titre remained constant (2.03) throughout the experimental schedule.

### Discussion

Vaccination against HS is widely practised in endemic areas but significant outbreaks still occur (SHIVACHANDRA et al., 2011). To develop a superior quality vaccine, components of bacterial cells, such as OMPs, LPS, capsule and fimbrial protein, have been studied. Amongst them, OMPs have been shown to play a predominant role in protective immunity and thus can be used as potential candidates.

In the present study, OmpH, in its native and recombinant form, was used as an immunogen in mice.

In our previous study, the *ompH* gene of *Pasteurella multocida* P<sub>52</sub> was cloned (pGEM-T Easy Vector, Qiagen), sequenced (Accession No. EU016232) and analysed

to obtain the mature OmpH as a 313 amino acid polypeptide, with a predicted molecular mass of 33,760 Da (SINGH et al., 2011). In the present study, OMP rich extract revealed 34 kDa as a major protein purified by preparative SDS-PAGE, as reported previously (BRAR et al., 2010; HAMID and JAIN, 2008).

No improvement in the expression profile of the desired 34 kDa protein was obtained while studying expression kinetics. BHAT and JAIN (2010) studied the induction kinetics of 49 kDa recombinant OMP protein of *Salmonella* Typhi, in order to determine the time for optimal induction of the expressed protein. They found that maximum induction was obtained at 4 h after addition of 1.0 mM IPTG to the medium.

Mice were chosen as an experimental model for protection studies and monitoring humoral immune response through i-ELISA, since there is a direct correlation between antibody response and protection (DUA et al., 1978; KHARB and SHIV CHARAN, 2013).

In the mouse protection test, survivability percentages of 83.33%, 100%, 50%, 63.67% and 83.33% were observed in Groups I-V respectively, against 100% mortality in Group VI. LUO et al. (1997) reported that purified native OmpH induced 100% protection but the recombinant induced little protection. In the present study, recombinant OmpH protein provided 63.67% protection in mice, as compared to native OmpH, which only induced 50% protection. LUO et al. (1997) purified native protein through size exclusion chromatography for the experiment, whereas in the present study it was gel eluted. It is possible that during elution from denaturing PAGE, native protein could have lost some immunogenic epitopes. LEE et al. (2007) also studied the protective efficacy of one full length and three short truncated fragments of recombinant OmpH protein in mice, and showed that the full length recombinant OmpH protein showed protection levels up to 70%, whereas, in truncated fragments the protection level was 30-50% against a homologous challenge. Moreover, BRAR et al. (2010) reported that 32 and 25 kDa gel eluted protein fractions, separately or in combination, did not provide any protection in mice.

In the present study, OMP fraction induced 100% protection. BASAGOUDANAVAR et al. (2006) reported 100% protection in mice using OMP- Montanide and OMP-liposome vaccines in challenge experiments, thus confirming the role of OMP in immunoprotection. KHARB and SHIV CHARAN (2010) investigated the role of OMPs in mice, showing 100% protection upon intranasal challenge, and 84% protection following subcutaneous challenge, as compared to 84% mortality in the control. Similarly, BRAR et al. (2010) reported 100 percent protection in mice using whole OMP as a vaccine. However, SRIVASTAVA (1998) observed in their study that mice immunized with OMP vaccine showed a survival rate of 67% and concluded that although the level of protection from OMP was lower than the whole cell vaccine, i.e. 84% survival rate, it was above the acceptable level. Commercial vaccine and sonicated whole cell lysate induced 83.33%

survivability in the present study that was comparable to an 84% survival rate with whole cell vaccine of *P. multocida*, as reported by SRIVASTAVA (1998).

In contrast to our findings, TAN et al. (2010) observed 100% protection with recombinant 37 kDa OmpH in mice. But the route of immunization and challenge was I/P. Immunization with major OMP of *Vibrio vulnificus* resulted in protective antibodies giving 100% protection until d28 in a murine model (JUNG et al., 2005). BHAT and JAIN (2010) also reported 100% protection in mice with recombinant and native 49 kDa protein in *Salmonella enterica* serovar Typhimurium.

BASAGOUDANAVAR et al. (2006) used OMP with adjuvants including dioleoyl phosphatidyl choline-based liposome and Montanide ISA206 water-in oil-in water emulsion, and observed that ELISA titres were similar on day-21 PI ( $\log_{10}$  titre 4.5) with an increase in titre one week after boost ( $\log_{10}$  titre 4.8). It increased further ( $\log_{10}$  titre 5.1) by day-45 PI before becoming stationary by day-68. BRAR et al. (2010) observed a direct relationship between antibody titres and survival rate following immunization of mice with 32 and 25 kDa eluted proteins and whole OMP. The mice vaccinated with whole OMP were 100 percent protected, showing an ELISA  $\log_{10}$  titre 3.1. In support of our findings, KHARB and SHIV CHARAN (2010) reported that antibody responses increased significantly on day 14 in their OMP and WCL groups ( $P < 0.05$ ). Antibody titres further increased significantly on 21 dpi ( $P < 0.01$ ). Anamnestic antibody response was observed on 28 dpi. It continued to increase on 35 dpi.

In our study, the commercial vaccine was initially found to be more immunogenic, giving the highest antibody titre until d14, as compared to sonicated whole cell lysate, OMP, native and recombinant OmpH, but in the later stages of the experiment, sonicated whole cell lysate, OMP and native OmpH were found to be as immunogenic as alum precipitated vaccine, with a sharp antibody response. On d28, all groups except Group IV (Recombinant OmpH), showed comparable titre values, indicating a similar antibody response. The challenge to the animals on d28 post immunization provided a booster to the immune response, resulting in an even higher antibody titre, as reported by PATI et al. (1996). Thus, it may be concluded that Native OmpH and OMP generated a delayed response. However, recombinant OmpH showed a weak antibody response in comparison to other preparations.

Taking these observations together, it may be concluded that OMPs play an immuno-protective role and possess strong potential for the development of a candidate subunit vaccine against HS. Further studies are required where this immunogenic fraction may be used as a booster dose after primary vaccination, to enhance the duration of protective immunity. The immunogenic protein may also be evaluated as a vaccine candidate through different routes of immunization, especially to induce mucosal immunity (KHARB and SHIV CHARAN, 2011).



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**JOSHI, S., K. TEWARI, R. SINGH: Poredbena imunogenost i zaštitna učinkovitost različitih pripravaka proteina vanjske membrane bakterije *Pasteurella multocida* (B:2) na mišjem modelu. *Vet. arhiv* 83, 665-676, 2013.**

**SAŽETAK**

Istraživanje je provedeno s ciljem da se procijeni zaštitni učinak rekombinantnog proteina (OmpH) vanjske membrane bakterije *Pasteurella multocida* (B:2). Prirodni protein OmpH od 34 kDa bio je pročišćen od iscrpka bogatog OMP-om. Provedeno je kloniranje, ekspresija i pročišćavanje rekombinantnog OmpH. Prvoj skupini miševa bio je primijenjen ultrazvučno obrađen stanični lizat. Drugoj skupini primijenjen je OMP, trećoj prirodni OmpH od 34 kDa, a četvrtoj rekombinantni OmpH. Peta skupina bila je pozitivna kontrola cijepljena komercijalnim cjepivom, dok je šesta skupina bila negativna kontrola. Stopa preživljavanja u I. skupini bila je 83,33%, u II. skupini 100%, u III. 50%, u IV. 63,67% te u V. također 83,33%. U VI. skupini mortalitet je iznosio 100%. Titar serumskih IgG određivan je i-ELISA-om u mješavini uzoraka prikupljenih jednom tjedno od 30 životinja iz svake skupine. U I. skupini ustanovljen je porast srednje vrijednosti titra specifičnih protutijela 7. dana kada je iznosila 2,58 (titar log<sub>10</sub>), 14. dana kada je iznosila 3,93 i 21. dana kada je iznosila 4,49. U II. skupini značajni porast srednje vrijednosti titra specifičnih protutijela dokazan je sedmoga dana 3,0, zatim 21. dana 4,04 te 28. dana 4,41. U III. skupini dokazan je značajan porast srednje vrijednosti titra specifičnih protutijela 7., 21. i 28. dana s vrijednostima 3,70, 4,23 i 4,67. U IV. skupini značajan porast titra dokazan je 7. i 14. dana sa srednjim vrijednostima 3,36 i 3,58. U kontrolnih životinja značajan porast titra dokazan je 7. i 14. dana s vrijednostima 4,06 i 4,41. Na osnovi prikazanih rezultata može se zaključiti da OMP ima zaštitnu ulogu i veliku mogućnost da bude kandidat za razvitek pojedinih cjepiva protiv hemoragijske septicemije.

**Ključne riječi:** *Pasteurella multocida*, OMP, OmpH, rekombinanta, cjepivo

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