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Immunoprotection in mice immunized with native OmpH, recombinant OmpH and HS alum precipitated vaccine of *Pasteurella multocida* P52 against *P. multocida* challenge

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Outer membrane proteins (OMPs) of *Pasteurella multocida* play an important role in virulence and are important for vaccine development due to immunogenicity. Here, we evaluated immunoprotection in mice, immunized with native OmpH, recombinant OmpH and HS alum precipitated vaccine of *P. multocida* P52 against *P. multocida* challenge. Protection studies were performed in mice of groups I (OmpH); II (recombinant OmpH); and III (HS alum precipitated vaccine). Group IV was control group administered with sterile PBS. The protection percentage was recorded as 83.33% protection in Gr. I & III, and 66.66% in Gr. II. The control group (Gr. IV) showed 100% mortality. The mean antibodies titers were detected at day 7, 14, 21 and 28 postimmunization, and compared with the control group. All immunized groups showed significantly high immune protection compared to the control group.

Keywords: Buffaloes, Cattle, Hemorrhagic septicemia, Indirect ELISA, Outer membrane proteins, Poultry

Pasteurella multocida (P. multocida), a Gram-negative coccobacilli (family *Pasteurellaceae)* is known to be highly contagious leading to a wide range of diseases in animals[1]. It leads to some of the economically important infections such as avian fowl cholera, rabbit snuffles, and hemorrhagic septicemia in cattle, goats and pigs[1]. It is generalized into five serogroups A, B, D, E, and F, based on their capsular typing and 16 serotypes based on somatic typing[2]. Hemorrhagic septicemia (HS), an acute and highly fatal disease of cattle and buffalo, is caused by two serotypes of *P. multocida*, viz. Asian serotype 6:B and the African serotype 6:E (Namioka-Carter)[3], leading to high mortality and morbidity[4,5].

In India, HS is mostly caused by serotype B:2. *Pasteurella multocida* causes diseases in animals (bovine, porcine, rabbits and poultry) immune suppressed by stresses such as viral infections and extreme climate with the aerosol transmission of infection between animals. The pathogenesis of infection is a result of complex interactions between host factors (species, age, immune status) and specific bacterial virulence determinants which include lipopolysaccharide (LPS), capsule, adhesin, outer membrane, etc.[6]. The passive mouse protection test using specific B:2 and E:2 immune rabbit sera have been used in Asia and Africa to identify different serotypes of *P. multocida*[7]. In Asia, HS is a major killer disease in buffaloes. Buffaloes are three times more susceptible than cattle[8].

Various strategies have been used to develop HS vaccines such as cellular vaccines, killed vaccines (bacterins), live-attenuated and genetically-engineered vaccines[5,9]. The most widely used vaccine in Asia is the whole cell formalin-killed *P. multocida* P52 bacterin precipitated with alum or emulsified in aluminium hydroxide gel. The alum precipitated and the aluminium hydroxide gel vaccines are reported to confer immunity for four to six months. Oil adjuvant vaccine (OAV) reduced the incidence of HS[10] but a few disadvantages have often been reported, like difficulty in injecting the OAV due to its high viscosity, abscess formation at the site of injection and post-vaccination shock reactions[3].

The outer membrane proteins (OMPs) of Gram-negative bacteria play essential roles in host-pathogen interactions and in disease processes. In the pathogenesis of pasteurellosis, OMPs of *P. multocida* play a significant role as potent immunogens[11], as well as immunodominant antigens, and hence are.

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responsible for cross-protective immunity, whereas lipopolysaccharides (LPS) alone induces only partial protection against pasteurellosis in mice (Data not provided). The inactivated bacterial vaccines provide serotype-specific protection whereas, the live vaccines composed of attenuated strains, confers protection against both homologous and heterologous serotypes[12].

Here, we did mechanistic and immunogenic study of bovine diseases in mice as these animals are preferred laboratory animals for experimentation, and as they mimic the approximate 30-40% bovine genotype, the findings can be extrapolated in the large animals[13,14]. Precisely, we evaluated the efficacy of OmpH and alum precipitate vaccine in providing protection against *P. multocida* serotype B:2 and comparative studies on protection conferred by OmpH and whole cell HS vaccine.

Material and Methods

Animals

Adult albino rats of either sex weighing 100-200 g were maintained at room temperature (RT) (25 ± 3°C) and were fed with a rodent lab diet and tap water *ad libitum*. The protocol for the study was approved by the Institutional Animal Ethical Committee (Ref. No. 46/IAEC/Vety/2017). Details of goups may be given here.

Culture

Pasteurella multocida serotype B:2 (vaccine strain P52) obtained from Institute of Animal Health and Veterinary Biologicals, Rasalpura, Mhow, was used in this study and maintained on blood agar medium. It was routinely cultured in brain-heart infusion (BHI) broth. The culture was stored at 4ºC in blood agar media and BHI agar slants. Molecular characterization of P52 was also performed. Molecular characterization of *P. multocida* serotype B:2 strain was done by the employment of polymerase chain reaction (PCR) targeting *ompH* using the forward and reverse primers[15]. Glycerol stocks were maintained at −20ºC.

Procurement of haemorrhagic septicaemia vaccine

The HS alum precipitated vaccine obtained from the Institute of Animal Health and Veterinary Biologicals Rasalpura, Mhow, India, was kept at both 4 and 37ºC.

Isolation and purification of immunogenic OMPs of *Pasteurella multocida*

OMPs of *P. multocida* B:2 vaccine strain P52 was characterized by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. Potential immunogens of *P. multocida* P52 were identified by electroblot immunoassay.

Expression and purification of *P. multocida* recombinant OmpH protein

Pfu DNA polymerase was used to ensure the appropriate sequence. Restriction enzyme (RE) digestion of insert DNA was carried out using two restriction endonucleases BamHI and SmaIfragment was ligated to pQE30-Xa vector between BamHI (cohesive ends) and SmaI (blunt ends) restriction sites. Ligation was carried out with T4 DNA ligase. HiPurA™ *E. coli* DH5α ready to use competent cells (HiMedia) were used to clone and express the OmpH protein of *P. multocida* B:2.

Preparation of vaccines, sterility and safety testing

OMPs of *Pasteurella multocida* B:2 vaccine strain P52 were characterized using the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique. The *P. multocida* P52 OMP demonstrated the presence of six OMP bands. The major bands were of 20, 34, 48, 72, 89 and 91 kDa. Potential immunogens of *P. multocida* P52 were identified by electroblot immunoassay[16]. The OMP vaccines (OmpH and OmpH recombinant) were tested before inoculation for their sterility by inoculating them on 5 to 10% sheep blood agar and Sabouraud's dextrose agar media. Both the OMP vaccines were subjected to safety testing by inoculating 0.1 mL of each preparation subcutaneously (S/C) to mice as per the method of Wijewardana and Sutherland[17] as shown in El-Tayeb *et al*.[18]. The inoculated mice were observed three days for mortality or other abnormality, while no abnormality was observed.

Production of mouse antiserum

Immunization of mice

A 100 µg of crude OMPs were emulsified in Freund's complete adjuvant (100 µL) and injected via sub-cutaneous (s/c) to mice (6-8 weeks of age). After 14 days, the same amount of OMPs (50 µg) was emulsified in Freund's incomplete adjuvant (50 µL) and injected s/c into mice. Four groups (I, II, III and IV) of mice consisting of six in each group were taken for immunization trial with OMPs preparations and HS alum precipitated vaccine. The animals of Gr. I (OmpH) & II (recombinant OmpH) were immunized s/c with 0.2 mL containing 100 µg of purified OMPs of *P. multocida* P52 with an equal volume of Freund's complete adjuvant, respectively. Group III (HS alum precipitated vaccine) was immunized with 0.2 mL of

HS vaccine as such and Gr. IV was administered 0.2 mL of sterile PBS (pH=7.4) and considered as the control. On the 14th day after primary immunization, the booster dose of 50 µg of respective OMPs with an equal volume of Freund's incomplete adjuvant was administered through the s/c route. Groups III & IV were administered HS alum precipitated vaccine and with 0.2 mL of sterile PBS (pH=7.4) through the s/c route, respectively. Immunized mice of all the groups were observed for any untoward signs and symptoms during the entire immunization trial.

Protective and cross-protective efficacy of the immunogenic OMPs of *P. multocida* type B:2 were evaluated by immunizing mice with vaccines prepared from the respective OMPs as per the method described by Tan *et al.*[19] and Azam *et al*.[20]. Evaluation of protection in mice immunized with native OmpH, recombinant OmpH and HS alum precipitated vaccine of *P. multocida* P52 against *Pasteurella multocida* challenge (10 cfu of *P. multocida* P52 strain) was carried out with three sub groups of mice and compared with the control group[21]. The mortality patterns of mice in all the subgroups were recorded daily up to 10 days following the challenge.

Indirect enzyme-linked immunosorbent assay (*Indirect- ELISA*)

Blood was collected 07 days after the booster dose. The blood was also collected from the control group. These sera were ready to be used in the development of the I-ELISA assay. Pooled sera from healthy (non-immunized: Gr. IV) and immunized mice were used as standard negative and positive controls, respectively. A checkerboard titration was performed for optimization of the working dilution of antigens and antibodies as per standard protocols. The antigen and serum dilutions which have shown a maximum difference in absorbance at 450 nm between positive and negative were selected. ELISA plates (Nunc, USA) were coated with 1 µg/mL of antigens (100 µL/well) by two-fold serial dilution in coating buffer (pH-9.6). Antigen was added to all the wells except antigen-negative control wells, where 100 µL of coating buffer was added. The plates were incubated at 4°C for overnight and then washed thrice using washing buffer, PBST (0.002 mol/L diluted PBS containing 0.1% Tween 20). The nonspecific sites on the antigen were blocked using 5% sodium meta periodate in PBS in all the wells (200 µL/well). The plates were incubated for 1 h at 37°C. Unbound antigens were washed thrice using a washing buffer. Meanwhile, dilutions of serum samples were made in 96-well plates (1:200 in blocking buffer). After incubation, plates were washed thrice using a washing buffer. Diluted serum samples (1:200-1:6400) were added to the sample wells in duplicate and mixed well. The contents were mixed properly by tapping the sides of the plate gently. The plates were again incubated at 37°C for one h and washed thrice. A 100 µL of secondary antibody (1:12000 in blocking buffer) rabbit antimouse IgG HRPO conjugate (Genei) was added to each well and again incubated for 1 h at 37°C. After washing, 100 µL of freshly constituted substrate solution was added to each well and kept at 37°C. 4 µL of 30% hydrogen peroxide (H_2O_2) per 10 mL of the substrate was also added just before use. After 10-12 min (maintained uniformity throughout) colour reaction was stopped by adding an equal volume of 1M H_2SO_4. The optical density of the wells was measured at 450 nm by the ELISA reader. Histograms were constructed with A450 of antibody titer vs. day post-vaccination to evaluate the humoral immune responses.

Statistical analysis

Antibody responses, as measured by I-ELISA, were analyzed by two-way ANOVA to find the statistical significance of the differences between the groups of mice with Minitab version 17.

Results and Discussion

In the present study, OmpH, in its native and recombinant form, was used as an immunogen in mice. OMP-rich extract revealed 34 kDa as a major protein purified by preparative SDS-PAGE, as reported earlier[22]. The humoral immune response of sera from mice inoculated with native OmpH, recombinant OmpH and HS alum precipitated vaccine were measured by I-ELISA (Fig. 1). Following vaccination with native OmpH, mice of Gr. I showed a sharp increase in the mean serum antibody titer on 7th day (1.853±0.019). The increasing trend of the mean serum antibody titer continued till it reached its peak on the 21st day (2.743±0.020) and showed a non-significant decline in antibody titer from day 21 to day 28 (2.320^{c}±0.012), post-immunization. The increase in serum antibody titer was found to be statistically significant ($P<0.01$). Similarly, mice in Gr. II vaccinated with recombinant OmpH also showed a significant increase in antibody titer (1.740^{d}±0.025) on the 7th day, with a rising trend till day 21 (2.647^{a}±0.018) ($P<0.01$, one-way Anova) and reduced slightly (non-significant) on day 28 post-

immunization. Similar is the case with Gr. III, vaccinated with HS alum precipitated vaccine, showing a significant increase in titer values from day 7 (1.817±0.012) to day 21 (2.653±0.013), with a non-significant reduction on day 28 post-immunization. The mean serum antibody titer (0.543[a]±0.009), observed in the control group (Gr. IV) was almost static during the entire experimental period, which was not statistically different at any of the time intervals during the study. Means with different superscripts differed significantly ($P \leq 0.01$) at different time intervals

The statistical analysis of overall humoral response in vaccinated groups showed a non-significant difference ($P \leq 0.01$) in antibody titers between groups. The antibody titer was found significant in Gr. I (native OmpH) (2.366[a]±0.100) and Gr. III (HS alum precipitated vaccine) (2.362[a]±0.098) as compared to Gr. II (Recombinant OmpH) (2.276[a]±0.102). The antibody titer in Gr. IV (0.543[b]±0.004) was the lowest which significantly differed from the rest of the groups. Figure 2 shows the overall humoral immune response in vaccinated groups post vaccination (Fig. 2).

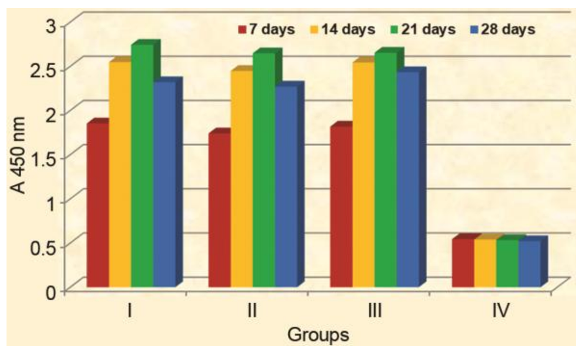

Fig. 1 — Antigen specific Humoral immune response in vaccinated groups at different time intervals post vaccination. [Gr. I, OmpH; Gr. II, recombinant OmpH; Gr. III, alum precipitated vaccine; and Gr. IV, sterile PBS (control)]

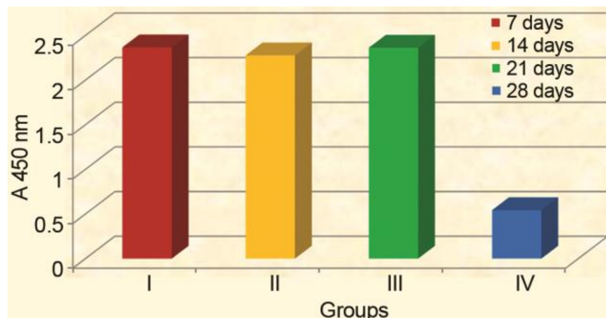

Fig. 2 — Overall humoral immune response in vaccinated groups post vaccination

Protection studies

The protection conferred by different immunogens in the challenge study showed 83.33% protection in Gr. I & III immunized with native OmpH and HS alum precipitated vaccine, respectively, with a reduction of survival to 66.66% in Gr. II, immunized with recombinant OmpH. Control group, recorded 100% mortality.

The present study evaluated the immunogenic ability of native OMPs and recombinant OMPs and their comparison with the HS alum precipitated vaccine in mice. The mean antibody titers were compared at day 7, 14, 21 and day 28 post-immunization and compared with respect to the control group, administered with sterile PBS. All immunized groups showed significantly high immune protection than the control group. In a study reported that purified native OmpH induced 100% protection but the recombinant induced little protection[20]. It was observed that recombinant OmpH protein provided 63.67% protection in mice, as compared to native OmpH, which only induced 50% protection[23].

In the present study, the protective ability of different immunogens in the challenge study showed 83.33% protection with native OMP used as a vaccine. The recombinant OmpH group showed 66.6% survivability and the groups immunized with HS alum precipitated vaccine showed a survival rate of 83.33%, whereas 100% mortality was recorded in the control group mice. The purified native protein through size exclusion chromatography for the experiment was also done[20], 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. 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% was also studied, whereas, in truncated fragments the protection level was 30-50% against a homologous challenge[16]. Moreover, it was 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, native OmpH fraction induced 83.33% protection.

The 100% protection in mice using OMP-montanide and OMP-liposome vaccines in challenge experiments was reported confirming the role of OMP

in immune protection[15]. The role of OMPs in mice was investigated showing 100% protection upon intranasal challenge, and 84% protection following s/c challenge, as compared to 84% mortality in the control[23]. Similarly, 100% protection in mice using whole OMP as a vaccine was also noted[21]. However, a study mice immunized with the 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[12]. Commercial vaccine and sonicated whole-cell lysate induced 83.33% survivability in the present study which was comparable to the 84% survival rate with whole cell vaccine of *P. multocida*, as reported by Muangthai *et al*.[12]. In contrast to our findings, a study reported 100% protection with recombinant 37 kDa OmpH in mice. But the route of immunization and challenge was inraperitoneal[18].

Immunization with major OMP of *Vibrio vulnificus* resulted in protective antibodies giving 100% protection until day 28 in a murine model. The 100% protection in mice with recombinant and native 49 kDa protein in *Salmonella enterica* serovar Typhimurium[22]. The OMP with adjuvants including dioleoyl phosphatidyl choline-based liposome and Montanide ISA206 water-in oil-in-water emulsion, and observed that ELISA titers were similar on day 21 post-immunization (log10 titer 4.5) with an increase in titer one week after booster dose (log10 titer 4.8). It increased further (log10 titer 5.1) by day 45 post-immunization before becoming stationary by day 68[15]. A direct relationship between antibody titers 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% protected, showing an ELISA log10 titer 3.1. Similar to our above findings, Azam *et al*.[23] has also observed that antibody responses increased significantly on day 14 in their OMP and WCL groups (P <0.05). Antibody titers further increased significantly on 21 day post-immunization (P <0.01), and the anamnestic antibody response was observed on 28-day post-immunization which continued to increase to 35 day post-inoculation[23].

In present study, the commercial vaccine was initially found to be more immunogenic, giving the highest antibody titer until day 21, 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 day 28, all groups, except Gr. IV (control), showed comparable titer values, indicating a similar antibody response. The challenge to the animals on day 14 post immunization provided a booster to the immune response, resulting in an even higher antibody titer[15]. Thus, it may be concluded that native OmpH and HS alum precipitated vaccine showed a comparable response.

Conclusion

The native OmpH protein was found capable of generating better immune response and protection against homologous challenge of *Pasteurella multocida* than recombinant OmpH protein. Taking these observations together, it may be concluded that OMPs play an immune-protective role and possess strong potential for the development of a candidate subunit vaccine against HS. Further studies are required with immunogenic fraction 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.

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Conflict of Interest

Authors declare no competing interests.

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