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Immunogenicity of recombinant Omp16 protein of *Pasteurella multocida* B:2 in mouse model

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ABSTRACT

Bacterial peptidoglycan-associated lipoproteins (PAL) are potential targets for the development of diagnostics/ subunit vaccines for infectious diseases. Most commonly prevalent Omp16 lipoprotein is absolutely conserved among *Pasteurella multocida* strains, which are involved in multiple infectious diseases of livestock worldwide. In the present study, we cloned *omp16* gene encoding for mature Omp16 of *P. multocida* B:2 strain P52 and over-expressed as a fusion protein in *Escherichia coli.* Mice immunized with purified recombinant non-lipidated Omp16 fusion protein (~32 kDa) resulted in elicitation of significant antigen specific serum antibody titres (total IgG and subtypes). A more pronounced increase in Th2 response (IgG1) was noticed. The study indicated the potential possibilities to use lipidated recombinant Omp16 protein in developing a composite subunit vaccine along with suitable adjuvant for haemorrhagic septicaemia/ pasteurellosis in livestock.

Key words: Immunogenicity, Mouse model, *Pasteurella multocida,* Peptidoglycan-associated lipoprotein (PAL), Recombinant Omp16

Pasteurella multocida, a Gram-negative bacterial pathogen, is associated with a variety of infectious diseases collectively termed as 'pasteurellosis' in a wide range of animals/birds including haemorrhagic septicaemia (HS) in cattle and buffaloes (Kumar *et al.* 2004, Biswas *et al*. 2004, Shivachandra *et al.* 2011). A considerable variation was observed between different strains belonging to capsular (A, B, D, E and F) and somatic (1–16) serogroups with respect to host predilection, virulent factors, mechanism of pathogenesis and immunity (Wilkie *et al*. 2012). Despite several vaccine formulations developed in the past to control pasteurellosis, till date, none of them was found to elicit broadly reactive, cross-protective and long lasting immunity (Shivachandra *et al*. 2011, Ahmad *et al*. 2014). Hence, there is a need to design and develop a novel vaccine formulation involving highly conserved and immunogenic antigens.

The surface antigens or outer membrane proteins (OMPs) of *P. multocida* are considered as potential immunogens (Hatfaludi *et al.* 2010). Among them, Omp16 (~16 to 18 kDa) was commonly present across different *P. multocida* strains irrespective of growth conditions (Wheeler 2009). Several Gram-negative bacterial lipoproteins are considered as potential target antigens for vaccine development against many infectious diseases including pasteurellosis (Kovacs-Simon *et al*. 2011, Shivachandra *et al*. 2014a). Moreover, Omp16 and its related peptidoglycan-associated lipoproteins (PAL), viz. P6 and 18 kDa proteins from other Gram negative bacterial species have been extensively studied and employed in the development of either species specific diagnostic assay or recombinant subunit vaccines against infectious diseases (Kasten *et al.* 1997, Godlewska *et al*. 2009).

On the basis of preliminary analysis indicating wide prevalence of Omp16 gene among various *P. multocida* strains, it is presumed that Omp16 protein could be developed as potential candidate antigen for subunit vaccine. However, the immune response and protective efficacy of Omp16 of *P. multocida* has not yet been described till date. We describe a convenient method for the over-expression, purification of rOmp16 of *P. multocida* B:2 in bulk, assessment of its purity/yield, protein structure and immunogenicity in mouse model.

MATERIALS AND METHODS

Bacteria, primers, vector and mice: Pasteurella multocida serogroup B:2 strain P52, a vaccine strain of HS, maintained in the Clinical Bacteriology Laboratory, ICAR-Indian Veterinary Research Institute (IVRI), Mukteswar, Uttarakhand (UK), India, was used. For recombinant clone construction, pET32a vector, *Escherichia coli* TOP10 and *E. coli* Origami(DE3)pLacI cells were used. One set of

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primers required for target gene amplification was designed and synthesized. Healthy Swiss albino adult mice of either sex and aged 6–8 week old, reared cum maintained in pathogen-free environment at laboratory at Mukteswar, were used in immunization trials. All the mice experiments were conducted according to the norms of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, Ministry of Environment and Forests, Animal Welfare Division and approval by the Institute Biosafety Committee (IBSC) as well as Institutional Animal Ethics Committee (IAEC), ICAR-IVRI, Mukteswar, India.

Construction of recombinant Omp16 clone (pOmp16): A design of primer set targeting omp16 gene sequence (nucleotide region-Nt:61–77 and Nt:-433–453) without signal peptide region (1M-C20 aa) encoding for mature Omp16 protein (21G-Y150 aa) was based on Omp16 gene sequence (GenBank Acc. No. AJ271673) from *P. multocida* B:2 strain P52 (Goswami *et al.* 2004). The oligonucleotides sequences had added restriction enzyme sites (underlined) for *Bam*HI and *Xho*I in forward and reverse primer at 5' end along with primer tags (small letters) respectively, as mentioned below:

O16F: 5'-cgcGGATCCggttcatctaaaaaaga–3' and
O16R: 5'-gtgCTCGAGttaGTATGCTAACACAGCAC–3'.

PCR mixture (25 µl) consisted of 25 pmol of each primer (O16F and O16R), template DNA along with other reagents and standard amplification conditions (Kasten *et al.* 1995). The amplified PCR product as well as pET32a vector were digested with *Bam*HI and *Xho*I. Following purification of both insert and vector by gel elution using gel extraction kit, ligation reaction was carried out as per standard protocol mentioned elsewhere. The resultant recombinant plasmid (pOmp16) was initially transformed into *E. coli* TOP10 cells and later into *E. coli* Origami(DE3)pLacI cells.

Expression, purification and western blot of rOmp16 fusion protein: Recombinant *E. coli* cells were grown in 1 litre LB broth containing antibiotics; ampicillin (50 mg/ml) and chloramphenicol (35 mg/ml), and kept in shaking incubator at 37°C. The cells were induced with 1mM IPTG at appropriate growth pace and harvested by centrifugation at 6,000 rpm for 20 min at 3 h post-induction. The cell pellet was resuspended in buffer (50 mM Tris-HCl, pH 7.8, 100 mM NaCl and lysozyme) and suspension was subjected for lysis by sonication. Soluble/insoluble fractions were separated by centrifugation at 18,000 rpm for 30 min. The insoluble fraction containing rOmp16 in the pellet was collected and solubilized by treatment with the denaturing buffer (8 M urea, 50 mM Tris-HCl, pH 7.8; 100 mM NaCl, 10 mM imidazole). The rOmp16 protein was purified by affinity chromatography using Ni-NTA superflow cartridges as per the method described earlier (Shivachandra *et al.* 2012, 2014b, Kumar *et al.* 2013). Briefly, following column binding, renaturation on column with gradual decreasing urea buffer (8–0 M) and washing, rOmp16 was eluted with elution buffer and rOmp16 protein fractions were analyzed by 10% SDS-PAGE. All the peak fractions were pooled and dialyzed in a buffer (50 mM Tris-HCl, pH 7.8; 100 mM NaCl) and concentrated before quantification using a NanoDrop 2000 Spectrophotometer.

For confirming purified rOmp16, Western blot was carried out using semidry immunoblot system as per Kumar *et al.* (2013). Briefly, transferred and blocked membrane was treated with primary antibody (anti-*P. multocida* B:2 strain P52 polyclonal hyperimmune rabbit serum) at 1:100 dilution and secondary antibody (goat anti-rabbit IgG HRPO conjugate) at 1:16,000 dilution. The blot was developed using DAB substrate solution and dried blots were photographed.

Prediction of rOmp16 fusion protein characteristics: The characteristics of rOmp16 fusion protein were predicted using the PROTEAN program (DNASTAR), as well as proteomics tools (PSIPRED) from the ExPASy website.

Mice immunization: Healthy Swiss albino mice (20) found negative for *P. multocida* antibody-free status were divided into 2 groups with 6 mice in each. Following bulk purification and quantification of rOmp16 protein, each dose (rOmp16 = 50 µg/mice) of 100 µl volume mixed with complete Freund's adjuvant (CFA) was injected separately in 6 mice subcutaneously. After 21 days, a booster dose with rOmp16 (50 µg/mice) was given to all the immunized mice using incomplete Freund's adjuvant (IFA). A control group mice (n=6) received 1× PBS, pH 7.4. All the animals were provided with feed and water *ad lib.* and monitored for any adverse reactions/morbidity/mortality subsequent to immunization.

Evaluation of immune response: All the mice including immunized and control group were bled on 0, 21, 42 dpi and analyzed for the serum presence of rOmp16 specific IgG as well as subtypes (IgG1 and IgG2a) by an indirect-ELISA as per the standard protocol described previously (Kumar *et al.* 2013, Shivachandra *et al*. 2014b). Briefly, rOmp16 antigen (100 ng/well) coated 96-well flat–bottomed Nunc Maxisorp plates were washed and blocked with PBS-T containing 5% skimmed milk powder (PBS-SMP). Dilutions of serum antibodies (1:400) in PBS-SMP were added to wells, the plates were incubated for 1 h at room temperature, washed 3 times with PBS-T, and binding detected with anti-mouse IgG (1:10,000) / IgG1 (1:8,000)/ IgG2a (1:8,000) horseradish peroxidase (HRP)-conjugated secondary antibodies and substrate OPD. Plates were read at 492 nm on a Sunrise–360063 plate reader.

All the data were expressed as mean±standard error (SE) and Student's *t*-test was carried out using SPSS 16.1 software for determining significance of observations. P values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Cloning, expression, purification and western blot of rOmp16 fusion protein: The schematic designs of Omp16 of *P. multocida* and rOmp16 fusion construct are depicted in Fig. 1 A,B. PCR amplification of omp16 gene resulted

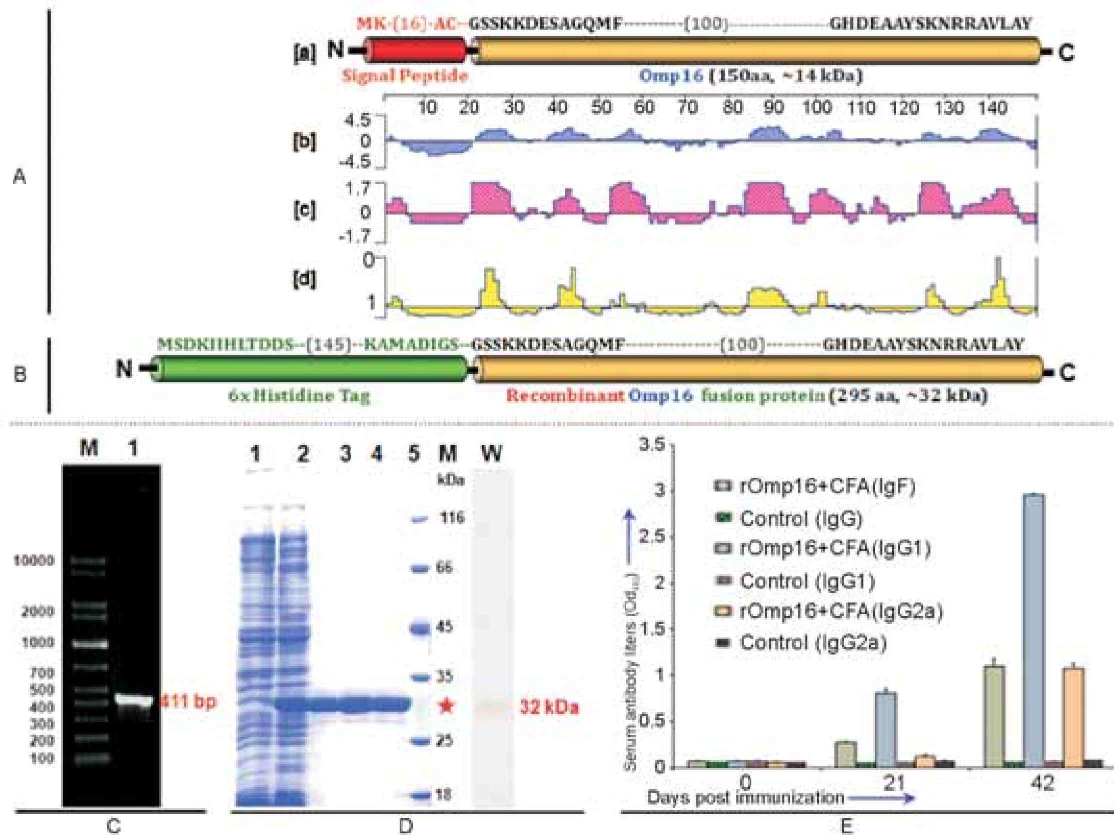

Fig. 1. A-E. Schematic design of clone construct, production and immunogenicity of rOmp16. A, Full length Omp16 protein of *P. multocida* B: 2 strain P52. [a] Hydrophilicity plot as per Kyte-Doolittle; [b] Antigenic index as per Jameson-Wolf; [c] Surface probability plot as per Emini. B, Schematic of rOmp16 construct design with N-terminus histidine tag region. C, Construction of clone (pOmp16). Lane M, 1 kb plus DNA ladder; Lane 1, PCR amplified *omp16* gene product (~411 bp). D, Production and Western blot of recombinant Omp16 fusion protein. Lane-1, Un-induced *E. coli* cell lysate; lane-2, induced *E. coli* cell lysate showing expressed rOmp16 (~32 kDa); lane–3, 4, 5, Purified fractions of rOmp16 using affinity chromatography; lane-M, protein standard marker; lane-W, immunoblot of lane 5, using rabbit hyperimmune sera against *P. multocida* B:2 strain P52. E, Immunogenicity of rOmp16 in immunized mice. The bar diagram indicating the rOmp16 specific serum antibody titers (IgG, IgG1 and IgG2a) of sera collected from all the mice at 0, 21 and 42 days post-immunization with rOmp16 as measured by indirect ELISA and recorded at OD 492 nm. The absorbance values are means±standard error [SE] of 6 mice/group. N, amino terminus; C, carboxyl terminus; aa, amino acids; bp, base pairs; kDa, kilo Daltons; IgG, immunoglobulin; Omp16, Outer membrane protein 16 kDa; A red star denotes a position of rOmp16.

in an amplicon of ~411 bp in size. The restriction enzyme digested and gel purified PCR product was successfully ligated to a linearised pET32a. Later, the resultant recombinant plasmid (pOmp16) was successfully transformed into *E. coli* Origami (DE3) pLacI cells.

Induced recombinant *E. coli* cells expressed rOmp16 protein having a total molecular weight ~32 kDa as observed on 10% SDS-PAGE (Fig. 1D, Lane 2). Recombinant cell lysis and solubility analysis indicated the presence of high amount of over-expressed rOmp16 protein in insoluble fraction. Further, the rOmp16 protein was purified under denaturing condition and renatured on column before elution and dialysis, which resulted in purified single band of rOmp16 protein on 10% SDS-PAGE at expected size of ~32 kDa (Fig. 1D, Lanes 3–5). A total yield accounting for >20 mg/l of purified rOmp16 was obtained. Immunoblot using polyclonal antibodies against *P. multocida* B:2 detected the presence of rOmp16 protein by development of brown colour in a chromogenic reaction on nitrocellulose membrane (Fig. 1D, Lane W).

Omp16, despite being an antigenically conserved surface antigen, appears not to be abundantly expressed on the bacterial surface and requires its bulk production in a heterologous host system. Hence, we successfully cloned, over-expressed and purified recombinant non-lipidated Omp16 of *P. multocida* from *E. coli* with high purity in sufficient amount, a necessary quality required for a good antigen. It was noted in the past that the majority of over-expressed recombinant lipoproteins were associated with intrinsic problems such as lipid modification either being incomplete or entirely absent, which led to inefficient immunogenicity trials. Despite several recent approaches including post-translational modification and target gene fusions which improved over-expression, the production of acylated heterologous recombinant lipoproteins within *E. coli* with a near native conformation and greater immunogenic potential, is always a concern. In view of these, the purified rOmp16 protein in our study, lacked

lipidation at its N-terminus as the design of rOmp16 construct was without a Cysteine residue (C20) and fused with hexa-histidine tag region encoded by a vector. The production of non-lipidated Omp16 without protein acylation at its N-terminus has an added advantage in vaccine development, especially in the cost efficient scale-up for bulk production than the lipidated Omp16 which appear to be more time-consuming and expensive during down-stream production process.

Predicted characteristics of rOmp16 fusion protein: The schematic of Omp16 protein with its predicted characteristic by PROTEAN are shown in Fig. 1A, subpanels [a] to [d], which indicated hydrophilicity, high antigenic index and surface probability. Recently, our preliminary omp16 gene sequence analysis of available *P. multocida* strains revealed an absolute homogeneity (100%) at amino acid level, however, at nucleotide level, the percentage of identity and divergence varied from 99.3–100% and 0–0.7%, respectively (Kumar *et al.* 2014). In an earlier report also, a wide distribution of omp16 gene was noticed among different serotypes of *P. multocida* and found to localize in 6 kb *Hind* III fragment of *P. multocida* genome (Goswami *et al.* 2004). Hence, we hypothesized that a highly conserved and widely prevalent Omp16 protein of *P. multocida* strains would be a suitable candidate antigen for inclusion in subunit vaccine.

Proteomic analysis revealed that rOmp16 fusion protein composed of Omp16 part (~14.21 kDa; 130 aa) along with N-terminus hexa-histidine tag (~17.72 kDa; 165 aa) accounting for a total molecular weight ~32 kDa (295 aa). The rOmp16 was predominantly composed of hydrophobic (97) residues followed by polar (68), strongly acidic (41) and basic (30) residues. Structurally, rOmp16 fusion protein was composed of N-terminus pET32 vector encoded region, which was found to posses 5 β-strands (β1 to β4) and 6 helix (α1 to α6). The C-terminus Omp16 formed a monomeric α/β sandwich composed of 4 β-strands and 4 α-helices with secondary elements arranged in the order; α1–β1–α2–β2–α3–β3–α4–β4. N-terminus of Omp16 was linked to C-terminus of vector region via flexible loop region. A point of lipidation (cysteine residue) was absent at the beginning of N-terminus sequence of rOmp16.

Immunogenecity of rOmp16 in mouse model: The serum antibody titres of rOmp16 specific total IgG and subtypes (IgG1and IgG2a) at various dpi are indicated in Fig. 1E. The change of content of serum antibody titers (IgG, IgG1 and IgG2a) in the immunized mice sera showed that there was a significant ($P<0.05$) difference between the immunized mice and the control group from 21 to 42 dpi. The rOmp16 specific IgG antibody as well as subtypes titres showed increasing trend (~3 fold) following booster dose. Between IgG1 and IgG2a, more pronounced rise in titre was observed for IgG1 in 42 dpi in immunized mice group. The ratio of IgG1/IgG2a was 2.74 and 0.75 in immunized and control mice groups, respectively, at 42 dpi. Absence of appreciable adverse reactions/symptoms was noticed in all mice during the course of immunization trials.

Recombinant vaccines also known as third generation vaccines are playing a distinct role in producing novel candidate vaccines in recent times especially against pasteurellosis (Hatfaludi *et al.* 2010, Ahmed *et al.* 2014). Although, a protective immunity appears to result from a more complex interplay of a number of factors, including antibodies to various antigens, a number of bacterial lipoproteins especially PALs, viz. P6, Omp16, 18 kDa proteins in several Gram-negative bacteria are considered as potential target antigens (Kovacs-Simon *et al.* 2011, Godlewska *et al.* 2009).

Mice immunized with non-lipidated rOmp16 elicited significantly stronger immune responses in the current study. A modest antigen dose (50 µg/mice) was used to find out the immunogenicity potential. It ascertained the immunogenicity potential of rOmp16 fusion protein. Follow-up experiments on the protective efficacy of varied rOmp16 antigen dose and suitable adjuvants need to be conducted. On the contrary, protective immunity elicited against P6 protein of other bacterial species, a baculovirus expressed recombinant P6-like protein failed to protect turkeys placed in contact with turkeys infected with *P. multocida* (Kasten *et al.* 1997). Vaccination of mice/chickens with either the lipidated or non-lipidated recombinant PCP also induced no protection against *P. multocida* challenge (Lo *et al*. 2004). Another conceivable fact is that proteins on the surface of *P. multocida* would not be accessible to antibodies due to presence of capsule produced by microbe. Moreover, Omp16 being a member of PAL superfamily, structurally similar to other bacterial lipoproteins involved in maintaining cell wall integrity, the surface exposed specific epitopes may be involved in eliciting protective immunity. Since, a single immuno-dominant T cell epitope in P6 protein of *H. influenzae* plays an important role in protective immune responses, a similar epitope mapping studies on Omp16 of *P. multocida* by immunoelectron microscopy of intact cells using monoclonal antibodies would likely to provide insights on mechanism of immunity and future antigen design.

The mechanism of protective immunity afforded by several candidate lipoprotein based subunit vaccine still remains unclear. Nevertheless, bacterial lipoproteins have been demonstrated to bind Toll-like-receptor (TLR2) present on antigen presenting cells (APCs), leading to their maturation and subsequent activation of helper T-cells and antibody producing B-cells (Kovacs-Simon *et al.* 2011). As noted by a recent study (Lugade *et al.* 2011), immunization of mice with non-lipidated antigen resulted in reduced antigen uptake by APCs and subsequently lowered cytokine releasing T-cell response. In our study, it was more likely that absence of either the lipid motif endowed immuno-stimulatory properties with rOmp16 antigen or reduced TLR2 stimulation by decreased endocytosis might have resulted in diminished cytokine production from sensitized cells. However, in future trials, a higher antigen dose may probably lead to relatively enhanced uptake and processing by APCs leading to

enhanced antibody production and better protective immunity.

Mice immunized subcutaneously with non-lipidated rOmp16 resulted in a more pronounced increase in Th2 response (IgG1) compared to Th1 (IgG2a) mediated immunity, indicating a predominant humoral response, a most commonly observed phenomenon in immunization trials with other bacterial lipoproteins (Kovacs-Simon *et al*. 2011, Lugade *et al*. 2011). Additionally, CFA/IFA used along with rOmp16 was well known to predominantly contribute for Th2 mediated immunity (Cox and Coulter 1997). Nevertheless, capability of rOmp16 to stimulate the cellular immunity against *P. multocida* infections, if any, requires further investigations.

Bacterial PALs are known to activate inflammation through TLR2. Since PAL and LPS known to synergistically activate APCs, the presence of lipid motif with Omp16 would be beneficial. Hence, we presume that an enhanced stimulation of protective immune response could be achieved either by direct conjugation of lipid motif or co-administration of Pam3Cys, an exogenous lipid adjuvant, along with non-lipidated rOmp16 antigen. As noted in the past (Godlewska *et al.* 2009, Kovacs-Simon *et al.* 2011), it is more likely that rOmp16 adjuvanted with lipid motif may lead to enhanced antigen uptake, robust and long lasting immune response, which could provide much higher protection against challenge. Secondly, on the basis of fact that in pasteurellosis, the infection route usually involves entry of the microbe through mucosal surfaces, induction of a robust mucosal immune response either by intranasal or aerosol vaccination may be an effective control strategy. Notably, an intranasal vaccination of mice with recombinant P6 of *P. pneumotropica* produced protection against pneumonia. Further, it was also noted that protection could be partially transferred with CD4(+) T cells and pulmonary challenge with the P6 antigen induced interferon-γ and the Th17 cytokine IL-21. Similarly, intranasal vaccination of mice with DNA vaccine containing P6 protein of *H. influenzae* also induced specific protective immunity in the upper respiratory tract. These studies implied that related homologous proteins like rOmp16 could also provide enhanced protective immunity against respective pathogens in their natural hosts.

Conclusively, the study demonstrated the immunogenicity of rOmp16 in mouse model. Further, studies could also be focused on developing live attenuated *P. multocida* strains or live bacterial vectors expressing *Pasteurella* antigens or carrying target genes in DNA vaccine for delivery at the mucosal surface so as to stimulate cross-protective immunity against haemorrhagic septicaemia/ pasteurellosis in livestock.

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REFERENCES

Ahmad T A, Rammah S S, Sheweita S A, Haroun M and El-Sayed L H. 2014. Development of immunization trials against *Pasteurella multocida*. *Vaccine* **32**: 909–17.

Biswas A, Shivachandra S B, Saxena M K, Kumar A A, Singh V P and Srivastava S K. 2004. Molecular variability among strains of *Pasteurella multocida* isolated from an outbreak of haemorrhagic septicaemia in India. *Veterinary Research Communications* **28**: 287–98.

Cox J C and Coulter A R. 1997. Adjuvants-a classification and review of their modes of action. *Vaccine* **15**: 248–56.

Godlewska R, Wisniewska K, Pietras Z and Jagusztyn-Krynicka E K. 2009. Peptidoglycan-associated lipoprotein (Pal) of Gram-negative bacteria: function, structure, role in pathogenesis and potential application in immunoprophylaxis. *FEMS Microbiology Letters* **298**: 1–11.

Goswami P P, Chaudhuri P, Tiwari V, Parihar N S and Harbola P C. 2004. Cloning and sequencing of a 16 kDa outer membrane protein gene of *Pasteurella multocida* P52. *Veterinary Research Communications* **28**: 17–25.

Hatfaludi T, Al-Hasani K, Boyce J D and Adler B. 2010. Outer membrane proteins of *Pasteurella multocida. Veterinary Microbiology* **144**: 1–17.

Kasten R W, Hansen L M, Hinojoza J, Bieber D, Ruehl W W and Hirshi D C. 1995. *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae. Infection and Immunity* **63**: 989–93.

Kasten R W, Wakenell P S, Ahmad S, Yilma T D and Hirsh D C. 1997. Lack of protection against avian cholera by vaccination with recombinant P6-like protein from *Pasteurella multocida*. *Avian Diseases* **41**: 972–76.

Kovacs-Simon A, Titball R W and Michell S L. 2011. Lipoproteins of bacterial pathogens. *Infection and Immunity* **79**: 548–61.

Kumar A A, Shivachandra S B, Biswas A, Singh V P, Singh V P and Srivastava S K. 2004. Prevalent serotypes of *Pasteurella multocida* isolated from different animal and avian species in India. *Veterinary Research Communications* **28**: 657–67.

Kumar A, Mohanty N N, Yogisharadhya R, Chacko N and Shivachandra S B. 2014. Structural features of a highly conserved Omp16 protein of *Pasteurella multocida* and comparison with related peptidoglycans-associated lipoproteins (PAL). *Indian Journal of Microbiology* **55**(1): 50–56.

Kumar A, Yogisharadhya R, Ramakrishnan M A, Viswas K N and Shivachandra S B. 2013. Structural analysis and cross-protective efficacy of recombinant 87 kDa outer membrane protein (Omp87) of *Pasteurella multocida* serogroup B:2. *Microbial Pathogenesis* **65**: 48–56.

Lo M, Boyce J D, Wilkie I W and Adler B. 2004. Characterization of two lipoproteins in *Pasteurella multocida*. *Microbiology and Infection* **6**: 58–67.

Lugade A A, Bianchi-Smiraglia A, Pradhan V, Elkin G, Murphy T F and Thanavala Y. 2011. Lipid motif of a bacterial antigen mediates immune responses via TLR2 signaling. *PLoS One* **6**: e19781.

Shivachandra S B, Kumar A A, Gautam R, Singh V P, Saxena M K and Srivastava S K. 2006. Identification of avian strains of

Pasteurella multocida in India by conventional and PCR assays. *Veterinary Journal* **172**: 561–64.

Shivachandra S B, Viswas K N and Kumar A A. 2011. A review of haemorrhagic septicaemia in cattle and buffalo. *Animal Health Research Reviews* **12**: 67–82 .

Shivachandra S B, Yogisharadhya R, Ahuja A and Bhanuprakash V. 2012. Expression and purification of recombinant type IV fimbrial subunit protein of *Pasteurella multocida* serogroup B:2 in *Escherichia coli*. *Research in Veterinary Science* **93**: 1128–31.

Shivachandra S B, Kumar A, Yogisharadhya R, Ramakrishnan M A and Viswas K N. 2013. Carboxyl terminus heterogeneity of type IV fimbrial subunit protein of *Pasteurella multocida* isolates. *Veterinary Research Communications* **37**: 269–75.

Shivachandra S B, Kumar A, Mohanty N N, Yogisharadhya R, Chacko N, Viswas K N and Ramakrishnan M A. 2014a. Homogeneity of VacJ outer membrane lipoproteins among *Pasteurella multocida* strains and heterogeneity among members of *Pasteurellaceae*. *Research in Veterinary Science* **96**: 415–21.

Shivachandra S B, Kumar A, Yogisharadhya R and Viswas K N. 2014b. Immunogenicity of highly conserved recombinant VacJ outer membrane lipoprotein of *Pasteurella multocida. Vaccine* **32**: 290–96.

Shivachandra S B, Yogisharadhya R, Kumar A, Mohanty N N and Nagaleekar V K. 2015. Recombinant transferrin binding protein A (TbpA) fragments of *Pasteurella multocida* serogroup B:2 provide variable protection following homologous challenge in mouse model. *Research in Veterinary Science* **98** (1): 1–6.

Wheeler R. 2009. Outer membrane proteomics of *Pasteurella multocida* isolates to identify putative host-specificity determinants. *Bioscience Horizons* **2**: 1–12.

Wilkie I W, Harper M, Boyce J D and Adler B. 2012. *Pasteurella multocida*: diseases and pathogenesis. *Current Topics in Microbiology and Immunology* **361**: 1–22.