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Immune Protective Efficacy of Recombinant Outer Membrane Protein H of *Pasteurella multocida* B:2 Associated with Hemorrhagic Septicemia in Pakistan

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AAS and AG conceptualized the idea. AM worked on research and methodology. WS and NA assisted in purification of protein.

Key words
***Pasteurella multocida* B:2, Hemorrhagic septicemia, Recombinant OmpH protein, SDS-PAGE, Immunoblotting**

ABSTRACT

P. multocida B: 2 is a Gram-negative, small, non-flagellated, pleomorphic coccobacillus. It does possess different types of outer membrane proteins (OMP's) which assist in interaction with host cells. The outer membrane protein H (OmpH) of *Pasteurella multocida* B:2 is a major transmembrane porin that can be used as a subunit vaccine and development of diagnostic kit against hemorrhagic septicemia (HS) because of its immunogenic nature. *Pasteurella multocida* has economic importance because of endemic and epizootic diseases in domestic and wild animals. This bacterium is an opportunistic pathogen of upper respiratory tracts of wild and domestic animals and birds as well as in livestock . The *ompH* gene was amplified with in-house designed primers, the amplified gene (1002 bp) was cloned into pET 40-b (+) vector and transformed into *E. coli* BL21 (DE3) competent cells using heat shock transformation method to get expression. The product size was confirmed by restriction digestion. The rOmpH protein was purified by immobilized metal affinity chromatography by using His-Ni resins to get desired protein and characterized qualitatively by SDS-PAGE by using His antibodies and western blotting. Once purified the immunogenicity of rOmpH was evaluated in BALB/c mice. The expression of recombinant OmpH protein (rOmpH) resulted in its accumulation in cytosol. The purified rOmpH protein had a molecular weight of 36 kDa and concentration around 0.973 mg/mL. The BALB/c mice group injected with 100 ng of purified rOmpH exhibited significant protection (80%) when challenged with LD50 of *P. multocida* B:2. From the obtained results it is evident that rOmpH can be used as subunit vaccine against *P. multocida* B:2 and the purified rOmpH can also be further used to develop diagnostic test i.e., ELISA to screen and assess the immune response of cattle and buffaloes against *P. multocida* B:2 in developing countries like Pakistan.

INTRODUCTION

Hemorrhagic septicemia (HS) is an acute and fatal disease of bovines especially cattle, water buffalo, other domestic and wild ruminants (Farooq *et al*., 2007; Sting *et al*., 2020). The etiology of HS is *Pasteurella multocida;* a gram negative, coccobacillus and facultative anaerobe bacterium (Basagoudanavar *et al*., 2006). HS is endemic in Pakistan and has been recently ranked as one of the major constraints in bovine health and production. The results of a recently conducted survey revealed that HS is prevalent in all agroclimatic zones and is a leading cause of mortality in cattle and buffaloes (Ghafar *et al*., 2020). Agriculture sector is one of the major contributors in Pakistan's economy where livestock sector plays a key role in socioeconomic development in rural area. The diseases like HS are associated with huge economic loses in Pakistan and a study conducted in 2007 revealed an estimated loss of USD20 million due to HS related morbidity and mortality of livestock (Farooq *et al.,* 2007). The two serotypes of the bacterium that are most prevalent in Southern and Southeastern Asian and tropical Africa include B:2 and E:2 (Orynbayev *et al*., 2019; Cuevas *et al*., 2020; Kutzer *et al*., 2021). The disease outbreaks in

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Pakistan are mostly recorded in monsoon season when both temperature and humidity are very high.

In the Asian countries' vaccination is the major disease control strategy used to control HS. The oil adjuvant and live attenuated vaccines are commonly used in South and Southeast Asia, but the vaccine coverage is very low and not enough to provide effective control of HS in the susceptible population (Zamri-Saad and Annas, 2016; Magyar *et al*., 2017). The main reason of the low vaccination coverage includes the difficulties in injecting the oil adjuvant vaccine since there are many types of cattle managements practiced across the country (Benkirane and De Alwis, 2002).

The diagnosis of HS disease is based on indirect hemagglutination (IHA), and enzyme linked immunosorbent assay (ELISA). Researchers have developed in house indirect ELISA tests using various antigens extracted from *P. multocida* (B: 2) but there is no standard test available to compare the sensitivity and specificity of the available tests (Singhla *et al*., 2020).

The ongoing work to improve the immunogenicity of HS vaccine, the bacterium is fractionated, and various cell surface components have already been identified and studied in this regard. Outer membrane proteins (OMPs) are considered as potential candidates due to their antigenicity and immunogenicity (Boyce *et al*., 2006; Kumar *et al*., 2013; Lin *et al*., 2002). Outer membrane protein H (OmpH) is one such major protein in the envelope of *P. multocida* B:2 that has been purified and characterized as a transmembrane porin protein (Chevalier *et al*., 1993). The recombinant OmpH protein has been analyzed for its protective ability against *P. multocida* in mice and pig models (Lee *et al*., 2007; Luo *et al*., 1997), but there is no comprehensive study available in Pakistan exploring the immunogenic potential of OmpH protein. Hence, in the present study, we cloned and expressed the immunogenic OmpH protein of *P. multocida* B:2 in *E. coli* BL21 (*DE3*) cells and after purification the rOmpH protein was tested in mice for its immune protectivity when mice were challenged with LD50 of *P. multocida* B:2.

MATERIALS AND METHODS

Molecular confirmation of P. multocida *serotype B:2*

P. multocida serotype B:2 isolated from a recent outbreak in Pakistan was obtained from Veterinary Research Institute, Lahore, Pakistan. The received culture was propagated using brain heart infusion broth and incubated at 37 °C for 24 h 200 rpm. The isolate was grown on BHI blood agar, the characteristic grayish pinpointed colonies emerged after 24 h of incubation at 37 °C. One single isolated colony was used to further inoculate BHI broth, and the overnight culture was used for genomic DNA isolation. Genomic DNA was extracted by using bacterial genomic DNA extraction kit (GF-1, Vivantis) following manufacturer's protocol. To confirm the isolate polymerase chain reaction (PCR) was performed using (Sensoquest lab cycler, Germany) two sets of primers i.e., *P. multocida* -specific primer pair KMT1T7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3', KMT1SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3' and the serotype B:2 specific pair of primers i.e. KTT72 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3'KTSP61 and 5'-ATC-CGC-TAA-CAC-ACT-CTC-3' (Townsend *et al*., 1998; Lugtenberg *et al*., 1986).

Amplification of ompH *gene*

The PCR amplification of *ompH* gene was carried out using a primer pair (forward AAA AAA G ^ GA TCC ATG AAA AAG ACA ATC GTA GCA, reverse primer AAA AAA G ^ TC GAC TTA GAA GTG TAC GCG TAA AC) designed to amplify and introduce restriction sites of *BamHI* and *SalI* flanking the *ompH* gene. PCR product was amplified using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific, MAN0012393). The amplicon was analyzed on 1% (w/v) agarose gel stained with GelRed (Biotium) and visualized by UV shadowing using GelDoc system (BioRad, Hercules, CA, USA).

Cloning of ompH *gene*

The amplified *ompH* gene product was purified by Gene JET gel extraction kit (Thermo Fisher Scientific, K0691). The poly-A tailed *ompH* gene product was then cloned into the pTZ57R/T using InsTAclone PCR cloning kit (Thermo Fisher Scientific, MAN0012706).

Sequencing of cloned ompH

Cloned PCR product was sent to Advance Bioscience International for sequencing using M15 forward and reverses primers. The *ompH* sequence obtained was analyzed and compared with the *ompH* sequences available in GenBank using NCBI blastn tool "blastn" (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression of rOmpH protein

The recombinant plasmid was extracted from transformed *E. coli* DH5α cells using GeneJET plasmid miniprep kit. The extracted plasmid was digested with *BamHI* and *SalI* and the digested product was ligated in pET-40b (+) expression vector and transformed into *E. coli* BL21 (*DE3*) competent cells. For protein expression, the single confirmed recombinant colony was picked up and inoculated in 5 mL of Luria Bertani (LB) broth supplemented with kanamycin (50 µg/mL) and incubated

at 37 °C for 24 h at 200 rpm. The 3 mL of culture was used to inoculate next 100 mL of kanamycin supplemented LB media that is further incubated at 37 °C for 24 h at 200 rpm. Then 1-liter LB broth with kanamycin was prepared and divided into two flasks of 1 liter with 450 mL of media and added 50 ml of previously growth culture in each flask and incubated for 8 h at 37 °C of 200 rpm. After 4 h of incubation, the culture was induced with sterile IPTG (1 mM final concentration) to one of the 450 mL of broth while other flask was kept uninduced as negative control. Both cultures were incubated again for 8 h at 37 °C of 200 rpm.

Purification of expressed rOmpH protein

The rOmpH was purified by HisPur™ Ni-NTA Spin Columns (Thermo Scientific 88226). The eluted fractions were analyzed by 12% SDS-PAGE for detection of proteins. To quantify the protein Bradford method was used (Bradford, 1976).

Immunostaining

The immunostaining was performed by transferring the proteins from SDS-polyacrylamide gel to nitrocellulose membrane by method described by Burnette (1981).

Immunogenicity testing in mice

The BALB/c mice were purchased from the colony managed in University of Veterinary and Animal Sciences. The mice were divided into four groups (G1, G2, G3 and G4) with each group having 5 mice. The G1 and G2 groups were injected subcutaneously with 50 and 100 µg adjuvant mixed rOmpH protein respectively. Whereas the control group (G3 and G4) was administered with 100uL of phosphate buffer saline (PBS). Booster dose was given on 7-, 14- and 28- days post-priming and blood was collected from immunized mice. After immunization the mice were challenged with *P. multocida* B:2 with LD_{50}-$10^{6.6}$ (0.1 mL) calculated by using the method described by Reed and Muench (1938). After 5 and 10 days of challenge the number of survivors was recorded. Repeat this experiment 3 times.

RESULTS

Molecular confirmation of isolate

The isolate was confirmed as *P. multocida* by having an amplified region of 480 bps (Fig. 1A) with species-specific primer pair (KMT1T7 and KMT1SP6). The serotype B:2 was confirmed by having an amplified region of 620 bps (Fig. 1B) using serotype specific primers (KTT72 and KTSP61).

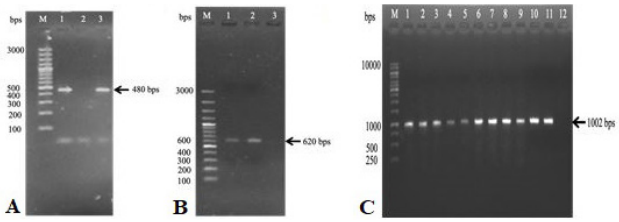

Fig. 1. Molecular identification of *P. multocida* B:2 and *Omph* (A) M: O'Gene Ruler 100bp Plus DNA ladder, lane 1: the 480 bps species conserver region amplified by using KMT1T7 and KMT1SP6 primers, lane 2: negative control, lane 3: positive control; (B) M: O'Gene Ruler 100bp Plus DNA ladder, lane 1: the 620 bps serotype specific region amplified by using KMT1T7 and KMT1SP6 primers, lane 2: positive control, lane 3: negitive control. (C) M: O'Gene Ruler 1 kb DNA ladder, lane 1-10: amplified *omph* amplificaon, lane 11: positive control, lane 12: negitive control.

Amplification and cloning of ompH *gene*

For amplifying *ompH* gene of *P. multocida* B:2 in-house designed primers were used. The Figure 1C shows 1002bp amplicons were matching the size of *ompH* gene of *P. multocida* B:2. The purified *ompH* gene fragment was cloned in pTZ57R/T cloning vector. The cloning was confirmed by double digestion of pTZ57R/T vector with *BamHI* and *SalI*. The appearance of two bands - one around 1002 bps (*ompH*) and other around 2886 bps confirmed the presence of *ompH* gene fragment and pTZ57R/T vector, respectively (Fig. 2A). The PCR product was sequenced. The obtained *ompH* gene sequence was analyzed for homology through "blastn" tool of NCBI. The *ompH* sequence exhibited 99.9% identity with outer membrane (*omph*) gene of *P. multocida* strain P52 (EU016232.1) with sequence coverage of 95%. Once the gene fragment was confirmed by sequencing, the *ompH* gene and expression vector i.e., pET-40b (+) was digested with *BamHI* and *SalI*. After digestion the *ompH* gene and pET-40b (+) vector were ligated together using DNA ligase. The ligated vector was transformed in *E. coli* BL21 (*DE3*) cells and positive recombinants were selected on kanamycin (50 µg/mL) supple mented agar plates. The recombinant colony was inoculated in kanamycin supplemented LB broth and overnight culture was used to extract plasmid. The extracted plasmid was digested with *BamHI* and *SalI* to further confirm the cloning. The presence of two bands of 1002 and 6190 bps, confirmed the successful cloning of *omph* in pET-40b (+) vector (Fig. 2B).

Expression of rOmpH protein

For expression of rOmpH protein, the recombinant BL21 (*DE3*) cells were grown in kanamycin supplemented

LB broth and once the culture reached to an OD_{600} of 0.6-0.8, IPTG (final concentration of 1mM) was added to induce the expression of rOmpH protein in one flask whereas another flask was kept uninduced. One milliliter culture was taken from induced and uninduced culture to compare total cell protein profile of both induced and uninduced cells. The presence of a band around 36 kDa in induced cell samples (Lane 1-3, Fig. 3A) can be attributable to rOmpH protein as this particular band was absent in uninduced cell (Lane 4, Fig. 3A).

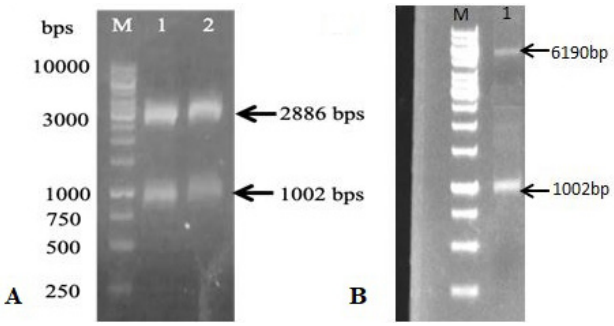

Fig. 2. (A) Restriction digestion of pTZ57R/T plasmid cloned with *ompH* gene. The enzyme digesation confirmed the presence of gene of interest *omph* characterized by appearance of 1002 bps band and pTZ57R/T plasmid characterized by 2886 bps band (lane 1 and 2), M: O'Gene Ruler 1 kb DNA ladder. (B) the plasmid (pET-40b(+):omph) was digested with *BamHI* and *SalI* to futher confirm the cloning. The presence of two bands of 1002 and 6190 bps, confirmed the successful cloning of *omph*inpET-40(+) vector.

N purification of expressed rOmpH protein

Around 3g of induced BL21 (*DE3*) cell mass was sonicated and the supernatant was separated after centrifugation. The rOmpH protein was purified by Ni^{+2} affinity chromatography and purified fractions were used to run on 12% reducing polyacrylamide gel. The elution fractions contained single band of 36 kDa (Fig. 3B). For quantifying the purified protein, the eluted fractions were combined and analyzed by Bradford assay. The protein concentration was quantified to be 0.973 mg/mL.

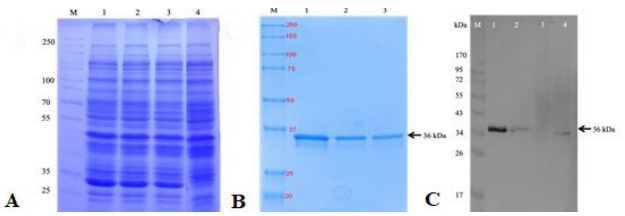

Fig. 3. Total cell protein analysis of induced and uninduced transformed BL21 (*DE3*). (A) M is Page ruler plus prestained protein ladder, Lane 1-3 correspond to the total cell protein of induced BL21 (*DE3*) transformed cells and lane 4 corresponds to the total cell protein of uninduced BL21 (*DE3*) cells termed as negative control. (B) Qualitative analysis of purified rOmph protein (Lane 1, 2 and 3). (C) Immunoblotting of rOmpH protein. M: PageRuler protein ladder, lane 1-2: purified rOmph protein, lane 3: negative control, lane 4: histidine tag containing protein used as positive control.

Immunostaining

The immunoblotting was performed using primary anti-histidine antibody and secondary horse reddish peroxidase conjugated goat antibody. The membrane was developed by BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium) enzyme substrate. The emergence of band around 36 kDa (Fig. 3C, Lane 1-2) confirmed the presence of histide tagged rOmpH protein.

Immunogenicity testing in mice

It was observed that in G1, immunized with 50 µg of rOmpH, 11 mice were survived out of 15 by the end of 10^{th} day and exhibited 73 % protection against *P. multocida* B:2. Whereas in G2, immunized with 100 µg of rOmpH, 13 mice were survived and showed 86 % protection against *P. multocida* on 10^{th} day of post challenge. The control group (G3) injected PBS and challenged with 0.1 mL of *P. multocida* B:2 and exhibited 100 % mortality while control group (G4) have 100% survival.

Table I. The immunogenic efficacy testing of rOmpH in mice.

Groups	Hyperimmune sera rOmpH [a](ug)	Challenged with PM[b] (mL)	No. of mice	Recovery[c] (%) in 5 days	Recovery (%) in 10 days
G1	50	0.1	15	12(80)	11(73)
G2	100	0.1	15	14(93)	13(86)
G3	PBS control group with challenged	0.1	15	0(0)	0(0)
G4	PBS Control group without challenged	0	15	15(100)	15(100)

a, 100uL of rOmpH protein was injected subcutaneously; b, mice were challenged with 0.1 mL of *P. multocida* B:2; c, is percentage recovery.

DISCUSSION

In Pakistan, HS is considered an important disease of bovine and it causes major economic losses due to high mortality in the infected cattle and buffalos. As control strategy, animals are vaccinated with alum-precipitated HS vaccine. Despite the vaccination, the death toll due to HS is still high. The major reasons of vaccine failure in developing protection against HS can be attributed to low antigenicity and degraded quality of vaccine due to poor storage conditions. Any vaccine that can induce sufficient immunogenicity and easy to store is highly desirable. This study was designed to seek the immunogenic potential of rOmpH protein of *P. multocida* B:2 for its prospective use as subunit vaccine against HS.

The procured culture isolated from HS infected animal during an outbreak in Pakistan was confirmed molecularly by using two sets of species and serotype specific primers designed and optimized already by (Townsend *et al*., 1998). The appearance of 480 bps band with KMT1T7 and KMT1SP6 primer pair confirmed the *P. multocida* and the amplification of 620 bps region with KTT72 and KTSP61 primers verified the B:2 serotype of *P. multocida*. The similar results were reported by Lugtenberg (1986). Once the isolate was confirmed to be *P. multocida* B:2 the *ompH* gene was amplified by using in-house designed primers and a 1002 bp amplicon was analyzed on agarose gel. Luo in 1999 also reported similar molecular size of *ompH* gene fragments amplified from *P. multocida* serotype associated with fowl cholera (Luo *et al*., 1999; Peng *et al*., 2019)

The *ompH* gene was further cloned into multiple cloning sites (MCS) of pTZ57R/T vector and selected via blue-white screening which is a commonly used method of screening cloning (Sambrook *et al*., 1989; Singh *et al*., 2011). The white colonies were considered positive. After confirming the *ompH* gene fragment via sequencing, the gene was cloned in expression vector pET-40b (+). The pET-40b (+) was used with some specification as 6x histidine tag helped in the purification of protein have an affinity with nickel column and dsbc signals help in localization of the protein to a soluble cytoplasmic fraction. The pET-40b (+): *ompH* plasmid was transferred to BL21 (*DE3*) cells for expression of rOmpH protein. The recombinant protein was expressed intracellular and extracted by the sonication of BL21 (*DE3*) cells. The supernatant after centrifugation of cell lysate was used for purification of protein by immobilized-nickel affinity chromatography. The similar expression and purification of rOmpH protein is described previously (Singh *et al*., 2011). As the target protein rOmpH has 6x histidine tag and have higher affinity with nickel charged resin and was eluted by applying 1 M imidazole. The eluted fractions were analyzed by 12 % SDS-PAGE. The purified rOmpH protein was observed to have a molecular weight of 36 kDa. In native form porin protein remains stable as a homotrimer and boiling the protein in the presence of detergent (i.e., SDS) denatures it and convert it to monomer. The size of denatured monomer porin protein varies from 34 to 42 KDa size depending upon serotypes and the method used for analysis (Barlowe *et al*., 1993; Lubke *et al*., 1994)

The protein was further confirmed by immunoblotting using anti-histidine antibodies. The anti-histidine antibody binds specifically with 6x histidine tag and hence the expressed rOmpH was characterized by immunoblotting. Immunoblotting is routinely used to characterize and confirm proteins. Singh *et al*. (2009) also used immunoblotting to characterize 37 KDa recombinant porin protein (rOmpH) of *P. multocida* B:2 using anti-histidine antibodies (Singh *et al*., 2009). Similarly in another study two porin proteins of *P. multocida* B:2 (30 and 37 kDa) was characterized by immunoblotting.

The immunogenic potential of rOmpH protein was studied by injecting mice with two different doses of rOmpH (50 and 100 g/kg body weight) and a booster dose given on 7-, 14- and 28 days post priming. The immunized mice were then challenged with 10 LD50 of *P. multocida* B:2 to analyze the extent of protection each group received. The results exhibited a dose dependent protection against *P. multocida* B:2 where the group of mice injected with 100 µg rOmpH/kg body weight of mice (G2) secured 86 % protection whereas group injected with 50 µg rOmpH/ kg body weight of mice (G1) received 73 % protection. The control group injected with PBS (G3) and challenged with 100 LD_{50} of *P. multocida* B:2 exhibited 100% mortality. The similar extent of protection was reported in previously published studies (Basagoudanavar *et al*., 2006; Bhat and Jain, 2010; Kharb and Charan, 2011). Tan *et al*. (2010) reported 100% protection in mice immunized and challenged intraperitonially (IP) whereas the mice which were immunized subcutaneously and challenged IP exhibited 86% protection similar to what was observed in this study (Tan *et al*., 2010).

CONCLUSION

In conclusion the rOmpH of *P. multocida* B:2 exhibited effective immunogenicity and protection against infection and will be used to develop an indirect ELISA test for diagnosis of *P. multocida* B:2.

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IRB approval

The BALB/c mice were cared for using protocol approved by the institution review committee for Animal Handling of the University of Veterinary and Animal Sciences, Pakistan.

Ethics statement

The study was carried out in compliance with guidelines issued by ethical review board committee of University of Veterinary and Animal Sciences, Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Barlowe, C., d'Enfert, C., and Schekman, R., 1993. Purification and characterization of SAR1p, a small GTP binding protein required for transport vesicle formation from the endoplasmic reticulum. *J. biol. Chem*., **268**: 873-879. https://doi.org/10.1016/S0021-9258(18)54015-1

Basagoudanavar, S., Singh, D. and Varshney, B., 2006. Immunization with outer membrane proteins of *Pasteurella multocida* (6: B) provides protection in mice. *J. Vet. Med*., **53:** 524-530. https://doi.org/10.1111/j.1439-0442.2006.00900.x

Benkirane, A. and De Alwis, M.C.L., 2002. *Haemorrhagic septicaemia*,: its significance, prevention and control in Asia. *Vet. Med. (Praha).,* **47**: 234-240.

Bhat, NH. and Jain, S., 2010. Immunogenic evaluation of a recombinant 49-kilodalton outer membrane protein of *Salmonella typhi* as a candidate for a subunit vaccine against typhoid. *J. Infect. Dis. Immun*., **2**: 30-40.

Boyce, J.D., Cullen, P.A., Nguyen, V., Wilkie, I. and Adler, B.J.P., 2006. Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the *in vivo* environment of the natural host. *J. Proteomics*, **6**: 870-880. https://doi.org/10.1002/pmic.200401342

Bradford, M.,1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.,* **2**: 248-254.

Burnette, N.W., 1981. Western blotting: Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem.,* **112**: 195-203.

Chevalier, G., Duclohier, H., Thomas, D., Shechter, E. and Wroblewski, H., 1993. Purification and characterization of protein H, the major porin of *Pasteurella multocida*. *J. Bacteriol. Res*., **175**: 266-276. https://doi.org/10.1128/jb.175.1.266-276.1993

Cuevas, I., Carbonero, A., Cano, D., Pacheco, IL., Marín, JC. and Borge, C., 2020. First outbreak of bovine haemorrhagic septicaemia caused by *Pasteurella multocida* type B in Spain. Short communication. *Acta Vet. Hung*., **68**: 8-11. https://doi.org/10.1556/004.2020.00014

Farooq, U., Irshad, H., Badar, N. and Ali, R., 2007. Status of haemorrhagic septicaemia based on epidemiology in Pakistan. *Pak. Vet. J*., **27**: 67-72.

Ghafar, A., McGill, D., Stevenson, MA., Badar, M., Kumbher, A., Warriach, HM., Gasser, RB. and Jabbar, A., 2020. A participatory investigation of bovine health and production issues in Pakistan. *Front. Vet. Sci*., **7**: 248. https://doi.org/10.3389/fvets.2020.00248

Kharb, S. and Charan, S., 2011. Mucosal immunization provides better protection than subcutaneous immunization against *Pasteurella multocida* (B: 2) in mice preimmunized with the outer membrane proteins. *Vet. Res. Commun*., **35**: 457-461. https://doi.org/10.1007/s11259-011-9484-8

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. *Microb. Pathog*., **65**: 48-56. https://doi.org/10.1016/j.micpath.2013.09.007

Kutzer, P., Szentiks, C.A., Bock, S., Fritsch, G., Magyar, T., Schulze, C., Semmler, T. and Ewers, C., 2021. Re-emergence and spread of haemorrhagic septicaemia in Germany: The wolf as a vector? *J. Microorganisms*, **9**: 1999. https://doi.org/10.3390/microorganisms9091999

Laemmli, UK., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685. https://doi.org/10.1038/227680a0

Lee, J., Kim, Y.B. And Kwon, M., 2007. Outer

membrane protein H for protective immunity against *Pasteurella multocida*. *J. Microbiol*., **45**: 179-184.

Lin, J., Huang, S. and Zhang, Q., 2002. Outer membrane proteins: key players for bacterial adaptation in host niches. *Microbes Infect*., **4**: 325-331. https://doi.org/10.1016/S1286-4579(02)01545-9

Lubke, A., Hartmann, L., Schronder, W. and Hellmann, E., 1994. Isolation and partial characterization of the major protein of one outer membrane of *Pasteurella haemolytica* and *Pasteurella multocida*. *Zent. Bakteriol*., **281**: 45-54. https://doi.org/10.1016/S0934-8840(11)80636-5

Lugtenberg, B., van Boxtel, R., Evenberg, D., de Jong, M., Storm, P. and Frik, J., 1986. Biochemical and immunological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect. Immun*., **52**: 175-182. https://doi.org/10.1128/iai.52.1.175-182.1986

Luo, Y., Glisson, J.R., Jackwood, M.W., Hancock, R., Bains, M, Cheng, I.H, and Wang, C., 1997. Cloning and characterization of the major outer membrane protein gene (*ompH*) of *Pasteurella multocida* X-73. *J. Bacteriol*., **179**: 7856-7864. https://doi.org/10.1128/jb.179.24.7856-7864.1997

Luo, Y., Zeng, Q., Glisson, J.R., Jackwood, M.W., Cheng, I.H., and Wang, C., 1999. Sequence analysis of *Pasteurella multocida* major outer membrane protein (OmpH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. *J. Vaccine*, **17**: 821-831. https://doi.org/10.1016/S0264-410X(98)00266-7

Magyar, T., Ujvári, B., Szeredi, L., Virsinger, N., Albert, E., Német, Z., Csuka, E. and Biksi, I., 2017. Re-emergence of bovine haemorrhagic septicaemia in Hungary. *Acta Vet. Hung*., **65**: 41-49. https://doi.org/10.1556/004.2017.004

Orynbayev, M., Sultankulova, K., Sansyzbay, A., Rystayeva, R., Shorayeva, K., Namet, A., Fereidouni, S., Ilgekbayeva, G., Barakbayev, K. and Kopeyev, S., 2019. Biological characterization of *Pasteurella multocida* present in the Saiga population. *BMC Microbiol*., **19**: 1-10. https://doi.org/10.1186/s12866-019-1407-9

Pati, U.S., Srivastava, S.K., Roy, S.C. and More, T., 1996. Immunogenicity of outer membrane protein of *Pasteurella multocida* in buffalo calves. *Vet Microbiol.,* **4**: 301-311.

Peng, Z., Wang, X., Zhou, R., Chen, H., Wilson, BA. and Wu, B., 2019. *Pasteurella multocida*: genotypes and genomics. *Microbiol. mol. Biol. Rev*., **83**: e00014-00019. https://doi.org/10.1128/MMBR.00014-19

Reed, L.V. and Muench, H., 1938. A simple method of estimating fifty per cent end points. Am. *J. Hyg*., **27**: 493. https://doi.org/10.1093/oxfordjournals.aje.a118408

Sambrook, J., Fritsch, EF. and Maniatis, T., 1989. *Molecular cloning: A laboratory manual.* CSHL press.

Singh, R., Kumar Gupta, P. and Prasad Rao, V.D., 2009. Expression and purification of the major outer membrane protein (OmpH) of *Pasteurella multocida* P 52 from *Escherichia coli*. *Vet. Arh*., **79**: 591-600.

Singh, R., Tewari, K., Packiriswamy, N., Marla, S. and Rao, VDP., 2011. Molecular characterization and computational analysis of the major outer membrane protein (*ompH*) gene of *Pasteurella multocida* P52. *Vet. Arh*., **81**: 211-222.

Singhla, T., Tankaew, P. and Sthitmatee, N., 2020. Validation of a novel ELISA for the diagnosis of hemorrhagic septicemia in dairy cattle from Thailand using a Bayesian approach. *Vet. Sci*., **7**: 163. https://doi.org/10.3390/vetsci7040163

Sting, R., Blazey, B., Schwabe, I., Schwalm, A., Müller, S., Sprague, L. and Rau, J., 2020. Haemorrhagic septicaemia (septicaemic pasteurellosis) in cattle in Baden-Wuerttemberg (Germany). *Berl. Munch. Tierarztl. Wochenschr*., **133**.

Tan, H.Y., Nagoor, N.H. and Sekaran, S.D., 2010. Cloning, expression and protective capacity of 37 kDa outer membrane protein gene (ompH) of *Pasteurella multocida* serotype B: 2. *Trop. Biomed.,* **27**: 430-441.

Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M. and Dawkins, H.J., 1998. Development of PCR assays for species-and type-specific identification of *Pasteurella multocida* isolates. *J. clin. Microbiol*., **36**: 1096-1100. https://doi.org/10.1128/JCM.36.4.1096-1100.1998

Zamri-Saad, M. and Annas, S., 2016. Vaccination against hemorrhagic septicemia of bovines. A review. *Pak. Vet. J*., **36**: 1-5.