Weber Medicine & Clinical Case Reports
ISSN:2449-1624
http://www.weberpub.org/wmccr.htm

International Open Acces Journal
Wesley & Eber Publishing

Research Article **Volume 1-4(2015)**

Protective Potential of High Molecular Weight Outer Membrane Protein of *Pasteurella multocida* Type 6:B

M. RAMLAN[1] Ph D, N. MUNIANDY[2] Ph D, Y. No NA[3] & A. RASEDEE[4] B.V.Sc, PhD

[1] Veterinary Services Department Malaysia, Wisma Tani, Block Podium 4G1, Precinth 4, Federal Government Administration Centre, 62630 Putrajaya, Malaysia.
[2] Faculty of Medicine, Universiti Kuala Lumpur-Royal College of Medicine Perak, Ipoh, Perak, Malaysia.
[3] Veterinary Research Institute, 59 Jalan Sultan Azlan Shah, 31400 Ipoh, Perak, Malaysia.
[4] Faculty of Veterinary Medicine, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Accepted 20th August, 2015

Abstract

Iron-regulated outer membrane proteins (OMPs) of *Pasteurella multocida* have been shown previously to elicit superior protective immunity. We have identified that high molecular weight OMP 116 kDa was expressed when *Pasteurella multocida* type 6:B was grown under iron-restricted condition. The highly purified 116 kDa protein was found to be only 60% protective to mice against experimental pasteurellosis. Two other proteins with high molecular weight (MW) (87 and 71 kDa) were shown to be expressed slightly more when grown under iron-restricted condition; however, the protection afforded by 87 kDa protein was is only 40%.

Keywords: *Pasteurella multocida*, outer membrane protein, protection, mice

1. Introduction

Haemorrhagic septicaemia (HS) in cattle and buffaloes is caused by two serotypes of *P. multocida*, viz. Asian serotype 6:B and the African serotype 6:E (Namioka-Carter)(Verna and Jaiswal (1997). *P. multocida* live in the tonsils of cattle and buffaloes and the organism appears intermittently in the nasopharynx of apparently healthy animals (Wijewardane T.G., *et al.*,(1993).The serotype B organism has been recognised as the etiological agent of HS of cattle and buffaloes in Asia. Besides lipopolysacharides (LPS), outer membrane proteins (OMPs) of *P. multocida* have also been studied as potential immunogens. The immunogenic role of OMPs from *P.multocida* 3:A has been characterised in rabbits by Lu *et al.*,(1998).They demonstrated that rabbits mounted major antibody responses against five OMPs (27, 37.5, 49.5, 58.7 and 64.4 kDa) which also protected rabbit against homologous challenge (Lu Y.S. *et al.*, (1991a).

Similarly, others have shown that iron-regulated OMPs or IROMPs with molecular weights (MWs) 76, 84 and 94 kDa expressed in *P.multocida* A:3 in iron-depleted medium play a significant role in cross-protective immunity(Srivastava, 1998; Choi-Kim *et al.*, 1991; Harper *et al*., 2006). Further, the virulence attributes, in-vivo expression, immunogenic and bactericidal properties of several *P. multocida* 3:A OMPs has been well studied (Confer *et al*, 1996; Dabo *et al*, 1997, 2003; Gatto *et al*., 2002; Davies *et al.*, 2004). More than 60 different OMPs ranging from 16 to 104 kDa from different *P.multocida* strains have been reported to play significant role in pathogenesis and immunity (Halfaludi *et al.*, 2010). The potential of OmpA (Confer and Ayalew 2013), OmpH (Boyce *et al.*, 2006, Dabo *et al.*, 2007; Shivachandra *et al*., 2013) as vaccine candidates was well explored against *P.multocida* challenged in mice (Vasfi Mirandi and Mittal(1997) and chicken (Luo Y. *et al.*, 1999).

Truscott and Hirsh (1988) demonstrated a 50 kDa *P. multocida* OMP that was antiphagocytic. Abdullahi *et al.*, (1990). however, failed to demonstrate any correlation between protection and antibody response to OMP from bovine isolates of *P. multocida* in challenged mice. This failure was most probably due to the use of mice instead of rabbits as there is similarity between *P. multocida* induced respiratory disease in cattle and that seen in the rabbit. It was not clear if the major OMPs in the range 19-36.5 kDa would serve as candidates for vaccine. Due to these discrepancies, OMPs were further investigated as potential immunogens for rabbit (Lu Y.S. *et al.,* 1991) and cattle [9, Srivastava SK., (1998). In addition, Dabo *et al* showed that *P.multocida* OmpA was a major immunogenic antigen that was conserved, surface-exposed and expressed in vivo. This was attributed to resistance of cattle to challenge with virulent *P.multocida* (Dabo *et al*, 1997; 2007; Shivachandra *et al.*, 2013). Calves vaccinated with OMPs or IROMPs developed high antibody response to a 96kDa protein (Prado *et al.*, 2005). This 96kDa OMP was found to be a homologue to the iron-regulated HasR, a heme acquisition receptor protein. Recently, Abubakar M.A (2014) has reviewed on the past efforts aimed at developing recombinant vaccines against *P. multocida*. Though recombinant proteins used as subunit vaccines have been reported to be safe (Hussaini, *et al.*, 2012), the cross- protective antigens still remain elusive. Stronger and broad spectrum vaccine can be achieved through identifying conserved immunogens of *P.multocida* (Shivachandra *et al*., 2011). Thus, the immunogenic and protective role of iron-regulated OMPs of *P. multocida* needs further investigation.

2. The purpose of the present study was;

- To determine the differences in the expression of OMPs under iron regulated conditions

Corresponding Author: N. Muniandy
Faculty of Medicine, Universiti Kuala Lumpur-Royal College of Medicine Perak, Ipoh, Perak, Malaysia.
E-mail:muniandy@unikl.edu.my

- To evaluate the immunogenic and protective potential of the major OMPs separated and isolated by preparative SDS-PAGE.

3. Materials and Methods

Bacteria strain

Pasteurella multocida type 6:B strain C82 (a vaccine strain) used at the Veterinary Research Institute (VRI), Ipoh was obtained through the courtesy of Dr S. Chandrasekaran. The seed culture was stored frozen in cattle blood in a biofreezer at -70°C and sub-cultured onto sheep blood agar (SBA) when required.

Preparation of outer membrane proteins

Outer membrane proteins were prepared from two liters of bacterial culture grown in the presence of α , α" -dipyridyl (20ug/ml) as according to Snipes *et al.*,(1988). Another batch of OMPs was also prepared from bacterial culture grown in the presence of 0.2 M ferric chloride.

Preparative SDS-PAGE on cylindrical gel, staining, excision and electro-elution

Preparative electrophoresis was performed on Prep Cell column (Model 491, Bio Rad) using 10% SDS-PAGE gel measuring 28 mm diameter x 10 cm length. The OMP sample (1,500 ug/ml) was diluted 1:4 in sample buffer and electrophoresis was performed for 4 hours at constant voltage (100V) until the dye front migrated to the end of the column. The cylindrical gel was forced out of the column using gentle pressure with tap water. The gel was then stained for one hour with 0.1% Coomassie brilliant blue-R250 and destained for 15 minutes only in methanol: acetic acid: water (4:1:5, v/v/v).

The stained protein bands of varying molecular weights that appeared as cylindrical discs along the gel were sliced out horizontally with a sharp scalpel. The stained external portion measuring about 1 mm along the circumference of the slice discs were excised and discarded. This ensured that the stained proteins of the gel were removed completely. The unstained inner portions were cut to small pieces for subsequent electro-elution. Elution of proteins from the pieces of gel was performed using Bio-Rad Model 422 electro-eluter following the manufacturer's instructions, for 90 minutes at 100 volts under cooling condition.

Preparative SDS-PAGE on slab gels and electroelution of the separated OMP component proteins were carried out according to Gilleland *et al.,*(1984). Individually separated bands in each lane were excised with a sharp scalpel and further cut into smaller pieces for subsequent electro-elution. Elution of stained proteins from these gel pieces was performed using a Bio Rad Model 422 electro-eluter following the manufacturer's instructions, for 60 mins at 100 volts under cooling condition.

Mini-gel SDS-PAGE

Discontinuous SDS-PAGE was performed according to LaemmLi (1970) using Bio-Rad Mini-Protein II gel slabs, 70 (L) x 80 (W) x 0.75 mm following the manufactures instructions (Bio Rad, USA). The resolving and stacking gel with 12% and 4% acrylamide, respectively, were used in all mini SDS-PAGE. Samples were diluted at least 1:4 with sample buffer and heated at 95°C for 5 minutes. Electrophoresis was performed at room temperature with constant voltage of 50 volts for the first 15 mins followed with 150 volts for 45 mins using running buffer.

Western blotting

Immunoblotting was carried out according to the method described by Towbin *et al.*, (1979). Proteins electrophoresed during SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane sheet (pore size; 0.2um, Bio-Rad Laboratories) in transfer buffer at 100 volts for 1 hour using a Mini Protein blotting system (Bio Rad Laboratories). The membrane containing the transferred proteins was probed with serum samples of mice against OMP and whole cell bacteria then with goat anti-mouse IgG conjugated with alkaline phosphatase. Reactivity was visualised by developing in a substrate system prepared by adding 50ul of 5% nitroblue tetrazolium (NBT) and 50ul of 7.5% bromochloroindoyl phosphate (BCIP) to 25ml of alkaline phosphatase substrate buffer (prepared just before use) until the desired intensity was achieved.

Active protection studies in mice

Groups of 6 mice were injected intra-peritoneally (i/p) with 50ug of purified protein on days 0, 7 and 14. The vaccinated mice were challenged subcutaneously (s/c) on day 28 post-vaccination at doses ranging 100 to 200 CPU with virulent *P. multocida* type 6:B grown for 10 hours in BHI. Mice were observed and mortality recorded for 5 days. Another group of 6 mice (control) were injected with PBS only and were challenged as above.

Determination of serum antibody titres by ELISA

Antibody titres of all serum samples, obtained by bleeding mice from the retro orbital plexus two days prior to challenge were determined using a direct ELISA as originally described by Engval and Perlman (1972) and modified by Mukkur *et al.*,(1991). The OMP prepared according to Snipes *et al.,* (1988) was used as antigen at a concentration of 15 ug/ml. Test sera samples were diluted 1/100 with dilution buffer (5% skim milk). Goat anti-mouse IgG conjugated alkaline phosphatase diluted 1:500 was used as secondary antibody. The bound conjugate was detected by the addition of a chromogenic substrate, 0.1% p-nitrophenyl phosphate, in 1 M diethanolamine buffer containing 0.02% MgCl. The optical density of the solution was then read at 405 nm using Titertek Multiskan MCC/340 MKII microtitre spectrophotometer (Labsystem, Finland).

4. Results

The expression of high MW proteins in bacteria grown under iron-regulated condition is shown in Figures 1 and 2. Different amounts of Sarkosyl extracted OMPs of *P. multocida* grown under iron-repleted condition were loaded on lanes 1 and 3 (Figure 1). It could be seen that the 116 kDa protein was not expressed under iron-replete condition whereas the 116 kDa protein band could be seen in lanes 2 and 4 for OMPs *of P. multocida* grown under iron limiting condition. Based on the thickness or intensity of the band it can also be seen that other high MW proteins (71, and 87 kDa) were also well expressed when the organisms were grown under iron-limiting condition (lanes 2 and 4).

The expression of high MW proteins was also shown by immunoblotting OMP or whole cell lysates with cattle *anti-P multocida* (whole cell) antiserum. As could be clearly seen in figure

3, the 116 kDa protein was only present in *P. multocida* grown under iron-limiting condition and not under the iron-repleted condition.

The protective capacity in mice against active challenge of each single protein obtained from preparative SDS-PAGE on cylindrical gel is shown in Table 1. Mice immunised separately with extracts containing 150ug proteins with molecular weights 48, 87 and 116 kDa were conferred 50-66% protections on active challenge with live organism. Similarly, the protective capacity of each stained protein after excision and electroelution was evaluated and the results are shown in Table 2. Mice immunised with approximately 150ug of stained proteins with molecular weights 87 and 116 kDa were also conferred 40 - 60% protections against active challenge. All mice immunised with purified proteins developed significantly ($p < 0.05$) higher antibody levels against the OMPs than controls.

5. Discussion

Iron-regulated OMPs were expressed by *P. multocida* grown in iron-restricted media (Snipes *et al*., 1998) High MW 76, 84 and 94 kDa OMPs, with affinity for siderophore binding were also expressed *by P. multocida* type A grown *in-vivo (Cho-Kim K et al.,* 1991). However, the actual immunogenic role of these proteins expressed under iron-regulated condition has not been fully investigated although comparison was made on the protective capacities of whole-cell killed vaccines grown under iron-depleted and iron-repleted conditions. Earlier studies have shown that whole-cell vaccine of *P. multocida* type 6:B grown under iron-restricted condition, in the presence of α, α"-dipyridyl as iron chelator, imparted superior protection against pasteurellosis in mice than the bacteria grown under iron-replete conditions (Muniandy and Mukkur , Personal communications).

Pasteurella multocida was shown to express unique proteins when grown in iron-restricted condition (Snipes *et al*.,(1988) and it has been postulated that *P. multocida* expresses antigens *in vivo* that were not expressed *in vitro* and these antigens induced heterologous serotype immunity(Rimler R.B. and Rhoades(1981). Iron is essential for bacterial growth and pathogenic bacteria, such as *P. multocida,* compete with host tissues *in vivo* to obtain iron. Proteins that were expressed under iron-limiting conditions were shown to be of outer membrane origin (Davies et al.,1994). The present study has shown that a high MW protein (116 kDa) was expressed when the bacteria was grown under iron limiting condition. Two other high MW proteins, i.e. 71 and 87 kDa proteins were also better expressed under iron-limitating condition than the 116 kDa protein.

Little is known about the mechanism by which *P. multocida* acquires iron *in vivo,* but a number of high MWOMPs with molecular masses 96, 84 and 80 kDa (Snipes et al., 1988) and 94, 84 and 76 kDa (Davies et al.,1994). were implicated by different researchers. It was reported that *P. multocida* serotype 3A expressed a 84 kDa OMP when grown in brain-heart broth containing α, α' -dipyridyl as iron-chelator. Comparison of OMPs of P *haemolytica* expressed *in vitro* and *in vivo* in cattle was also made by Davies *et al*.(1994) Lung-grown bacteria differed from iron-sufficient *in vitro* grown bacteria in having enhanced expression of 71, 77 and 100 kDa iron-regulated proteins. Differences were also apparent in the Western blot profiles of OMPs of *in vitro* and lung-grown bacteria. In addition, Gilmour *et al.,* (1991) demonstrated that vaccine containing iron-regulated protein of *P. haemolytica* type A2 enhances protection against experimental pasteurellosis in lambs. This strongly indicated that some cell membrane associated antigens that were apparently expressed under iron-limiting conditions could be responsible for conferring better protective immunity. Immunoblotting sonicated cell extracts and OMP preparation with sera obtained from calves vaccinated with live attenuated aroA derivative of *P.multocida* type2:B showed 37 and 50 kDa bands as strongly immunogenic. The 30 and 35 kDa bands were identified as OmpH and OmpA by MASCOT analysia, respectively. (Saeed Ataei *et al.,* 2009). However, these authors did not report on the immunogenicity of these OMPs.

By the modified procedure of preparative electrophoresis using Prep cell (Ramlan M. *et al*., 1998), eight protein bands were clearly seen in the cylindrical gel after staining with Coomassie blue. Three protein bands viz., 48, 87 and 116 kDa were separated into almost single bands as was confirmed after excision, electro-elution and re-electrophoresis in mini SDS-PAGE. However, the low MWproteins were not well separated and still contained other proteins as contaminants. Active protection studies of single proteins obtained after preparative polyacrylamide gel electrophoresis on cylindrical gel revealed that proteins with MWs 87 and 116 kDa conferred protection for up to 60% in immunized mice against experimental challenge. Ruffolo and Adler (1996) also showed that Oma87 was a protective outer membrane antigen as specific Oma87 antiserum protected mice against homologous lethal *P. multocida* challenge. Improved immunogenicity, due to enhanced production of some 84KDa protein was also shown when bacteria were grown under restriction conditions. (Srivastava, S.K. 1998).

Immunoblotting studies by Pati *et al*, (1996) suggested that the polypeptides of MWs 30, 37 and 44 kDa from OMP of *P. multocida* serotype B:2 were major immunogens. However, active protection studies on these individual proteins was not carried out by the latter. Also, passive mouse protection study by Ramdani and Adler (1991) showed that monoclonal antibodies of proteins with MWs 29, 33, 36 and 42 were opsonic and phagocytic but not protective.

It was very difficult to isolate pure proteins from crude OMP in large amounts from unstained gels. As such, electrophoresed disc gels were partially stained and proteins that could be seen in stained slab gels after SDS-PAGE were excised and electro-eluted. This procedure ensured that the purity of individual protein as judged visually was not in doubt. Active protection studies of the stained proteins showed that high MW proteins (116 kDa) conferred 60% protection. Few OMPs of *P. multocida* have been studied by other workers: a 50 kDa OMP *of P. multocida* type A was reported to have antiphagocytic activity (Truscott and Hirsh, 1988) ; a 37 kDa OMP of *P. multocida* type A was thought to be the major porin; a monoclonal antibody against 37.5 kDa OMP of *P. multocida* type 3A was protective in animal models (Lu ,Y.S,1991) and the highly purified 33 kDa protein prepared by Muniandy (1993), following solubilisation with Zwittergent and ion-exchange chromatography, provided only partial protection (25%) to immunised mice. The present study revealed a 116 kDa OMP was expressed in *P. multocida* under iron limiting condition. Active protection studies showed that 150 ug of such highly purified protein afforded 66% protection on active challenge.

Thus, the 48, 87 and 116 kDa OMPs of *P.multocida* type 6:B could be potential vaccine candidates.

6. Conclusion

While the protective and immunological properties of the isolated 71, 87 and 116 kDa OMPs in mouse model showed some promise as potential vaccine candidates, further purification, genome sequencing and characterization to elucidate the role of their related genes in health and disease, is necessary before their genes could be cloned and expressed on carrier vectors for immunization.

Acknowledgements

The authors wish to express their thanks to Prof. TKS Mukkur of School of Biomedical Sciences, Curtin University, Bently, Australia for his advice and guidance in the preparative SDS-PAGE techniques and active protection studies.

Recommendations for Further Studies

References

1. Abdullahi, M.Z., Gilmour, N.J.L. and Poxton, I.R. (1990). Outer membrane proteins of bovine strains of *Pesteurella multocida* type A and their doubtful role as protective antigens. J. Med. Microbiol, 32:3626-3629.

2. Abubakar Musa Ahmad (2014). Efforts towards the development of recombinant vaccines against *Pasteurella Multocida*. Science World J. Vol 9 (No2)

3. Choi-Kim, K., Maheswaran, S.K., Felice, L.J. and Molitor, T.W. (1991). Relationship between the iron- regulated outer membrane proteins and the outer membrane proteins of in vivo grown *Pasteurella multocida*. Vet. Microbiol., 28:75-92.

4. Confer, A.W. and Ayalew, S. (2013). The OmpA family of proteins: Roles in bacterial pathogenesis and immunity. Vet.Microbiol.163:207-222.

5. Dabo, S.M., Confer, A.W., and Murphy, G.L. (1997). "Outer membrane proteins of bovine *Pasteurella multocida* serogroup A isolates". Vet. Microbiol. 54 (2): 167 - 183.

6. Dabo, S.M., Taylor, J.D., and Confer, A.W. (2007). "*Pasteurella multocida* and bovine respiration diseases" Animal Health Res Rev. 8:129-150.

7. Davies, R.L., McCluskey, J., Gibbs, H.A., Coote, J.G., Freer, J.H. and Parton, R. (1994). Comparison of outer-membrane proteins of *Pasteurella haemolytica* expressed in vitro and in vivo in cattle. Microbiology, 140:3293-3300.

8. Engval, E. and Perlman, P. (1972). Enzyme-link immunosorbent assay, ELISA III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. J. ImmunoL, 109:129-135

9. Gatto, N.T.,Dabo, S.M.,Hancock, R.E., Confer, A.W.(2002). Characterisation of, and immune responses of mice to, the purified equivalent outer membrane protein of *Pasteurella multocida* serotype A:3 (Omp28). Vet Microbiol.87:221-235

10. Gilleland, H.E., Parker, M.G., Matthews, J.M. and Berg, R.D. (1984), Use of a purified outer membrane protein F (porin) preparation of Psedomonas aeruginosa as a protective vaccine in mice. Inf. Imm., 44:49-54.

11. Gilmour, N.J.L., Donachie, W., Sutherland, A.D., Gilmour, J.S., Jones, G.E. and Quirie, (1991). Vaccine containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. Vaccine. 9:137-140.

12. Hatfaludi, T., Al - Hasani, K., Boyce, J.D., and Adler, B. (2010). Outer membrane proteins of *Pasteurella multocida*. Vet. Microbiol. 144-1 -17

13. Harper, M., Boyce J.D, Adler B. (2006). "*Pasteurella multocida* pathogenesis" 125 years after Pasteur. FEMS Microbiology.. 265:1 - 10.

14. LaemmLi, U.K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, 277:680-685.

15.] Luo Y, Zeng Q, Glisson JR, Jackwood MW, Cheng IH 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. Vaccine. 17:821-831.

16. Lu, Y.S., Afendis, S.J. and Pakes, S.P. (1988). Identification of immunogenic outer membrane proteins of *Pasteurella multocida* 3:A in rabbits. Infect. Immun., 56:532-37.

17. Lu, Y.S., Lai, W.C., Pa kes, S.P. and Nie, L.C. (199la). A monoclonal antibody against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against pasteurellosis. Infect. Immun., 59:172- 80.

18. Lu, Y.W., Aguila, H.N., Lai, W.C. and Pakes, S.P. (1991 Ib). Antibodies to outer membrane proteins but not to lipopolysaccharide inhibit pulmonary proliferation of *Pasteurella multocida* in mice. Infect. Immun., 59:1470-475.

19. Mukkur, T.K.S., Walker, K.H., Jones, D., Wronksi, E. and Love, D.N. (1991). Immunizing efficacy of aromatic- dependent *Salmonella dublin* in mice and calves. Comp. Microbiol. Immunol. Inf. Dis., 14:243-256.

20. Muniandy, N. (1993). Immunochemical studies on protective antigens of *Pasteurella multocida* type 6:B. Ph. D ,Thesis, University of Sydney, p. 226-258.

21. Patid, 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., 52:301-311.

22. Prado ME., Dabo SM., Confer., AW. (2005). Immunogenicity of iron-regulated outer membrane proteins of Pasteurella multocida A:3 in cattle: molecular characterization of the immunodominant heme acquisition system receptor (HasR) protein. Vet. Microbiol. 105:269-280.

23. Ramdani and Adler, B. (1991) Opsonic monoclonal antibodies against lipopolysaccharide antigens of *Pasteurella multocida* and the role of LPS in immunity. Vet. Microibiol, 26:335-347.

24. Ramlan, M., Muniandy, N., No Na, Y. and Rogayah, M. (1998) Purification of high molecular weight outer membrane proteins of *Pasteurella multocida* type 2:B. In: Proc. 10th National Biotechnology Seminar, SIRIM Bhd,p.\85- 189.

25. Rimler, R.B. and Rhoades, K.R. (1981). Lysates of turkey-grown *Pasteurella multocida:* protection against homologous and

heterologous serotype challenge exposure. Am. J. Vet. Res., 42:2117-2121.

26. Rishendra Verna and Jaiswal T.N. (1997). Protection, humoral and cell-mediated immune responses in calves immunized with multiple emulsion haemorrhagic septicaemia vaccine. Vaccine, 15(11):1254-1260.

27. Ruffulo C. G and Ben Adler (1996). Cloning, sequencing, expression and protective capacity of the oma87 gene encoding the *Pasterella multocida* 87-kilodalton outer membrane antigen. Inf and Immunity. 64: (8) 3161-3167.

28. Snipes, K.D., Hansen, L.M. and Hirsch, D. (1988). Plasma and iron-regulated expression of high molecular weight outer membrane protein by *P. multocida*. Amer. J. Vet. Res., 49:1336-1338.

29. Shivachandra S.B., Viswas, K.N., Kumar, A.A. (2011). A review of hemorrhagic septicaemia in cattle and buffalo. Animl Health Res. Reviews 12(1):67-82.

30. Srivastava, S.K. (1998). Immunogenicity of *Pasteurella multocida* grown in iron restricted medium. J. Appl. Anim. Res., 13: 137-144.

31. Towbin, H., Stachelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. In: Proc. Natl. Acad. Sci., USA., 76:4350-4354.

32. Truscott, W. and Hirsh, D. (1988). Demonstration of an outer membrane protein with antiphagocytic activity from *Pesteurella multocida* of avian origin. Inf. Immun., 56:1538-1544.

33. Wijewardane T.G., Horadagoda, N.V., Vipulasiri, A.A. and Thalagoda,S.A. (1993). Isolation and characterisation of *P. multocida* from tonsils of apparently healthy cattle. In Pasteurellosis in production animals: ACIAR Proceedings No: 43. Patten, B.E., Spencer T.L., Johnson R.B, Hoffman D. and Lehane (Eds.), p. 209-214.

Figures and Tables

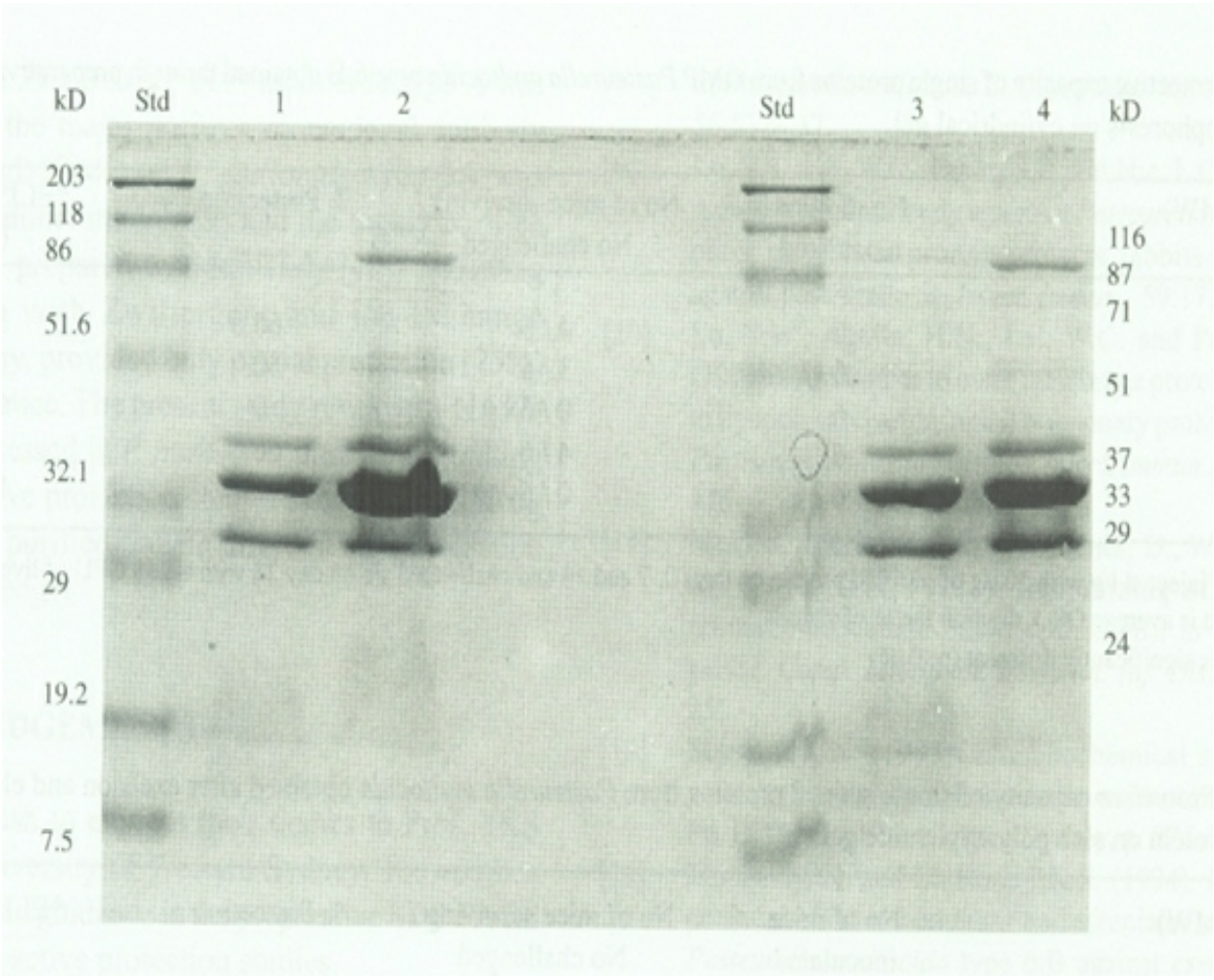

Figure 1. SDS-PAGE of OMPs from *P. multocida* grown under iron- regulated conditions stained with Coomassie blue. Lanes 1 and 2; 8 µg OMP of *P. multocida* grown under iron-repleted and iron-limiting conditions, respectively. Lane 3 and 4; 10 µg OMP of *P. multocida* grown under iron repleted and depleted conditions, respectively. Broad range molecular weight marker standard shown on the left.

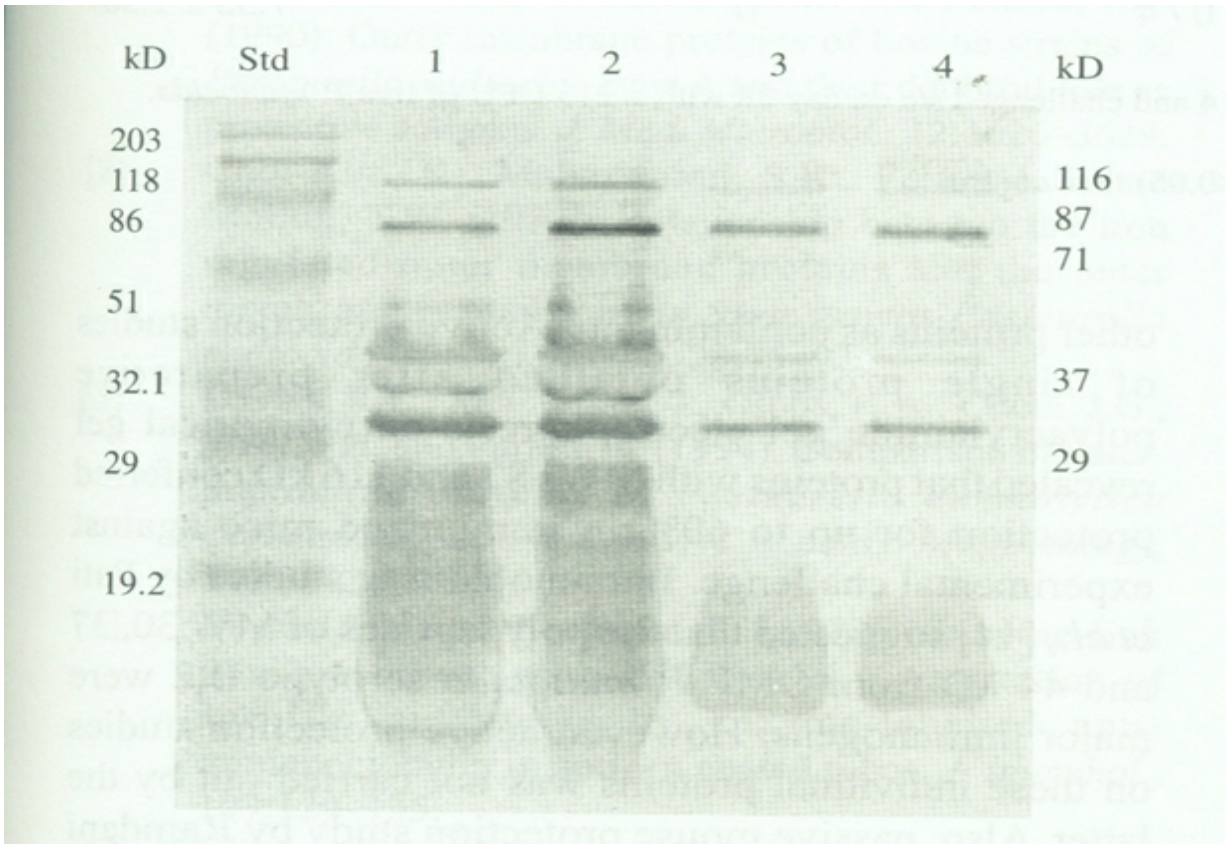

Figure 2. Immunoblot of OMP and Whole cell lysate (WC) from *P. multocida* grown under iron-repleted and iron-depleted conditions. Lanes 1 and 2; OMP from bacteria grown in the presence of ferric chloride and dipyridyl, respectively. Lanes 3 and 4; WC from ferric and DIP grown. Broad range molecular weight marker standard shown on the left.

Table 1. Protective capacity of single proteins from OMP *Pasteurella multocida* type 6:B obtained through preparative polyacrylamide |gel electrophoresis on cylindrical gel.

Protein (MW)	No of mice inoculated	No of mice surviving / No challenged	% Protection	*ELISA antibody litre (ΔΔ > ± SEM)
116kDa	6	4/6	66.6	30.32 ± 20.67 [b]
87 kDa	6	3/6	50	12.70 ± 2.69 [b]
71 kDa	6	0/6	0	1 0.54 ± 4.46 [b]
48 kDa	6	4/6	66.6	16.04 ± 9.04 [b]
Control	6	0/6	0	8.43 ± 3.56 [a]

Mice were injected i/p with 50 ug of purified protein on days 0, 7 and 14 and challenged s/c on day 28 with = 268 CPU of live *P. Multocida*

* ELISA unit is average OD x dilution factor of sample a,b - means significantly different ($p < 0.05$)

Table 2. Protective capacity of single stained proteins from *Pasteurella multocida* obtained after excision and electroelution of stained protein on slab polyacrylamide gel.

Protein (MW)	No of mice inoculated	No of mice surviving / No challenged	% Protection	*ELISA antibody litre (ΔΔ > ± SEM)
116kDa	5	3/5	60	10.33 ± 1.00 [a]
87 kDa	5	2/5	40	16.01 ± 2.82 [a]
71 kDa	5	0/5	0	18.34 ± 4.23 [a]
48 kDa	5	1/5	20	27.44 ± 4.72 [a]
Control	4	0/4	0	7.33 ± 2.50

Mice were injected i/p with 50ug of purified protein on days 0, 7 and 14 and challenged s/c on day 28 with = 213 CPU of live *P. Multocida*.

* - ELISA unit is average OD x dilution factor of sample a - Mean antibody litres with superscript 'a' are significantly higher ($p<0.05$) then control