Research in Veterinary Science 87 (2009) 207–210

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Identification of immunogenic proteins associated with protection against haemorrhagic septicaemia after vaccination of calves with a live-attenuated *aroA* derivative of *Pasteurella multocida* B:2

Saeed Ataei [a,1], Richard Burchmore [a], J. Christopher Hodgson [b], Anna Finucane [a], Roger Parton [a], John G. Coote [a,*]

[a] Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom
[b] Moredun Research Institute, International Research Centre, Bush Loan, Penicuik, Midlothian, United Kingdom

ARTICLE INFO

Article history:
Accepted 21 January 2009

Keywords:
Pasteurella multocida
OmpA
OmpH
Immunogenic proteins
Attenuated vaccine

ABSTRACT

Pasteurella multocida serotype B:2 is the causative agent of haemorrhagic septicaemia (HS), a fatal disease of cattle and buffaloes. As a step towards the identification of individual antigens that may protect against HS, proteins present in a sonicated cell extract (SCE) and outer-membrane protein (OMP) preparation of a wild-type *P. multocida* serotype B:2 were investigated by immunoblotting with sera from calves which had been protected against challenge with a virulent strain of *P. multocida* B:2 by vaccination with a live-attenuated *aroA* derivative of the challenge strain. Five proteins in SCE, of approximately 50, 37, 30, 26 and 16 kDa, were recognised by the sera. In an OMP preparation, two bands, at 37 and 50 kDa, were recognised as strongly immunogenic. Mass spectrometry analysis of proteins corresponding in size to those detected by immunoblotting identified the 37 kDa band as OmpA, but the band at 50 kDa was not identified with certainty. A major 30 kDa OMP, identified as OmpH, was not strongly immunogenic.
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Haemorrhagic septicaemia (HS) is an acute, fatal disease of cattle and buffaloes (*Bubalus bubalis*) characterized by terminal septicaemia and shock (Horadagoda et al., 2001). The disease is the most economically important contagious disease of these species in South and South–east Asia and the Middle East, where *Pasteurella multocida* serotype B:2 is the causative agent (Wijewardana, 1992; De Alwis, 1992, 1999). HS is generally controlled by vaccination with inactivated whole-cell preparations, but these have problems of reactogenicity, due probably to high endotoxin content, and do not provide long-lasting immunity (Verma and Jaiswal, 1998; De Alwis, 1999). The key antigens of *P. multocida* B:2 that evoke protective immunity to HS in cattle have not been defined, but *P. multocida* B:2 outer-membrane proteins (OMPs) have been implicated as protective antigens (Vasfri Marandi and Mittal, 1997; Srivastava, 1998; Pati et al., 1996; Basagoudanavar et al., 2006; Lee et al., 2007). For other serotypes of *P. multocida,* OMPs are recognised as important immunogens (Rimler, 2001; Gatto et al., 2002; Prado et al., 2005; Al-Hasani et al., 2007; Wu et al., 2007; Dabo et al., 2008). As a step towards identification of individual OMPs of serotype B:2 that may confer protection against HS, sera obtained at different times following vaccination intramuscularly (IM) of calves with an attenuated *aroA* derivative (JRMT12) of *P. multocida* B:2 (Tabatabaei et al., 2002) were used. Calves vaccinated with the *aroA* strian were fully protected against subcutaneous (SC) challenge with the virulent parent strain 85020 (Hodgson et al., 2005).

Sonicated cell extract (SCE) was prepared from strain 85020 after static growth in Brain Heart Infusion broth (BHI, Oxoid) for 18 h at 37 °C. Bacteria were collected by centrifugation at 3000xg for 30 min at 4 °C (RC-5B, Sorvall), washed three times with PBS, resuspended in PBS and disrupted by sonication using a Vibra Cell ultrasonic processor (Jencons-PLS, Leighton Buzzard) for three 60-s bursts with intermittent cooling on ice. The resulting suspension was centrifuged at 3000xg for 30 min at 4 °C, and the supernate was filtered through a 0.2 μm-pore-size membrane (Sartorius). The protein concentration was determined by a modified Lowry procedure (Markwell et al., 1978). OMPs of strain 85020 were obtained by Sarkosyl extraction as described by Davies et al. (1992) with modifications. A suspension of sonicated bacteria in 20 mM Tris–HCl (pH 7.2) was centrifuged at 10,000xg for 30 min at 4 °C. The supernate was then centrifuged at 1,00,000xg for 1 h at 4 °C (Combi, Sorvall) and the pellet, containing the cell envelopes, was resuspended in 0.5% (w/v) *N*-lauroylsarcosine (Sarkosyl, Sigma) and left for 20 min at room temperature to solublise the cytoplasmic membranes. OMPs (Sarkosyl-insoluble membrane

* Corresponding author. Tel.: +44 (0) 141 330 5845; fax: +44 (0) 141 330 4600.
E-mail address: j.coote@bio.gla.ac.uk (J.G. Coote).
[1] Present address: Razi Vaccine and Serum Research Institute, Karaj, Tehran, Islamic Republic of Iran.

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doi:10.1016/j.rvsc.2009.01.007

fraction) were then pelleted by centrifugation at 1,00,000xg for 30 min at 4 °C, washed once in distilled water and resuspended in PBS (pH 7.2).

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was done on 10% (w/v) acrylamide gels and protein bands were visualised with Coomassie blue. Immunoblotting was done as described previously (Hodgson et al., 2005). Protein identification by mass spectrometry (MS) followed standard protocols (Mutapi et al., 2005). MS/MS ions data were extracted using Analyst QS software and proteins identified using the Mascot search engine (Matrix Science). Only protein matches with scores that exceeded the 95% confidence threshold were accepted as positive identifications with a high 'hit score' providing greater confidence that the identification was correct.

SCE of strain 85020 showed numerous protein bands by SDS–PAGE ranging from 16 to 90 kDa with a prominent band at 30 kDa (Fig. 1A). For immunoblotting, sera collected at intervals from calves vaccinated with the *aroA* strain JRMT12 were used (Hodgson et al., 2005). Groups of four calves were vaccinated twice 4 weeks apart with 10^9 CFU of strain JRMT12 and challenged subcutaneously with 10^7 CFU of strain 85020 at 8 weeks of age. Fig. 1B shows the results obtained using sera collected at different times from one of the calves as representative of the group. The sera were obtained on the day of vaccination (pre-vaccination), 4 weeks after first vaccination, 1 week after the second vaccination and 1 week after challenge and the IgG titres in these sera were 59, 126, 2753 and 5244 ELISA units ml^{-1}, respectively (Hodgson et al., 2005). Sera obtained from each of the four calves reacted with proteins of a similar size, although some slight variation in individual band intensities was apparent between the blots for the four different sera (data not shown). The pre-vaccination serum showed some reactivity against SCE, for example with a band of approximately 53 kDa (Fig. 1B, lane 1), and serum obtained 4 weeks after the first vaccination reacted with an additional band at approximately 44 kDa (Fig. 1B, lane 2). The 44 and 53 kDa bands were not considered as putative protective antigens because vaccination IM with a single dose of 10^9 cfu of the *aroA* strain showed no protection against challenge (unpublished observations). Sera obtained after the second vaccination and after challenge, when IgG titres had increased markedly, reacted strongly with additional SCE bands at 50, 37, 30, 26 and 16 kDa (Fig. 1B, lanes 3 and 4), identifying these as possible protective antigens as calves receiving the booster vaccination IM were protected against challenge (Hodgson et al., 2005).

The SDS–PAGE profile of the OMP preparation revealed two major bands of 30 and 37 kDa and several minor bands (Fig. 2A). An immunoblot using calf immune serum obtained one week after the second IM vaccination revealed two strong bands, of 37 and 50 kDa (Fig. 2B) which appeared to correspond to a major OMP band (number 2 in Fig. 2A) and a minor OMP band (number 1 in Fig. 2A), respectively. The most dominant band in the Coomassie blue stained OMP preparation at 30 kDa (number 3 in Fig. 2A) showed little or no reaction in the corresponding blot (Fig. 2B). It is possible that the bands at 37 and 50 kDa identified in the OMP immunoblot (Fig. 2B) are the same bands at 37 and 50 kDa identified in the SCE immunoblot (Fig. 1B, lane 3). No immunogenic OMPs equivalent to the 16, 26, 30 and 44 kDa bands visible in the immunoblot of SCE with the same serum (Fig. 1B, lane 3) were evident in the immunoblot of the OMP preparation (Fig. 2B).

As the OMP preparation contained fewer proteins than the SCE, which minimised the possibility that an excised band might contain more than one protein of similar MWs, the protein bands labelled 1–3 in the OMP preparation (Fig. 2A) were chosen for identification. The most dominant, but poorly immunogenic, band of the OMP preparation at 30 kDa (band 3, Fig. 2A) was identified as OmpH of *P. multocida* by MASCOT analysis (highest hit score 718, sequence coverage 41% (percentage of sequence covered by the matching peptides), 35 kDa, NCBI accession number 2853240,

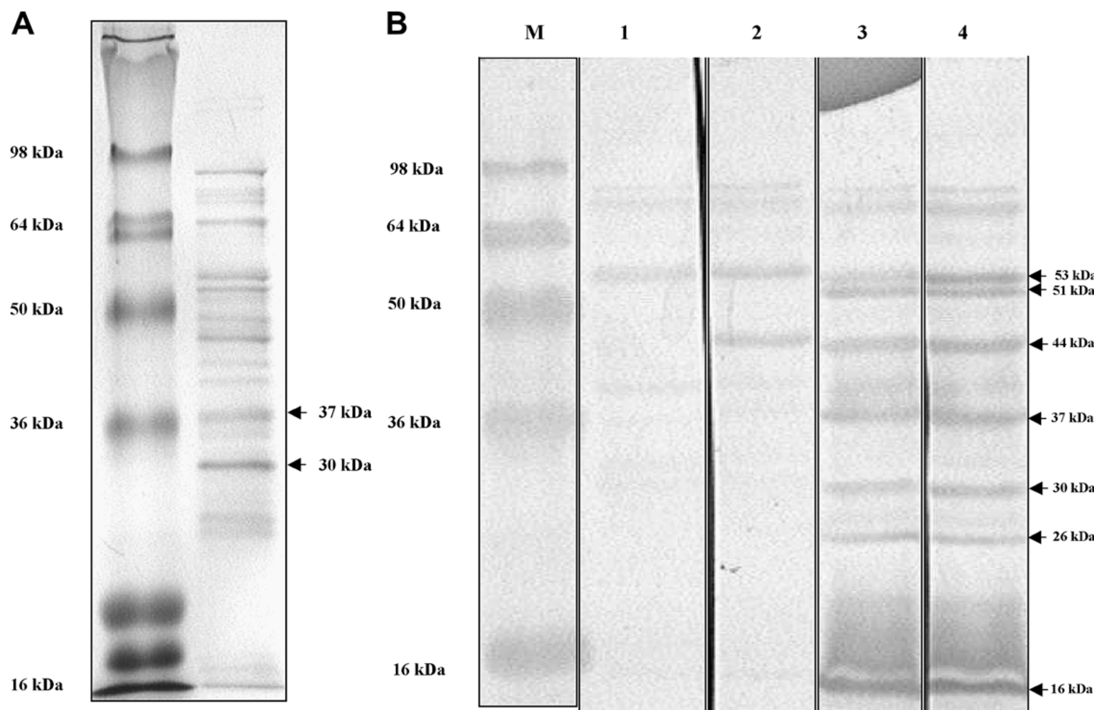

Fig. 1. (A) Coomassie Blue stained SDS–PAGE profile of a sonicated cell extract (SCE) preparation of *P. multocida* 85020 grown in BHI broth (right lane). SCE proteins were loaded at 10 µg per well. MW markers (left lane). (B) Immunoblot of *P. multocida* SCE with sera taken at intervals from a calf after vaccination IM with *P. multocida* strain JRMT12 and challenge with the *P. multocida* strain 85020 parent strain, as representative of the group of four vaccinated and challenged animals. M; MW marker; Lane 1, Before vaccination; Lane 2, Four weeks after first vaccination; Lane 3, One week after second vaccination; Lane 4, One week after challenge.

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