Identification of immunodominant proteins from *Mannheimia haemolytica* and *Histophilus somni* by an immunoproteomic approach

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Abstract

Mannheimia haemolytica and *Histophilus somni* are frequently isolated from diseased cattle with bovine respiratory disease (BRD). They compromise animal lung function and the immune responses generated are not sufficient to limit infection. Identification of specific immunogenic antigens for vaccine development represents a great challenge. Immunogenic proteins were identified by immunoproteomic approach with sera from cattle immunized with a commercial cellular vaccine of *M. haemolytica* and *H. somni.* Proteins of *M. haemolytica* were identified as solute ABC transporter, iron-binding protein, and hypothetical protein of capsular biosynthesis. *Histophilus somni* proteins correspond to porin, amino acid ABC transporter, hypothetical outer membrane protein, cysteine synthase, and outer membrane protein P6. Although these antigens share strong similarities with other proteins from animal pathogens, the ABC system proteins have been associated with virulence and these proteins could be considered as potential vaccine candidates for BRD.

Résumé

Mannheimia haemolytica *et* Histophilus somni *sont fréquemment isolées de bovins atteints de maladies respiratoires bovines (MRB). Ces agents compromettent la fonction pulmonaire et les réponses immunitaires générées ne permettent pas de limiter l'infection. L'identification d'antigènes spécifiques et immunogènes qui permettraient le développement de vaccins, représente un grand défi actuellement. Les protéines immunogènes ont été identifiées par une approche immunoproteomique en utilisant des sérums provenant de bovins immunisés par des vaccins commerciaux de* M. haemolytica *et* H. somni. *Les protéines de* M. haemolytica *ont été identifiées comme étant un transporteur ABC, une protéine de liaison du fer et une hypothétique protéine impliquée dans la biosynthèse de la capsule. Celles de* H. somni *correspondent à une porine, à un transporteur ABC d'acides aminés, à une hypothétique protéine de membrane externe, à la cystéine synthase et à la protéine membranaire P6. Bien que ces antigènes présentent une forte homologie avec des protéines provenant d'autres pathogènes d'animaux, les protéines du système ABC sont associées à la virulence et pourraient être considérées comme des candidats potentiels pour l'élaboration de vaccins contre les MBR.*

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Pneumonia caused by opportunistic bacterial pathogens is common and important in all domestic animal species and in humans. Bovine respiratory diseases (BRD) occur as shipping fever pneumonia of feedlot cattle, enzootic pneumonia of dairy and veal calves, and pneumonia of adult cows (1). This is a polymicrobial disease syndrome with any of a number of bovine respiratory viruses, predisposing cattle to secondary bacterial pneumonia. *Mannheimia haemolytica* and *Histophilus somni* are the most commonly isolated pathogens from the respiratory tract of diseased cattle which can establish infection in the lungs of cattle subjected to a variety of stresses (2).

At the time of arrival to feedlots, prevalence of serum antibody titers to *M. haemolytica* and *H. somni* vary considerably but are generally high, presumably because of prior exposure or vaccination. Besides protective immune responses, lung defenses are compromised and exacerbated infection prolongs disease (1). Immune sera from naturally infected cattle have been shown to contain antibodies that recognize OmpA of *M. haemolytica* (3). Protective antibodies to *H. somni* immunoglobulin-binding protein A IbpA virulence factor have been detected in experimentally infected calves (4). Immunization schemes with recombinant proteins have identified some important surface antigens as OmpA, OmpP2, PlpF, Plp4, and LppB in these bacteria (5,6), and vaccines against *M. haemolytica* include virulence factor leukotoxin Lkt and surface lipoproteins Gs60 and PlpE (7). In spite of available information about the dynamics of preventive immune responses in BRD (8), challenges to contain infection prevail, and there is no evidence of the effectiveness of feedlot cattle vaccination (9). The objective of this study was to compare vaccine protein immunodominance and identify immunogenic proteins in related BRD causing bacteria with a whole cell bovine immunization protocol, by using an immunoproteomic approach.

Mannheimia haemolytica A1 (ATCC BAA-410) and *H. somni* (ATCC 43626) were grown in brain heart infusion broth (BHI) at 37°C for 36 h and 48 h, respectively. *Histophilus somni* was grown in presence

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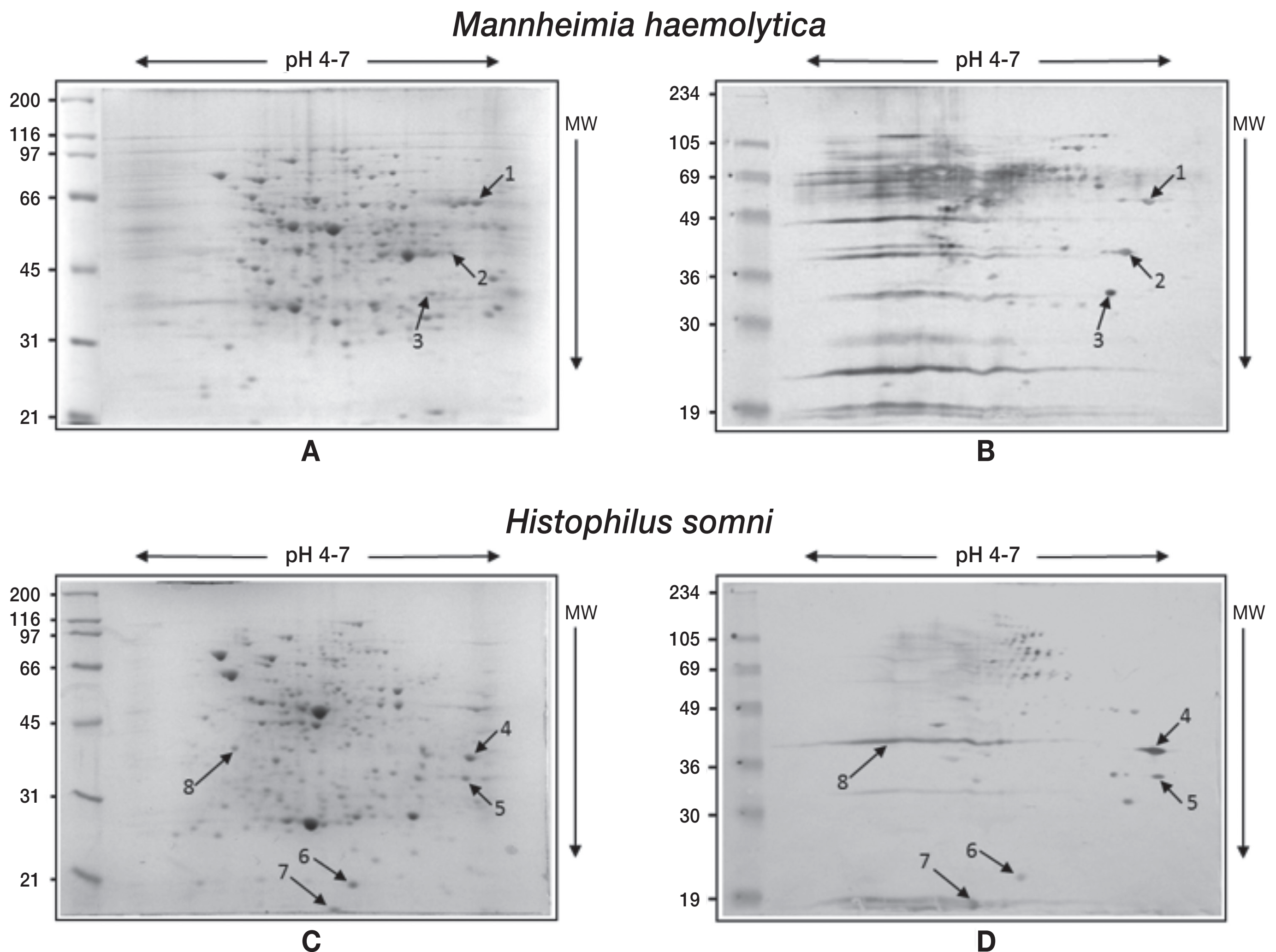

Figure 1. 2-D map and Western blot of cell homogenates from *M. haemolytica* and *H. somni:* (A and C) — 2-D maps (pH 4–7) of total proteins from *M. haemolytica* and *H. somni*, respectively, stained by Coomassie blue R250. (B and D) — Replica from 2-D gels over nitrocellulose sheets incubated with pooled sera from four calves exsanguinated after 42 days post primo-immunization with commercial vaccine. MW, size standards (kDa). Arrows, protein spots identified by LC-MS/MS.

of 10% of CO_2. Bacterial cells were harvested at 6500 × g for 10 min, then the cellular pellet was washed twice in phosphate buffer saline (PBS) and lysed with 2-D Protein Extraction buffer (GE Healthcare Life Sciences, Uppsala-Sweden). Immobiline DryStrip Gels (pH 4–7 and 11 cm) were rehydrated overnight with 40 μL of protein sample, 85 μL of Destreak rehydration solution, and IPG buffer pH = 7 (GE Healthcare Life Sciences). Isoelectric focusing was carried out with an Ettan IPGphor III system for 40 000 V/h as recommended by the manufacturer (GE Healthcare Life Sciences). Afterwards, strips were equilibrated with DTT 1% (w/v) and iodoacetamide 2.5% (w/v), and two-dimensional (2-D) PAGE was carried out with a Mini-PROTEAN® Tetra Electrophoresis system (Bio-Rad. California, USA) in 10% polyacrilamide gels. Proteins were then electroblotted onto nitrocellulose sheets (Hybond ECL, GE Healthcare Life Sciences).

Immunoblot was carried out with a pool of bovine immune sera obtained from calves (between 5- to 7-months-old) previously immunized intramuscularly twice (1 month between immunizations) using a commercial vaccine (Biobac 11 Vias; BioZoo Laboratories, Zapopan, Jalisco., México) containing *M. haemolytica* and *H. somni* bacterins. Animal protocols were followed according to current Mexican policy (NOM-062-ZOO-1999) for experimental animal regulations. Serum at 1:20 dilution was used as a source of primary antibodies to independently detect proteins from *M. haemolytica* and *H. somni* antigens, and then with anti-bovine mouse IgG-POD (Bio-Rad). Membranes were revealed by using the peroxidase method. Immunogenic proteins were selected by 2-D Western blotting (Figures 1B and D) and protein spots were excised from a replica of 2-D gel visualized by Coomassie blue staining (Figures 1A and C). Three main proteins from *M. haemolytica* (Figure 1A) were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS (Protein Chemistry Core Facility, Columbia University, Table 1) as ATP binding cassette transporter, iron-binding ABC transporter protein YfeA, and hypothetical capsular biosynthesis protein. However, 5 main proteins from *H. somni* (Figure 1C), were identified as peptidoglycan-associated OmpP6, amino acid ABC transporter substrate-binding protein, porin, cysteine synthase, and hypothetical OMP. Interestingly, some of the

Table 1. Protein spots identified by MALDI-TOF MS

Spot number	NCBI reference sequence	Annotation/species	Theoretical MW[a] (kDa)/p*I*	Experimental MW[b] (kDa)/p*I*
1	YP_002171386.1	ABC transporter substrate-binding protein/*M. haemolytica* PHL213	58/6.8	56/6.7
2	YP_002173390.1	Hypothetical protein MHA_0524/*M. haemolytica* PHL213	41.1/6	39/6.5
3	YP_002171469.1	ABC transporter Fe^{3+}-binding protein YfeA/*M. haemolytica* PHL213	32/5.91	32.5/6.3
4	YP_001784767.1	Porin/*H. somnus* 2336	41.8/8.9	43/6.7
5	YP_718419.1	Amino acid ABC transporter binding protein/*H. somnus* 129PT	28.5/8.8	30/6.7
6	YP_001783864.1	Hypothetical protein HSM_0520/*H. somnus* 2336	22.1/9.5	23/5.6
7	YP_718471.1	Peptidoglycan-associated outer membrane lipoprotein P6/*H. somnus* 129PT	14.8/5.6	17/5.2
8	YP_718579.1	Cysteine synthase/*H. somnus* 129PT	33.6/6.4	36/5

[a] According Swiss Institute of Bioinformatics SIB database.

[b] Observed MW and p*I* of protein spot in the 2-D gel.

identified proteins from *H. somni* share a high similarity with their counterparts present in other livestock pathogens. BLAST analysis revealed that peptidoglycan-associated OmpP6 has a 70% to 77% similarity with *Avibacterium paragallinarum*, *Haemophilus parasuis*, and *Pasteurella multocida* Omp proteins. In addition, amino acid ABC transporter substrate-binding protein has 75% to 85% similarity with *Avibacterium paragallinarum*, *Haemophilus parasuis*, *Actinobacillus pleuroneumoniae*, and *M. haemolytica* ABC transporter proteins. This finding suggests that the identified immunogenic proteins are common in a wide range of animal pathogens and may be evaluated as vaccine antigens. No protein spots were immunodetected by sera from non-vaccinated animals (data not shown).

Commercial vaccines against bovine pneumonic pasteurellosis consisting of cell-free supernatants from *M. haemolytica* cultures induce good antibody titers in calves to surface proteins as lipoprotein GS60, and it has been proposed to include antigenic proteins in experimental vaccines (10). In this work, with a multivalent cellular vaccine immunization against *M. haemolytica* and *H. somni*, some surface associated antigenic proteins were identified as it has been previously documented (11,12). In a similar proteomic analysis performed with sera from vaccinated animals with OMP-enriched fractions of *M. haemolytica*, some ATP-binding cassette (ABC) transporter proteins, including the iron-binding protein YfeA found in this work, and hypothetical conserved proteins, were reported (13). This confirms that these are good immunogenic proteins that should be considered in vaccine formulations. Previously, it has been described that cell extracts of enriched iron-regulated protein fractions from *P. haemolytica* induced good protection against experimental infection in vaccinated lambs (14). Iron-regulated OMPs have also been described as immunogenic antigens in naturally infected calves (15). Two different ABC transporter proteins from both microorganisms were immunodetected in this study. ABC systems are known to play roles in virulence and pathogenicity because they are associated with many physiological processes, such as uptake of nutrients as peptides, sugars, and scarcely abundant metals, being in consequence attractive targets for new vaccines in other pathogenic bacteria (16).

In this work, a similar 2-D Western-blotting pattern was observed for both bacterial protein lysates incubated with sera from vaccinated animals. As both bacteria are members of the Pasteurellaceae family, they probably induce similar immune responses to orthologous proteins in cattle. These results suggest that the immunodominant proteins detected for *M. haemolytica* and *H. somni* may be good candidates for development of a multivalent subunit vaccine as has been proposed for sheep pneumonia (17). Interestingly, an antibody response against a hypothetical protein related to capsular biosynthesis was observed and it would be important to confirm if this specific protein may be a good antigen for vaccine formulations. An early work indicated that administration of capsular polysaccharide of *M. haemolytica* was related to a high degree of anaphylaxis in calves (18). Chimeric proteins consisting of immunodominant epitopes of PlpE and Lkt, however, developed good antibody responses against *M. haemolytica* in mice and cattle (19). It would, therefore, be interesting to find out if chimeric proteins with immunogenic epitopes from 2 related ABC transporter proteins identified from both bacteria would elicit an immunological response against BRD. According to this hypothesis, antigenicity analysis of the identified proteins with CLC Main Workbench 6.0, based on 2 different algorithms, was carried out and predicted antigenic regions were found. Residues 325–439 and 15–42 from *M. haemolytica* ATP binding cassette transporter and hypothetical capsular biosynthesis protein, respectively, were highly antigenic, as well as residues 165–210 and 35–78 from *H. somni* amino acid ABC transporter substrate-binding protein and hypothetical Omp, respectively. Further research will be necessary in order to study the immunogenicity of the resulting recombinant protein. In addition, it must be established whether the immunodetected proteins have a role in protection *versus* increasing susceptibility. Finally, an immunoproteomic approach using sera from convalescent animals, naturally or experimentally infected, should be performed in order to confirm or identify new antigens for vaccine formulations.

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References

1. Caswell JL. Failure of respiratory defenses in the pathogenesis of bacterial pneumonia of cattle. Vet Pathol 2014;51:393–409.

2. Fulton RW, Shawn-Blood K, Panciera RJ, et al. Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. J Vet Diagn Invest 2009;21:464–477.
3. Zeng H, Pandher K, Murphy GL. Molecular cloning of the *Pasteurella haemolytica* pomA gene and identification of bovine antibodies against PomA surface domains. Infect Immun 1999; 67:4968–4973.
4. Lo KL, Kimball RA, Lehmann J, Gershwin LJ, Worby C, Corbeil LB. Antibody responses of calves to *Histophilus somni* recombinant IbpA subunits. Comp Immunol Microbiol Infect Dis 2012;35:453–459.
5. Ayalew S, Shrestha B, Montelongo M, Wilson AE, Confer AW. Immunogenicity of *Mannheimia haemolytica* recombinant outer membrane proteins serotype 1-specific antigen, OmpA, OmpP2, and OmpD15. Clin Vacc Immunol 2011;18:2067–2074.
6. Guzmán-Brambila C, Rojas-Mayorquín AE, Flores-Samaniego B, Ortuño-Sahagún D. Two outer membrane lipoproteins from *Histophilus somni* are immunogenic in rabbits and sheep and induce protection against bacterial challenge in mice. Clin Vaccine Immunol 2012;19:1826–1832.
7. Orouji S, Hodgins DC, Lo RY, Shewen PE. Serum IgG response in calves to the putative pneumonic virulence factor Gs60 of *Mannheimia haemolytica* A1. Can J Vet Res 2012;76:292–300.
8. Confer AW, Fulton RW, Clinkenbeard KD, Driskel BA. Duration of serum antibody responses following vaccination and revaccination of cattle with non-living commercial *Pasteurella haemolytica* vaccines. Vaccine 1998;16:1962–1970.
9. Larson RL, Step DL. Evidence-based effectiveness of vaccination against *Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni* in feedlot cattle for mitigating the incidence and effect of bovine respiratory disease complex. Vet Clin North Am Food Anim Pract 2012;28:97–106.
10. Lee RW, Cornelisse M, Ziauddin A, et al. Expression of a modified *Mannheimia haemolytica* GS60 outer membrane lipoprotein in transgenic alfalfa for the development of an edible vaccine against bovine pneumonic pasteurellosis. J Biotechnol 2008;135:224–231.
11. Pandher K, Murphy GL, Confer AW. Identification of immunogenic, surface-exposed outer membrane proteins of *Pasteurella haemolytica* serotype 1. Vet Microbiol 1999;65:215–226.
12. Tagawa Y, Bastida-Corcuera F, Corbeil LB. Immunological characterization of the major outer membrane protein of *Haemophilus somnus.* Vet Microbiol 2000;71:245–254.
13. Ayalew S, Confer AW, Hartson SD, Shrestha B. Immunoproteomic analyses of outer membrane proteins of *Mannheimia haemolytica* and identification of potential vaccine candidates. Proteomics 2010;10:2151–2164.
14. Gilmour NJL, Donachie W, Sutherland AD, Gilmour JS, Jones GE, Quirie M. Vaccine containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. Vaccine 1991;9:137–140.
15. Puchalski A, Urban-Chmiel R, Dec M, Wernicki A. An electrophoretic characterization of iron-transporting proteins in *Mannheimia haemolytica* A1. Pol J Vet Sci 2013;16:527–532.
16. Wu Z, Zhang W, Shao J, Wang T, Lu Y, Lu C. Immunoproteomic assay of secreted proteins of *Streptococcus suis* serotype 9 with convalescent sera from pigs. Folia Microbiol 2011;56:423–430.
17. Subramaniam R, Shanthalingam S, Bavananthasivam J, et al. A multivalent *Mannheimia-Bibersteinia* vaccine protects bighorn sheep against *Mannheimia haemolytica* challenge. Clin Vacc Immunol 2011;18:1689–1694.
18. Conlon JA, Shewen PE. Clinical and serological evaluation of a *Pasteurella haemolytica* A1 capsular polysaccharide vaccine. Vaccine 1993;11:767–772.
19. Confer AW, Ayalew S, Montelongo M, et al. Immunity of cattle following vaccination with a *Mannheimia haemolytica* chimeric PlpE–LKT (SAC89) protein. Vaccine 2009;27:1771–1776.