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Characterization of Microvesicles of *Mannheimia haemolytica* Serotype A1 (Reference Strain) and Serotype A2 (Field Isolate)

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Abstract: *Mannheimia haemolytica* is a commensal of cattle, sheep and other ruminants, but it can also cause bovine and ovine pneumonic Pasteurellosis (infecting the respiratory tract), which is responsible for considerable economic losses in cattle and sheep industries. *M. haemolytica* shows different antigens. Each one of these antigens could also contribute to the development of disease and induce a specific immune response by the host. Gram negative bacteria contain outer membrane vesicles that are secreted into the medium under certain growth conditions. These vesicles retain outer membrane and cell wall constitutive components such as proteins, Lipo-Polysaccharides (LPS), muramic acid, capsule, fimbria and others. These components play a crucial role in the development of the illness as well as in the immune response activation. Antigens of immunological interest could be inside of the small Microvesicles (MVs) released by *M. haemolytica* under stress conditions. The immunogenicity of these MVs could stimulate an adequate humoral response, in the defense of individuals exposed to virulent strains and prevent animals from developing pathological signs. The importance of obtaining an effective immunogen lays in the prevention and control of pneumonic ovine Pasteurellosis. The objective of this study was to obtain and characterize antigens from Microvesicles (MVs) of *Mannheimia haemolytica* A1 and A2 serotypes, grown under stress conditions using gentamycin during logarithmic growth. In this study 2 strains of *M. haemolytica* were used: Strain ATCC A1 and the second strain was the A2 serotype obtained from a pneumonic lung. Characterization of *M. haemolytica* MVs allows recognition of the main bacterial antigens in these structures, which makes them candidates for immunological evaluation in animal models.

Key words: Microvesicles, *Mannheimia haemolytica*, ovine, serotype, LPS

INTRODUCTION

Mannheimia haemolytica is a commensal bacterium of cattle, sheep and other ruminants, yet it can be the causal agent of bovine and ovine pneumonic pasteurellosis, by infecting the respiratory tract. This disease is responsible for considerable economic loses in cattle and sheep industries (Ackermann and Brogdena, 2000; Frank *et al*., 1996; Brogden *et al*., 1998).

M. haemolytica shows different antigens which could contribute to the development of the disease and in addition induce a specific immune response by the host (Leite *et al*., 2002). Gram negative bacteria such as *Porphyromonas gingivalis* (Ratchapin *et al*., 2001), *Actinobacillus pleuropneumoniae*, *Neisseria meningitides* (Moe *et al*., 2002), *Brucella ovis* and *Actinobacillus seminis* (Núñez *et al*., 2006), release Microvesicles (MVs) that are secreted into the medium under certain growth conditions or in the presence of some antibiotics. These vesicles retain constitutive components of the bacterial surface, such as proteins, Lipopolysaccharides (LPS), muramic acid, capsule, fimbria and others. These components play a crucial role in the development of illness as well as in the immune response activation (Kadurugamuwa, 1995).

Several vaccines against *M. haemolytica* serotypes have been unsuccessfully tried, against the specific serotypes that affect sheep (Srinand *et al*., 1996). Antigens of immunogenic interest could be carried by MVs released by *M. haemolytica* under stress conditions. Immunogenicity of these Mvs could stimulate an adequate humoral response, involved in the defense of

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individuals exposed to virulent strains, preventing animals from developing pathological signs. The importance of obtaining an effective immunogen lays in the prevention and control of ovine pneumonic pasteurellosis.

The aim of this study was to obtain microvesicles of *Mannheimia haemolytica* serotypes A1 and A2, grown under stress during exponential growth and characterize the different antigens.

MATERIALS AND METHODS

Strains: Two strains of *M. haemolytica* were used: strain ATCC serotype A1 and an isolate of serotype A2 obtained from an ovine pneumonic lung. The identification of the strains was realized by using biochemical tests and the capsular serotype was determined by indirect hemoagglutination using reference anti-sera in microplates (Frank and Wessman, 1978).

Microvesicles (MVs) collection: Bacteria were developed in 100 mL BHI at 37°C for 12 h. Fifty milliliter of culture medium were taken to be inoculated into 500 mL of BHI with 2 mL of 1M $CaCl_2$ added. It was incubated for 12-16 h that corresponds to the time in which the culture is in a full logarithmic growth phase and 20 μL of gentamycin are added per culture mL completing incubation up to 48 h. After that the culture was centrifuged at 3500 g for 15 min to eliminate biomass and obtain the supernatant that was then passed through 0.45 and 0.22 μm Millipore filters. The supernatant was ultracentrifuged at 150.000 g for 3 h. at 4°C. The supernatant was eliminated and the pellet was obtained in a total of 2 mL PBS and then frozen at -20°C until its use (Kadurugamuwa and Beveridge, 1995).

Kinetics of MVs collection: Given *M. haemolytica* growth characteristics, as well as microvesicles production, the microvesicles collection kinetics was established for 16, 24 and 48 h. Since, it has been observed by electrophoretic analysis that the bands that correspond to MVs disappeared in some of these incubation periods, probably because the cells stopped producing adequate amounts of microvesicles or else the bacteria died. Therefore, culture times and antibiotic concentrations were studied to find the combination that would give highest yield of these structures. Along with kinetics, the best observation by electron microscope was established in the 24 h of culture.

Electron microscopy: Grid preparation: Nickel grids were covered with formvar and shaded with carbon. Ten to fifteen microlitter of the sample, were placed on the grid on the darkest side, where the film was placed and it was allowed to sit for 15 min. After that it was stained removing excess of the sample with filter paper and carefully drying the grid, adding 10 - 15 μL of 1% Phosphotungstic Acid at pH 6.0 for 90 sec (Kadurugamuwa and Beveridge, 1995).

Collection and cuantification of Outer Membrane Proteins (OMP): Strains of *M. haemolytica* were spread in agar blood and incubated at 37°C for 12 h. Afterwards it was spread in half a liter of BHI broth, incubated at 37°C until an absorbance of 0.9-600 nm. Cells were harvested by centrifugation at 4 C, 3500 g for 20 min. The pellet was re-suspended in 60 mL of 10 mM HEPES added with 20 μL of 100 mM PMSF and 20 μL of 100 mM EDTA, as protease inhibitors. In order to obtain cell fractions, the culture cells were distributed in several tubes in the amount of 10-15 mL in an ice bath and then, there broke with aultrasonic processors, with three sets of 10 sec pulsars X 1 min. rest. Then it was centrifuged at 1000 g for 15 min to remove all large cell remains. The supernatant was recovered and centrifuged at 150.000 g for 2 h in 10 mM HEPES. It was considered that the obtained pellet corresponded to crude cell sheathing containing the outer and cytoplasmic membranes. Total membranes pellet was dissolved in 10 mL of 1% sarcosyl and softly stirred during 30 min at room temperature. Finally the sample was centrifuged at 150.000 g for 1 h and the resulting pellet corresponded to OMP that was then dissolved in deionized water, while the supernatant corresponded to the internal membrane protein fraction (Squire *et al*., 1994).

Protein purification in the presence of detergents and lipids: One hundred microlitter of *M. haemolytica* outer membrane protein solution were added to 400 μL of methanol and subjected to vortex action and then centrifuged during 10 sec at 9500 g in conic polyethylene microtubes. To this sample, 100 μL of chloroform were added and subjected again to vortex action and centrifuged 10 sec at 9000 g. For samples with high phospholipids content, such as the one obtained from *M. haemolytica,* 200 μL of chloroform were added. Phase separation was carried out adding 300 μL distilled water and subjecting it again to vortex action, centrifuging 1 min at 9000 g. The upper phase was carefully removed and discarded. Finally, 300 μL of methanol was added to the rest of the lower chloroformic phase and the precipitated protein inter-phase, those were mixed and centrifuged 2 min at 9000 g, the supernatant was removed and the protein pellet dried by air spray and kept at -70°C until its use (Riezu-Boj *et al*., 1986).

Protein quantification: Total protein content was determined by colorimeter, using the Bradford microtitration method. Once lipids and detergent were

eliminated, the pellet was dissolved in 60 μL of deionized water. Titration was carried out with a bovine serum albumin curve of 1-10 μg mL^{-1}, carrying out the technique in flat bottom ELISA plates, placing 10 μL of *M. haemolytica* protein sample plus 190 μL Bradford's reagent and reading at 590nm, determining the protein curve by linear regression (Bradford, 1976).

Electrophoresis in polyacrylamide gels with Sodium Dodecil Sulfate (SDS-PAGE): In order to determine what proteins of the outer membrane fraction are shared by the MVs, they were separated by molecular weights using SDS-PAGE according to the Laemmli method (Laemmli, 1970). Protein concentration, of the outer membrane fraction as well as MVs was adjusted to 25 μg of protein in each 10 μL. Antigens were dissolved mixing 50 μg of the antigen in 20 μL the sample buffer and boiled (95°C) during 10 min. After that, 10 μL of the sample were added to each lane, in 12% gels, run at a voltage of 80 V for the concentration gel and 100 V for the separation gel. Gels were stained with Coomassie blue during 1 h at room temperature, with soft stirring. After that they were washed until the desired bands could be clearly visualized and finally gels were kept in 10% acetic acid (Dabo *et al*., 1994). In other instances, the gels were silver stained for proteins, LPS or were transferred to a nitrocellulose membrane. Band molecular weight of the outer membrane fraction, as well as MVs was estimated by comparison of bands' position of a standard of known molecular weight proteins placed on the same gel and through specific software (GeneTools from SynGene, England; Cambridge).

Silver stain for proteins: The gel was set overnight in a methanol-acetic-water (5:1:4) solution. The setting solution was removed and the gel washed 3 times for 15 min in 500 mL of distilled water. Staining mix was prepared at that time with 56 mL of 0.1 M NaOH, 5 mL of (100%) NH_4OH, 2 gr of $AgNO_3$ dissolved in 10 mL of very cold deionized H_2O added drop by drop and finally 200 mL of deionized water, adding first 100 mL and then the other 100 mL. The gel was stirred vigorously for 10 min and then washed four times, 1 min each, with distilled water. Developing was carried out with 50 mg of citric acid dissolved in 200 mL of distilled water plus 1 mL of (38%) formaldehyde and then taken to 1000 mL. Approximately 250-500 mL were transferred to the crystallizer and stirring was maintained until protein bands appeared (10-15 min approximately). The solution was discarded and the reaction was stopped with 10 mL of 25% isopropanol, 7% acetic acid plus 200 mL of deionized water, stirring for 1 h and changing to distilled water to store at 4°C or dehydration of the gel (Sambrook and Russell, 2001).

Silver stain for LPS: The gel was set overnight in a fresh solution of 25% isopropanol and 7% acetic acid.The solution was decanted and the gel oxidized for 5 min in a solution of 150 mL deionized water, 1.05 g periodic acid and 4 mL of setting solution. It was washed 8 times for 15-20 min in 200 mL of distilled water each time. After that it was stained during 10 min with a fresh solution that contained 28 mL of 0.1N NaOH, 1.25 mL of 29.4% NH_4OH and 5 mL of 20% $AgNO_3$. Then the gel was washed 4 times in 200 mL of distilled water during 10 min each wash. After that, a solution was prepared containing 50 mg of citric acid in 0.5 mL of 37% formaldehyde. This solution was added to the gel waiting between 5 and 10 min until the desired intensity of the bands was obtained. In order to preserve the gel it was submerged for 60 min in 200 mL of a 0.35% acetic acid solution (Sambrook *et al*., 2001).

Western blot: Once proteins were separated by SDS-PAGE, protein bands were transferred to a nitrocellulose membrane by electrotransference at 400 mA during one hour. When the transference was verified, the membrane was treated 24 h with 5% skimmed milk in order to block non-specific antibody union points. After that, the membrane was washed on three occasions with 0.05% PBS-Tween 20 for 15 min each occasion and then incubated with the testing antibody for 2 h at 37°C or overnight at 4°C. The membrane was washed again three times in PBS-Tween 20 and incubated with the sec antibody (conjugated with peroxidase) for 2 h at 37°C or overnight at 4°C. Finally, the membrane was revealed with 0.05% 3´3 Diaminobencidine (DAB), Ni-Co and 0.1% H_2O_2, stirring softly until the desired band intensity was obtained and the reaction was stopped in running water (Toubin and Gordon, 1984).

Plasmid extraction (cleaning of bacterial strains): *Mannheimia haemolytica* was cultured in 25 mL BHI broth and then 5 mL aliquots were taken to culture them again in 50 mL BHI broth in a nephelometric matrass. The matrasses were worked in duplicate, one served as control and the other had Acridine Orange added to it as a mutagen to facilitate the loss of a possible plasmid. This was added directly to the culture in early logarithmic growth phase (2-3 h of culture) or by making a solution in PBS, filtered by 0.22 μm Millipore. Once the early log phase was passed, 100 μL aliquots of each one were taken at 30 min intervals, both from matrasses with different Acridine Orange concentrations added (10, 20, 40 and 60 μg mL^{-1} of culture), as well as control matrasses. Glass spatula was then used to inoculate BHI agar plates that were incubated at 37 °C overnight. Colonies that grew in

the presence of Acridine Orange as well as from the controls were collected. The colonies that grew in the presence of Acridine Orange were re-suspended in 5 mL of BHI to be harvested. From the harvest a sample was taken in 5mL PSS and this suspension was taken as 100% and from this initial step, logarithmic dilutions were performed up to 1×10^{-5}, 100 µL aliquots were taken from each one and inoculated into BHI agar plates and then incubated at 37°C overnight. The colonies were then selected and inoculated in BHI agar with and without ampicillin (50 µg mL^{-1}), antibiotic that was selected because of ampicillin resistance of the strain that was used, as determined by antibiogram sensidises of low, medium and wide spectrum. Colonies growing in the ampicillin agar were those that did not loose their plasmid and those that did not grow were those that lost their resistance plasmid. MVs were extracted from colonies that grew in BHI without ampicillin (Southwick *et al*., 1972; Tomas and Kay, 2005).

Plasmidic DNA extraction: An MVs pellet was obtained from the liquid medium culture by centrifugation at 1500 g for 15 min and it was washed with HEPES 10 mM pH 7.4. After that the pellet was re-suspended in 200 µL of Sol. I (50 mM glucose, 25 mM TRIS-HCL (pH 8) and 10 mM EDTA (pH 8)) at 4°C. Stirring softly, 400 µL of recently prepared Sol. II (0.2N NaOH and 1% SDS), were added and stirred by inversion. It was stored in tubes on ice for 5 min. Then 300 µL of Sol. III (60 mL 5M potassium acetate, 11.5 mL glacial acetic acid and 28.5 mL of sterile deionized H_2O) at 4°C were added. It was shaken until a clot was formed and maintained at -20°C for 30 min and then centrifuged at 9000 g for 10 min. It was washed with an equal volume, of supernatant that was obtained, consisting of chloroform, phenol and isoamilic alcohol in a proportion of 1:1:1. The upper phase was collected and 2 volumes of absolute ethanol were added and maintained at -70°C for 2 h. Finally the pellet was resuspended in 50 µL of sterile deionized water and the sample was run in 1% agarose gel (Sambrook and Russell, 2001).

RESULTS AND DISCUSSION

Outer Membrane Proteins (OMP) and serotype A1 (reference strain) as well as serotype A2 (field strain) MVs were separated by 12% SDS-PAGE. Each run was performed with 2 gels, one was stained with Coomassie blue and the other with silver or it was used to perform immunotransference. OMP as well as MVs samples were run in the same gel to determine protein patterns of both OMP as well as MVs, observing great similitude between

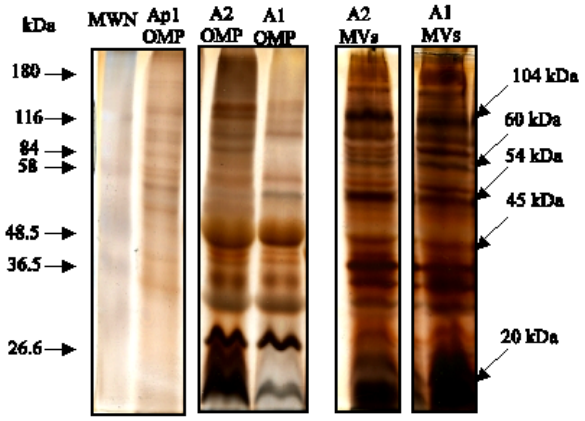

Fig. 1: 12% SDS-PAGE of *M. haemolytica* OMP and MVs, stained with silver

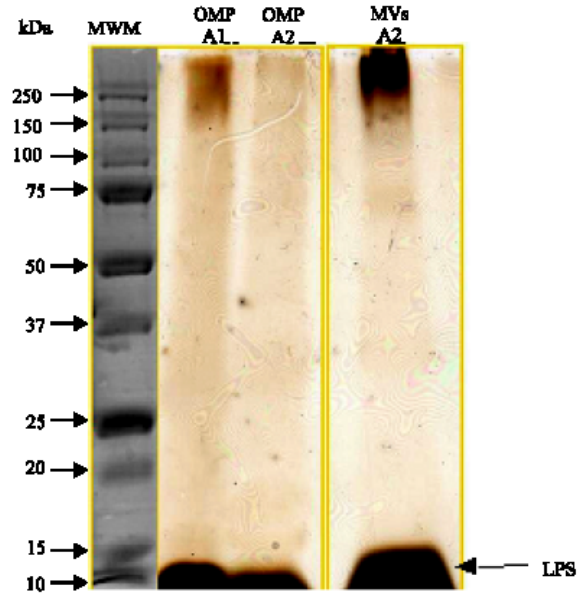

Fig. 2: 12% SDS-PAGE of *M. haemolytica* OMP and MVs, stained with silver for LPS

them in relation to the amount of protein bands that these structures share. In most of the cases a strain of A. *pleuropneumoniae* serotype 1 (Ap1) was used as control.

Silver staining was carried out of the OMP and MVs of serotypes A1 and A2, making more evident, the proteins that are shared between the outer membrane fractions and the microvesicles liberated by both serotypes. In Lane 1 are the molecular weight markers, in lane 2 are the *A.p*1 OMP that were used as control, in lanes 3 and 4 *M. haemolytica* A2 and A1 OMPs are seen respectively. The protein pattern of this fraction shares a large amount of bands with the MVs of the respective serotypes; lanes 5 and 6. Both correspond to MVs that

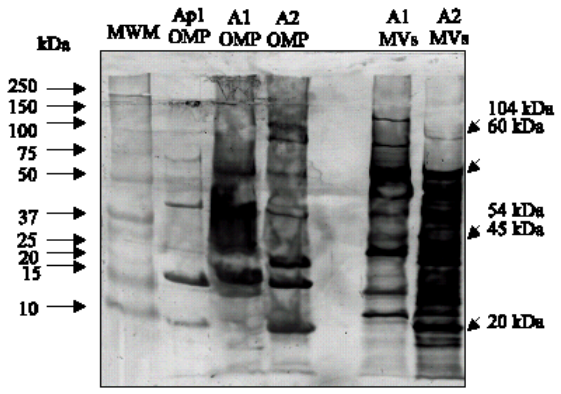

Fig. 3: Western Blot with sera of "clinically healthy" lambs, sampled in the field

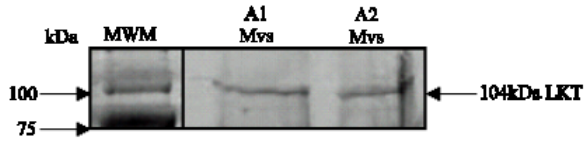

Fig. 4: Western Blot with an anti-Apx1 *A. pleuropneumoniae* serum that has crossed reaction with LKT

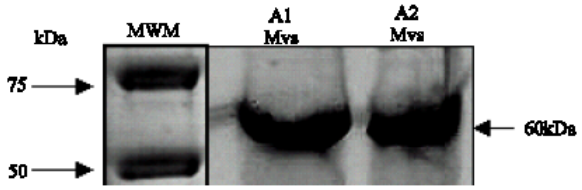

Fig. 5: Western Blot with *A. pleuropneumoniae* anti-adhesin serum

were extracted from cultures treated with antibiotic and in the last lane, arrows show the main identified MVs proteins (Fig. 1).

Once the OMPs are obtained, as well as the respective MVs for each serotype, the presence of LPS was determined in both fractions by silver staining modified for LPS. As the image shows (Fig. 2), OMPs as well as MVs, have LPS, as it can be seen at the bottom of the lanes at the level of markers 10-15 kDa in dark color. In the case of the MVs, in some cases the top part of the lane is stained because it contains components of the outer membrane, giving a positive tincture reaction in the upper part of the lane. In the first lane the molecular weight markers are found, in lanes 2nd and 3rd the OMP of serotype A1 and serotype A2 respectively, in lane 4 MVs pf *M.h* serotype A2, where LPS are stained at the bottom of the lane at the level of the 10-15 kDa markers (lane 4, Fig. 2).

Immunoblot for OMP and MVs, of serotype A1 as well as A2 were carried out, challenged with clinically ill and clinically healthy animal sera, observing in both cases an intense recognition reaction to different MVs proteins of both serotypes, mainly MVs of serotype A2; in this case it is important to mention that most of the "clinically healthy" lambs that were sampled in the field had large amounts of antibodies against *M. haemolytica* specifically serotype A2 antigens. In lane 1 the molecular weight markers are found, in lane 2 OMP of *A. pleuropneumoniae* serotype 1 (*A.p* 1) used as control, in lanes 3 and 4 OMP of serotypes A1 and A2, respectively and finally in lanes 5 and 6 MVs proteins of serotype A1 as well as serotype A2 respectively are seen (Fig. 3).

In order to determine the presence of Leukotoxin (LKT) in microvesicles, an anti-Apx1 *A. pleuropneumoniae* serum was used since those bacteria have hemolysin, with weight (105 kDa) and RTX structure that are very similar to *M. haemolytica* LKT (Fig. 5). On Immunoblot, a band can be clearly seen slightly above the 100 kDa marker that is being recognized by the anti-Apx1 *A. pleuropneumoniae* serum, which could correspond to *M. haemolytica* leukotoxin giving a positive crossed reaction. In lane 1 molecular weight markers are found and in lanes 2 and 3, MVs LKT of serotypes A1 and A2 of *M. haemolytica*, respectively (lane 2 and 3, Fig. 4).

To determine presence of adhesin an Immunoblot was carried out with MVs, of *M. haemolytica* serotype A1 as well as serotype A2, with an anti–adhesion serum against a 60 kDa protein of *A. pleuropneumoniae* previously identified in that agent (Enríquez *et al*., 2004), (Fig. 6). In that case, a clear recognition of a protein of similar weight in *M. haemolytica* MVs was observed. In both cases, a recognition of a 60 kDa band was observed between the 50 and 75 kDa marker, that reacted with the anti-adhesin serum of *A. pleuropneumoniae* and could correspond to a protein of similar structure in *M. haemolytica* A2. In lane 1 the molecular weight markers are found and in lanes 2 and 3 MVs of *M. haemolytica* serotypes A1 and A2 respectively where a crossed reaction is observed (lane 2 and 3, Fig. 5).

Once the procedure has been carried out to eliminate resistance plasmids, 2% agarose gels were run to determine the presence or absence of said structure, beginning from clean strains with Acridine Orange and strains without treatment.

Electron microscopy: Different field observations revealed the presence of spherical regular sized structures that could suggest microvesicles coalescence, likewise

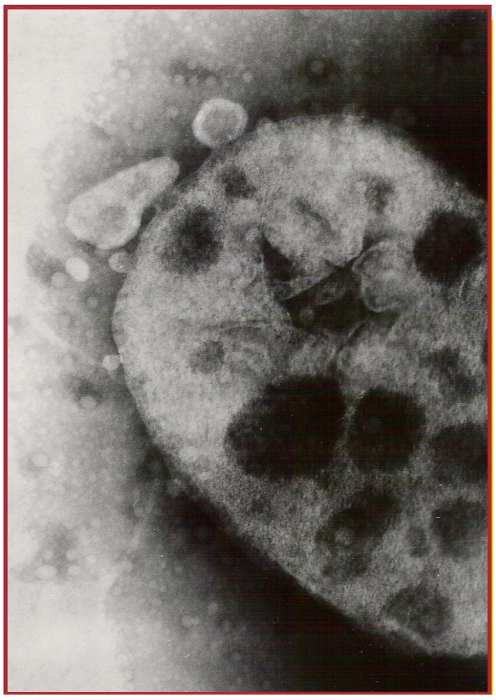

Fig. 6: In the image, the bacterial body of *M. haemolytica* is seen liberating multiple spherical structures of approximately 100 nm in diameter. Those structures tend to coalesce to form larger size structures

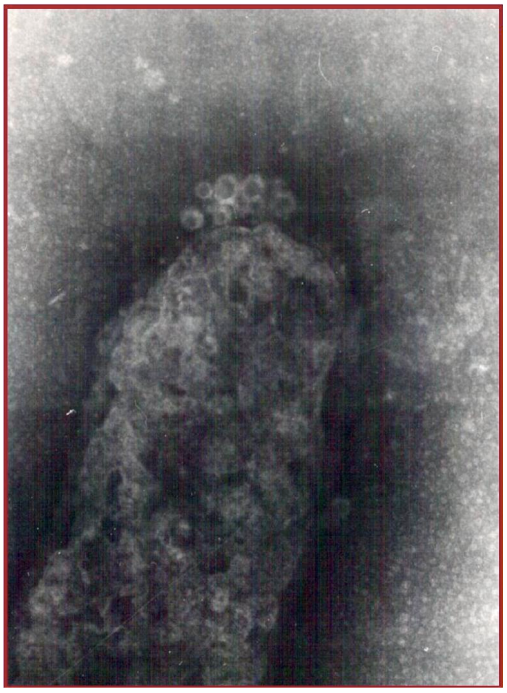

Fig. 7: A clump of MVs is seen in one of the poles of the bacterial body, most of them of a uniform size with a very clear halo surrounding them marking the double membrane

numerous MVs were observed separating themselves from the bacterial outer membrane. Those structures are approximately 100 nm in diameter. In some cases, there is a tendency to coalesce forming structures of larger diameter. In Fig. 6, the coalescence can be observed, as well as the formation of multiple MVs coming from the bacterial surface, the double membrane characteristic of them is clearly seen. Finally, in Fig. 7, a clump of MVs are observed in one of poles of the bacterial body, most of them of a uniform size.

Extracellular secretion of products is the main mechanism by which Gram-negative pathogens communicate with and intoxicate host cell. Vesicle released from the envelope of growing bacteria serve as secretory vehicle for proteins, lipids and DNA, amongst other products. Vesicles released occurs in infected tissues and this liberation is by environmental factors. Vesicles plays roles in establishing a colonization niches, carrying and transmitting virulence factors in to the tissues of the host, as well as modulating the defense responses of the host. The liberation of toxins mediated by vesicles is a potent attack mechanism exhibited by diverse Gram negative bacteria. Biochemical and functional properties of the vesicles that are liberated by pathogens into the environment reveal their critical potential and impact on the diseases they produce (Kuehn and Kesty, 2005).

Virulence factor secretion by Gram negative pathogens is complicated by the fact that the bacterial envelope consists of 2 lipid double bilayers, the inner and outer membrane and the periplasma in between. These bacteria have developed many strategies, some specific to pathogen, to enable active virulence factors to gain access to the extracellular environment, typically the tissues or bloodstream of the host organism (Henderson *et al*., 2004).

Pathogenic as well as non-pathogenic Gram negative microorganisms secrete vesicles (Mayrand and Grenier, 1989; Kadurugamuwa and Beveridge, 1997; Li *et al*., 1998; Beveridge, 1999.) including *Escherichia coli* (Hoekstra *et al*., 1976; Gankeman *et al*., 1980), *Shigella* sp. (Kadurugamuwa and Beveridge, 1999; Dutta *et al*., 2004), *Neisseria* sp. (Devoe and Gilchrist, 1973; Dorward *et al*., 1989), *Bacteroides* (including *Porphyromonas*) sp. (Grenier and Mayrand, 1987; Mayrandand Holt, 1988; Zhou *et al*., 1998), *Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge, 1995), *Helicobater pylori*

(Fiocca *et al*., 1999), *Vibrio* sp. (Chatterjee and Das, 1967; Kondo *et al*., 1993), *Salmonella* sp. (Vesy *et al*., 2000; Wai *et al*., 2003), *Brucella mellitensis* (Gamazo and Moriyon, 1987), *Campylobacter jejuni* (Logan and Trust, 1982; Blaser *et al*., 1983), *Actinobacillus actinomycetemcomitans* (Nowothy *et al*., 1982), *Xenorhabdus nematophilus* (Khandelwal and Banerjee-Bhatnagar, 2003), *Borrelia burgdorferi* (Shoberg and Thomas, 1993), *Actinobacillus pleuropneumoniae* (Negrete *et al*., 2000) and *Actinobacillus seminis* (Núñez del Arco *et al*., 2006).

Studies made on microvesicles of diverse bacterial origins support the idea that they have a common function: vesicles are the means by which the bacteria interact with the prokaryotic, eukaryotic cell and its environment (Kuehn and Kesty, 2005).

Biochemical analysis and the functional characterization of the pathogen-derived outer membrane vesicles, demostrated that this secretory pathway has been usurped by pathogens for the transport of active virulence factors to the host cells. Outer membrane vesicles contain adhesins, toxins and immunomodulator components and they directly mediated bacterial binding and invasion, causing cytotoxicity and modulate the immune response of the host. Microvesicles of the outer membrane are considered a potent factor of bacterial virulence due to their participation in such diverse aspects of the interaction host-pathogen (Kuehn and Kesty, 2005).

Mannheimia species can express a large amount of potent surface antigens including Lipopolysaccharides (LPS), Outer Membrane Proteins (OMP), iron mediated proteins (HOM), fimbria and capsular polysaccharides. These bacteria secrete a cytolysine that is specific for leukocytes called Leukotoxin (LKT) that kills phagocytes. Different antigens expression occurs in different growth conditions. This increases the possibility of altering antigen expression *in vivo* and contributes to disease pathogenesis. Each one of these antigens contributes to the clinical presentation of the disease, in combination with the secretions and cell components that favor the characteristic presentation of the disease (Whiteley *et al*., 1992).

There are studies that indicate that antibodies against outer membrane proteins of *M. haemolytica* play a very important role in pneumonic Pasteurellosis immunity in cattle, since they are the most important antigens in promoting resistance to that disease. Those studies were carried out by Morton *et al*. (1994), Moiser (1989) and Confer (1993). They all indicated a statistically significant correlation between resistance to pneumonic Pasteurellosis and the presence of serum antibodies directed against a large amount of proteins present in saline extracts of the whole bacterial cell (Morton *et al*., 1994).

Since MVs, are formed from the outer membrane, they carry then in their structure a large amount of these OMP that enrich them antigenically.

In a study performed by Rosenqvist *et al*.(1995) working with the human antibody response against outer membrane MVs vaccines of *Neisseria meningitidis*, observed that the antibody response against outer membrane proteins during vaccination was mainly of IgG isotype, when a third dose, was applied 4 or 5 years late.

Mannheimia haemolytica, Leukotoxin (LKT) as well as LPS play an important role in pneumonic Pasteurellosis pathogenesis. It is believed that those 2 antigens are the main bacterial virulence factors during the bacterial invasion that contribute to develop the characteristic pathology of this disease. The LKT, is secreted when the bacteria is in the logarithmic growth phase and acts as a pore forming cytolysine inserting itself in the membrane of the target cells (Clinkenbeard *et al*., 1989) this results in osmotic unbalance that generates the death of the host cell. LKT begins a cascade of events that allow damage to tissue, pneumonia and death of the animals (Sreevatsan and Maheswaran, 2002).

Mannheimia genus LPS comprises 10-25% of the bacterial dry weight. This antigen generally is found associated to the bacterial cell, but it has been possible to detect it in lung infections (Breider *et al*., 1990). It is considered one of the bacterial components that have a high capacity of inducing inflammatory response (Brogden *et al*., 1995). It is known that LKT as well as LPS induce gene expression for pro-inflammation cytokines, including IL-1 and FNT in bovine alveolar macrophages (Yoo *et al*., 1995a, b).

On the other hand, it is known that the parenteral administration of high concentrations of purified proteins, peptides or hapten carrier conjugates allow a high immune response and these when administered with an adjuvant, induce a much higher humoral as well as cellular response in the individual. Adjuvant activity of DNA and Oligodeoxinucleotides (ODN) was described at the beginning of 1960. Hechtel *et al*. (1965) showed for the first time that the injection of DNA from bovine digested thymus cells and DNA from Sheep Red Blood Cells (SRBC) in AKR mice gave way to 2 or 3 times more cell plate formation than when administering an alone antigen.

Different researches have proven that ODN may stimulate T cells. Katz *et al*. (1973) showed that ODN may induce the expansion of surface carriers of T cells and

Allison (1973), showed that these are capable of elevating mixed lymphocytes reactions.

Bacterial DNA is not endowed with polyclonal stimulating properties. Messina *et al.* (1991) and Sun *et al.* (1997) demonstrated that bacterial DNA induces independent proliferation of T cells and polyclonal synthesis of B lymphocyte immunoglobulins in mice. This effect was observed with ss-, ds-DNA and 100 pb DNA fragments. The DNA effect is quite similar to that of endotoxins and therefore it was proposed that both substances stimulate innate immunity acting directly on the inflammatory response. Plasmid DNA activates Dendritic Cell (DC) maturity (Sparwasser *et al.*, 1998). These authors demonstrated that bacterial DNA, or CpG ODN, cause immature DC maturation, activating them and producing cytotoxins such as IL-12, IL-6 and FNT∝. DC induction and maturation and the production of cytotoxins demonstrates that bacterial DNA or CpG ODN are capable of contributing to the DC efficiency to initiate a cell mediated immune response by T cells. Bacterial plasmids induce also the expression of IL-1B and PAI-2 genes, characteristic phenomenon of macrophage activation.

Mannheimia haemolytica carries a DNA plasmid fragment in its MVs that strengthens the antigenic and immunological effect of the same. Up until know the specific function of this MVs identified plasmid is unknown. Nevertheless, it is true that when accompanying Mvs antigens they may generate a better immune response in those individuals that are exposed to them.

CONCLUSION

Identification of the main antigens (LKT, LPS, OMP, DNAp) in *Mannheimia haemolytica* MVs, gave way to the detection of the immunological potential of those structures, which makes them important immunogens that can be tested in animal models, evaluating thus their capacity to incite an adequate humoral immune response in individuals exposed to them.

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