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Microvesicles of *Actinobacillus seminis* Show Proteolytic Activity

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Abstract: The objective of the present study was to identify proteolytic activity in microvesicles (MVs) from *Actinobacillus seminis*. A reference strain *A. seminis* ATCC 15768 and a field strain isolated were used. The microvesicles were obtained from ultracentrifugation from 48 h cultures. The proteolytic activity was determined in PAGE-SDS (12%) copolimerized with gelatin at 1%. The protein profile of the microvesicles was also established. The size of MVs was determined with transmission electron microscopy and it was determined to range from 100-150 nm. The protein profile rendered a 75 kDa protein, previously reported to be specific for *A. seminis*. Proteolytic activity was detected in the bands located at 79.5, 59, 40, 27 and 13.5 kDa. These results demonstrated the presence of proteolytic activity in *A. seminis* MVs. Further investigation on protease characterization and assessment of their importance in ovine epididimitis pathogenesis is needed.

Key words: *Actinobacillus seminis*, proteases, microvesicles, proleolytic activity

INTRODUCTION

Ovine epididimitis is an infectious process affecting rams. It is a contagious and progressive disease with chronic or acute course resulting in testes degeneration and infertility. The illness has an economical impact due to the unfavorable effects on the fertility of the flock.

Aactinobacillus seminis is considered part of the normal microflora of the penis and epididimus mucous membranes in the ovine. Nevertheless, *A. seminis* has been isolated from semen of ram with epididimitis by several authors (Baynes and Symmons, 1960; Livingston and Hardy, 1964; Gamazo *et al*., 1989; Low *et al*., 1995; Mbai *et al*., 1996; De la Puente *et al*., 2000). The bacterium provokes an ascending epididimitis that can progress to orchitis and irreversible infertility. *Actinobacillus seminis* together with *Brucella ovis* are considered the main pathogenic agents of epididimitis and ovine infertility. In contrast to *B. ovis*, there is little knowledge about *A. seminis* mechanism of infection (Mbai *et al*., 1996).

Gram-negative bacteria possess two different membranes, cytoplasmic membrane and outer membrane, separated by the periplasmic space containing several structural components and enzymes. These bacteria have developed at least 5 different secretion systems in order to release into the extracellular medium molecules that allow them to colonize tissues, communicate and to obtain nutrients. When Gram negative bacteria divide, the outer membrane is able to release microvesicles (MVs) to the medium (Ramón *et al*., 2006; Li *et al*., 1996). They are formed mainly by outer membrane protein, associated with toxins and enzymes such as proteases (Zhou *et al*., 1998). The formation of MVs has been reported in *Actinobacillus seminis* and other *Actinobacillus* such as *A. actinomycetemcomitans*, *A. pleuropneumoniae* and in *Brucella melitensis*, *Brucella ovis*, *Bacteroides burgdorferi*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Núñez *et al*., 2006; Li *et al*., 1996; Gamazo *et al*., 1989; Zhou *et al*., 1998).

Microbial proteases have been considered a virulence factor during pathogenesis in diverse diseases caused by microorganisms. Proteases can travel outside bacteria inside vesicles released to the medium. Importantly, proteolytic activity has been identified in supernatants of *A. pleuropneumniae* cultures (Negrete *et al*., 2000). No information is available about proteolytic activity in *A. seminis* vesicles.

The aim of this study was to identify proteolytic activity in *A. seminis* Mvs.

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MATERIALS AND METHODS

Bacteria and growth conditions: *A. seminis* ATCC 15768 and a field strain were growth in *Brucella* agar (BA; Beckton-Dickinson, Franklin Lakes, New Jersey, USA) supplemented with 10% of ovine blood during 48 h and 37°C.

Microvesicles isolation: The bacterial cells were harvested from 48 h growth plates with saline. They were centrifuged at 10,000 X g, for 20 min at 4°C to separate the cells. The supernatant was collected and filtered through a 0.22 µm membrane (Millipore) to remove residual cells. The MVs were recovered by ultracentrifugation (150,000 X g, 3 h at 4°C) (Li *et al*., 1996; Zhou *et al*., 1998). The samples were maintained frozen (-20°C) until its usage. The process was repeated several times for both strains.

Electron microscopy: The MVs were observed by negative staining. Total 25 µL were put in a nickel lattice with Formvar covering, with 20 min adsorption time. Staining was done with 25% (w/v) phosphotungstic acid (Sigma Chemical Company, St. Louis, Missouri, USA) for 3 min at pH 8 and observed under the transmission electron microscope JEM 2000 EX at 40,000 X.

Electrophoresis and zymogram: The MVs were separated in PAGE- SDS, preparing a 1:1 sample with Laemmli buffer for samples and using a 12% polyacrylamide gel. The gel was stained with Coomassie Blue R-250 (Sigma Chemical Company, St. Louis, Missouri, USA) (Laemmli, 1970).

The proteolytic activity in microvesicles was assessed by copolymerizing PAGE-SDS with porcine gelatin at 1% in the separating gel (Negrete *et al*., 1994). Two microliters of sample were put in each well and electrophoresed at 100 V at 4°C. The gel was then put in 2.5% Triton X 100 solution during 1 h, then in 1 M Tris + $CaCl_2$ for 24 h at 37°C. The gel was stained with Coomassie Blue R-250 and unstained to reveal the bands with enzymatic activity.

Lipopolysaccharide (LPS) determination: To evidence outer membrane origin, the presence of LPS in the MVs was assessed by the Limulus Amebocyte Lysate test (LAL) test (QCL-1000 Chromogenic *Limulus* amebocyte lysate, Bio-Whittaker, East Rutherford, New Jersey, USA) (Young *et al*., 1972).

RESULTS

Microvesicles: *A. seminis* MVs were obtained by ultracentrifugation. In Fig. 1, electron microscope images of the microvesicles can be observed, obtained from a 48 h culture. The structures s howed a size of 100-150 nm.

***A. seminis* microvesicles PAGE-SDS:** The protein profile of MVs from *A. seminis* strains was determined by Coomassie Blue staining. The electrophoretic patterns were very similar between the two strains studied and showed a wide array of protein activity between 15 and 200 kDa (Fig. 2). It is possible to observe a 75 kDa protein in both strains and it was consistently found in the different assays conducted.

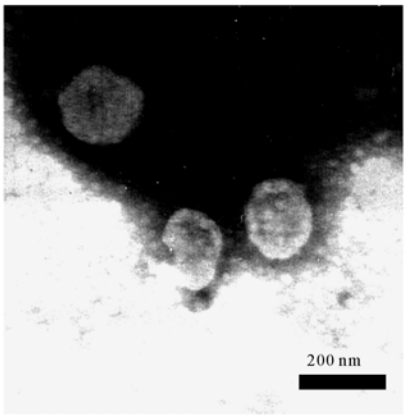

Fig. 1: Transmission Electron Microscopy showing *A. seminis* microvesicles Bar = 200 nm

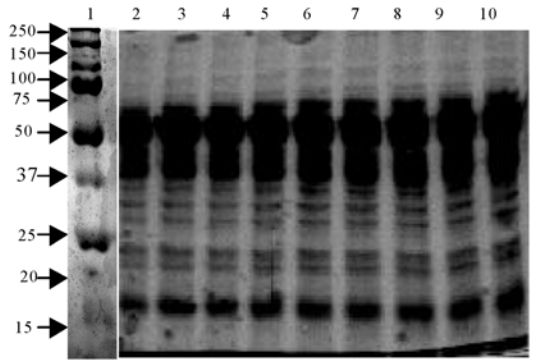

Fig. 2: PAGE SDS at 12% of *A. seminis* microvesicles stained with Coomassie blue. Each well contained 15 µg of protein. Line 1, it shows the molecular weight markers (kDa); lines 2 to 6 *A. seminis* ATCC 15768 microvesicles and lines 7 to 10, field strain. Observe the presence of the 75kDa protein specific to *A. seminis*

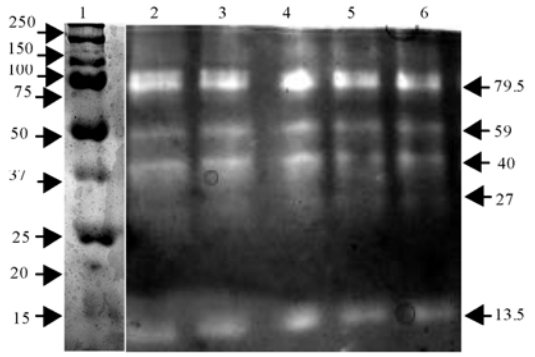

Fig. 3: Zymogram of *A. seminis* microvesicles. Two µL of sample were used to reveal the proteolytic activity bands. Line 1: molecular weight markers (kDa); line 2 and 3 *A. seminis* field strain; Lines 4, 5 and 6 *A. seminis* ATCC 15768. The right side of the gel indicates the molecular weights where proteolytic activity was detected

***A. seminis* microvesicles zymogram:** Figure 3 shows the protease activity in PAGE SDS copolymerized with gelatin. The reference strain (ATCC 15768) and a field strain showed similar proteolytic activity. There are five different proteolytic activity bands with approximate molecular weight of 79.5, 59, 40, 27 and 13.5 kDa.

LPS determination in *A. seminis* microvesicles: LPS was determined in the MVs (LAL Test). LPS was detected in microvesicles from both *A. seminis* ATCC 15768 and the field strain, showing an activity of 0.12 and 0.1 uE mL^{-1}, respectively.

DISCUSSION

In 1989, Mayrand and Grenier (Meyer and Fives-Taylor, 1993) reported that extracellular vesicles are part of the outer membrane from bacteria; then showing the same compounds found in outer membranes, such as proteins and lipopolysaccharides. Gram-negative bacteria produce MVs during normal or stressing growt conditions. The release of these structures has been considered by several authors as a new secretion mechanism by which bacteria can damage host tissues because of virulence factors contained in or associated with MVs, gain access to colonization tissues by killing other bacteria, or can promote the adhesion of nanoadherent bacteria favouring biofilm formation (Ramón *et al*., 2006). MVs can contribute to the pathogenic processes and become an important virulence factor as reported by Negrete *et al.* (1992), they described the presence of metalloproteases and Apx toxins in the microvesicles released by *Actinobacillus pleuropneumoniae*. *A. seminis* MVs, released to the medium and concentrated afterwards (Fig. 1) showed an approximate size between 100-150 nm. They were observed in 48 h cultures. It is reported that the size of MVs can vary from 20-500 nm, as observed in *Actinobacillus pleuropneumoniae* (Negrete *et al*., 2000), *Actinobacillus actinomycetemcomitans* (Meyer and Fives-Taylor, 1993), *Brucella melitensis* y *Brucella ovis* (Gamazo *et al*., 1989) y *Pseudomonas* (Kadurugamuwa and Beveridge, 1995). Figure 2 shows the protein profile of vesicles obtained in different occasions for both, the reference sample *A. seminis* ATCC 15768 and a field strain as separated by 12% PAGE-SDS. A wide array of different molecular weight proteins are observed. The profile shown herein is close to that reported by Núñez *et al*. (2006), for the subcellular fraction of *A. seminis* outer membrane. Núñez *et al*. (2006), evidenced a 75 kDa protein found only in *A. seminis*, the one was also found in the study reported herein. The outer membrane origin of this protein was demonstrated by LPS presence in both *A. seminis* strains ranging from 0.2 and 0.25 UE mL^{-1}. This demonstrates that the origin of these structures is the outer membrane of *A. seminis*.

In the present study, Fig. 3, the bands with proteolytic activity had the following weights 79.5, 59, 40, 27 and 13.5 kDa, A sample of supernatant free from microvesicles was tested to demonstrate that proteolytic activity is associated with microvesicles only (data not shown). In this regard, Zhou *et al.* (1998) demonstrated that the reported endotoxic activity from filtrates of *Veillonella* sp., was due to the presence of microvesicles and detected LPS activity.

It is necessary to continue the characterization of proteases released by *A. seminis* and to investigate their importance in the pathogenesis of ovine epididimitis. Moreover, the microvesicles can be a source of molecules with antigenic activity which, after purification and characterization, could be used to elucidate the pathogenesis and to develop immunogens and diagnostic tests.

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