Identification of an immunogenic protein of *Actinobacillus seminis* that is present in microvesicles

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Abstract

Actinobacillus seminis is a gram-negative bacterium of the *Pasteurellaceae* family that is involved in ovine epididymitis. Looking for a protein specific to this species, we determined the protein profile of subcellular fractions of *A. seminis* (American Type Culture Collection number 15768): proteins from the outer membrane (OMPs), inner membrane (IMPs), and cytoplasm (CPs). These profiles provide the first data, to our knowledge, regarding subcellular fractions of *A. seminis.* In the OMP fraction, we identified a protein with a molecular mass of 75 kDa that proved to be immunogenic and apparently specific for *A. seminis.* This conclusion was based on the reaction of hyperimmune serum of rabbits inoculated with whole cells of *A. seminis* that was tested against sonicated complete cells of reference strains and field isolates of *Brucella ovis, Mannheimia haemolytica, Pasteurella multocida,* and *Histophilus somni.* No protein of these bacteria cross-reacted with the 75-kDa protein of *A. seminis.* Furthermore, when each type of hyperimmune serum was tested against the sonicated cells and each of the subcellular fractions of *A. seminis,* it did not recognize the *A. seminis* 75-kDa protein. We also isolated and identified this protein in microvesicles released to the culture supernatant. The results suggest that the 75-kDa protein could be used to establish a diagnostic test specific for ovine epididymitis caused by *A. seminis.*

Résumé

Actinobacillus seminis *est une bactérie à Gram négatif de la famille des* Pasteurellaceae *associée à l'épididymite ovine. En cherchant pour une protéine spécifique à cette espèce, nous avons déterminé le profil de protéine de fractions sub-cellulaires d'*A. seminis *(ATCC 15768) : des protéines de la membrane externe (OMPs), de la membrane interne (IMPs) et du cytoplasme (CPs). Ces profils fournissent selon nous, les premiers résultats concernant les fractions sub-cellulaires d'*A. seminis. *Dans la fraction OMP, nous avons identifié une protéine avec un poids moléculaire de 75 kDa qui s'est avérée être immunogène et apparemment spécifique à* A. seminis. *Cette conclusion était basée sur la réaction de sérum hyperimmun de lapins inoculés avec des cellules entières d'*A. seminis *et qui a été testé avec des cellules entières soniquées de souches de référence et d'isolats cliniques de* Brucella ovis, Mannheimia haemolytica, Pasteurella multocida *et* Histophilus somni. *Aucune protéine des ces bactéries n'a donné de réaction croisée avec la protéine de 75 kDa d'*A. seminis. *De plus, lorsque des sérums hyperimmuns produits contre les autres bactéries mentionnées ci-dessus ont été testés avec les cellules soniquées et chacune des fractions sub-cellulaires d'*A. seminis, *aucun n'a reconnu la protéine de 75 Kda d'*A. seminis. *Nous avons également isolé et identifié cette protéine dans des micro-vésicules relâchées dans le surnageant de culture. Ces résultats suggèrent que la protéine de 75 kDa pourrait être utilisée pour mettre en place un test diagnostique spécifique pour l'épididymite ovine causée par* A. seminis.*

(Traduit par Docteur Serge Messier)*

Introduction

Contagious ovine epididymitis is defined as infection of the testicle and epididymis in rams. This condition may occur in acute, subacute, and chronic phases and is clinically detected when the testicle degenerates and atrophies, resulting in subfertility or sterility (1). Ovine epididymitis damages young and adult animals, causing important losses to the sheep industry. This disease is a serious reproductive disorder, with marked pathological changes in the genital tract (2).

Among the etiologic agents of epididymitis are several bacterial species, such as *Brucella ovis, Actinobacillus seminis, Mannheimia haemolytica (Pasteurella haemolytica), Pasteurella multocida,* and *Histophilus somni (Haemophilus somnus)* (3). The route of entry is commonly a wound in the genital mucosa; however, for *A. seminis* the route has not been adequately investigated.

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A pleomorphic gram-negative coccobacillus 1×1 to 1.4 μm, *A. seminis* is found in chains or palisades. It is nonmotile and non-sporulated. This facultative anaerobe has fermentative metabolism; it grows optimally in 10% CO_2 at 37°C in media with blood or serum, producing small colonies after 24 h and large, white colonies (1 to 2 mm in diameter) after 48 h (4,5).

Reports of epididymitis caused by *A. seminis* are scarce. The first isolation was reported from Australia in 1960 by Baynes and Simmons (6), who described suppurative epididymitis caused by a gram-negative pleomorphic microorganism in a ram. In 1968 Baynes and Simmons (7) reported having isolated a microorganism from 3 rams with epididymitis. The same year, in New Zealand, *A. seminis* was isolated from a ram with epididymitis and polyarthritis (8). In Mexico, the first case of epididymitis produced by *A. seminis,* in animals imported from New Zealand, was reported in 1979 (9).

In 1988, Healey et al (10) reported having separated proteins from strains of *A. seminis* and *A. actinomycetemcomitans,* both isolated from rams with epididymitis. The proteins from the 2 isolates had a similar molecular mass. However, other isolates identified as *Actinobacillus* spp. had different protein profiles, leading to the conclusion that diverse members of the *Actinobacillus* genus can probably be isolated from rams with epididymitis. The objective of our study was to identify a protein specific to *A. seminis.*

Materials and methods

Bacterial strains and growth conditions

The bacterial species used in this study are shown in Table I. All strains were grown on *Brucella* agar (BA; Becton Dickinson, Rutherford, New Jersey, USA) in 10% CO_2 at 37°C.

Subcellular fractions of *A. seminis*

All reagents were from Sigma Chemical Company, St. Louis, Missouri, USA. Bacterial subcellular fractions were obtained according to the method described by Morton et al (11) in 1996, with some modifications. American Type Culture Collection (ATCC) *A. seminis* 15768 was grown on BA for 24 h, suspended in 10 mM HEPES pH 7.2, and centrifuged at $1000 \times g$ for 30 min. The pellet was washed twice and resuspended in 10 mM HEPES containing 0.1 M phenylmethyl-sulfonyl fluoride, 0.1 M *p*-hydroxymercury benzoate (PHMB), and 3% ethylene diamine tetraacetic acid. Cells was sonicated by 6 cycles of 1 min in a Vibra-Cell (Sonics & Materials; Newtown, Connecticut, USA), and cellular detritus was removed by centrifugation at $10\,000 \times g$ for 20 min. The supernatant was centrifuged at $150\,000 \times g$ for 1 h; the new supernatant contained the cytoplasmic proteins (CPs), and the pellet contained the total membranes. The membranes were resuspended in 10 mM HEPES containing 1% (w/v) sodium N-lauryl sarcosine and agitated for 30 min at 37°C (12). They were then centrifuged at $150\,000 \times g$ for 1 h. The pellet contained the outer membrane proteins (OMPs) and the supernatant the inner membrane proteins (IMPs). The OMPs were treated to remove lipids and detergent and were suspended in water according to the method of Wessel and Flügge (13). The purity of the OMPs was verified with the QCL-1000 Chromogenic *Limulus* Amebocyte

Table I. Bacterial strains and isolates used in this study

Bacterium	Isolate	Origin
Actinobacillus seminis	15768	ATCC
	Isolate	INIFAP–SAGARPA
Brucella ovis	BP 2001	BUAP
	BP 2002	
Mannheimia haemolytica	GM31	FESC, UNAM
	EH 28	
	CA22	
	MA16	
	SC17	
Pasteurella multocida	SM13	FESC, UNAM
	AC14	
	SA15	
	AE28	
	SC17	
Histophilus somni	Isolate	INIFAP–SAGARPA

ATCC — American Type Culture Collection; INIFAP–SAGARPA — Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias. Secretaría de Agricultura Ganadería, Desarrollo Rural, Pesca y Alimentación; BUAP — Benemérita Universidad Autónoma de Puebla; FESC, UNAM — Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México

Lysate (LAL) test for lipopolysaccharide (LPS) (BioWhittaker, East Rutherford, New Jersey, USA) (14), and LPS was stained with silver by the method of Tsai and Frasch (15). The IMPs and CPs were separately precipitated overnight with cold ethanol 1:4 (v/v) at 4°C. They were then centrifuged at $1000 \times g$ for 20 min, and the sediment was dried by air flow and kept frozen until used. Protein concentration was determined by the method of Bradford (16).

Rabbit antiserum

Two New Zealand rabbits weighing 2 kg each were intramuscularly inoculated with *A. seminis* 15768 cells (1.5×10^8 colony-forming units [CFU]) in saline solution, initially with Freund's complete adjuvant and 20 d later with Freund's incomplete adjuvant. After 7 d, the rabbits were given intramuscular booster inoculations without adjuvant 3 times, at 1-wk intervals. A titer of 1:2050 for the antibody against *A. seminis* was obtained by dot enzyme-linked immunosorbent assay (ELISA-dot) with *A. seminis* sonicated cells used as antigen.

Serum antibody against *B. ovis* was donated by Facultad de Estudios Superiores Cuautitlán, Universidad Nacional Autónoma de México, and antibody against *M. haemolytica, P. multocida,* and *H. somni* was donated by Centro Nacional de Investigaciones Disciplinarias en Microbiología Animal, Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias, Secretaría de Agricultura Ganadería, Desarrollo Rural, Pesca y Alimentación, México. All antibodies were produced in rabbits with the use of sonicated cells. The titers (1:2000 for *B. ovis* and 1:1000 for the other bacteria) were determined by ELISA-dot with use of the sonicated cells as antigen.

Two New Zealand rabbits weighing 2 kg each were intramuscularly inoculated with the 75-kDa protein of *A. seminis* 15768, obtained
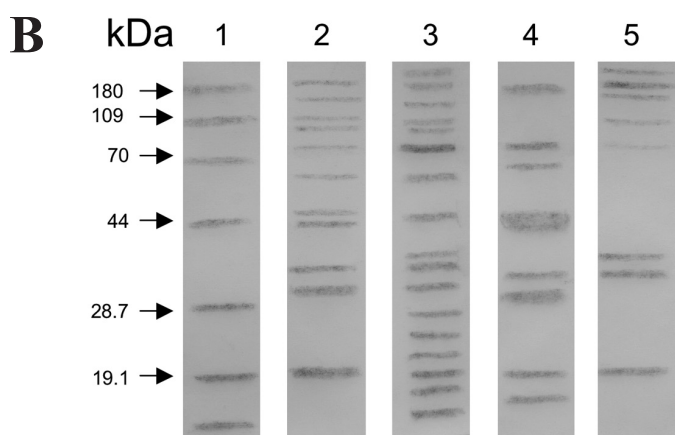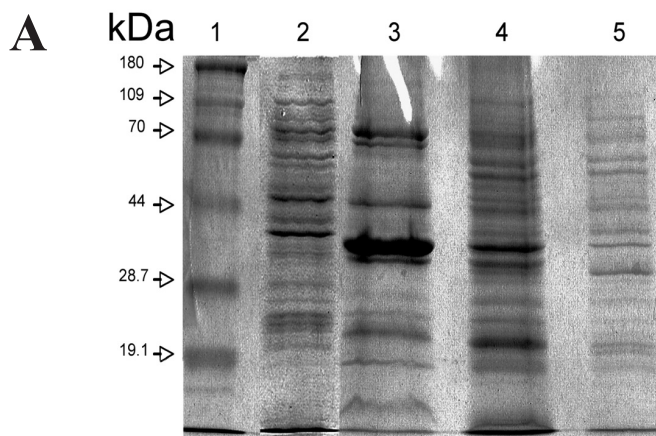
Figure 1. A, proteins of *Actinobacillus seminis* separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane 2, whole-cell extracts; lane 3, outer membrane proteins (OMPs); lane 4, cytoplasmic membrane proteins (CPs); lane 5, inner membrane proteins (IMPs). B, corresponding results of Western blot testing with rabbit antibody against *A. seminis*. In all the figures, lane 1 consists of molecular mass markers.

Figure 2. Demonstration of the 75-kDa protein that is apparently specific for *Actinobacillus seminis* (A, lane 2) by Western blot testing of extracts from whole cells of bacteria associated with contagious epididymitis. A, extracts of each bacterium were exposed to antibody against *A. seminis*: lane 2, *A. seminis*; lane 3, *Brucella ovis*; lane 4, *Histophilus somni*; lane 5, *Mannheimia haemolytica*; lane 6, *Pasteurella multocida*. B, extract from *A. seminis* was exposed to antibody against each bacterium: lane 2, antibody against *A. seminis*; lane 3, antibody against *B. ovis*; lane 4, antibody against *H. somni*; lane 5, antibody against *M. haemolytica*; lane 6, antibody against *P. multocida*.

by elution with 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A titer of 1:4000 was obtained by ELISA-dot using the eluted protein as antigen.

Protein preparation, purification, and testing

Whole cells and each subcellular fraction of *A. seminis* were separated by 10% SDS-PAGE (17); each well contained 10 μg of protein. Proteins were electrotransferred (18) to 0.45-μm nitrocellulose membranes (Gibco Laboratories, Carlsbad, California, USA) for 1 h at 400 mA by means of a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad Laboratories, Hercules, California, USA). To visualize the *A. seminis* proteins recognized by the rabbit antiserum, a peroxidase-labeled goat IgG conjugate against rabbit antigen, diluted 1:1000 (Sigma), was used.

To determine which proteins of *A. seminis* are species-specific, whole cells of *A. seminis* were separated by 10% SDS-PAGE, transferred, and exposed to rabbit hyperimmune serum against the other bacteria, with use of the same enzyme conjugate.

To purify the 75-kDa protein from *A. seminis* 15768, the OMPs were separated by 12% SDS-PAGE in a Hoefer chamber 16 × 0.2 × 18 cm
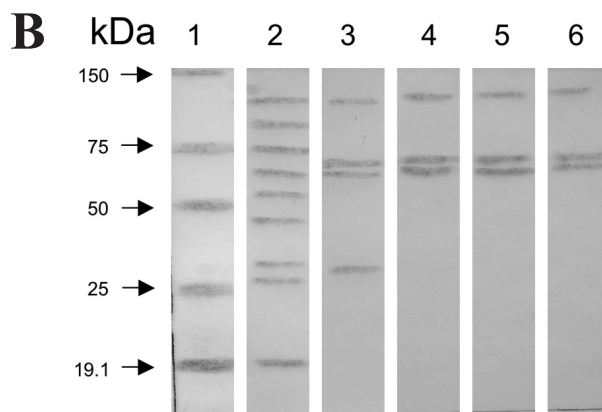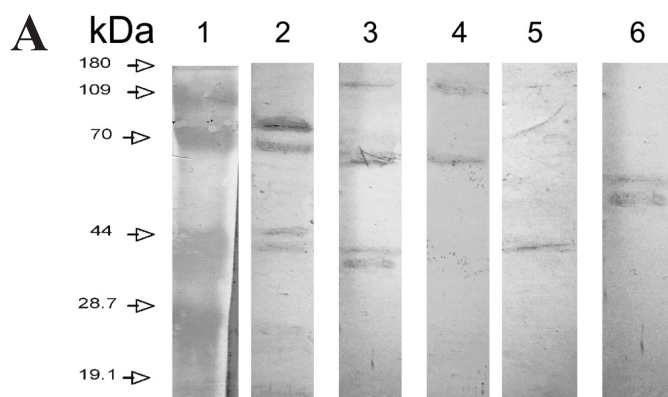
and stained with Coomassie blue (Hoefer Scientific Instruments, San Francisco, California, USA). The 75-kDa OMP was cut from the gel and electroeluted in a MiniProtean III chamber (BioRad) for 3 h at 60 mA. The eluted material was precipitated with 4 volumes of ethanol at 4°C, centrifuged at 10 000 × *g* for 20 min, and then resuspended in deionized water. The protein concentration was measured and purity checked with the use of 12% SDS-PAGE and Western-blot testing with the rabbit antiserum to the 75-kDa protein.

To determine if the 75-kDa protein was species-specific for *A. seminis*, field isolates were obtained from epididymal tissues of rams infected with *A. seminis, B. ovis, M. haemolytica, P. multocida*, and *H. somni*. Whole cells were separated by 12% SDS-PAGE (17), and then the proteins were electrotransferred to 0.45-μm nitrocellulose membranes for 1 h at 400 mA and exposed to the rabbit antiserum to the *A. seminis* 75-kDa protein, with use of the same enzyme conjugate.

Isolation of *A. seminis* membrane microvesicles

After centrifugation of cultured *A. seminis* 15768 at 10 000 × *g* for 20 min at 4°C, the supernatant was filtered sequentially through

membranes of pore diameters 0.45 μm and then 0.22 μm (Millipore, Billerica, Massachusetts, USA) to remove residual cells. Vesicles were recovered by ultracentrifugation (150 000 × g for 3 h at 5°C) and kept at −20°C until used (19).

Electron microscopy

For negative staining, cultured *A. seminis* 15768 was harvested by centrifugation at 10 000 × g for 20 min at 4°C. Whole cells or vesicles (15-μL) were placed onto carbon and copper grids coated with polyvinyl formal (Formvar), stained with 25% (v/v) phosphotungstic acid, and observed with an electron transmission microscope (JEM-100S; JEOL, Tokyo, Japan) at a magnification of × 40 000 (20).

For scanning electron microscopy, cultured *A. seminis* 15768 with vesicles were dehydrated in graded alcohols, dried, coated with gold, and observed with a scanning electron microscope (JSM-5410LV; JEOL) at a magnification of × 20 000 (20).

Immunoblotting of membrane microvesicles

To detect the 75-kDa protein in membrane microvesicles of *A. seminis,* after electrophoresis the vesicle proteins were transferred to a nitrocellulose membrane for 1 h at 400 mA and then exposed to the rabbit antiserum to the *A. seminis* 75-kDa protein, with use of the same enzyme conjugate.

Results

Protein profiles of *A. seminis* subcellular fractions

Figure 1A shows the protein patterns of the whole cells, OMPs, CPs, and IMPs separated by SDS-PAGE (lanes 2, 3, 4, and 5, respectively). A pattern corresponding to smooth LPS was found in *A. seminis* in a gel with urea (not shown). The amebocyte LAL test showed that the OMP fraction had a low level of contamination with LPS (0.17 endotoxin units/mL), but contamination was not found in the IMP and CP fractions, which demonstrated their purity. Figure 1B shows the corresponding Western-blot results, which were different for each fraction, showing high antigenic reactivity by the 3 subcellular fractions when tested with the antiserum against whole cells of *A. seminis.* A 75-kDa polypeptide was observed in the OMP and CP fractions (lanes 3 and 4, respectively). These antigenic protein profiles provide the first data, to our knowledge, regarding various subcellular fractions of *A. seminis.*

Specific protein of *A. seminis*

As Figure 2A shows, Western-blot tests of whole-cell extracts with rabbit hyperimmune serum against *A. seminis* revealed a protein of approximately 75 kDa in *A. seminis* (lane 2) but not in *B. ovis, M. haemolytica, P. multocida,* or *H. somni.* On the other hand, antibodies against the other bacteria did not recognize the 75-kDa protein in whole-cell extracts (Figure 2B). These results suggest that the 75-kDa protein could be specific to *A. seminis.*

When the 75-kDa protein was purified from cellular extracts by electroelution (Figure 3A, lane 3) and then exposed to hyperimmune serum against *A. seminis* and the other microorganisms, Western-blot testing revealed that the eluted protein was recognized only
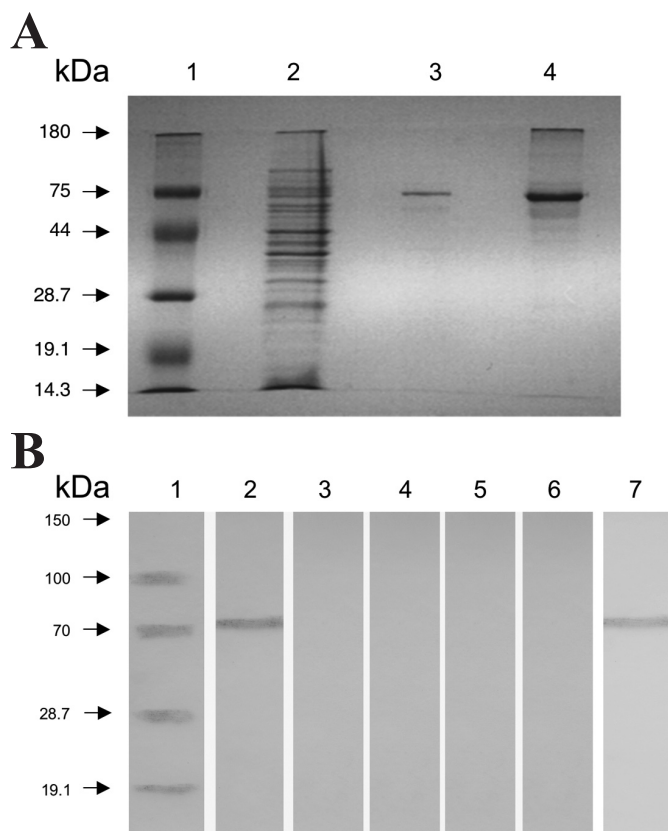

Figure 3. A, separation by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of whole cells (lane 2), the 75-kDa protein (lane 3), and microvesicles (lane 4) of *Actinobacillus seminis.* B, Western-blot results, showing recognition of the 75-kDa protein in *A. seminis* microvesicles only by *A. seminis* whole cells (lane 2) and not by antibodies against field isolates of *Brucella ovis* (lane 3), *Histophilus somni* (lane 4), *Mannheimia haemolytica* (lane 5), *Pasteurella multocida* (lane 6), and *A. seminis* (lane 7).

by the hyperimmune serum against *A. seminis* (Figure 3B, lane 2). Also, Western-blot testing with serum against whole-cell and field isolates of the same bacteria (Table I), obtained from epididymal tissues of rams, revealed that the 75-kDa protein was recognized by serum against isolates of *A. seminis* but not by serum against the other bacterial species (data not shown). These results confirm the specificity of the 75-kDa protein of *A. seminis.*

Membrane microvesicles of *A. seminis*

Membrane vesicles associated with cells from *A. seminis* cultures in the early stationary phase were observed by scanning electron microscopy (Figure 4A) and negative-staining transmission electron microscopy (Figures 4B and 4C). The vesicle diameter varied from 20 to 100 nm, as was described for *A. acinomycetemcomitans* (21) and *A. pleuropneumoniae* (19). After concentration by centrifugation and analysis by SDS-PAGE, the vesicles were found to be rich in the 75-kDa protein and other proteins (Figure 3A, lane 4). The 75-kDa protein was recognized in the microvesicles by antibodies against *A. seminis* whole cells (Figure 5, lane 2) and field isolate but not by antibodies against the other bacteria (Figure 5, lanes 3, 4, 5, and 6).
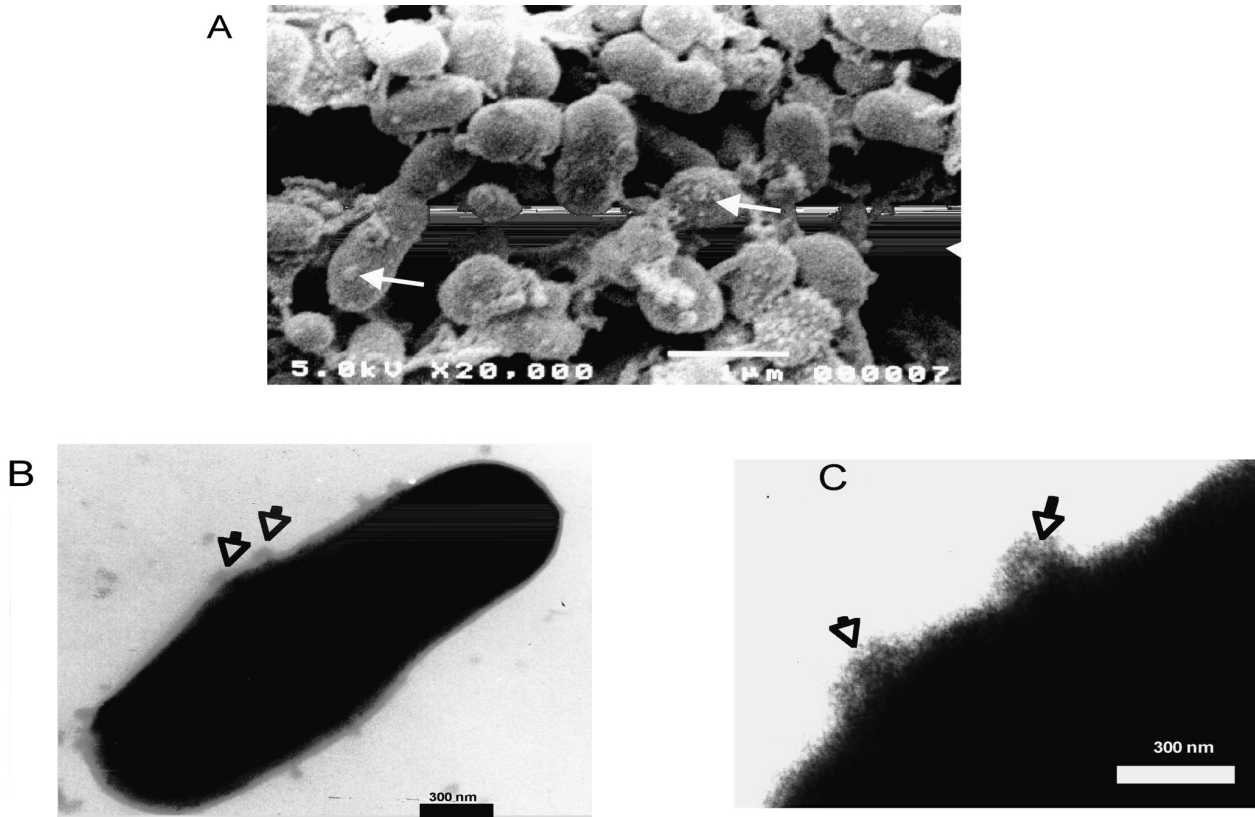
Figure 4. Vesicles (arrows) on the surface of *Actinobacillus seminis* cells, shown by (A) scanning electron microscopy ($\times$ 20 000; bar = 1 μm) and (B, C) transmission electron microscopy after staining with phosphotungstic acid ($\times$ 40 000; bars = 300 nm).
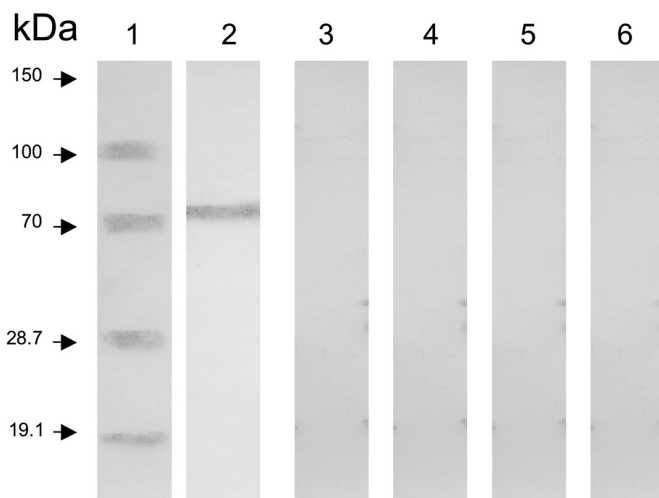

Figure 5. Immunoblot analysis of *Actinobacillus seminis* microvesicles, showing recognition of the 75-kDa protein by antibody against *A. seminis* (lane 2) but not by antibody to *Brucella ovis* (lane 3), *Histophilus somni* (lane 4), *Mannheimia haemolytica* (lane 5), or *Pasteurella multocida* (lane 6).

Discussion

Infectious epididymitis in rams is a major disease in countries devoted to the industrialized breeding of sheep. *Brucella ovis* was reported as the main etiologic agent in Australia in 1960; however,

A. seminis and *H. somni* are also commonly isolated from lesions and produce clinical signs and reproductive problems in rams (3). Analysis of the protein profiles and outer membrane antigens of *A. seminis* and *A. actinomycetemcomitans,* which are considered to be transient ovine genital flora, suggests that these opportunistic pathogens constitute a diverse group of bacteria (10). However, *A. seminis* is one of the most common isolates and produces clinical signs, lesions, and reproductive problems in rams that may be similar to those of *B. ovis.* Transmission of *A. seminis* is assumed to be venereal, through direct contact between mucous membranes of all natural orifices when crowding occurs during the premating period owing to increased homosexual activity. Ewes are considered to be possible intermediate carriers, promoting ewe-to-lamb transmission (6).

Identification and characterization of microorganisms has historically been based on morphologic, biochemical, serologic, toxigenic, and genetic characteristics. More recently, examination of CP patterns by means of PAGE has been used to distinguish taxonomically among strains within a species and among organisms within a genus or family. Currently the diagnostic technique used to identify *A. seminis* is bacteriologic and includes Gram's and modified Ziehl–Nielsen staining. The latter differentiates *A. seminis* and *B. ovis.* This technique is performed until the animal shows the characteristic signs of the infection. There is no specific biochemical test to distinguish *A. seminis* from the other bacterial species involved in epididymitis.

The OMPs of many gram-negative bacteria have been studied. Some have been identified as porins, Fe-receptors, invasins, and

factors in resistance to serum opsonization and killing in *Escherichia coli* and in the genera *Neisseria, Yersinia, Actinobacillus,* and others (22). Healey et al (10) added *n*-octyl-β-D-glucopyranoside to *A. seminis* isolates and used SDS-PAGE and spectrophotometric scanning of gels to analyze protein extracts from *A. seminis* (ATCC 15768) and *A. actinomycetemcomitans.* Bacterial isolates were grouped according to protein profile. Group 1 consisted of *A. seminis, A. actinomycetemcomitans,* and 7 field isolates of *Actinobacillus* spp., all of which had common protein bands of approximate molecular masses of 94, 64, 60, 52, 44, and 26 kDa. Group 2 had a unique protein profile, with relatively few protein bands in common.

To the best of our knowledge, this is the 1st report of data on the proteins in subcellular fractions of *A. seminis.* The polypeptide with a molecular mass of 75 kDa identified in the fractions and had an apparently specific immunogenic reaction: there was no cross-reaction of the hyperimmune serum from rabbits inoculated with whole cells of *A. seminis* against sonicated complete cells of *B. ovis, M. haemolytica, P. multocida,* or *H. somni.* Furthermore, when each type of hyperimmune serum of rabbits inoculated with whole cells of *B. ovis, M. haemolytica, P. multocida,* and *H. somni* was exposed to sonicated cells and each of the subcellular fractions of *A. seminis,* there was no recognition of the *A. seminis* 75-kDa protein by the other species.

Gram-negative bacteria form microvesicles, or blebs, as a result of outer membrane budding. These structures range in diameter from 20 to 500 nm and are released into the culture medium. They contain mainly outer membrane components and associated toxins and enzymes (19,21). Their release could therefore be a mechanism of tissue damage in epididymitis caused by *A. seminis.* They also provide an excellent source for the purification and characterization of biologically active molecules. However, it is still necessary to conduct tests with serum from animals naturally infected with microorganisms other than *A. seminis* that cause epididymitis. In addition, it is important to demonstrate a lack of cross-reaction with other members of the *Actinobacillus* group and to prove the specificity of the 75-kDa protein for *A. seminis.* Validation tests are necessary before the 75-kDa protein can be used in a diagnostic test for ovine epididymitis caused by *A. seminis.*

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