IDENTIFICATION OF POTENTIAL ADHESINS SHARED AMONG ISOLATES OF ACTINOBACILLUS SPECIES AND ACTINOBACILLUS-LIKE BACTERIA CULTURED FROM RAM LAMBS WITH CLINICAL EPIDIDYMİTIS

by

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ACKNOWLEDGEMENTS

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Yu-Wen Liu
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ABSTRACT

Identification of Potential Adhesins Shared Among Isolates of Actinobacillus Species and Actinobacillus-like Bacteria Cultured from Ram Lambs with Clinical Epididymitis

by

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Utah State University, 1991

Major Professor: Dr. Mark C. Healey
Department: Animal, Dairy, and Veterinary Sciences

Ram lamb epididymitis, a serious reproductive disease of sheep, is caused principally by bacteria belonging to the genera Haemophilus and Actinobacillus. Six bacteria were studied: the American Type Culture Collection (ATCC) of Actinobacillus seminis (ATCC 15768), ATCC of Actinobacillus actinomycetemcomitans (ATCC 29522), field isolates of A seminis (86722 and 4101) and field isolates of Actinobacillus-like bacteria (Y136 and D107). The objectives of this study were to quantitate the adhesion of these 6 bacteria to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs), evaluate the effect of rabbit polyclonal antibody prepared against ATCC 15768 (PoAb 15768) on bacterial adherence to BKECs and REECs, and partially characterize the adhesins present on these bacteria.
In a bacterial adhesion assay (BAA), strain and species differences were noted. The number of bacteria adhering to each BKEC ranged from a low of 4.27 ± 1.00 (Actinobacillus-like D107) to a high of 31.84 ± 2.00 (A. seminis 86722). The number of bacteria adhering to each REEC ranged from a low of 3.05 ± 0.34 (Actinobacillus-like D107) to a high of 21.61 ± 2.03 (Actinobacillus-like Y136). In a bacterial inhibition assay (BIA), PoAb 15768 inhibited the adhesion of ATCC 15768 to both BKECs and REECs by 5%. This same antiserum inhibited the adhesion of ATCC 29522 to BKECs by 14.5% and to REECs by 22%. The inhibition of A. seminis 86722 adherence to BKECs and to REECs was less than 14% and 35%, respectively. For A. seminis 4101, Actinobacillus-like Y136, and Actinobacillus-like D107, PoAb 15768 failed to prevent adhesion to either BKECs or REECs. When the 6 bacteria were analyzed by autoradiography, 2 (Actinobacillus-like D107) to 8 (ATCC 29522) potential adhesins were identified. However, the pathogenicity has not been firmly established for many Actinobacillus species and Actinobacillus-like bacteria. The potential adhesins identified in this study were not unequivocally confirmed as bacterial adhesins. An in vitro model may facilitate the recognition of potential adhesins used by Actinobacillus species and Actinobacillus-like bacteria and may eventually lead to the development of an efficacious bacterin to prevent epididymitis in ram lambs at risk.
INTRODUCTION

Ram epididymitis represents a reproductive disorder which is economically devastating to the sheep industry in Australia, New Zealand, Europe, South Africa and western America because the disease occurs frequently in these areas. Reduced fertility and sterility in clinically infected rams causes a serious problem in sheep production. Culling affected rams from the flock and annual vaccination of rams at risk are the primary methods for controlling the spread of the disease. Recent studies indicate that putting antibiotics in the feed may prevent epididymitis in the flock.

There are 2 recognized forms of ram epididymitis. In mature adult rams from range flocks, Brucella ovis is the pathogenic bacterium involved. However, in purebred flocks consisting of virgin ram lambs 6-15 months of age, epididymitis is most frequently caused by Actinobacillus seminis and Haemophilus somnus. These latter 2 bacteria are considered to be normal flora in these young lambs. While these bacteria can be cultured from 90 to 95% of all purebred ram lambs less than 1 year of age, they cause disease in only a small percentage, usually less than 20%. Both A seminis and H. somnus are gram-negative pleomorphic bacteria. Current information suggests that while A seminis is the most common pathogen involved, additional species and strains within this genus may also cause ram lamb epididymitis.

The gross and histologic lesions of epididymitis in mature range rams and in purebred ram lambs are very similar. Current evidence indicates that the pathogenic bacteria invade the ram
epididymides possibly via the urine, semen, blood or a testicular injury. When the pathogenic bacteria infect the epididymis, the epithelium of the tubules become infiltrated with lymphocytes and plasma cells and subsequently undergo hyperplasia. As the disease progresses, plasma cells and neutrophils can be found in the resultant exudate, while the capillary endothelium undergoes hyperplasia. Concurrent with the tubular changes is the production of intraepithelial cysts. Spermatostasis develops due to luminal stenosis and the destruction of tubules caused by interstitial fibrosis and alterations in the intratubular epithelium. In most cases, the lesion is in the tail rather than the head of the epididymis. Generally, lesions are unilateral, with both epididymides only rarely infected. In about 90% of the cases, the tail of the epididymis is enlarged. This enlargement may be extensive and involve the entire length of the epididymis. The amount of increase in size varies from essentially none to 4-5 times that of a normal configuration. In the early stages when fibrosis is minimal, the affected tail of the epididymis, though enlarged, is still soft tissue. In advanced stages, the involved area of the epididymis is hard, and the normal globular shape is distorted. Eventually, the epididymis becomes fibrotic. Most infected rams are unable to produce good quality semen. Moreover, these infected rams may become infertile and even sterile.

The adherence of bacteria to target host cells is known to be a prerequisite for the development of many diseases of man and animals. Many bacteria use adhesins located on the bacterial
outer membrane, to adhere to host cell receptors as a necessary step for colonization and subsequent infection of the host cells. The capability of bacteria to multiply and produce a stable bacterial population, whether they are invasive or not, is dependent upon bacterial adhesion. For most bacterial pathogens, adhesion is advantageous, both for the survival of the bacteria and the efficient delivery of injurious toxins to host tissues. If bacteria cannot maintain close proximity with host cells, they will eventually be swept away or destroyed by various host defenses.

The mechanism by which bacteria infect host tissue can be loosely divided into 3 stages. In the initial or association stage, there is a weak reversible interaction between the bacteria and the host cell. In addition to small numbers of noncovalent bonds which may form between the bacteria and host mucosal surface, chemotaxis is also a significant virulence mechanism used by bacterial pathogens. In the second or adhesion stage, there is a stable and irreversible interaction between the bacteria and host cells. Noncovalent bonds form between the bacterial adhesins and the host cell receptors. In the final or invasion stage, bacteria penetrate the host cell barrier and colonize inside the cell. This intracellular colonization causes many morphological changes to occur within the host cell. Although bacteria belonging to the genus Actinobacillus are believed to adhere to host cells, there is no evidence to suggest that these bacteria enter into epithelial cells of the ram lamb epididymides.
Bacterial adhesins are molecular structures that specifically adhere to host cell receptors.\textsuperscript{25,27,30} Recent studies have indicated that these adhesins may consist of either proteins, polysaccharides, lipoteichoic acids, or conjugates of these substances.\textsuperscript{24,25,27,30} For example, bacteria-secreted polysaccharides (glycocalyx), an important part of the bacterial extracellular matrix, mediate both firm adhesion and provide for colony formation.\textsuperscript{31,32} Lipoteichoic acid is a component of the cell wall of certain bacteria and may also contribute to adhesion. The majority of adhesins which have been characterized at the molecular and genetic level are surface proteins of gram-negative bacteria.\textsuperscript{30,33} Proteinaceous bacterial adhesins can be divided into those with fimbrial morphology and those lacking a definite size and shape.\textsuperscript{27,30} For instance, type 1 and 987p fimbriae of \textit{Escherichia coli} are elongated structures of 7 nm in diameter and have a molecular mass of 20 kilodaltons (kD).\textsuperscript{27,30} However, \textit{Bordetella pertussis} and \textit{Staphylococcus aureus} lack fimbrial adhesins, and must rely on a different type of adhesion to effect adherence to target host cells.\textsuperscript{34-36}

Theoretically, a bacterial infection may be prevented by utilizing an agent that blocks the specific interaction between the bacterial adhesin and the host cell receptor.\textsuperscript{36-38} Today, there are many successful examples of this methodology.\textsuperscript{28,29,36-41} However, at the present time, there is no bacterin commercially available which can be used to prevent ram lamb epididymitis. Although there is a commercially available bacterin for use against \textit{B. ovis} infections, bacterial pathogens which cause epididymitis in ram lambs remain a serious problem to the sheep industry. Twenty-
seven different isolates of *Actinobacillus* species and *Actinobacillus*-like bacteria cultured from the lesions or semen of ram lambs with clinical epididymitis, were taxonomically identified by Scanlan et al\textsuperscript{18} using cultural and biochemical techniques. However, the adhesins on these particular bacterial isolates have not been identified, isolated and purified.\textsuperscript{42,43} For this reason, an efficacious bacterin to prevent ram lamb epididymitis has not yet been developed.

Most of the traditional bacterins are whole, attenuated or killed bacterial cells. Recently, a few bacterins have been prepared from bacterial outer membrane extracts.\textsuperscript{44-49} Some of these extract-derived bacterins have been demonstrated to be more efficacious than the traditional whole-cell bacterins.\textsuperscript{48,49} If researchers could analyze the outer membrane antigens of the bacterial pathogens causing ram lamb epididymitis and identify the conserved adhesins used to adhere to host epithelial cells, an efficacious bacterin to prevent this disease would be a likely possibility.

The objectives of the present study were to (1) quantitate the adhesion of 6 bacterial isolates (ATCC 15768, ATCC 29522, *A. seminis* 86722, *A. seminis* 4101, *Actinobacillus*-like Y136 and *Actinobacillus*-like D107) to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs), (2) evaluate the effect of polyclonal antibody prepared against ATCC 15768 (PoAb 15768) on preventing the adherence of these 6 bacteria to BKECs and REECs, and (3) identify and partially characterize the protein profiles of
these 6 bacteria and the adhesins used by these 6 bacteria to adhere to BKEC and REEC receptors.
MATERIALS AND METHODS

Bacterial source and culture conditions—The *Actinobacillus* species and *Actinobacillus*-like bacteria used in this study were obtained from different sources.\(^a\)\(^b\) Six bacterial isolates of either *Actinobacillus* species or *Actinobacillus*-like bacteria were used (Table 1). Two species were obtained from the American Type Culture Collection (ATCC), which included *A. seminis* 15768 and *A. actinomycetemcomitans* 29522. *Actinobacillus seminis* 86722 and *A. seminis* 4101 were cultured from epididymal lesions of ram lambs with clinical epididymitis, while the *Actinobacillus*-like bacteria (Y136 and D107) were cultured from the semen of ram lambs with epididymitis. All of these bacterial isolates were passed to Columbia agar base\(^c\) plates which contained 5% fetal bovine serum (FBS)\(^d\) and 10 μCi/ml of tritiated sodium acetate (TSA)\(^e\). After 24 hours of incubation in a GasPak jar\(^e\) at 37°C under an atmosphere of 15% CO\(_2\) and 85% N\(_2\), the radiolabeled bacteria were harvested under sterile conditions. The bacteria were washed twice with a phosphate buffer saline solution (PBSS, pH 7.2) and collected by centrifugation at 1,450 x g for 15 minutes.

\(^a\) American Type Culture Collection, Rockville, MD.
\(^b\) Provided by Drs. Marie S. Bulgin, Veterinary Teaching and Research Center, Caldwell, ID; Leroy R. Maki, University of Wyoming, Laramie; and Clell V. Bagley, Utah State University, Logan, UT.
\(^c\) Becton Dickinson & Co., Cockeysville, MD.
\(^d\) Hyclone Laboratories, Logan, UT.
\(^e\) DuPont, Wilmington, DE.
Table 1—Source of 2 American Type Culture Collection (ATCC) and 4 field isolates of *Actinobacillus* species and *Actinobacillus*-like bacteria

<table>
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<th>Source</th>
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<tr>
<td>ATCC</td>
<td>15768*, 29522†</td>
</tr>
<tr>
<td>Lesions</td>
<td>86722‡, 4101‡</td>
</tr>
<tr>
<td>Semen</td>
<td>Y136§, D107§</td>
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* ATCC of *Actinobacillus seminis*.  
† ATCC of *A. actinomycetemcomitans*.  
‡ Field isolates of *A. seminis*.  
§ Field isolates of *Actinobacillus*-like bacteria.

Following the final washing, radioisotopic labeled bacteria were resuspended in PBSS until a final concentration of $1 \times 10^9$ bacteria/ml was reached. Bacterial concentration was determined by a spectrophotometer\(^f\) at an absorbance of 1 and read at 550 nm. Negative controls consisted of the same bacterial isolates grown on Columbia agar plates without TSA (nonlabeled bacteria). A stock suspension of nonlabeled bacteria was prepared as described above. All of the labeled and nonlabeled bacterial stock suspensions were freshly prepared before use and diluted 1:4 with 90% Eagle's minimum essential medium (MEM)\(^g\) and 10% FBS

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\(^f\) Baush & Lomb Inc, Rochester, NY.  
\(^g\) Gibco Laboratories, Grand Island, NY.
culture medium to give working suspensions of 2.5 x 10^8 bacteria/ml.

Epithelial cell source and culture conditions—Two cell lines (BKECs and REECs) were used as the source of epithelial cells for developing a bacterial adhesion model in this study. The BKECs were obtained from the American Type Culture Collection [ATCC: CCL 22, MDBK (NBL-1)]. The REECs were prepared by castrating young ram lambs 4 weeks of age and processing the epididymis as described by Hwang et al. The epithelial cell lines were grown separately in T-75 tissue culture flasks containing MEM. The culture medium was supplemented with L-glutamine (0.1 mM), sodium pyruvate (0.1 mM), 10% FBS and 90% MEM. The cells were maintained at 37°C in a humidified atmosphere of 6% CO₂. The culture medium of each flask was changed every 3 days and cells were subcultured every 5 days. After a confluent monolayer of epithelial cells had formed in a T-75 flask and was confirmed by phase-contrast microscopy, the cells were harvested by using 10 ml of a 0.05% trypsin-EDTA solution at 37°C on a model 25 shaking water bath at 50 oscillations/minute for 10 minutes.

The dissociated epithelial cells were planted into individual wells of 24-well plates. The 24-well plates were incubated overnight at 37°C in an atmosphere of 6% CO₂. Experiments using BKECs and REECs were performed after overnight incubation, when

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h Corning Glass Works, Corning, NY.
i Olympus Corporation, Lake Success, NY.
j Flow Laboratories, Inc, McLean, VA.
k Precision Instrument, GCA Co, Chicago, IL.
l CA Housser & Son, Philadelphia, PA.
a confluent monolayer of epithelial cells had formed on the bottom of each well. The culture medium in each well was aspirated and the monolayer of epithelial cells was washed twice with 0.5 ml of 100% MEM (pH 7.2). The plates were gently rocked back and forth a few times to insure adequate washing. Epithelial cells in at least 3 wells in each plate were detached by washing with 0.5 ml of a 0.05% trypsin-EDTA solution. The cells were then quantitated by counting in a hemacytometer and the number of cells per well estimated.

Production of antiserum—Preimmunization blood used to prepare normal rabbit serum (NRS) was drawn from a New Zealand white rabbit. The NRS was prepared by allowing the blood to clot at room temperature for 2 hours, followed by centrifugation at 1,300 x g for 15 minutes at 4-6 C. The rabbit was then immunized with an emulsion prepared by mixing 1 ml of 5 x 10^9 ATCC 15768 whole bacterial cells in PBSS and 1 ml of complete Freund’s adjuvant (CFA). One milliliter of this emulsion containing 2.5 x 10^9 bacteria was injected intramuscularly into each hind leg. The rabbit was given an intramuscular booster injection containing 2 x 10^9 ATCC 15768 in 0.5 ml of PBSS emulsified with 0.5 ml of incomplete Freund’s adjuvant (IFA) 1 month later. During the next 6 weeks, 3 identical booster injections were administered at 2 week intervals. Before every booster, 10 ml of blood was drawn from the central ear artery and the PoAb 15768 titer was confirmed, using the enzyme-linked immunosorbent assay (ELISA). One week after

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m RaR Rabbitry, Stawood, WO.
n Difco Laboratories, Detroit, MI.
the last booster injection, a 10 ml blood sample was collected. Three days later, the rabbit was exsanguinated via cardiac puncture. Approximately 100 ml of blood was collected and allowed to clot at room temperature for 2 hours. The resulting serum (rabbit anti-ATCC 15768 serum = PoAb 15768) was collected by centrifugation at 1,300 x g for 15 minutes at 4-6 C. The antiserum was aliquoted into 1 ml volumes, pipetted into 2 ml sterile cryogenic vials, and stored at -80 C.

**Enzyme-linked immunosorbent assay (ELISA)—Antisera containing PoAb 15768 was obtained via cardiac puncture and titered using the ELISA. Sixteen wells in a 96-well, flat-bottom plate were coated with 100 µl of whole cells from each of the 6 bacterial isolates (7.5 x 10^7 bacteria/ml) suspended in 0.06M carbonate buffer (NaHCO₃, pH 9.6). The plate was covered with parafilm and incubated overnight at 4 C. Following incubation, the unattached bacteria were removed by washing the plate several times with ELISA diluent (0.025M PBSS and 0.05% Tween 20, pH 6.8). A 100 µl volume of serially diluted antiserum (diluted from 1:6,400 to 1:819,200) were added to individual wells of each of the even numbered columns in the plate. Normal rabbit serum was used as a negative control and added to the wells of the odd numbered columns in the plate. The plate was incubated for 2 hours and then washed several times by immersion in distilled water. One hundred microliters of a 1:2,000 dilution of horseradish peroxidase (HRPO)-conjugated goat anti-rabbit IgG in ELISA

⁰ Bio-Rad Laboratories, Richmond, CA.
diluent was pipetted into each well. The plate was again covered and incubated for 2 hours at 37°C. Following incubation, the plate was washed several times in distilled water as before. Finally, 100 μl of 0-phenylenediamine (OPD, 0.4 mg/ml, in 0.01M pH 5.0 citrate buffer with 0.012% H₂O₂) substrate was added to each well. The enzymatic reaction was allowed to proceed for 20 minutes in the dark at room temperature, before being stopped by adding 50 μl of 1N HCl to each well. The optical density (OD) was measured for each well using a model EL309 microplate autoreader at 450 nm wavelength on mode 4.

**Bacterial adhesion assay (BAA)—**A model for demonstrating the adhesion of *A. seminis* to epithelial cells has been reported by Healey et al. 40 This model was employed in the present study to demonstrate the adhesion of the 6 bacterial isolates to BKECs and REECs. Briefly, a 0.5 ml volume of 1.25 x 10⁸ [³H]-labeled bacteria was added to each of 3 wells in 6 separate 24-well plates. Each plate contained a confluent monolayer of BKECs or REECs on the bottom of each well. The same volume of nonlabeled bacteria was added to 3 different wells in the same 6 plates. The plates were incubated at 37°C in an atmosphere of 6% CO₂ for 10, 20, 30, 40, 50, and 60 minutes. At the end of each incubation period, 1 plate was selected and the supernatant containing any unattached bacteria was aspirated by gently washing the individual wells 3 times with 0.5 ml 100% MEM at 37°C on a shaking water bath at 50 oscillations/minute for 5 minutes. After the wash, 0.5 ml of a

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p Sigma Chemical Co, St. Louis, MO.
q Bio-Tek Instrument Inc, Burlington, VI.
tissue solubilizing solution [2% sodium dodecyl sulfate (SDS)\textsuperscript{r} and 2N tetramethyl ammonium\textsuperscript{s} mixture (1:1)] was added to each well to lyze the adhering bacteria and epithelial cells. The plates were placed on a clinical rotator\textsuperscript{t} adjusted to minimum speed at room temperature (20-25 °C) for 2 hours. Two hundred microliters of lysate were then removed from each well and mixed with 5 ml of liquid scintillation cocktail\textsuperscript{u} in a linear copolymer plastic liquid scintillation vial.\textsuperscript{v} A Packard 1500 liquid scintillation counter (LSC)\textsuperscript{w} was used to count the radioactivity in disintegrations per minute (dpm) of the lysate. The dpm for each of the 3 vials was averaged for every 10 minute period for both the labeled and nonlabeled bacteria. The radioactivity of the lysate from the nonlabeled bacteria was used to adjust the LSC for background for every 10 minute period. Specific bacterial radioactivity (SBR) was determined by preparing control vials that contained 200 μl of a labeled or nonlabeled suspension of bacteria (3 vials each), 200 μl of tissue solubilizing solution and 5 ml of biodegradable counting cocktail. The SBR for individual bacteria was calculated by the formula:

\[
\text{dpm of 0.2 ml labeled bacteria} \over 0.2 \times [2.5 \times 10^8 \text{ bacteria/ml}]
\]

The number of bacteria adhering to each BKEC or REEC was calculated by the formula:

\textit{\textsuperscript{r} Parke-Davis, Morris Plains, NJ.}\textit{\textsuperscript{s} Aldrich Chemical Co, Inc, Milwaukee, WI.}\textit{\textsuperscript{t} Fisher Scientific Co, Pittsburgh, PA.}\textit{\textsuperscript{u} Beckman Instrument, Inc, Fullerton, CA.}\textit{\textsuperscript{v} Research Products International Corp., Mount Prospect, IL.}\textit{\textsuperscript{w} Packard Instrument Co, Downer Grove, IL.}
Bacterial inhibition assay (BIA)—Antiserum harvested from New Zealand white rabbit was used to test the effect of PoAb 15768 on bacterial adhesion to BKECs and REECs. Both antiserum and NRS were heat-inactivated at 56°C for 30 minutes and sterilized by passage through a 0.2 μm filter prior to use. The PoAb 15768 were diluted with culture medium containing 90% MEM and 10% FBS (1:50, 1:100, 1:200, 1:500, 1:1,000 dilution). The NRS was 1:100 with the same culture medium. All 6 bacterial isolates were incubated separately with each dilution of PoAb 15768 at 37°C for 30 minutes under 6% CO₂ and 100% relative humidity. Treatment and control groups of bacteria were incubated under the same conditions (Table 2). After a 30 minute incubation period, the treatment and control groups of bacteria were processed through the BAA as described previously. The only modification to the original BAA procedure was the time period allowed for bacterial adherence to the BKECs and REECs. This period was set at 1 hour.

Iodination and preparation of bacterial outer membrane proteins (OMPs)—The [¹²⁵I] labeling of the OMPs of the 6 bacteria used in this study was performed in accordance with the method described by Krause and Baseman,³⁷ and Baseman and Hayes.³⁸ Briefly, the bacteria were plated, harvested and washed as previously described. The concentration of each bacterial suspension was adjusted to 1 x 10¹⁰ bacteria/ml in PBSS.

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xBelman Sciences Inc, Ann Arbor, MI.
Table 2—Experimental design followed for the bacterial inhibition assay

<table>
<thead>
<tr>
<th>Bacterial group</th>
<th>[³H] bacteria</th>
<th>PoAb 15768†</th>
<th>NRS§</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2†</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3†</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Treatment group.
† Control group.
‡ Polyclonal antibody 15768.
§ Normal rabbit serum.

Iodination of bacterial OMPs was accomplished by treating 1 ml of a $1 \times 10^{10}$ suspension of bacteria with 0.5 μCi of carrier-free Na$^{125}$I,$^{\dagger}$ and mixing on a vortex for 1 minute. Twenty five microliters of 20mM chloramine-T in 50mM phosphate buffer (PO$_4$$_3^-$, pH 7.5) was added to the iodinated bacteria and incubated for 1 minute at room temperature. Another 25 μl of 20mM chloramine-T was added for an additional minute. This labeling reaction was terminated by the addition of 50 μl of 40mM sodium metabisulfite (Na$_2$S$_2$O$_5$) in 50mM phosphate buffer (pH 7.5). After 2 minutes, 0.3 ml of cold phosphate buffer iodine (PBI),§ supplemented with 2mM phenylmethyl-sulfonyl fluoride (PMSF),§ was added. The iodinated

$^{\dagger}$ Biomedical, Inc, Costa Mesa, CA.
bacteria were centrifuged at 8,000 x g for 5 minutes, then washed 3 time with PBI-PMSF and once with PBS-PMSF. The bacteria were then centrifuged, washed, and processed for gel electrophoresis.

In this study, 10% of the iodinated bacteria were solubilized in Tris-SDS (0.025M Tris and 2% SDS, pH 8.0) in a boiling water bath for 10 minutes. The remaining 90% of the labeled bacteria were extracted in 50mM Tris buffer containing 0.2% SDS and 2% sodium deoxycholate (NaDoC, pH 8.2) plus 2mM PMSF at 37 C for 1 hour. After incubation, the preparation was centrifuged and the supernatant was retained as the bacterial extract. The extract was used in the [125I]-BAA. The pellet was solubilized in Tris-SDS buffer in a boiling water bath for 10 minutes. All of the iodinated samples (whole cell, extract and pellet) used for gel electrophoresis were prepared by this procedure.

Fixation of epithelial cells—A 2.5 x 10^6 suspension of BKECs or REECs was planted into separate T-25 flasks containing 5 ml of 90% MEM and 10% FBS. A confluent monolayer of epithelial cells was allowed to grow overnight. Each monolayer of cells was fixed with 2.5% glutaraldehyde at 4 C for 1 hour. The glutaraldehyde fixation permitted BKECs and REECs to be incubated in a mixture of 0.2% SDS plus 2% NaDoC without noticeable surface damage and loss of cellular integrity. The fixed epithelial cells were then washed 3 times with PBSS and incubated with 5 ml of bovine serum albumin (BSA, 2 mg/ml) in PBSS at 37 C for 1 hour. After washing with PBI twice and PBSS once, the cells were used in the [125I]-bacterial OMPs adhesion assay.
125I-bacterial OMPs adhesion assay—One hundred and fifty microliters of bacterial extract, obtained from each of the 6 [125I]-labeled bacteria, were diluted with PBSS to 1 ml, then incubated separately with a confluent monolayer of REECs at 37°C for 1 hour. Following incubation, the epithelial cells were washed with PBI 4 times. The bound proteins and epithelial cells were then placed in 1 ml of Tris-SDS buffer in a boiling water bath for 5 minutes. The supernatant was analyzed by SDS-PAGE.2

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)—The protein profiles of the 6 bacterial isolates were compared by SDS-PAGE according to the procedure described by Healey et al.42,43 Briefly, 0.5 ml of each isolate was extracted in 9.5 ml of 2% n-octyl-β-D-glucopyranoside (n-octyl-glucoside)9 solution for 2 hours at room temperature. The n-octyl-glucoside is a nonionic detergent used for nondenaturing, solubilization and purification of cellular outer membrane antigens.51,52 The extracted proteins were pretreated in 0.5% 2-mercaptoethanol9 and 0.1% SDS in a boiling water bath for 5 minutes. The tracking dye used was 0.1% bromphenol blue solution.aa The extracted proteins were evaluated by a discontinuous gradient SDS-PAGE. The gel consisted of a 10 to 20% gradient running gel and a 5% stacking gel. Electrophoresis was carried out for 6 to 7 hours at 60 mA and 250 V in 0.025M Tris-0.192M glycine buffer containing 1% SDS. Following electrophoresis, the gel was stained with Coomassie brilliant blue R250.9

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2 Pharmacia Fine Chemicals, Piscataway, NJ.

aa Eastman-Kodak Co, Rochester, NY.
Autoradiography—The potential adhesins derived from the 6 bacterial isolates were analyzed by autoradiography.\textsuperscript{38,53-56} Iodinated samples of the bacterial isolates were prepared as described in the "Iodination and Preparation of Bacterial OMPs" and "[\textsuperscript{125}I]--Bacterial OMPs Adhesion Assay" sections of the materials and methods. The different iodinated bacterial samples were separated by SDS-PAGE. Following electrophoresis, the gradient gel was dried using a Gel slab drier GSD-4\textsuperscript{z} and the dried gel was processed through the autoradiography procedure.\textsuperscript{53-56} Briefly, the dried gel was first exposed to Kodak diagnostic film (8 x 10 inches, 20.3 x 25.4 cm)\textsuperscript{aa} at -80 C for 24-36 hours. After exposure, the screen and film were thawed at room temperature for 30 minutes. The film was then developed and fixed in a dark room. From [\textsuperscript{125}I]-autoradiography, the banding patterns of the bacterial OMPs and the potential bacterial adhesins were visualized.
RESULTS

BKECs and REECs—A monolayer of BKECs or REECs on the bottom of each well in 24-well plates were allowed to become confluent prior to counting in a hemocytometer. The average number of epithelial cells in each well was $1 \times 10^5$. This value was used to calculate the number of bacteria adhering to each BKEC or REEC (see formula in materials and methods).

ELISA—Table 3 shows the ELISA titers of PoAb 15768 against the 6 bacterial isolates employed in this study. Titers ranged from a low of 6,400 for *Actinobacillus*-like Y136, to a high of 102,400 for *A. seminis* 86722. A titer was not detectable when PoAb 15768 was incubated with *Actinobacillus*-like D107.

BAA—For any given *Actinobacillus* species or *Actinobacillus*-like bacteria, the amount of radioactivity recovered from each set of triplicate wells was consistent. However, the amount of $[^{3}H]$ acetate incorporated by the bacteria, as well as the actual number of bacteria added per well varied somewhat from experiment to experiment. Also variable was the number of counts per minute per well and CFU per well. The reproducibility of the assay was very good in that it was never difficult to distinguish adherent isolates from poorly adherent or nonadherent isolates. After incubation of $[^{3}H]$ labeled *Actinobacillus* species and *Actinobacillus*-like bacteria with BKECs or REECs, the bacteria which adhered to the surface of the epithelial cells were monitored by LSC. The results of the BAA are provided in Table 4. Bacterial were observed to
Table 3—Results of the enzyme-linked immunosorbent assay (ELISA) using polyclonal antibody (PoAb) 15768* against 2 American Type Culture Collection (ATCC) and 4 field isolates of *Actinobacillus* species and *Actinobacillus*-like bacteria

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>PoAb 15768 titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>15768†</td>
<td>51,200</td>
</tr>
<tr>
<td>29522‡</td>
<td>51,200</td>
</tr>
<tr>
<td>86722§</td>
<td>102,400</td>
</tr>
<tr>
<td>4101§</td>
<td>51,200</td>
</tr>
<tr>
<td>Y136I</td>
<td>6,400</td>
</tr>
<tr>
<td>D107l</td>
<td>-</td>
</tr>
</tbody>
</table>

* Serum sample used was obtained via cardiac puncture.
† ATCC of *A. seminis*.
‡ ATCC of *A. actinomycetemcomitans*.
§ Field isolates of *A. seminis*.
I Field isolates of *Actinobacillus*-like bacteria.
- = Negative.
Table 4—Results of the bacterial adhesion assay after 60 minutes, using *Actinobacillus* species and *Actinobacillus*-like bacteria incubated with bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Bacteria/BKEC*</th>
<th>Bacteria/REEC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>15768†</td>
<td>12.60 ± 1.08</td>
<td>11.12 ± 1.01</td>
</tr>
<tr>
<td>29522‡</td>
<td>10.01 ± 1.56</td>
<td>6.47 ± 1.30</td>
</tr>
<tr>
<td>86722§</td>
<td>31.84 ± 2.00</td>
<td>19.58 ± 0.96</td>
</tr>
<tr>
<td>4101§</td>
<td>16.61 ± 1.30</td>
<td>14.05 ± 1.81</td>
</tr>
<tr>
<td>Y136l</td>
<td>24.50 ± 0.99</td>
<td>21.16 ± 2.03</td>
</tr>
<tr>
<td>D107l</td>
<td>4.27 ± 1.00</td>
<td>3.05 ± 0.34</td>
</tr>
</tbody>
</table>

* Mean of triplicate experiments ± standard deviation.
† ATCC of *A. seminis*.
‡ ATCC of *A. actinomycetemcomitans*.
§ Field isolates of *A. seminis*.
l Field isolates of *Actinobacillus*-like bacteria.
adhere to the BKECs and REECs in a stepwise fashion during the course of the experiment (Figs 1-6). When $2.5 \times 10^8$ bacteria were incubated for 60 minutes with $1 \times 10^5$ epithelial cells, the mean number of bacteria adhering to a single BKEC ranged from a low of $4.27 \pm 1.00$ (Actinobacillus-like D107) to a high of $31.84 \pm 2.00$ (A seminis 86722). Bacteria adhering to a single REEC ranged from a low of $3.05 \pm 0.34$ (Actinobacillus-like D107) to a high of $21.16 \pm 2.03$ (Actinobacillus-like Y136). All experiments for each of the 6 bacterial isolates were repeated in triplicate.

BIA—The BIA demonstrated that preincubating the 6 bacterial isolates with different dilutions of PoAb 15768 (1:50 to 1:1,000) had variable results (Figs 7-9). The ATCC 15768 isolate was inhibited by a maximum of 5% from adhering to both the BKECs and REECs (Fig 7). The adhesion of ATCC 29522 was inhibited by a maximum of 14.5% and 22.0% to BKECs and REECs, respectively (Fig 8). The percent inhibition of A seminis 86722 to the BKECs was less than 14% (Fig 9). The percent inhibition of A seminis 86722 adhering to REECs reached 35% when this isolate was incubated in PoAb 15768 (Fig 9). No inhibition in the adherence of A seminis 4101 and Actinobacillus-like Y136 and D107 to either BKECs and REECs was noted when these 3 bacterial isolates were first incubated with PoAb 15768 (data not shown).
Figure 1—Adhesion of American Type Culture Collection (ATCC) 15768 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacterial adhesion assay (BAA), 12.60 ± 1.08 ATCC 15768 were estimated to adhere to each BKEC and 11.12 ± 1.01 to each REEC after 60 minutes. The values listed for each 10 minute period represent the mean ± the standard deviation (determined in triplicate) of the number of bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
Figure 2—Adhesion of American Type Culture Collection (ATCC) 29522 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacterial adhesion assay (BAA), 10.01 ± 1.56 ATCC 29522 were estimated to adhere to each BKEC and 6.74 ± 1.30 to each REEC after 60 minutes. The values listed for each 10 minute period represent the mean ± the standard deviation (determined in triplicate) of the number of bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
Figure 3—Adhesion of *Actinobacillus seminis* 86722 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacterial adhesion assay (BAA), 31.84 ± 2.00 *A. seminis* 86722 were estimated to adhere to each BKEC and 19.58 ± 0.96 to each REEC after 60 minutes. The values listed for each 10 minute period represent the mean ± the standard deviation (determined in triplicate) of the number of bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
Figure 4—Adhesion of *Actinobacillus seminis* 4101 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacterial adhesion assay (BAA), $16.61 \pm 1.30$ *A. seminis* 4101 were estimated to adhere to each BKEC and $14.05 \pm 1.81$ to each REEC after 60 minutes. The values listed for each 10 minute period represent the mean ± the standard deviation (determined in triplicate) of the number of bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
Figure 5—Adhesion of *Actinobacillus*-like Y136 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacterial adhesion assay (BAA), $24.05 \pm 0.99$ *Actinobacillus*-like Y136 were estimated to adhere to each BKEC and $21.16 \pm 2.03$ to each REEC after 60 minutes. The values listed for each 10 minute period represent the mean ± the standard deviation (determined in triplicate) of the number of bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
Figure 6—Adhesion of *Actinobacillus*-like D107 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacterial adhesion assay (BAA), $4.27 \pm 1.00$ *Actinobacillus*-like D107 were estimated to adhere to each BKEC and $3.05 \pm 0.34$ to each REEC after 60 minutes. The values listed for each 10 minute period represent the mean ± the standard deviation (determined in triplicate) of the number of bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
Figure 7—Inhibition of American Type Culture Collection (ATCC) 15768 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacteria inhibition assay (BIA), radioisotopic labeled ATCC 15768 were first incubated with different dilutions of polyclonal antibody (PoAb) (1:50 to 1:1,000). The adherence of ATCC 15768 to both BKECs and REECs was inhibited by a maximum of 5%. The values listed for the different dilution of PoAb 15768 represent the mean ± the standard deviation (determined in triplicate) of the percent inhibition.
Figure 8—Inhibition of American Type Culture Collection (ATCC) 29522 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacteria inhibition assay (BIA), radioisotopic labeled ATCC 29522 were first incubated with different dilutions of polyclonal antibody (PoAb) (1:50 to 1:1,000). The adherence of ATCC 29522 to BKECs and REECs was inhibited by a maximum of 14.5% and 22.0%, respectively. The values listed for the different dilution of PoAb 15768 represent the mean ± the standard deviation (determined in triplicate) of the percent inhibition.
Figure 9—Inhibition of *Actinobacillus seminis* 86722 to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs). In a bacteria inhibition assay (BIA), radioisotopic labeled *A. seminis* 86722 were first incubated with different dilutions of polyclonal antibody (PoAb) (1:50 to 1:1,000). The adherence of *A. seminis* 86722 to BKECs and REECs was inhibited by a maximum of 14% and 35%, respectively. The values listed for the different dilution of PoAb 15768 represent the mean ± the standard deviation (determined in triplicate) of the percent inhibition.
SDS-PAGE and autoradiography—The profiles of the bacterial OMPs as discerned by SDS-PAGE was done for comparative purposes. After separation by discontinuous gradient SDS-PAGE and staining with Coomassie brilliant blue R250, the n-octyl-glucoside extracts from the 6 bacterial isolates consisted of more than 25 protein containing fractions for each isolate (Fig 10). Although many of the major bands present in the outer membrane extracts of ATCC 15768 and 29522 as well as A seminis 86722 and 4101 were shared, there were some differences among these 4 bacteria. The protein profiles were similar for the outer membrane extracts and the whole cell preparations for each of the 6 bacterial isolates (Fig 11).

Radioiodination combined with SDS-PAGE and autoradiography were used to demonstrate the potential bacterial adhesins of the 6 isolates. The bands observed by autoradiography were not confirmed as bacterial adhesins, so we consider them to be only "potential adhesins". After an exposure time of 24-36 hours, the diagnostic film of the gel of potential protein adhesins was developed (Fig 12). The ATCC 15768 isolate showed 5 potential adhesins (14.4 kD, 21 kD, 29 kD, 36 kD, 40 kD), while ATCC 29522 had 8 potential adhesins visible on the film. Two of the bands were very faint (84 kD, 155 kD), and may or may not represent potential adhesins. But the remaining 6 bands were quite obvious (14.4 kD, 21 kD, 29 kD, 33 kD, 36 kD, 52 kD). These latter 6 potential adhesins were shared with A seminis 86722 and 4 potential adhesins were shared with A seminis 15768 (14.4 kD, 21 kD, 29 kD, 36 kD). However, the band in A seminis 86722 with a
molecular mass of 52 kD was very faint, and may not represent a potential adhesin. In *A. seminis* 4101, potential adhesins 14.4 kD, 21 kD, 29 kD and 36 kD were present. However, in *Actinobacillus*-like Y136, only 2 potential adhesins (14.4 kD, 24 kD) were noted. *Actinobacillus*-like D107 had 5 potential adhesins (14.4 kD, 21 kD, 26 kD, 30 kD, 32 kD). Two of the bands (26 kD, 30 kD) may or may not be potential adhesins. Only 1 potential adhesin (14.4 kD) was common to the 6 bacterial isolates. Four potential adhesins (14.4 kD, 21 kD, 29 kD, 36 kD) were shared among the 2 ATCC and 2 *A. seminis* isolates.
Figure 10—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of \(n\)-octyl-\(\beta\)-D-glucopyranoside extracts of 2 American Type Culture Collection (ATCC) and 4 field isolates of *Actinobacillus* species and *Actinobacillus*-like bacteria. Lanes A and B are ATCC 15768 and ATCC 29522, respectively. Lanes C through F are field isolates of *A. seminis* 86722, *A. seminis* 4101, *Actinobacillus*-like Y136 and *Actinobacillus*-like D107, respectively. The molecular masses in kilodaltons (K) is indicated to the left.
Figure 11—Autoradiograph of whole cell preparations and n-octyl-β-D-glucopyranoside extracts from 2 American Type Culture Collection (ATCC) and 4 field isolates of Actinobacillus species and Actinobacillus-like bacteria. Lanes A and B are ATCC 15768 whole cell and extract, respectively. Lanes C and D are ATCC 29522 whole cell and extract, respectively. Lanes E and F are A seminis 86722 whole cell and extract, respectively. Lanes G and H are A seminis 4101 whole cell and extract, respectively. Lanes I and J are Actinobacillus-like Y136 whole cell and extract, respectively. Lanes K and L are Actinobacillus-like D107 whole cell and extract, respectively. The molecular mass in kilodaltons (K) is indicated to the left.
Figure 12—Autoradiograph of the potential bacterial adhesins of 2 American Type Culture Collection (ATCC) and 4 field isolates of *Actinobacillus* species and *Actinobacillus*-like bacteria. This experiment was done by incubating the 6 bacterial extracts separately with a glutaraldehyde fixed monolayer of epithelial cells. The potential bacterial adhesins were then analyzed by SDS-PAGE and autoradiography. Lanes A and B are the potential adhesins of ATCC 15768 and ATCC 29522, respectively. Lanes C through F are potential adhesins of field isolates *A. seminis* 86722, *A. seminis* 4101, *Actinobacillus*-like Y136 and *Actinobacillus*-like D107, respectively. Faint bands may or may not represent potential adhesins (arrows). The molecular mass in kilodaltons (K) is indicated to the left.
DISCUSSION

Ram lamb epididymitis is a reproductive disorder of purebred sheep 6 to 15 months old.\textsuperscript{12} Recent studies indicate that 1 of the principal pathogens of this disease is the gram-negative, pleomorphic bacterium \textit{A seminis}.\textsuperscript{3,12,14,16-18} This bacterium can be cultured from the preputial cavity of essentially all ram lambs in a flock.\textsuperscript{13} Lamb epididymitis may cause infected ram lambs to experience reduced breeding potential as well as sterility.\textsuperscript{1,23} It is unclear as to why this disease only rarely reaches 20\%.\textsuperscript{10} One possible explanation is that only certain strains of \textit{A seminis} are pathogenic.\textsuperscript{43} Another possibility is that the genetic and/or immune status of infected ram lambs permits the adhesion of pathogenic bacteria to target epididymal epithelial cells. The specific relationship between bacterial pathogenicity and adherent capacity is still unknown, although adhesion to host cells is considered to be a prerequisite for the pathogenesis of several human and animal diseases.\textsuperscript{24-28} Information obtained from the BAA suggests that strain and species differences may influence the capability of select \textit{Actinobacillus} species and \textit{Actinobacillus}-like bacteria to adhere to the epithelial cells \textit{in vitro}. The 6 bacterial isolates used in this study showed differing capacities to adhere to epithelial cells (Fig 13). For example, \textit{A seminis} 86722 and \textit{Actinobacillus}-like Y136 adhered to BKECs and REECs in the largest numbers, while ATCC 29522 and \textit{Actinobacillus}-like D107 adhered in the fewest numbers. It is possible that because different bacterial isolates have different surface adhesins, some isolates
Figure 13—Combined results of the bacterial adhesion assay (BAA). Strain and species differences influenced the adhesion of *Actinobacillus* species and *Actinobacillus*-like bacteria to bovine kidney epithelial cells (BKECs) and ram epididymal epithelial cells (REECs) in vitro. In the BAA, *Actinobacillus seminis* 86722 and *Actinobacillus*-like Y136 adhered to BKECs and REECs in the largest numbers, while American Type Culture Collection 29522 and *Actinobacillus*-like D107 adhered in the fewest numbers. The values listed for each isolate represent the the highest number (determined in triplicate) of the bacteria adhering to each BKEC or REEC, using the formula provided in the materials and methods.
readily adhere to REECs and BKECs while others do not. Another possibility is that continued passage of the bacteria in vitro may result in a decrease or loss of surface adhesins.\textsuperscript{24} The 6 bacterial isolates used in this study were passaged an indeterminant number of times. When comparing the adherence among the 6 bacterial isolates, ATCC 29522 and \textit{A. seminis} 4101 not only showed a lower adhering number, but also showed a higher standard deviation. This may suggest that these two isolates lost some of their surface adhesins following continued passage in vitro.

In many bacterial infections, successful colonization and infectivity rely on the adhesion of bacteria to host epithelial cells.\textsuperscript{24-28} This process is believed to require the relatively stable, irreversible attachment of bacterial surface adhesins to epithelial cell receptors.\textsuperscript{24,27} It is possible that \textit{A. seminis} may initially adhere to ram lamb epididymal epithelial cells by specific surface adhesins in association with host cell receptors. However, because of the difficulty of performing \textit{in vivo} experiments to confirm \textit{in vitro} results, very little is known about the ability of \textit{A. seminis} to adhere to epididymal epithelial cells in sheep or the mechanisms by which they may do so.

A bacterial \textit{in vitro} adhesion model has been developed by using BKECs as the source of target epithelial cells.\textsuperscript{40} Both BKECs and REECs were used in the present study. The BKECs were used for comparative purposes because they afforded an established, continuous cell line which was significantly easier to maintain than REECs. However, the REECs were used in the \textit{in vitro} model because
they are probably more closely allied to the pathogenesis of lamb epididymitis as it occurs in vivo.

It has been reported that bacterial adhesion to host cells is a specific interaction between bacterial surface adhesins and molecules of the host cell membrane in a ligand-receptor fashion. Moreover, earlier reports suggest that the interaction may be blocked by interrupting the adhesion process. Unfortunately, preincubating the 6 bacterial isolates used in this study with different dilutions of PoAb 15768 did not completely inhibit bacterial adhesion to BKECs or REECs for any of these isolates. However, PoAb 15768 decreased the adherence of ATCC 15768, ATCC 29522 and A seminis 86722 by less than 35% (Figs 7-9), whereas A seminis 4101, Actinobacillus-like Y136 and Actinobacillus-like D107 remained essentially unaffected. One possible reason that the PoAb 15768 failed to block the adhesion of Actinobacillus-like Y136 and Actinobacillus-like D107 is that these 2 bacteria are not truly Actinobacillus species. These bacteria are only classified as Actinobacillus-like bacteria based on their cultural and biochemical characteristics. Moreover, SDS-PAGE and autoradiography revealed that the protein profiles of A seminis 4101, Actinobacillus-like Y136 and Actinobacillus-like D107 are different from ATCC 15768, ATCC 29522 and A seminis 86722 (Figs 10 and 11). Although A seminis 4101 is classified as an A seminis, its protein profile is noticeably different from ATCC 15768, ATCC 29522 and A seminis 86722 (Fig 10). As a result, it is very likely that this dissimilar profile may result in the presence or absence of different surface adhesins. Therefore, PoAb 15768 may not be
capable of blocking the adhesins present on *A seminis* 4101. Additionally, some of the bacterial adhesins may be only weakly antigenic, such that high affinity antibodies are not produced against them.

Recent studies have described adhesins which are composed of proteins, polysaccharides, lipoteichoic acids or conjugates of these. Lipopolysaccharides of gram-negative bacteria are necessary to stabilize the protective outer membrane and are essential for the biological activity of several OMPs. Lipoteichoic acid is not only a component of some bacterial cell walls, but also a functional element in bacterial adhesion. Some OMPs clearly have a structural role, while others are involved with the transport of low molecular weight solutes into the periplasm. Other OMPs determine bacterial virulence. The present study focused on bacterial OMPs. The methods of SDS-PAGE and autoradiography were used to analyze and evaluate OMPs as potential bacterial adhesins. According to the autoradiography results, the 6 bacterial isolates contained from 2 to 8 potential protein adhesins (Table 5). Only 1 band (14.4 kD) was shared between all 6 bacterial isolates. Four bands (14.4 kD, 21 kD, 29 kD, 36 kD) were shared between ATCC 15768, ATCC 29522, *A seminis* 86722 and *A seminis* 4101. Except for *Actinobacillus*-like Y136, 1 protein band with a molecular mass of 21 kD was common to the other 5 bacterial isolates.

The results obtained by the ELISA, BAA, BIA and autoradiography from the 6 isolates of *Actinobacillus* species and *Actinobacillus*-like bacteria are given in Table 5. Positive ELISA
Table 5—Combined results from the enzyme-linked immunosorbent assay (ELISA)*, bacterial adhesion assay (BAA), bacterial inhibition assay (BIA), and autoradiography

<table>
<thead>
<tr>
<th>Isolates</th>
<th>ELISA</th>
<th>BAA</th>
<th>BIA</th>
<th>Autoradiography</th>
</tr>
</thead>
<tbody>
<tr>
<td>15768†</td>
<td>++++</td>
<td>+</td>
<td>+/ -</td>
<td>5 (14.4, 21, 29, 36, 40)</td>
</tr>
<tr>
<td>29522‡</td>
<td>++++</td>
<td>++</td>
<td>+</td>
<td>8 (14.4, 21, 29, 33, 36, 52, 84§, 155¶)</td>
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<tr>
<td>86722§</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>6 (14.4, 21, 29, 33, 36, 52¶)</td>
</tr>
<tr>
<td>4101§</td>
<td>++++</td>
<td>++</td>
<td>-</td>
<td>4 (14.4, 21, 29, 36)</td>
</tr>
<tr>
<td>Y136¶</td>
<td>+/ -</td>
<td>+++</td>
<td>-</td>
<td>2 (14.4, 24)</td>
</tr>
<tr>
<td>D107¶</td>
<td>-</td>
<td>+/ -</td>
<td>-</td>
<td>5 (14.4, 21, 26¶, 30¶, 32)</td>
</tr>
</tbody>
</table>

* Serum sample used was obtained via cardiac puncture.
† ATCC of A seminis.
‡ ATCC of A actinomycetemcomitans.
§ Field isolates of A seminis.
¶ Field isolates of Actinobacillus-like bacteria.
§ Questionable banding pattern.
+++ , +++ , ++ , + , +/ - = positive reactions ranging from strong to weak, - = negative.
and BAA results were observed for ATCC 15768, ATCC 29522, A seminis 86722 and A seminis 4101. Only ATCC 29522 and A seminis 86722 showed specific inhibition in the BIA. One possible explanation is that the specificity of PoAb 15768 was for adhesins shared between ATCC 29522 and A seminis 86722 (ie: potential adhesins with a molecular mass of 33 kD or 52 kD), but not for proteins shared between these 4 Actinobacillus isolates (ie: 14.4 kD, 21 kD, 29 kD, 36 kD). This may also be the reason why PoAb 15768 could not inhibit the adhesion of A seminis 4101. The other possibility for the weak reaction to ATCC 15768 in the BIA is that PoAb 15768 could not block the adherence of potential adhesins having a molecular mass of 40 kD because of very weak adhesin antigenicity. It is also possible that this inhibition assay was not sufficiently sensitive to detect the adhesion of ATCC 15768 to BKECs and REECs. In contrast to ATCC 15768, A seminis 86722 seemed to have not only very strong reactions in the ELISA and the BAA, but also a high inhibition in the BIA. This result suggests that some of the 6 potential adhesins, identified by autoradiography, have strong antigenicity and that PoAb 15768 was directed against them. However, PoAb 15768 failed to recognize Actinobacillus-like Y136 in the ELISA and did not inhibit this bacterium in the BIA. Actinobacillus-like Y136 did show a positive result in the BAA. Autoradiography revealed that Actinobacillus-like Y136 contained only 2 potential adhesins. In accordance with the results reported here, perhaps PoAb 15768 only blocks the common adhesins shared between these 6 bacterial isolates (14.4 kD), not including the specific adhesins (24 kD) in the outer membrane of
Actinobacillus-like Y136. Moreover, the bacteria may be using some other specific adhesins to adhere to epithelial cells, but not the protein adhesins. These specific adhesins have either a weak antigenicity or did not appear on the ATCC 15768 surface. Actinobacillus-like D107 was negative in all 3 assay used, a finding which was not unexpected in view of the very different nature of this bacterium when compared to the others.

Many bacterial OMPs are surface expressed and almost all appear to be antigenic. Some OMPs of certain gram-negative bacteria isolated from infected animals and people have vaccinogenic potential. It has been reported that some bacterins prepared from outer membrane extracts of pathogenic bacteria had greater efficacy than bacterins prepared from whole bacterial cells. This suggests that there is merit in purifying bacterial surface antigens for use in some bacterins. The potential adhesins identified in this study may prove efficacious when incorporated into a bacterin for use against ram lamb epididymitis.

In conclusion, strain and species differences were noted in the adherence of ATCC 15768, ATCC 29522, A seminis 86722, A seminis 4101, Actinobacillus-like Y136 and Actinobacillus-like D107 to BKEC's and REEC's. In addition, various potential protein adhesins were identified. However, the pathogenicity has not been firmly established for many Actinobacillus species and Actinobacillus-like bacteria tested. The potential protein adhesins identified here were not unequivocally confirmed as bacterial adhesins. We speculate that the use of such an in vitro model may facilitate studies on the recognition of potential adhesins used by Actinobacillus species and
Actinobacillus-like bacteria and may eventually lead to the development of an efficacious bacterin to prevent epididymitis in ram lambs at risk.
REFERENCES


