Evaluation of three serological techniques for differentiating strains of *Moraxella bovis* by pilus antigens

by

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GENERAL INTRODUCTION

Moraxella bovis has been identified as the primary pathogen associated with infectious bovine keratoconjunctivitis (IBK) or "pinkeye" in cattle.[1,2,3] In animals with active infections, the clinical signs include blepharospasm, conjunctivitis, photophobia, and ulceration.[4] As IBK progresses, corneal ulcers and conjunctivitis occur. Perforation of the cornea can occur. As a sequelae to healing, partial or total corneal opacity can occur. The degree of opacity is dependent upon the amount of corneal involvement during the active phase of the disease.

The disease results in significant economic loss to farmers and ranchers.[5] IBK causes appreciable pain and distress to infected animals, results in loss of weight during the disease, and can leave the animal with permanent blindness. Decreased foraging ability, reduced usefulness of vision impaired animals, and the cost of treatment have a major economic impact on the livestock producer.

Moraxella bovis is a gram negative bacteria that is transmitted between animals by flies[6,7] and by contact.[8] The virulence factors of *M. bovis* are believed to be ß-hemolysin,[9,10,11] protease,[12] and pilus.[13,14] The major antigens eliciting protective antibody against reinfection with *M. bovis* have been proposed as being ß-hemolysin[15] and pilus.[16] The protective antigens identified in *M. bovis* bacterins by vaccination and challenge studies have been pilus[17,18,19] and "cornea-degrading enzymes".[20] Of the two protective antigens, more evidence for pilus as eliciting immunologic protection has been presented. In vaccination and challenge studies, bacterins containing pilus invoked protection against

experimental challenge with homologous *M. bovis* strains.[21]

The objective of this study was to evaluate 3 test methods to identify an appropriate system for categorizing pilus strains into serogroups. Anti-pilus rabbit sera were produced against three strains of piliated *M. bovis* using pilus enriched antigen. Anti-endotoxin antibody was removed from the sera by affinity chromatography. Enzyme linked immunosorbent assay (ELISA), nondenaturing discontinuous (native) polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic immunoblot (Western blot), and dissociative discontinuous sodium dodecyl sulfate (SDS) - PAGE/Western blot were used to evaluate the sera against crude preparations of homologous and heterologous *M. bovis*. Each method was evaluated and compared for the specificity and the relative significance of the information obtained.

EXPLANATION OF THESIS FORMAT

This thesis consists of an introduction, a literature review, a separate manuscript, a general summary, references, and acknowledgments. The master's candidate, Linn A. Wilbur, is the senior author and principal investigator for the manuscript.

LITERATURE REVIEW

Infectious Bovine Keratoconjunctivitis

Infectious bovine keratoconjunctivitis is a disease of cattle which occurs primarily during the 3 to 4 warmest months of the year.[22,23] Peak period of IBK lesions follows the period of highest ultraviolet (UV) light exposure[23] and maximum fly populations.[24] Incidence of disease can be as high as 45% of all cattle in a herd during an IBK epizootic,[25] but more typically the disease is endemic and remains at a lower level. One survey placed the endemic level of cattle with IBK at 11.2%.[20]

The primary infectious agent causing IBK has been identified as *M. bovis*. *Moraxella bovis* has been shown to cause IBK in gnotobiotic calves.[1,3] In experimental challenges the infection rate for gnotobiotic calves is lower than conventionally reared animals.[3] Although *M. bovis* is the primary agent in IBK there are additional factors that act in synergism with *M. bovis* to initiate and potentiate the disease. Infectious bovine rhinotracheitis virus[25] and *Mycoplasma bovoculi*[26] are infectious agents that have been identified as contributing to IBK. Other common ocular mycoplasmas do not appear to be significant in this respect.[26,27] There are reports that adenoviruses,[28] *Listeria*,[29] *Neisseria*,[30] and *Thelazia*[31] may be isolated from the eye during clinical IBK and may contribute to IBK lesions. Ultraviolet light,[23,32] rag weed pollen,[33] and face flies (*Musca autumnalis*)[6,7,24,34] have been associated as IBK predisposing, potentiating, and transmitting factors, respectively. None of these have been shown to produce IBK lesions unless *M. bovis* is present. Infectious bovine rhinotracheitis virus,[35] *Mycoplasma bovoculi*,[26]

and UV light[11,36,37] have been used in experimental challenge models, prior to *M. bovis* challenge, to enhance IBK lesions.

Moraxella bovis

Moraxella bovis is a gram negative diplobacillus, of the family *Neisseriaceae*. A gram negative diplobacillus, *Morax-Axenfeld bacillus*, was first described in 1915 by S. N. Mitter from a case of keratitis in cattle in Bengal, India. In 1919 J. A. Allen described a Canadian isolate from cattle with keratitis and showed it to be *Morax-Axenfeld bacillus*. In 1923 F. S. Jones and R. B. Little isolated a diplobacillus from an active keratitis outbreak.[38] Jones and Little's organism was classified in 1937 by Hauduroy et al.[39] as *Haemophilus bovis*.[40] In 1939 Lwoff proposed renaming *Haemophilus bovis* as *Moraxella bovis*. *Moraxella bovis* was included in the family of *Neisseriaceae* in 1968.[40] Two additional species have been described, *Moraxella equi*[41] and *Moraxella caprae*,[42] but both now are considered to be strains of *M. bovis*.

Identification of *M. bovis* is done by a negative nitrate reduction test, no fermentation of carbohydrates, a positive oxidase test, and characteristic 3 zone proteolysis of litmus milk.[38,43] A negative phenylalanine deaminase test and no growth on MacConkey agar[40] are also useful for identification. Catalase activity and ß-hemolysis is variable.

Virulence Mechanisms of *Moraxella bovis*

The primary virulence factors of *M. bovis* have been shown to be ß-hemolysin,[9,10,11] protease,[12] and pilus.[13,14] Virulent *M. bovis* organisms

adhere to and invade the cells of the corneal epithelium. A preference for the "dark", older corneal epithelial cells is noted.[1,14] Erosion of the corneal epithelial surface occurs and is attributable to a "pitting factor" which may be a protease. No direct correlation has been made between the "pitting factor" and the presence of pilus, although pitting on agar plates is characteristic of piliated colonies and is lacking in non-piliated colonies. It is believed that pili are the mechanism that *M. bovis* uses to adhere to the epithelial cells.[14] Only ß-hemolytic[11,44] and piliated[2] strains have been shown to be virulent in experimental infections.

Protection against Infectious Bovine Keratoconjunctivitis

The prevention of IBK is of major interest to cattle producers due to the economic impact of the disease. Various attempts have been made to produce a protective vaccine. Initial studies, under experimental conditions, suggested that live cultures or formalized vaccines of *M. bovis* would be protective.[45,46] Subsequently, it was shown that these vaccines failed to protect under natural conditions.[47]

Antibody against hemolysin is elicited during natural infection and can be measured.[15] Antibody to hemolysin cross-reacts to all strains of *M. bovis*. No conclusive evidence exists to show that anti-hemolysin activity is protective against subsequent infection. No method to stabilize the molecule has been discovered,[9,48] and whether vaccination with hemolysin would be protective is unknown.

One report, using a bacterin containing attachment antigen and a protease or "cornea-degrading enzyme," showed protection against homologous and heterologous experimental challenge.[20] In a field efficacy trial of 32 herds, the rate of infection was reduced from 11.2% to 4.3% with 1 vaccination and to 3.1% with 2 vaccinations.[20] Effectiveness of the bacterin was related more to the "cornea-degrading enzyme" than to the attachment antigen.

Vaccination with piliated whole cell (killed) or purified pilus as vaccines has proven relatively effective in protecting against IBK in homologous challenge systems,[16,17,18,21,49] but has failed to protect against heterologous challenges. Antibody against pilus has been shown to prevent adherence *in vitro* of homologous piliated *M. bovis* to monolayers of corneal epithelial cells.[50]

Pilus of *Moraxella bovis*

Examination of *M. bovis* by transmission electron microscopy has shown the presence of fimbriae or pili on the surface of the organism.[51] Pilus appear to have a polar orientation.[52,53] The presence of pilus has been associated with twitching motility[54,55,56] and competence for DNA transformation.[57] Colonies on blood agar that are agar corroding and friable have been shown to have pili when examined by electron microscopy (EM). Colonies that are mucoid and non-agar corroding fail to shown pilus expression by EM.

Various schemes to describe colonies associated with pilus production are reported in the literature. The association of "rough" or

"smooth" colony type has been used by different authors to describe the same colony type, leading to confusion when using this terminology. The use of spreading-corroding (SC type) and non-corroding (N type) nomenclature has also been proposed.[2] The SC colony type has been associated with pilus producing organisms and the N type colony with non-piliated organisms. The latter nomenclature (i.e., SC versus N type) is more consistent with what is observed on colony morphology. Others have used P^+ and P^- to denote piliated and non-piliated strains.[52,58,59]

Colonies that are composed of organisms expressing pili are characteristically smooth, convex, "egg shaped," and friable. These colonies corrode or pit the agar beneath the area of colony growth. These colonies do not disperse in saline and are described as being autoagglutinating. Autoagglutination can be inhibited by suspending the organism in a 10% magnesium chloride solution.[60] Colonies of this type retain crystal violet stain when the colonies are stained with a dilute solution.[61]

Non-piliated colonies appear as non-uniform, non-glistening, flat surfaced colonies and tend to be larger than the piliated colonies. No agar corrosion can be detected beneath the colony. The cells uniformly disperse in saline and the colonies do not retain the crystal violet stain.

Transformation from N to SC colony type has been reported to occur at a frequency between zero and 1 in 10^4 when grown on agar.[51] Transformation from piliated to non-piliated cells occurs, especially with extended time between passages. The process involved in conversion

from SC to N colony type is unknown. No pattern of genomic deletion for N versus SC type colonies has been observed.[59]

The pili of *M. bovis* are 65-85 Å in diameter[51] and may be up to 3 μm in length.[52] Each pilus strand is a polymeric unit composed of repeating monomeric or pilin units. By dissociation under SDS-PAGE conditions the pilin has been shown to vary in apparent molecular mass. Lepper and Power[62] reported three pilin apparent molecular masses, 16,500 dalton, 17,400 dalton, and 18,000 dalton. These were respectively designated alpha (α), beta (ß), and gamma. Ruehl et al.[52] reported two pilin apparent molecular masses, 17,000 and 20,000 dalton, which were designated ß and α, respectively. Ruehl et al. showed that a *M. bovis* strain possessing pilus composed of ß pilin subunits is more infective than the same strain with pilus made of α pilin.[52] Each strain produces primarily one pilin type, but evidence of more than one pilin type appearing on a lane of SDS-PAGE has been presented.[52,62] Phase variation between α and ß pilin types has been reported to occur due to an inversion of a 2 kilobase segment of DNA within the pilin encoding region of the *M. bovis* genome.[59]

Using an oligonucleotide probe and sonicated *M. bovis* DNA cloned into an expression vector, the portion of the DNA responsible for pilin production was identified. The probe was obtained from a portion of *Neisseria gonorrhea* that encodes for the amino terminal end of the pilin. The pilin gene was then subcloned and sequenced. The probe contained a segment of DNA that encoded for a six amino acid leader sequence. The leader sequence was identified as being at the amino terminal end. The

remainder of the pilin strand, designated as the mature protein, consisted of 151 other amino acids.[58]

Amino Terminal Homology of Pilins from Diverse Microbial Genera

The amino terminal end of the pilin from *M. bovis* shares amino acid homology with several other genera.[58] These conserved proteins are found in *Neisseria gonorrhoeae*,[63,64,65] *Neisseria meningitidis*,[65] *Moraxella nonliquefaciens*,[66] *Bacteroides nodosus*,[67,68] *Pseudomonas aeruginosa*,[69,70] and *Vibrio cholerae*.[71] Each genus or species has a modified amino acid N-methyl-phenylalanine (MePhe) as the first residue in the mature protein. Each member of the MePhe family have polymeric pilus with repeating homogeneous monomeric units, have two cysteine residues, share amino acid homology for 6 to 7 amino acids preceding the mature protein, and have high homology for the initial portion of the mature protein. The leader sequence for *M. bovis*[59] and *N. gonorrhoeae*[72] has been suggested as being responsible for transport of the pilin unit across the cell membrane. In addition this portion of the pilin is believed to be involved in binding of the pilin into the polymeric pilus unit.[64,67,69]

Grouping *Moraxella bovis* by Pilus

Protection against IBK with piliated bacterins has been primarily against challenge with homologous strains. This evidence has supported the perception that it should be possible to group strains of *M. bovis* by pilus antigens. One unpublished study (European patent application no. 83110511.9, 1984) of 16 United States strains from the culture collection

of the National Animal Disease Center, Ames, IA, showed that there were at least 8 strains of *M. bovis* possessing antigenically unique pili. Studies with British isolates showed 7 groups of pili.[73] Seven antigenic groups of pili have been associated with Australian strains.[53,62]

In the United States, strains of piliated *M. bovis* have not been grouped into serogroups based on pilus type. Typing *M. bovis* into antigenic groups would make it possible to investigate which strains are required in bacterins in the United States to provide the greatest protection against IBK. No studies in cattle have been done to show that serologically cross-reactive strains are also cross-protective. In cell culture, serum against *M. bovis* strains from Great Britain protected against attachment of other *M. bovis* strains within the same serogroup, but failed to prevent attachment of strains from other serogroups.[74]

A single strain of *N. gonorrheae* can undergo multiple phase variations due to multiple loci identified for pilus production.[75] Immunization with pili from one phase variation with low cross-reactivity to other phase variations has been shown to be non-protective in a guinea pig subcutaneous, open ended, chamber model against other phase variations of the same strain.[76] Immunization with phase variations that had cross-reactivity to other phase variations were shown to be protective against those alternative phase variations.[76] Although amino acid homology for α versus ß pilin for *M. bovis* strain EPP63 is approximately 70%,[59] there have been no reports to show that immunity against one phase variations of a strain of *M. bovis* will protect against another phase variation of the same strain.

Bacteroides nodosus, a member of the MePhe group of pilus producing organisms, has been shown to have 9 major serogroups and can be further divided into other serogroups or into sub-serogroups for a total of 18 possible groups.[77] The pilus of *B. nodosus* has not been associated with attachment but is believed to function in surface translocation due to twitching motility.[78] It is believed that by surface translocation cells are able to penetration into the hoof matrix. Protection has been demonstrated for pilus vaccines against the same serogroup and between certain selected serogroups but no universal protective pilus antigen has been demonstrated.[79] Pilus vaccines using 8 to 10 strains representing different serogroups and sub-serogroups have proven effective in preventing clinical footrot and have been configured based upon serological studies of the most prevalent strains in Great Britain and Western Europe.

The gene for *B. nodosus* pilus production has been cloned into *Escherichia coli* and *Pseudomonas aeruginosa*. The *E. coli* pilin product remained on the inner surface of the cell membrane and was not assembled into pilus. Vaccination with either whole or disrupted *E. coli* cells failed to provide protection. In *P. aeruginosa* the *B. nodosus* pilin gene produced assembled pilus on the outer membrane. When the genetic engineered *P. aerouginose* was used in vaccination studies protection against homologous challenge was demonstrated.[80]

Presently three federally licensed *M. bovis* vaccines are available in the United States.[81] Published information from two of the companies is available.[17,20] Both vaccines are inactivated whole cell bacterins.[82]

The published efficacy trials were done using a single strain for one bacterin[20] and two strains for the second bacterin.[17]

Techniques for Determining *Moraxella bovis* Pilus Cross-Reactivity

Cross-reactivity of *M. bovis* strains was initially done with a gel diffusion precipitin test.[83] The antisera used were prepared with crude whole cell preparations containing pili and other cell components. Antigen from homologous and heterologous *M. bovis* strains showed lines of identity as did antigen from organisms of other genera.

Vandergaast and Rosenbusch[22] examined *M. bovis* field isolates from an IBK epizootic in Iowa. By indirect immunofluorescence using rabbit antisera produced against extracted envelope material they identified a strain of *M. bovis* different from their 8 reference strains. Using gold-labeled goat anti-rabbit sera in a immunoelectron microscopy technique, they showed that the rabbit sera were reactive with pili.

Moore and Rutter[73] evaluated an indirect ELISA, a tandem crossed immunoelectrophoresis and a slide agglutination test to evaluate rabbit anti-pilus sera produced with purified pili. Purified pili were used as the antigens for the ELISA and the tandem crossed immunoelectrophoresis test. Whole cells were used as the antigens for the slide agglutination test. All three techniques gave comparable results. SDS-PAGE was used to determine the apparent molecular mass of pilin from each of the strains.

Lepper and Barton[53] used a double sandwich ELISA to serogroup *M. bovis*. Initially, anti-pilus rabbit or goat sera were analyzed by an

indirect ELISA and antibody against cross-reacting strains were adsorbed from the serums. The adsorbed anti-pilus sera were then used for a double sandwich ELISA to quantitate sonicated crude pilus preparations. No determination of the apparent molecular mass of the pilin was done.

Ruehl et al.[52] used SDS-PAGE/Western blotting to show the apparent molecular mass of pilin of strains of *M. bovis*. The antisera were specific for pilus but reacted with all pili groups.

EVALUATION OF THREE SEROLOGICAL TECHNIQUES FOR DIFFERENTIATING STRAINS OF *MORAXELLA BOVIS* BY PILUS ANTIGENS

Evaluation of three serological techniques for differentiating strains of *Moraxella bovis* by pilus antigens

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ABSTRACT

Three techniques, enzyme linked immunosorbent assay, non dissociative discontinuous polyacrylamide gel electrophoresis accompanied by electrophoretic immunoblot (Western blot) analysis, and sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) accompanied by Western blot analysis, were evaluated for their ability to differentiate three piliated strains of *Moraxella bovis*. Anti-pilus rabbit sera were produced against three strains of *Moraxella bovis* using pilus enriched preparations. Anti-endotoxin activity was removed from the sera by affinity chromatography. The three techniques were evaluated for their ability to differentiate and identify differences in the pilus antigens using the three sera and crude antigens from the same strains. All three techniques distinguish homologous anti-pilus serum and crude antigen when comparing homologous versus heterologous titers or serum extinction dilutions. The SDS-PAGE/Western blot allowed antigen identification and demonstrated the apparent molecular mass of the pilin. It was demonstrated that SDS-PAGE/Western blot analysis would allow serogrouping of *Moraxella bovis* strains by pilus differences when using predetermined dilutions of antigenically unique, strain specific anti-pilus sera.

INTRODUCTION

The primary pathogen of infectious bovine keratoconjunctivitis (IBK) has been identified as *Moraxella bovis*.[1,2,3] The virulence factors presently identified for *M. bovis* infections are protease[4], hemolysin[5,6,7], and pilus.[8] Pilus is believed to be an epithelial cell adherence factor.[9] Experimental challenge with non-piliated strains of *M. bovis* has not resulted in a significant percentage of infected cattle.[8] Protection with bacterins containing pilus[10] and protease[11] to prevent IBK has been demonstrated. More conclusive evidence has been presented for protection by bacterins containing piliated cells than for protease or "cornea-degrading enzyme" bacterins.[12,13,14] Pilus has been shown to induce protective immunity against homologous strains in experimental vaccination and challenge studies, but not against challenge with heterologous strains.[13,15]

Moore and Rutter[16] and Lepper and Power[17] grouped strains of *M. bovis* by pilus cross reactivity, with strains isolated from Great Britain and Australia, respectively. Moore and Rutter[16] used purified pilus to produce anti-*M. bovis* sera. The sera were evaluated against purified pilus in an indirect enzyme linked immunosorbent assay (ELISA) and a tandem crossed immunoelectrophoresis technique. In addition, a slide agglutination test using whole cells and the *M. bovis* anti-pilus sera were evaluated. Lepper and Power evaluated goat and rabbit anti-pilus sera produced against purified pilus in an indirect ELISA using purified pilus as the antigen. These sera were then used in an antigen capture, sandwich ELISA to evaluate purified pilus from *M. bovis* strains.

Vandergaast and Rosenbusch examined *M. bovis* field isolates from an IBK epizootic by indirect immunofluorescence using rabbit sera prepared against envelope preparations. They were able to demonstrate the emergence of a pilus type different from their reference strains.[18]

Three techniques that could be used to demonstrate strain specific *M. bovis* pilus reactions were evaluated in this study. An indirect ELISA technique, a non-dissociative discontinuous polyacrylamide gel electrophoresis (native PAGE) followed by electrophoretic immunoblotting (Western blot) technique, and a dissociative discontinuous sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting were compared.

MATERIALS AND METHODS

Bacterial Strains

Moraxella bovis strains EPP63(300), FLA64(6), and IBH68(748R) were obtained from Dr. George W. Pugh, Jr. (National Animal Disease Center, Ames, IA). All three strains were isolated from cattle with active IBK lesions. Strains EPP63 and IBH68 were isolated from individual cattle in Iowa and strain FLA64 isolated from a single animal from a Florida cattle herd. Two of the three strains (EPP63 and FLA64) were isolates used in previous work.[14,19] Each strain was evaluated by oxidase, catalase and phenylalanine deaminase tests, by glucose, maltose, and sucrose fermentation, and by litmus milk inoculation. Gas and H_2S production were also evaluated. The production of pili from each strain was determined by noting colony characteristics and observing morphology using electron microscopy (EM) techniques. Each strain was passaged daily on 5% bovine blood trypticase soy agar (TSA) plates (BBL Microbiology Systems, Cockeysville, MD) to maintain piliation. Cells were scraped from the agar, resuspended in trypticase soy broth (TSB) without dextrose (BBL Microbiology Systems, Cockeysville, MD), and stored frozen at -60 C. The strains were thawed and passaged daily for 3 to 5 days prior to use.

Pilus Enriched Antigen Preparation

Pilus enriched antigens were prepared by a modification of the technique of Brinton et al.[20] Eighteen-to-twenty-hour aerobically grown *M. bovis* cultures were used to inoculate multiple 5% bovine blood TSA pans, 22 by 32 cm. After incubation at 37 C for 18-20 hours, the cells

were scraped from the surface and placed into ice cold 0.15 M ethanolamine HCl (ETh) buffer, pH 10.3. The bacteria were sheared in a tissue homogenizer system (Tissumizer Ultra-turrax, Tekmar, Cincinnati, OH) for 15 minutes at 24,000 RPM. During shearing the vessel containing the ETh solution was kept in an ice bath.

Cells were removed by centrifugation at 10,000 x *g* for 15 minutes at 4 C. Cell debris was removed from the supernatant by centrifugation at 40,000 x *g* for 1 hour at 4 C. Pili were precipitated from the supernatant by the addition of sufficient saturated ammonium sulfate solution to make a final 10% ammonium sulfate solution. The solution was allowed to precipitate for 24 to 72 hours at 4 C. The solution was then centrifuged at 50,000 x *g* for 1 hour at 4 C. The pellet was resuspended in ETh buffer, mixed overnight and centrifuged at 23,000 x *g* for 1 hour at 4 C to remove insoluble membrane contaminants. The supernatant was transferred to 12,000 to 14,000 dalton dialysis tubing (Spectra/Por, Spectrum Medical Industries, Inc., Los Angeles, CA) and dialyzed against 0.05 M Tris buffered saline (TBS) pH 8.0 for 3 to 7 days. The buffer was changed a minimum of two times, at 24 to 48 hour intervals, during the dialysis. The contents of the dialysis tubing were centrifuged at 100,000 x *g* for 1 hour at 4 C and the pellet resuspended in ETh buffer.

The samples were examined by silver staining of a 10-15% gradient mini SDS-PAGE gel (Phast System, Pharmacia, Uppsala, Sweden). If multiple bands were evident after examination by SDS-PAGE, further purification was done by precipitation, centrifugation, and dialysis. The protein concentration of a sample from each pilus enrichment

preparation was determined by a commercial bicinchoninic acid/copper sulfate protein assay (BCA Protein Assay Reagent, Pierce, Rockford, IL). The final pilus enriched ETh solution was stored at 4 C without addition of preservatives.

Antiserum Production

New Zealand White rabbits, 6 to 8 pounds, were bled and then intravenously (IV) inoculated in the lateral ear vein with 1 mg of pilus protein. A second inoculation of the pilus preparation (1 mg) was given IV 3 weeks after initial inoculation. The rabbits were anesthetized with 50 mg ketamine hydrochloride (Ketaset, Ft. Dodge Labs., Inc., Ft. Dodge, IA) and 0.5 mg acepromazine maleate (PromAce, Aveco Co., Inc., Ft. Dodge, IA) and bled by cardiac puncture 2 weeks after the second IV injection. After clotting, serum was collected by centrifugation.

Anti-endotoxin Removal

Endotoxin extraction

Endotoxin was extracted and used to prepare an anti-endotoxin affinity column (personal communication, Dr. Richard B. Rimler, National Animal Disease Center, USDA, Ames, IA). Non-piliated colonies of EPP63 were identified. Trypticase soy broth without dextrose was inoculated with the non-piliated strains and allowed to grow for 48 to 72 hours at 37 C. Bacteria were harvested by centrifugation at 4600 x *g* for 30 minutes at 4 C. The pellet was resuspended in 20 mM Tris, 500 mM NaCl buffer, pH 7.5 made in sterile, pyrogen free water (Abbott Laboratories,

North Chicago, IL) (pyrogen free TBS). The pellet was washed 2 times and then resuspended in sterile, pyrogen free water. Approximately 2 mg/ml of proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and the solution incubated at 60 C for 1 hour. The solution was placed in a boiling water bath for 1 hour to inactivate the proteinase K. A 1:10 dilution of the solution in cold 95% ethanol was made and 0.3 mg/ml of sodium acetate was added. The solution was kept at -20 C for 24 to 48 hours, then centrifuged at 6,000 x *g* for 1 hour and the pellet washed with cold ethyl ether. The resulting pellet was resuspended in pyrogen-free TBS.

Affinity chromatography

One column volume of the EPP63 digested solution was loaded onto endotoxin removal gels (Detoxi-Gel, Pierce, Rockford, IL) and the crudely purified endotoxin allowed to bind overnight. The columns were washed with 3 column volumes of pyrogen-free TBS. Rabbit anti-pilus serum produced against EPP63 pilus enriched antigen was passed over three column volumes of the affinity column containing the bound homologous endotoxin. The resultant serum was diluted 1:8 by the treatment. The endotoxin extraction and affinity chromatography was repeated 2 more times, using non-piliated strains homologous for the remaining anti-pilus sera, FLA64 and IBH68.

Evaluation of endotoxin removal

The crude digest and a sample of purified *M. bovis* endotoxin (courtesy Dr. Ricardo F. Rosenbusch, VMRI, Iowa State University, Ames, IA) were evaluated by a silver stained SDS-PAGE gel. Removal of

anti-endotoxin activity from the sera was evaluated by Western blotting (see SDS-PAGE/Western blot technique below).

Crude Pilus Antigen

Each of the three strains were grown as previously described and individually harvested from 5% bovine blood TSA pans. The bacteria were sheared and centrifuged, as previously described to remove cells and large cell fragments. The supernatant was stored at 4 C for use as crude pilus antigen.

ELISA

An *M. bovis* indirect ELISA, with minor modification, as described by Lehr et al.[10] was used. *Moraxella bovis* crude antigen was used in place of purified pilus, a 0.05% Tween 20/0.005 M phosphate buffered saline solution was used as the wash solution, and a 1:250 dilution of peroxidase conjugated protein A (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was used as the conjugate. Two fold dilutions from 1:2 to 1:1024 of the preinoculation rabbit sera and two fold dilutions from 1:8 to 1:4096 of the adsorbed anti-pilus sera for all three strains were evaluated. The ELISA was repeated using the crude antigen for the remaining two strains.

Native PAGE and Western Blot

Native PAGE

A non-dissociative discontinuous 5-15% acrylamide gradient PAGE[21] was used. The stacking gel buffer was Tris-HCL, pH 6.8 and the resolving gel buffer Tris-HCl, pH 8.8. The reservoir buffer was tris glycine, pH 8.3.[21] Three repetitions of four gels were cast and electrophoresed. Each repetition included all three crude antigens and the pilus enriched preparation corresponding to the anti-pilus serum used for that repetition. The samples were mixed 1:1 with stacking gel buffer/10% glycerol. The gels were electrophoresed using 30 mA of constant current per gel at 12 C for 3 to 4 hours. One gel in each repetition was stained with a commercial silver stain kit (Bio-Rad Lab., Richmond, CA).

Western blot

Three gels of each set were electrophoretically transferred to 0.45 micron nitrocellulose (Bio-Rad Lab., Richmond, CA) by the method of Towbin et al.[22] Transfer was done at 100 volts and 10 C, for 1 hour in 10% methanol/25 mM Tris/192 mM glycine buffer. The nitrocellulose membrane was stored frozen at -20 C after electrophoresis.

The nitrocellulose membranes were later thawed and then blocked with 3% bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, MD) in 20 mM Tris/500 mM NaCl, ph 7.5 (TBS) for 30 minutes. All procedures were done at 25 C. Adsorbed anti-pilus serum was used as the first antibody, was diluted in TBS with 1% bovine serum albumin, and incubated on the membrane with a 10 lane manifold (Deca Probe, Hoefer Scientific Instruments, San Francisco, CA) for 1 hour. The membranes were washed 4

times with 0.05% Tween 20 in TBS. A 1:1000 dilution of peroxidase conjugated protein A was used as the second antibody and the membrane incubated for 1 hour. The membranes were washed four times in 0.05% Tween 20 in TBS. The color substrate, 4-chloro-1-naphthol (Bio-Rad Laboratories, Richmond, CA) was added and the membrane incubated until color development occurred. Extinction dilutions were measured for each serum and antigen combination. The extinction dilution was estimated to be the last dilution of serum that gave a visible signal on immunoblots.

In each repetition, adsorbed serum from one strain was used at 2-fold dilutions (1:8 to 1:4096) for evaluation of the specificity of the serum against homologous and heterologous antigens. After three repetitions all possible crosses were compared.

SDS-PAGE and Western Blot

SDS-PAGE

Antigens were separated by a 12.5% SDS-PAGE as described by Laemmli.[23] The crude and pilus enriched samples were mixed 1:1 with 2% SDS/5% 2-mercaptoethanol/10% glycerol sample buffer and boiled for 3 minutes. Apparent molecular mass markers (Bio-Rad Laboratories, Richmond, CA) were included with the gel that was silver stained. Gels were electrophoresed under 30 mA of constant current per gel at 12 C for 3 to 4 hours. Three repetitions of four gels as previously described were done.

Western blot

Electrotransfer and immuno-evaluation were as previously described for the Native PAGE/Western blot.

RESULTS

Bacterial Strains

Each strain was confirmed as being *M. bovis* by a positive oxidase test, by negative glucose, maltose, and sucrose fermentation, and by a characteristic three zone peptonization without fermentation of litmus milk. Gas and H_2S production were negative. Strain EPP63 was catalase positive. Strains FLA64 and IBH68 were catalase negative. All strains liquified nutrient gelatin. Hemolytic activity was observed for all three strains from both piliated and non-piliated colonies. Piliated colonies were identified by a friable colony characteristic with agar corrosion beneath the colony. Non-piliated colonies were identified by a mucoid consistency and lack of agar corrosion. It could be shown using electron microscopy that the agar corroding, friable colonies were composed of cells with pilus on their surface. No pilus was evident by EM from the mucoid, non-agar corroding colonies (Figure 1 and 2).

Pilus Enriched Antigen

Examination of each pilus enriched preparation by SDS-PAGE revealed single bands for each piliated strain, when SDS-PAGE/silver stained gels were optimally loaded. These bands corresponded to the apparent molecular mass for monomeric units of pilus, or pilin. The pilin bands were at approximately 17,000 dalton for strains EPP63 and FLA64 and at approximately 20,000 dalton for strain IBH68 (data not shown). Additional minor bands, over the entire range and exceeding the range of the apparent molecular mass markers, could be discerned by overloading

Figure 1. Electron photomicrograph of *Moraxella bovis*, strain FLA64, from a colony with friable and agar corroding characteristics, negative stained, and 30,400 X. Note pili in background

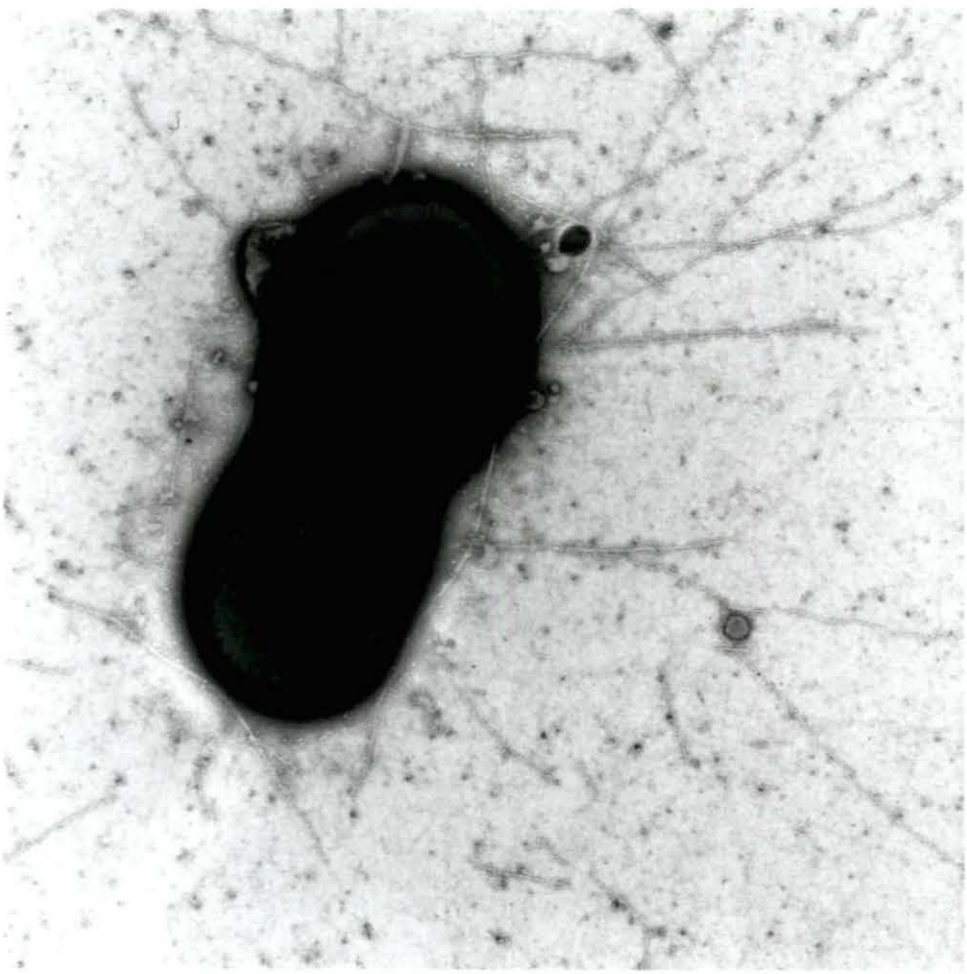
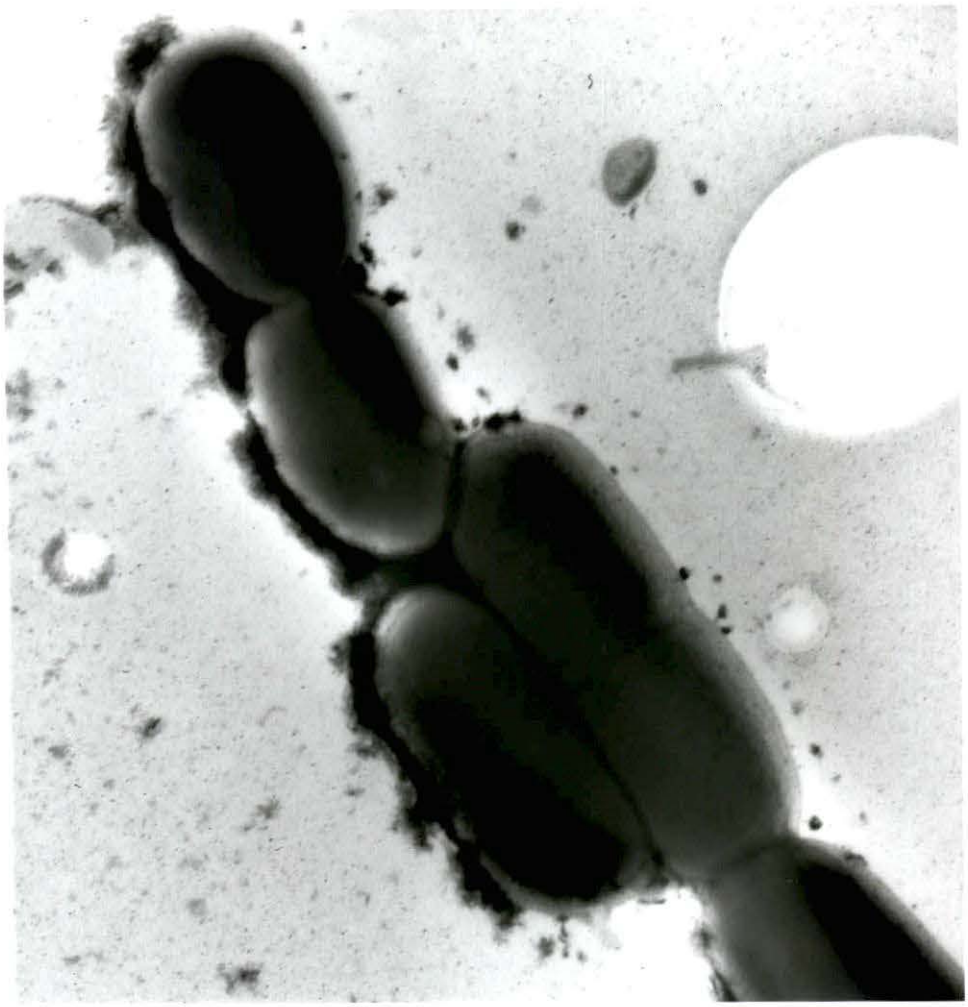


Figure 2. Electron photomicrograph of *Moraxella bovis*, strain FLA64, from a colony with mucoid and non-agar corroding characteristics, negative stained, and 30,400 X. Note lack of pili in background



the gel (Figure 3, Lane A for EPP63, data not shown for FLA64 and IBH68). Average pilus yield from approximately 10 grams (wet weight) of bacteria was 1 mg (dry weight). The pilus enriched preparations contained predominantly pili by EM evaluation (data not shown).

Antiserum

No adverse reactions were noted following initial antigen injection into rabbits. Sporadic anaphylactic reactions occurred on second injection, but were controlled by administering epinephrine. Evaluation of the non-adsorbed antisera by SDS-PAGE/Western blot showed reactivity with an apparent molecular mass band consistent with the apparent molecular mass of pilin and with bands in the less than the 14,400 dalton apparent molecular mass markers. Non-adsorbed sera, made against specific strains, cross-reacted with bands from crude antigens from both homologous and heterologous strains at the expected pilin apparent molecular mass (Figure 4, EPP63 homologous system; data not shown for IBH68 and FLA64).

Anti-endotoxin Removal

The purified *M. bovis* endotoxin had multiple bands when evaluated by silver stained SDS-PAGE gels. The bands ranged in apparent molecular mass from less than the 14,400 dalton to approximately 31,000 dalton (Figure 3, lane C). The crude *M. bovis* digest had a major band at less than 14,4000 dalton, multiple minor bands between 14,400 to 97,400 dalton, and multiple minor bands exceeding the apparent molecular mass

Figure 3. Twelve point five percent SDS-PAGE/silver stain of *Moraxella bovis* endotoxin removal antigens: Lane A (EPP63 pilus enriched preparation, 25 μg protein), lane B (EPP63 crude preparation, 54 μg protein), lane C (purified *M. bovis* endotoxin, 10 μg lipopolysaccharide), lane D (proteinase K digested EPP63, 8 μl loaded onto lane, lipopolysaccharide determination not done). Unmarked lane is apparent molecular mass markers, in kilodaltons

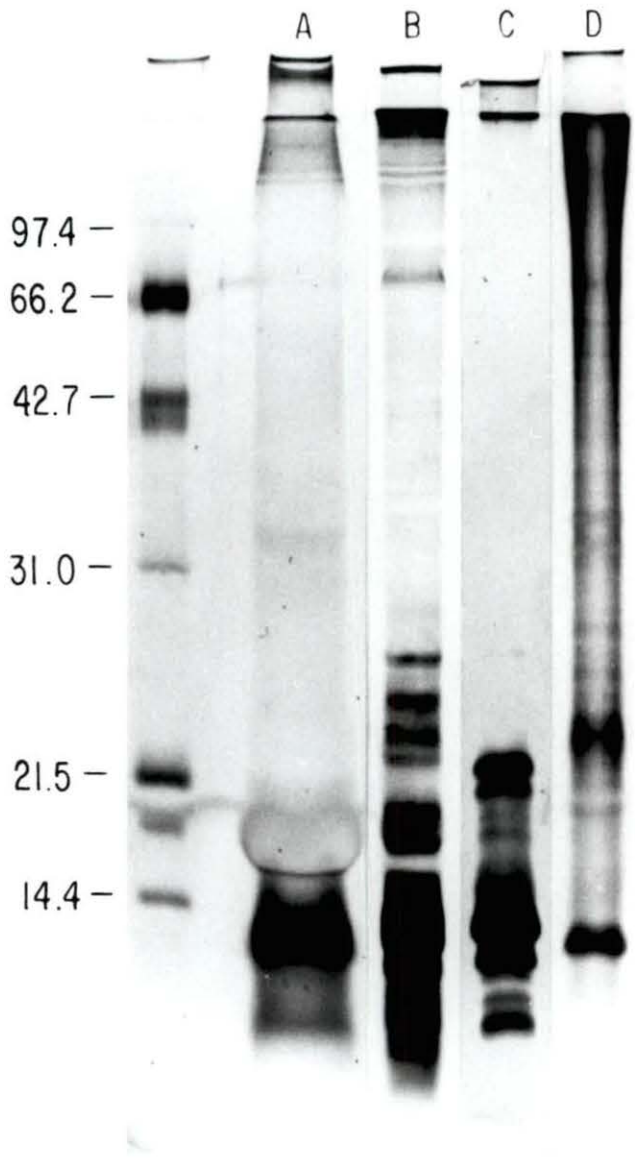
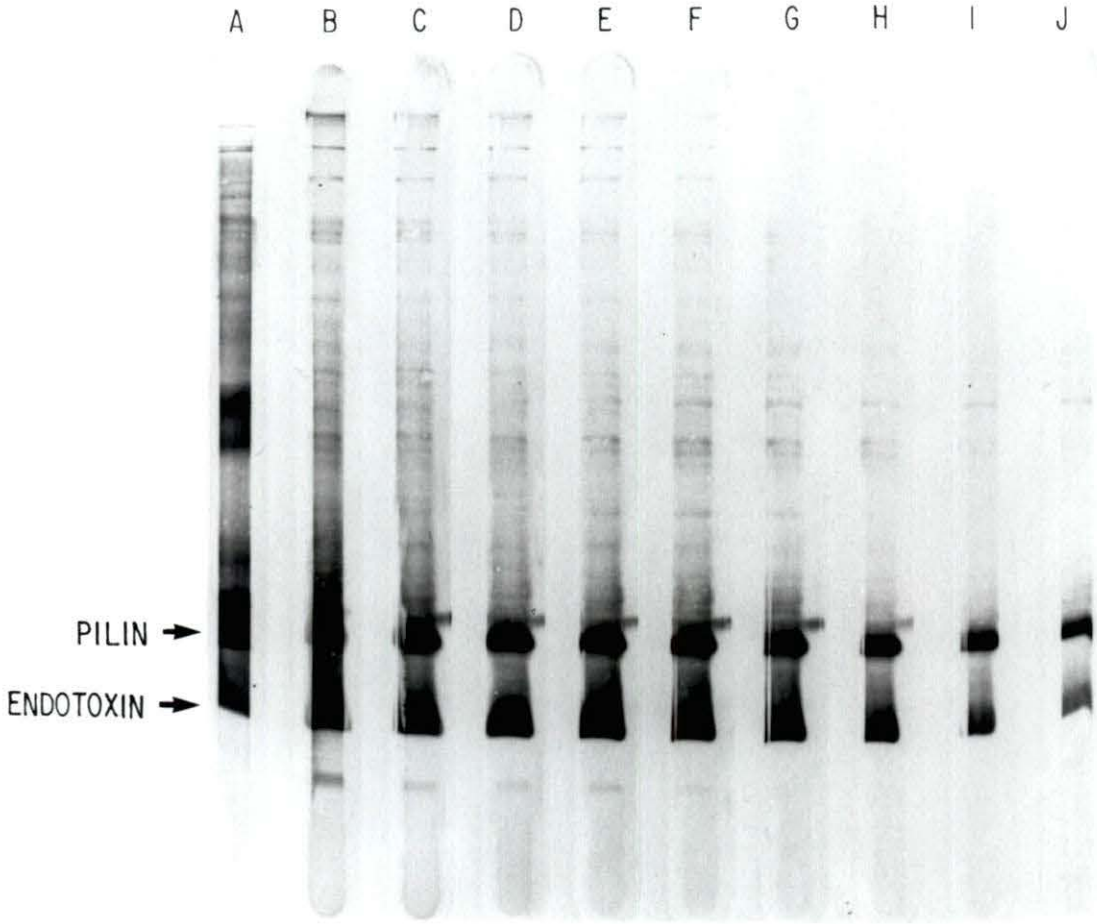

A
B
C
D
97.4
66.2
42.7
31.0
21.5
14.4

Figure 4. Twelve point five percent SDS-PAGE/Western blot using non-adsorbed EPP63 anti-pilus *Moraxella bovis* serum: Lane A (EPP63 pilus enriched antigen, 5 μg protein, reacted with a 1:256 dilution of non-adsorbed EPP63 anti-pilus serum) and lanes B-J (EPP63 crude antigen, 54 μg reacted with non-absorbed EPP63 anti-pilus serum diluted two-fold from 1:8 to 1:2048, respectively)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PILIN
ENDOTOXIN

markers (greater than 97,400 dalton) (Figure 3, lane D). After homologous endotoxin affinity chromatography the adsorbed sera had a single dominant band consistent with the apparent molecular mass of pilin for that strain (Figures 5).

ELISA

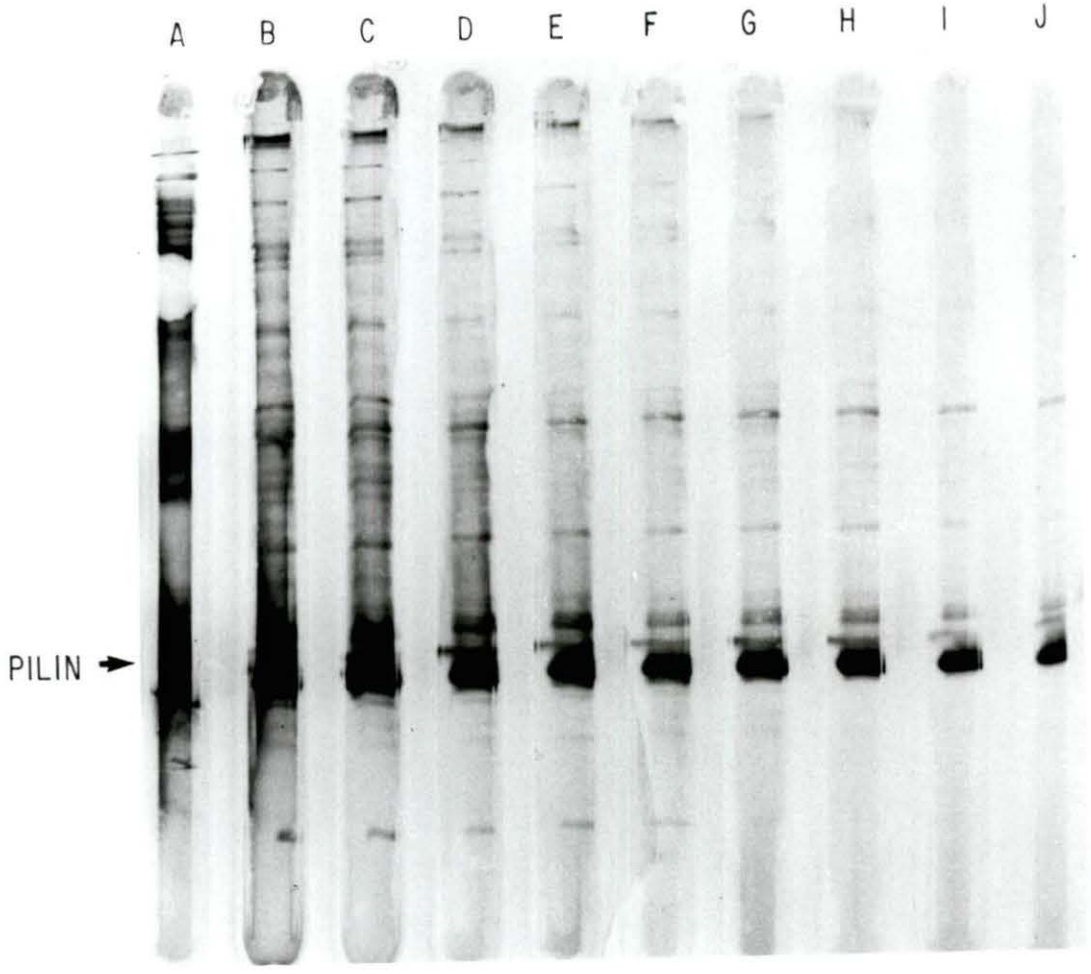
No significant pre-immunization titer was detected for any of the rabbits when evaluated against crude pilus preparations. Each of the anti-pilus sera had the highest endpoint titer against the homologous crude antigen. The homologous reactions were 8 to 32-fold higher than any of the heterologous reactions (Table 1).

Native PAGE and Western Blot

On the silver-stained native PAGE gel, pilus enriched antigens had a major band at the stacking/resolving gel interface and minor bands over the remainder of the lane. The crude antigens showed multiple major and minor bands over the entire lane (Figure 6).

The Western blot for the pilus enriched preparations, when using the homologous antigen/antibody system, reacted only at the stacking/resolving gel interface (Figure 7). The absorbed anti-pilus sera reacted with a major band at the stacking/resolving gel interface with the homologous antigen/antibody system. At low dilution the anti-pili sera reacted uniformly over the upper two-thirds of the lanes. At greater dilutions (ie. >1:256 dilution for EPP63), the homologous antigen/antibody system reactions gradually decreased and then disappeared, except for persistence of the reaction at the

Figure 5. Twelve point five percent SDS-PAGE/Western blot using adsorbed EPP63 anti-pilus *Moraxella bovis* serum: Lane A (EPP63 pilus enriched antigen, 5 μg protein, reacted with a 1:256 dilution of adsorbed EPP63 anti-pilus serum) and lanes B-J (EPP63 crude antigen, 54 μg, reacted with absorbed EPP63 anti-pilus serum diluted two-fold from 1:8 to 1:2048, respectively)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PILIN

Table 1. ELISA titer of pre-immunized and post-immunized rabbit sera tested with crude antigen from 3 strains of *Moraxella bovis*

	Rabbit serum					
	EPP63		FLA64		IBH68	
Crude antigen	Pre-imm.	Post imm. adsorbed	Pre-imm.	Post imm. adsorbed	Pre-imm.	Post imm. adsorbed
EPP63	1:2	1:4096	1:2	1:64	1:4	1:128
FLA64	1:4	1:64	1:8	1:2048	1:8	1:128
IBH68	1:2	1:2	1:2	1:8	1:2	1:64

Figure 6. Five to fifteen percent gradient native PAGE/silver stain of *Moraxella bovis* antigens: Lane A (EPP63 pilus enriched antigen, 5 μg protein), lane B (EPP63 crude antigen, 54 μg protein), lane C (FLA64 crude antigen, 47 μg protein), lane D (IBH68 crude antigen, 57 μg protein)

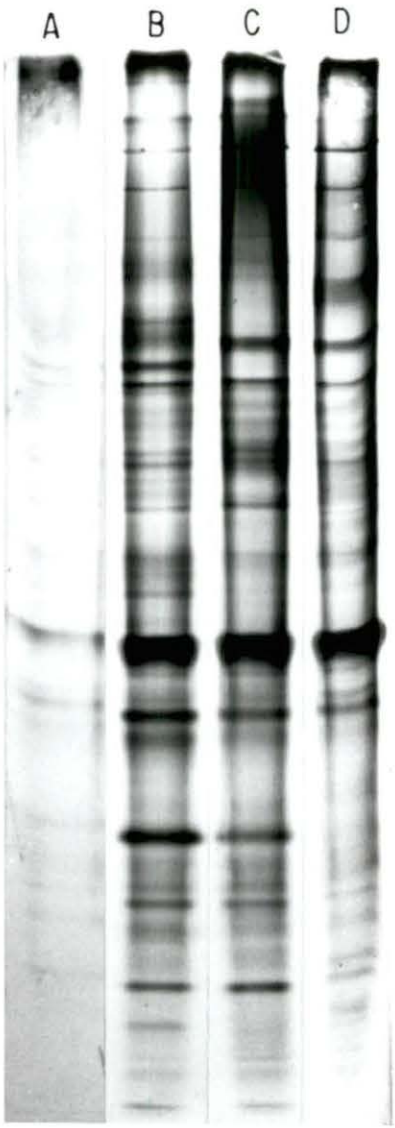
A
B
C
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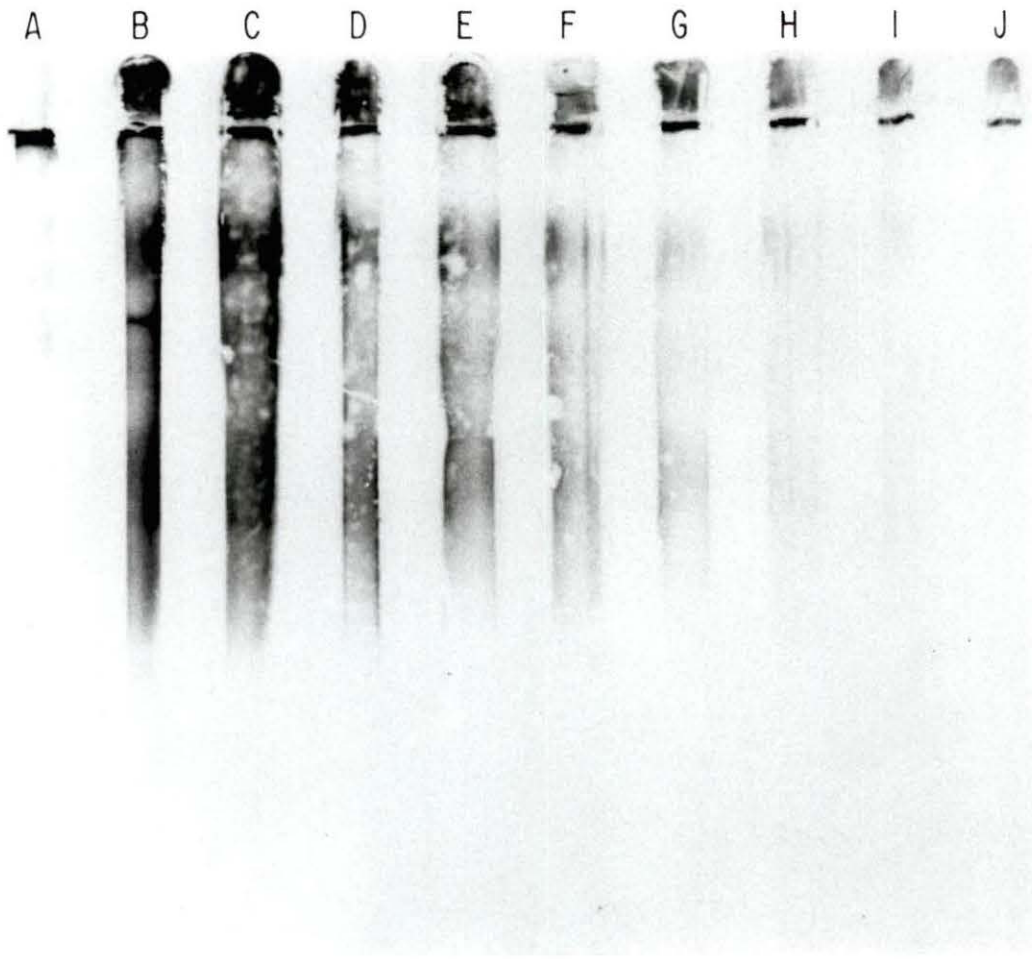
Figure 7. Five to fifteen percent gradient native PAGE/Western blot of *Moraxella bovis* homologous antigen/antibody: Lane A (EPP63 pilus enriched antigen, 5 μg protein, reacted with a 1:32 dilution of EPP63 adsorbed anti-pilus serum) and lane B-J (EPP63 crude antigen, 54 μg protein, reacted with absorbed EPP63 anti-pilus serum diluted two-fold from 1:8 to 1:2048, respectively)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stacking/resolving gel interface (Figure 7). On the heterologous antigen/antibody system only a band at the stacking/resolving gel interface was detected, and only at low dilutions (Figure 8). No single, defined band could be determined to be pilus for the crude or the pilus enriched antigen, either in the silver stain or the Western blot. If the anti-pilus serum reacted, it reacted uniformly along the upper two-thirds of the lanes. Strain specificity for the individual sera was demonstrated by endpoint titer comparison of homologous and heterologous reactions (Table 2). The endpoint titers for the homologous systems was 16 to 64 fold higher than for heterologous systems.

SDS-PAGE and Western Blot

The pilus enriched preparations when evaluated by silver staining of SDS-PAGE gels appeared as single bands (Figure 9). By overloading a gel lane, the preparations could be shown to have multiple bands (Figure 3). The predominant band in the pilus enriched preparations corresponded to a band at the same apparent molecular mass in the homologous crude antigen preparations. A single major reactive band was revealed by both homologous and heterologous antigen/antibody combinations at lower anti-pilus sera dilutions (Figure 10 and 11). As the serum was diluted minor bands could no longer be detected. Anti-pilus sera for EPP63 and FLA64 had extinction dilutions of 16-fold and IBH68 8-fold more reactive with homologous than heterologous antigen/antibody combinations (Table 3).

Figure 8. Five to fifteen percent gradient native PAGE/Western blot of *Moraxella bovis* homologous and heterologous antigen/antibody: Lane A (EPP63 crude antigen, 54 μg protein, reacted with a 1:32 dilution of EPP63 adsorbed anti-pilus serum) and lane B-J (IBH68 crude antigen, 57 μg protein, reacted with absorbed EPP63 anti-pilus serum diluted two-fold from 1:8 to 1:2048, respectively)

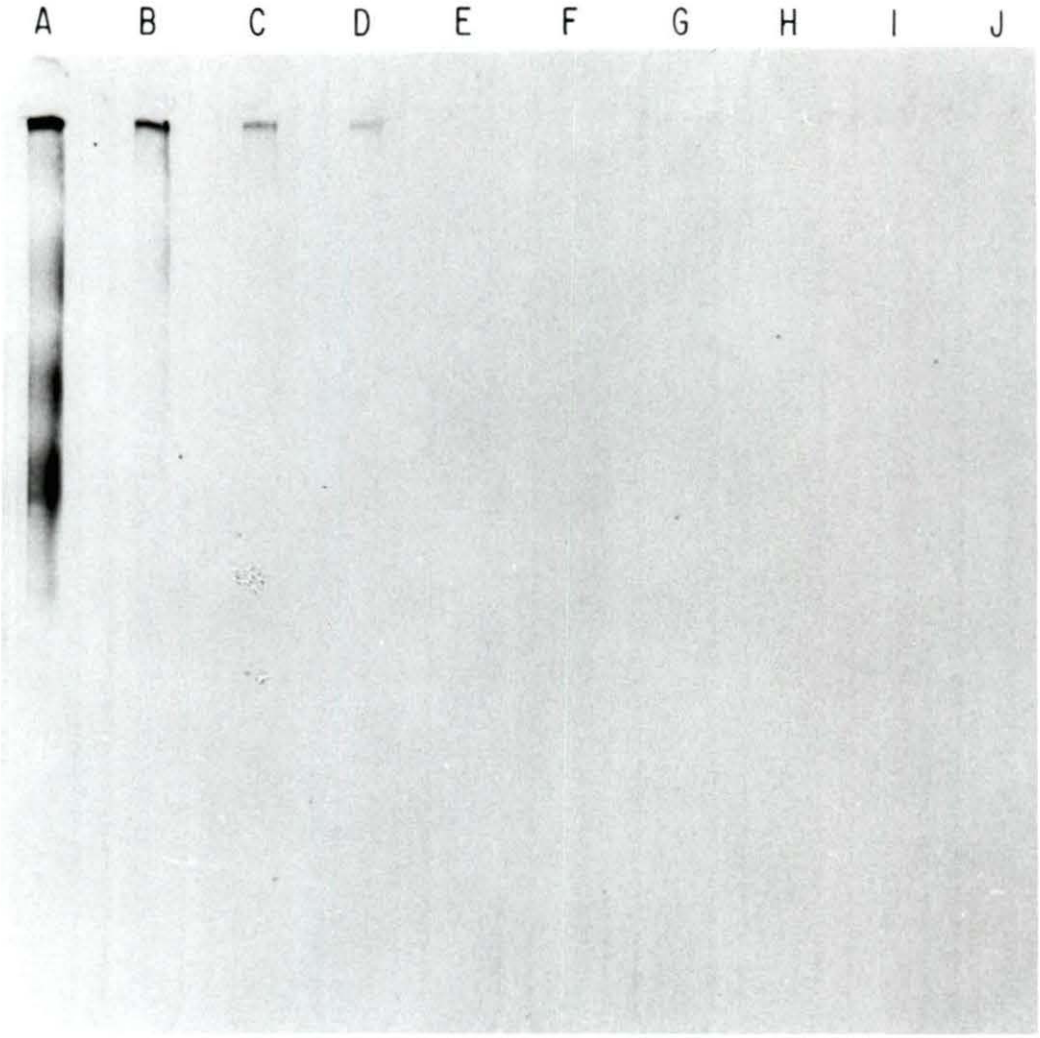
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Table 2. Native PAGE/Western blot extinction dilutions for *Moraxella bovis* anti-pili rabbit sera tested with crude antigen from 3 strains of *M. bovis*

Crude antigen	Adsorbed anti-pili sera		
	EPP63	FLA64	IBH68
EPP63	>1:2048	1:16	1:32
FLA64	1:16	1:256	<1:8
IBH68	1:32	1:8	1:256

Figure 9. Twelve point five percent SDS-PAGE/silver stain of *Moraxella bovis* antigens: Lane A (EPP63 pilus enriched antigen, 5 μg protein), lane B (EPP63 crude antigen, 54 μg protein), lane C (FLA64 crude antigen, 47 μg protein), and lane D (IBH68 crude antigen, 57 μg protein). Unmarked lane is apparent molecular mass markers, in kilodaltons

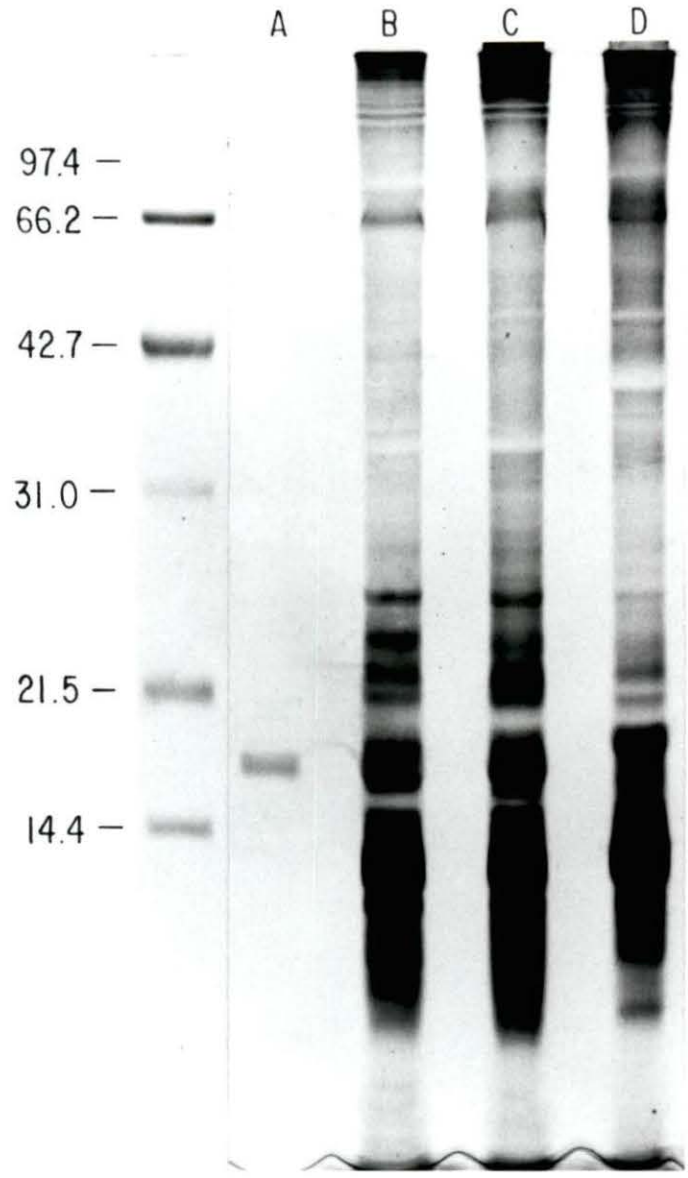
A
B
C
D
97.4
66.2
42.7
31.0
21.5
14.4

Figure 10. Twelve point five percent SDS-PAGE/Western blot of *Moraxella bovis* homologous antigen/antibody: Lane A (EPP63 pilus enriched antigen, 5 μg protein, reacted with a 1:32 EPP63 dilution of adsorbed anti-pilus serum) and lane B-J (EPP63 crude antigen, 54 μg protein, reacted with absorbed EPP63 anti-pilus serum diluted two-fold from 1:8 to 1:2048, respectively)

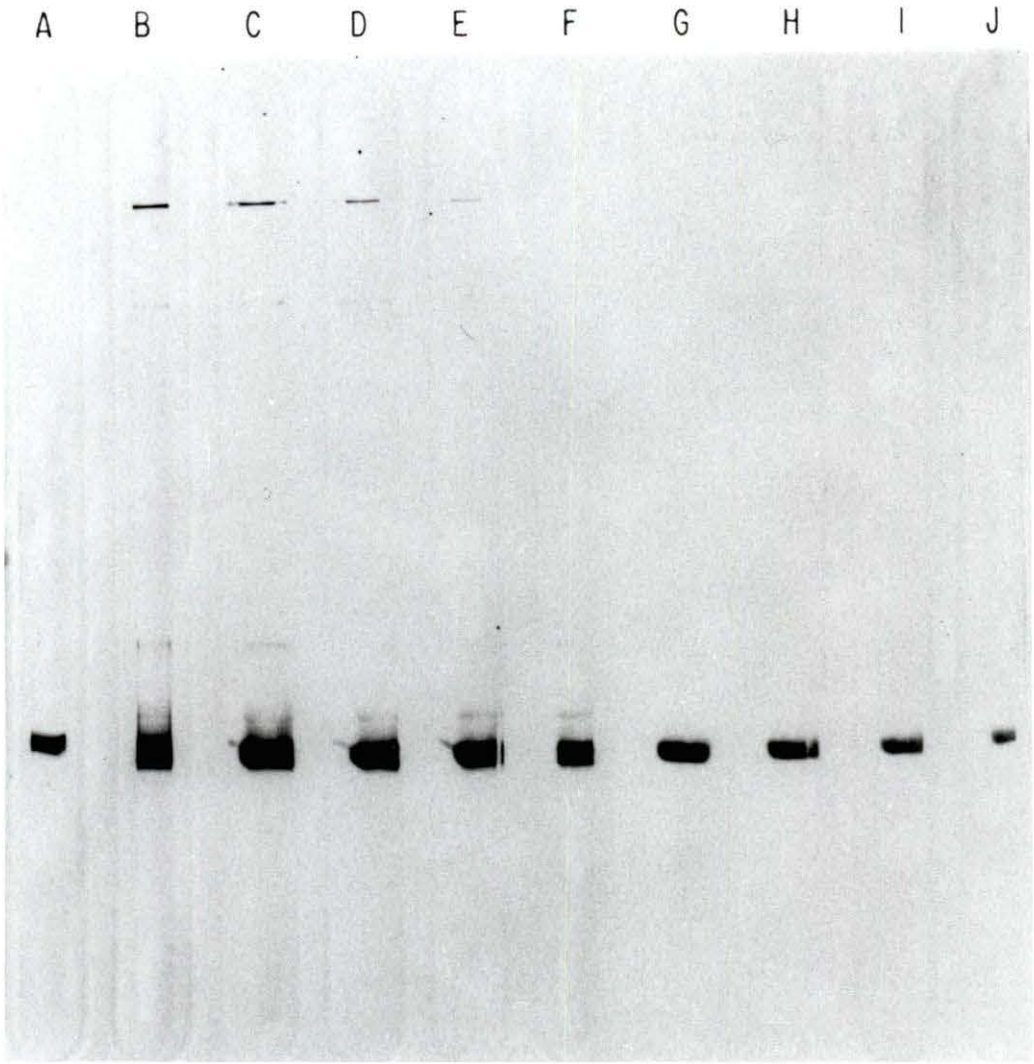
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Figure 11. Twelve point five percent SDS-PAGE/Western blot of *Moraxella bovis* homologous and heterologous antigen/antibody: Lane A (EPP63 crude antigen, 54 μg protein, reacted with a 1:32 dilution of EPP63 adsorbed anti-pilus serum) and lane B-J (FLA64 crude antigen, 47 μg protein, reacted with absorbed EPP63 anti-pilus serum diluted two-fold from 1:8 to 1:2048, respectively)

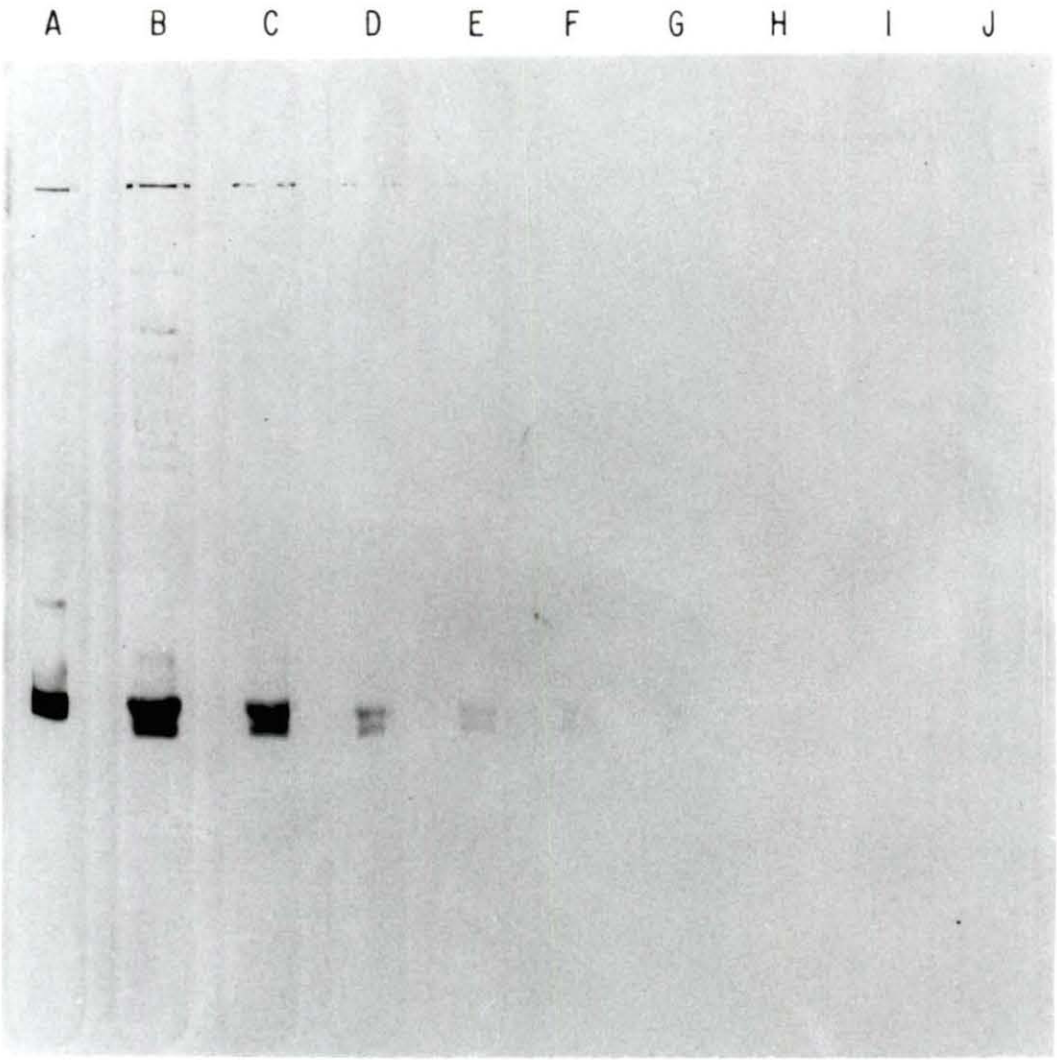
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Table 3. SDS-PAGE/Western blot extinction dilutions for *Moraxella bovis* anti-pili rabbit sera tested with crude antigen from 3 strains of *M. bovis*

Crude antigen	Adsorbed anti-pili sera		
	EPP63	FLA64	IBH68
EPP63	>1:2048	1:128	1:128
FLA64	1:64	1:1024	1:32
IBH68	1:64	1:64	1:512

DISCUSSION

Two ß (17,000 dalton pilin for EPP63 and FLA64) and one α (20,000 dalton pilin for IBH68) piliated *Moraxella bovis* strains were used in this study. Initial work with non-adsorbed rabbit sera failed to demonstrate strain specificity when used with SDS-PAGE/Western blots. After affinity column adsorption to remove reactivity to endotoxin, dilutions of the sera showed pili strain specificity against homologous crude preparations by the SDS-PAGE/Western blot technique.

All three techniques, ELISA, native PAGE/Western blot, and SDS-PAGE/Western blot, could be used to demonstrate *M. bovis* pilus strain specificity. The ELISA technique could not be used to demonstrate which antigens reacted with the antiserum and would therefore, to limit reactivity, require use of purified pilus as the antigens. Purification of pili is not suitable for rapid pilus serotyping of field strains of *M. bovis*, as it is a time consuming and expensive preparative technique. Use of native PAGE/Western blot analysis also failed to discriminate as to which antigen the antiserum reacted. The failure to produce a defined pilus band was not unexpected. A pilus strand is composed of a variable number of units of the monomeric pilin and therefore has variable apparent molecular mass. The native PAGE sample treatment does not degrade the polymeric configuration. The band demonstrated at the stacking/resolving junction is most likely due to the acrylamide pore size excluding the larger pilus strand fragments.

The SDS-PAGE/Western blot technique could be used to demonstrate the presence of pili, the apparent molecular mass of the pilin, and the

strain-specificity of pilin. By comparing extinction titers for sera, specificity against homologous pili was demonstrated.

Non-adsorbed anti-pilus sera failed to show strain specificity on SDS-PAGE/Western blot analysis. After the anti-endotoxin activity was removed, strain specificity of the serums was demonstrated. The lack of strain specificity prior to anti-endotoxin removal can be due to an endotoxin fraction with the same apparent molecular mass as the pilin. The SDS-PAGE/silver stain of the purified endotoxin demonstrated bands of endotoxin consistent with the same approximate apparent molecular mass as pilin.

Ruehl et al. have demonstrated that a strain possessing pili composed of ß pilin is significantly more infectious in an experimental challenge model when compared with the same strain expressing pili composed of α pilin.[24] It is presumed that protection against disease is best achieved by immunizing with homologous ß pilin antigens. It is therefore important to know the apparent molecular mass of pilin for selection of isolates used in vaccine production. Of the three techniques evaluated with the adsorbed anti-pilus sera only the SDS-PAGE/Western blot could be used to demonstrate the apparent molecular mass of the pilin and the degree of heterogeneity of the *M. bovis* strains.

The sera reacted in a comparable manner on the native PAGE/Western blot and the SDS-PAGE/Western blot since strain specificity could be demonstrated at high dilutions of serum for both techniques. From this it can be inferred that the major antigenic epitopes recognized by the anti-pilus sera on denatured pilus or pilin are the same as those exposed

on the native configuration of the pilus. This reactivity of the serums may well correlate to the circulating antibody reaction induced by natural infection or vaccination.

At lower serum dilutions, heterologous reactions occurred and can be explained by the fact that the pilus antigenic epitopes consist of both conserved and variable regions. Ruehl et al.[24] have demonstrated conserved and variable regions within the ß and α phase variation of one strain. It appears that the variable domains predominate as antigens and reactivity against the conserved regions can be eliminated by dilution of the antiserum.

A group of three unique non-cross-reactive anti-pilus sera were developed to begin categorizing unknown strains of *M. bovis* by pilus type. Using crude preparations of *M. bovis* as the SDS-PAGE antigen and the adsorbed anti-pili sera as the Western blot antibody, it was possible to demonstrate the apparent molecular mass of the pilin monomer and to determine the serotype of the pilus. A panel of strain-specific, adsorbed anti-pilus serums could then be used in a SDS-PAGE/Western blot to demonstrate the serogroup and pilin apparent molecular mass of a crude preparation of an unknown *M. bovis* strain.

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GENERAL SUMMARY

Moraxella bovis is the primary infectious agent of infectious bovine keratoconjunctivitis. Pilus and hemolysin are the primary virulence factors of *M. bovis*. Pilus is an adherence factor and antibodies against pilus prevent adherence of the organism to corneal epithelial cells in culture. Protection against *M. bovis* challenge can be elicited by vaccination with a piliated strain of *M. bovis*, but challenge-exposure with heterologous piliated strains will result in disease. An increased virulence is noted for pilus composed of 17,000 dalton pilin (ß pilin), therefore it is presumed that protection against disease is best achieved by immunizing with homologous ß pilin antigens.

Several techniques have been used to serogroup British and Australian strains of *M. bovis*. None of the techniques, by themselves, rapidly show the serogroup and pilin apparent molecular mass of the pili of a field strain. No serogrouping of United States strains has been reported.

An affinity column, with a crude *M. bovis* digest as the source of endotoxin antigen, was shown to be effective in removing *M. bovis* anti-endotoxin activity from serums. By adsorbing out the anti-endotoxin activity from rabbit serums prepared against pilus-enriched preparations, strain specificity could be demonstrated with these sera in a SDS-PAGE/Western blot assay.

A panel of strain-specific, adsorbed anti-pilus serums could then be used in a SDS-PAGE/Western blot to demonstrate the serogroup and pilin apparent molecular mass of a crude preparation of an unknown *M. bovis* strain.

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