March 2006

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JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2006, p. 772–776 Vol. 44, No. 3
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.3.772–776.2006

Effects of *Moraxella* (*Branhamella*) *ovis* Culture Filtrates on Bovine Erythrocytes, Peripheral Mononuclear Cells, and Corneal Epithelial Cells†

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Received 29 September 2003/Returned for modification 14 April 2004/Accepted 14 December 2005

Infectious bovine keratoconjunctivitis (IBK) is a highly contagious ocular disease that affects cattle of all ages and that occurs worldwide. Piliated hemolytic *Moraxella bovis* is recognized as the etiologic agent of IBK. According to data from the Nebraska Veterinary Diagnostic Laboratory System, however, *Moraxella* (*Branhamella*) *ovis* has been isolated with increasing frequency from cattle affected with IBK. The objective of this study was, therefore, to examine *M. ovis* field isolates for the presence of the putative virulence factors of *M. bovis*. Culture filtrates from selected *M. ovis* field isolates demonstrated hemolytic activity on bovine erythrocytes and cytotoxic activity on bovine peripheral blood mononuclear cells and corneal epithelial cells. The hemolytic activity of the culture filtrates was attenuated after heat treatment. Polyclonal antibodies raised against the *M. bovis* hemolysin-cytotoxin also recognized a protein of approximately 98 kDa in a Western blot assay. These data indicate that the *M. ovis* field isolates examined produce one or more heat-labile exotoxins and may suggest that *M. ovis* plays a role in the pathogenesis of IBK.

Infectious bovine keratoconjunctivitis (IBK) is a highly contagious disease that affects cattle of all ages and that occurs worldwide (27, 28, 31). The adverse economic impact of IBK due to decreased weight gain and costs of therapeutic treatment is estimated to be $150 million annually (27). Piliated hemolytic strains of *Moraxella bovis* are recognized as the etiologic agents of IBK (5, 10, 11, 23, 35, 36, 37). *Moraxella bovis*, however, cannot always be recovered from clinical cases; and other organisms, such as *Moraxella* (*Branhamella*) *ovis*, *Mycoplasma bovoculi*, infectious bovine rhinotracheitis virus, and adenovirus, have been suggested to play a role in the pathogenesis of IBK (17, 19, 31, 37).

According to data of the University of Nebraska Veterinary Diagnostic Laboratory System, *M. ovis* is isolated with increasing frequency from cases of IBK. From 1999 to 2002, *M. ovis* was isolated three times more often from field cases than *M. bovis*. Isolation of *M. ovis* from cases of keratoconjunctivitis has previously been reported in sheep, goats, deer, and moose (3, 14, 16, 17, 30, 33, 40, 41). In cattle, *M. ovis* has been isolated from IBK cases (17, 31); but the significance of its isolation is currently unclear because, in one study, experimental inoculation of calves with *M. ovis* isolated did not cause keratoconjunctivitis (17). These findings suggest that *M. ovis* is not a pathogen or that additional factors may be required for virulence (9, 17, 32, 43).

Isolates of *M. ovis* recovered from cattle with IBK have not been examined for potential virulence factors (6, 21, 26, 34, 39). In an attempt to elucidate whether *M. ovis* plays a role in the etiology and pathogenesis of IBK, the objective of this study was to investigate *M. ovis* field isolates for the presence of factors known to be virulence factors in *M. bovis* by examining the effects of *M. ovis* culture filtrates on different types of cells of bovine origin.

MATERIALS AND METHODS

Bacterial isolates. *Moraxella ovis* isolates recovered from cattle with IBK were passed twice on Trypticase soy agar containing 5% sheep blood (blood agar; Remel, Lenexa, KS) and were stored at −80°C in 15% glycerol. A known virulent isolate of *M. bovis*, designated Epp63 (20, 35, 36), and a reference isolate of *M. ovis* (ATCC 19575) recovered from a sheep with conjunctivitis were used in each assay for comparison. Field isolates of *M. ovis* were identified by phenotypic and biochemical criteria. Briefly, *M. ovis* colonies were 1 to 3 mm in diameter after 24 h of incubation, firm, and hemolytic on blood agar; and they tended to slide across the agar surface when they were touched with a Nichrome wire loop. Microscopically, the *M. ovis* isolates were gram-negative cocci (0.6 to 1.0 μm) arranged in pairs. All *M. ovis* isolates were aerobic and oxidase and catalase positive, and they reduced nitrate but did not ferment carbohydrates or liquefy gelatin (8, 13, 22, 30).

Preparation of culture supernatants. Aliquots of each isolate from frozen stock cultures were streaked onto blood agar and incubated at 37°C for 24 h. Culture for hemolysin production during early-logarithmic-phase growth was performed as described previously (20) but with modification. Briefly, three colonies of each isolate from the blood agar plate were transferred into 3 ml of brain heart infusion (BHI) broth (Remel) supplemented with 5 mM $CaCl_2$/ml (Sigma Chemical Company, St. Louis, MO), and the cultures were incubated for 18 h at 37°C. The cultures were then transferred to Erlenmeyer flasks containing 50 ml of BHI broth supplemented with 5 mM $CaCl_2$/ml and were incubated with shaking (200 rpm) for 4.5 h at 37°C. The bacterial cells were removed by centrifugation (12,000 × *g*) for 10 min at 4°C, and the culture supernatants were transferred to sterile centrifuge tubes. Each supernatant was filtered through a 0.22-μl polysulfone syringe filter (Pall Gelman Laboratory, Ann Arbor, MI), and the cell-free supernatants (CFSs) were either used immediately or frozen at −80°C until use. An aliquot of each CFS was plated on blood agar to verify sterility.

Hemolytic activity assay. Bovine erythrocytes were washed three times with sterile phosphate-buffered saline (PBS), and a 10% erythrocyte suspension was

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† This article is published as ARD Journal Series number 13909, with the approval of the University of Nebraska Agricultural Research Division.

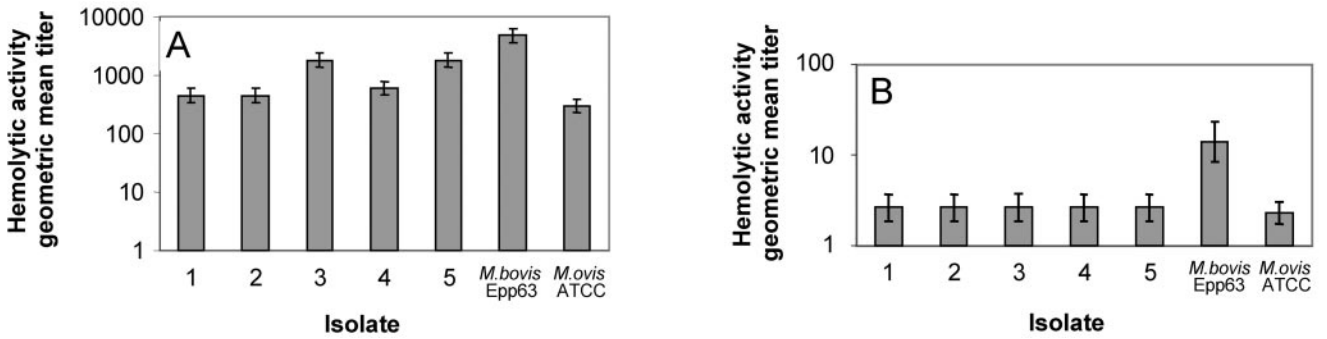

FIG. 1. Hemolytic activities of *M. bovis* and *M. ovis* isolates on bovine erythrocytes with and without heat treatment. Bars indicate the geometric mean titers of the hemolytic activities of the CFSs from *M. bovis* Epp63, *M. ovis* ATCC 19575, and five *M. ovis* field isolates without heat treatment (A). Heat treatment consisted of incubation of the CFSs at 56°C and cooling to room temperature prior to use in the hemolysin assay (B). Error bars represent the 95% confidence intervals of the means. The results shown represent data from five independent replications of each set of experiments.

prepared in PBS and stored at 4°C for no longer than 3 days before use. The hemolytic activity of each CFS was analyzed in 96-well microtiter plates as described previously (20) but with modification. Briefly, twofold dilutions (1:2 to 1:2,048) of each sample were run in duplicate in 100 μl of a 0.15 M sodium chloride (Sigma) solution containing 10 mM Tris-Cl buffer (Sigma) and 10 mM $CaCl_2$ (Sigma) buffer (NTC buffer). Wells with distilled water served as positive control wells, and wells with NTC buffer served as negative control wells. One hundred microliters of 2% washed bovine erythrocytes in PBS was added to each well, and the plates were incubated for 2 h at 37°C. Intact erythrocytes were pelleted by centrifugation (1,500 × *g*) for 5 min, and 100 μl of each supernatant was then transferred to a new plate. The absorbance (optical density [OD] at 410 nm) was determined for each well by using an enzyme-linked immunosorbent assay reader (Molecular Devices Corporation, Sunnyvale, CA). The mean absorbance was calculated for each set of wells, and the titers were determined by using the reciprocals of the last dilution with absorbance values above 5% of the mean of the positive control wells.

To determine whether the hemolytic activity of *M. ovis* CFS was heat labile, 1 ml of each CFS was incubated in a water bath for 30 min at 56°C and was then allowed to cool to room temperature. The hemolytic activity of each CFS was then analyzed in 96-well microtiter plates as described above. In addition, each CFS was stored at 4°C for 24 h and the hemolytic activity was determined as described above.

Preparation of bovine PBMCs. Bovine peripheral blood mononuclear cells (PBMCs) were prepared as described previously (20). Briefly, freshly collected blood in EDTA was centrifuged (700 × *g*) for 30 min at 4°C. The buffy coats were removed and diluted with PBS, and the mixture was layered onto Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) and then centrifuged (700 × *g*) for 40 min at 4°C. The PBMCs were then collected from the interface, washed three times in PBS containing 5 mM EDTA, and counted in a hemocytometer.

Cytotoxic activity assay. The density of the PBMCs was adjusted to a concentration of 5×10^6 cells/ml for the dimethylthiazol diphenyltetrazolium bromide (MTT) reduction cytotoxicity assay, and the cytoxic activity of each CFS was determined in 96-well microtiter plates as described previously (20). Briefly, twofold serial dilutions of each sample were run in duplicate in 100 μl of 1× RPMI 1640 medium (Gibco BRL, Rockville, MD) supplemented with 10% fetal calf serum. One hundred microliters of the PBMC suspension was added to each well, and the plates were incubated for 1 h at 37°C. Following incubation, the plates were centrifuged (700 × *g*) for 5 min and the supernatant was discarded. One hundred microliters of clear 1× RPMI 1640 medium and 20 μl of MTT (Sigma) dye solution (5 mg MTT in 1 ml distilled H_2O) were then added to each well, and plates were again incubated until a colored reaction product was visible (2 to 4 h). The plates were then centrifuged (700 × *g*) for 5 min, the supernatant was discarded, and 100 μl of acid isopropanol was added to each well. Gentle agitation was used to dissolve the formazan precipitate. The absorbance of each well was determined on an enzyme-linked immunosorbent assay reader by using a test wavelength of 570 nm and a reference wavelength of 630 nm. The percent cytotoxicity for each well was determined as follows: [1 − (OD of toxin-treated mononuclear cells/OD of untreated PBMCs)] × 100. The untreated PBMCs were used as cell viability controls.

The cytotoxic activity titer in each well was determined by using the reciprocal of the highest dilution that caused the cytolysis of 50% or more of the target cells compared to the level of cytolysis of the cells control wells. One hundred microliters of the PBMC suspension and 100 μl of RPMI 1640 medium were used to assess cell viability, and 100 μl of medium and 100 μl of undiluted CFS were used to assess nonspecific dye reduction.

Corneal epithelial cell culture. Corneas were collected from three clinically healthy calves immediately following euthanasia. After removal of the globes and the attached conjunctival sacs, the ocular tissues were briefly rinsed with 70% ethyl alcohol and were then rinsed twice with sterile 0.85% saline solution. A sterile scalpel blade was used to remove the cornea at the limbus, and the corneal epithelium was dissected away from the underlying stroma. The corneal epithelium was then rinsed three times in sterile 1× RPMI 1640 medium containing 0.003% gentamicin sulfate (Sigma). The corneal epithelium was minced with a sterile scalpel blade in a sterile glass petri dish and then transferred to culture flasks containing RPMI 1640 medium supplemented with 10% fetal calf serum and 0.003% gentamicin sulfate (c-RPMI). The flasks were incubated at 37°C in 7% CO_2 for 72 h. After incubation, the medium was decanted and the flasks were washed once with warm (37°C) sterile PBS. Fresh c-RPMI medium was then added to each flask, and the flasks were again incubated at 37°C in 7% CO_2. Fresh c-RPMI medium was added to each flask at 72-h intervals or until 100% confluent cell monolayers had formed. The confluent cells were detached with 2.5% trypsin (Sigma), resuspended in c-RPMI, transferred to 96-well culture plates, and incubated at 37°C in 7% CO_2 for approximately 72 h or until 90% confluent cell monolayers had formed in the wells.

Corneal epithelial cell cytotoxicity assay. Medium from each corneal epithelial cell monolayer plate was discarded, and 10 μl of fresh c-RPMI medium was added to each well. Fifty microliters of a CFS was then added to each well, while 50 μl of BHI broth was added to each control well. The plates were incubated at 37°C in 7% CO_2 for 2 h. Medium from each plate was then discarded, and 200 μl of fresh clear c-RPMI medium was added to each well. Each plate was then examined at ×400 magnification with an inverted microscope. The assay was performed three times in duplicate.

Western blot analyses of *M. bovis* Epp63 and *M. ovis* CFS. CFS proteins from two *M. ovis* field isolates, *M. ovis* ATCC 19575, and *M. bovis* Epp63 were separated by standard 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (38), and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). A kaleidoscope prestained standard (Bio-Rad) was used to assess transfer efficiency. The nonspecific binding sites on the membrane were blocked with 1% fish gelatin (Sigma) and 0.5% skim milk (Carnation) in PBS overnight at 4°C. The primary antibody, a polyclonal rabbit serum raised against a partially purified extract of hemolytic *M. bovis* strain T+ (2) (kindly provided by L. George, University of California—Davis), was used at a 1:250 dilution in PBS. After 1 h of incubation in primary antibody at ambient temperature, the membrane was washed (PBS containing 500 μl/liter Tween 20 [Bio-Rad]) and then incubated for 1 h in a 1:1,000 dilution of goat anti-rabbit immunoglobulin G horseradish peroxidase-labeled conjugate (Promega, Madison, WI). Detection of bound secondary antibody was by chemiluminescence (Pierce, Rockford, IL) and autoradiography. Normal rabbit serum was substituted for anti-*M. bovis* antibodies as a negative control in the protocol.

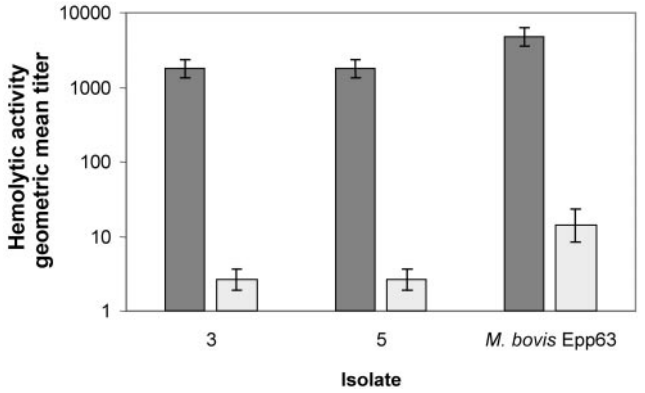

FIG. 2. Comparison of hemolytic activities of *M. bovis* and *M. ovis* field isolates after heat treatment. Prior to the assay, the CFSs were treated as described in the legend to Fig. 1, but heat-treated (light gray bars) and non-heat-treated (dark gray bars) CFSs were run side by side. Bars indicate the geometric mean titers of the hemolytic activities of the CFSs from *M. bovis* Epp63 and two of the five *M. ovis* field isolates (isolates 3 and 5), as in Fig. 1. Error bars represent the 95% confidence intervals of the means. The results shown represent data from three independent replications.

RESULTS

Hemolytic activities of CFSs on bovine erythrocytes. The CFSs from all isolates demonstrated hemolytic activities in the assay, with titers ranging from 256 to 2,048 for the five *M. ovis* field isolates and from 4,096 to 8,192 for the *M. bovis* Epp63 isolate (Fig. 1A). Heat treatment of the CFSs before the hemolysin assay was conducted attenuated the hemolytic activity, as the titers of the *M. ovis* CFSs observed after heat treatment ranged only from 2 to 4 (Fig. 1B). This effect was also evident when heat-treated and non-heat-treated CFSs were run side by side for direct comparison (Fig. 2). In this series of assays, non-heat-treated *M. ovis* CFSs had hemolytic activity titers of 1,024 to 2,048 that were reduced to titers of 2 to 4 after heat treatment. Non-heat-treated *M. bovis* CFS titers were 4,096 to 8,192, and the titers after heat treatment were 8 to 32. When the CFSs were stored at 4°C for 24 h prior to the assay, hemolytic activity was reduced by three- to fourfold dilutions in the *M. ovis* CFSs (titer range, 32 to 256) and one- to three fold dilutions in the *M. bovis* Epp63 CFSs (titer range, 1,024 to 2,048) (data not shown). Because of the consistent hemolytic activities of their CFSs, field isolates 3 and 5 were selected for use in the cytotoxicity assays and in Western blot analyses.

Cytotoxic activities of CFSs on bovine PBMCs. The CFSs of *M. ovis* field isolates 3 and 5, *M. ovis* ATCC 19575, and *M. bovis* Epp63 demonstrated cytotoxic activities on bovine PBMCs, with geometric mean titers ranging from 64 to 16 (data not shown).

Effects of CFSs on bovine corneal epithelial cells. Exposure of corneal cell monolayers to CFSs from *M. ovis* field isolates 3 and 5 and *M. bovis* Epp63 resulted in cell detachment and complete loss of the cell monolayers within 2 h. The CFS from *M. ovis* ATCC 19575, however, did not cause any noticeable monolayer disruption or the loss of cells (Fig. 3).

SDS-PAGE and Western blot analysis of CFSs. The polyclonal primary antibody recognizes a cluster of repeats in toxin (RTX) A proteins of *M. bovis* strain T+ (2). These antibodies also recognized several proteins in the *M. bovis* Epp63 CFS (Fig. 4, lane *M. bovis* Epp63). A cluster of strong bands migrated at about 90 to 100 kDa, and another cluster migrated at about 80 to 88 kDa. Several proteins were also detected in the *M. ovis* CFS (Fig. 4). The distribution of bands in all *M. ovis* lanes, however, was such that the more prominent bands migrated at lower molecular masses. A single band of about 98 kDa recognized in the *M. bovis* CFS was also recognized in all *M. ovis* CFSs. When preimmunization rabbit serum was used as the primary antibody, no bands were seen (data not shown), indicating that the bands detected by the anti-*M. bovis* serum are due to a specific antigen-antibody reaction and are not due to nonspecific binding of the antibody.

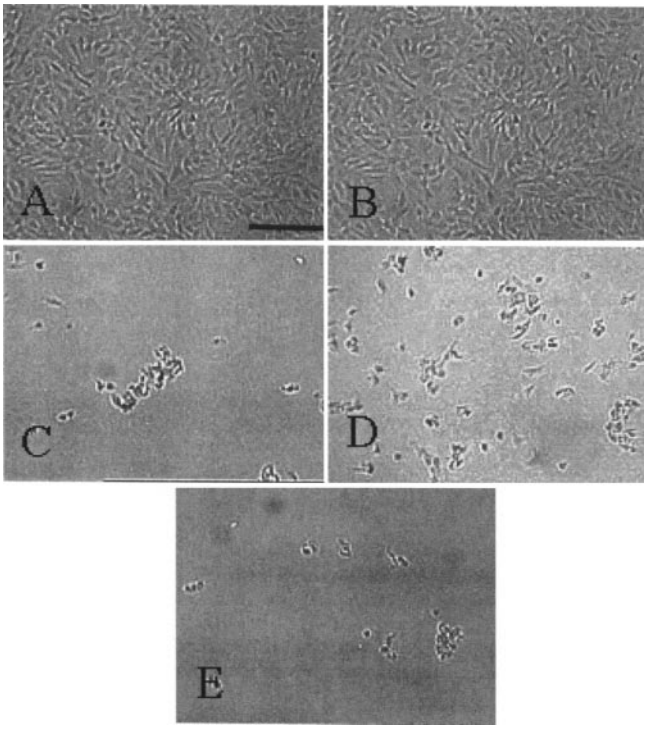

FIG. 3. Effects of CFSs on bovine corneal epithelial cell monolayers. Monolayers of corneal epithelial cells were either not exposed to any CFS (negative control) (A) or exposed to *M. ovis* ATCC 19575 CFS (B), exposed to *M. ovis* field isolates 3 (C) or 5 (D), or exposed to *M. bovis* Epp63 CFS (positive control) (E). Note the complete disruption of the monolayers exposed to CFSs produced by field isolates 3 and 5 and *M. bovis* Epp63. The photomicrographs shown are representative of three independent replications. Bar, 100 μm.

DISCUSSION

The results of this study indicate that *M. ovis* isolates recovered from cattle with IBK produce one or more heat-labile exotoxins that demonstrate hemolytic activity on bovine erythrocytes and cytotoxic activity on bovine PBMCs and corneal epithelial cells. The data indicate that, like the known virulent isolate *M. bovis* Epp63 (12, 20, 21, 25, 27), the *M. ovis* isolates tested also possess hemolytic and cytotoxic activities. The hemolytic and cytotoxic activities of *M. bovis* are mediated by a hemolysin, a calcium-dependent, pore-forming RTX (2, 5, 7, 12, 20). Although the definite nature of the toxic activities of

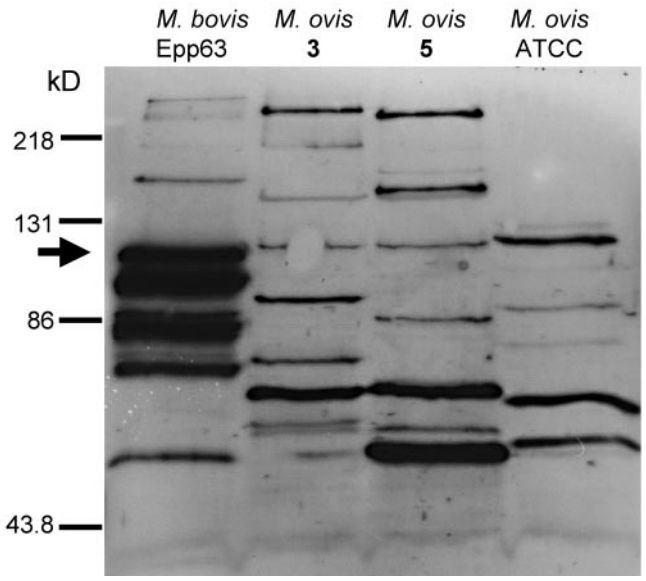

FIG. 4. Detection of *M. ovis* CFS proteins by Western blot analysis. CFS proteins were separated by SDS-PAGE on a standard 10% polyacrylamide gel and then subjected to Western blot analysis. The primary antibody was anti-*M. bovis* rabbit serum. Molecular masses (in kDa) are indicated on the left; the arrow denotes bands of about 98 kDa, the molecular mass recognized for different RTXs. The picture shown is representative of three independent assays.

M. ovis CFSs has yet to be determined, it is possible, based on the similarity of action, that these effects are mediated by an exotoxin qualitatively similar to the *M. bovis* RTX. Substantial attenuation of the hemolytic activity of the *M. ovis* CFS after heat treatment also suggests that the presumptive *M. ovis* exotoxin is proteinaceous, like the *M. bovis* RTX. Based on the observation that the titers of the *M. bovis* CFSs were slightly higher in this assay, it may be speculated that the *M. ovis* exotoxin is more readily inactivated. A similar tendency was evident when CFSs were incubated at 4°C for 24 h before the hemolysin assay was conducted. This difference could be due to structural or conformational differences in the proteins or to the activity of proteolytic enzymes released by *M. ovis* during logarithmic growth (4, 18). The objective of this study, however, was to examine selected *M. ovis* field isolates for the presence of potential virulence factors, and no attempt was made to quantify the hemolytic or the cytotoxic activity.

The primary antibody used in the Western blot assay specifically recognized the *M. bovis* toxin, but it also recognized several proteins in the *M. ovis* CFSs. A cluster of bands of approximately 95 to 100 kDa may be significant because this molecular mass is consistent with those of recognized RTXs (18, 42), including *M. bovis* RTX A (1, 2). A single band in all *M. ovis* CFSs migrated at this particular molecular mass, suggesting some structural similarity. The identities of the several lower-molecular-mass bands present in the *M. ovis* lanes are unknown at this time. Other investigators have shown that full-length RTXs degrade after only a few hours in culture, and specific antibodies recognize several smaller degradation products (2, 18). If it is assumed that a toxin is present in the *M. ovis* CFS, it is possible that those lower-molecular-mass bands represent the degradation products of a full-length toxin.

The *M. ovis* isolates examined in this study differed in their hemolytic and cytotoxic activities. This may suggest that the isolates are different strains, but no testing was done to determine if these isolates were indeed different strains. The number of animal passages of each of the *M. ovis* field isolates received prior to laboratory isolation is unknown, and the number of animal and laboratory passages of *M. ovis* ATCC 19575 is also unknown. Interestingly, *M. ovis* ATCC 19575 did not exhibit any cytotoxicity to bovine corneal epithelial cells, although toxicity to erythrocytes and peripheral blood mononuclear cells was observed. Intraspecies variation (e.g., strain differences), as suggested above, does not likely account for these results. It has been shown for *M. bovis* that only piliated isolates can adhere to bovine corneal epithelial cells (24, 29); and it is possible, therefore, that isolate ATCC 19575 was not piliated and could not adhere to the corneal cells to exert a toxic effect. Some RTXs have been shown to specifically recognize and bind to a host cell receptor (15, 41), and it is also possible that the presumptive hemolysin of the ATCC 19575 strain lacked this ability. It is also noteworthy that the ATCC 19575 isolate is of sheep origin, and a toxin produced during passages in sheep may act on the cornea in a strictly species-specific manner but may act more promiscuously on erythrocytes and PBMCs. The *M. ovis* isolates were not examined for piliation, and the reasons for differences in activity between the two *M. ovis* field isolates and the ATCC 19575 isolate remain speculative.

Currently, piliated and hemolytic *M. bovis* isolates are recognized as the etiologic agents of IBK. Data from the Nebraska Veterinary Diagnostic Laboratory System, however, indicate that *M. ovis* is isolated from the majority of cattle with IBK. From January 1999 through December 2002, samples from a total of 109 cases of IBK were received at the Veterinary Diagnostic Center. In 79 cases (72.5%), *M. ovis* was the only agent isolated, whereas in 18 cases (16.5%), *M. bovis* was the only pathogen isolated. In 12 cases (11%), both *M. ovis* and *M. bovis* isolates were recovered. Other investigators have isolated *M. ovis* from cattle with IBK (17, 31), but experimental inoculation of calves with *M. ovis* isolates did not cause keratoconjunctivitis in one study (17). Although the *M. ovis* isolates in another study exhibited hemolytic activity on blood agar (31), they were not examined for cytotoxic activity or piliation. These findings might suggest that *M. ovis*, if it is pathogenic, may require initial corneal damage or other predisposing factors to cause disease in cattle. *Moraxella bovis* isolates differ in their virulence; some isolates readily cause disease experimentally, while others require corneal damage to cause disease (11, 19, 34). Differences in virulence may also be a common to *M. ovis* isolates. Since only piliated, hemolytic isolates of *M. bovis* are pathogenic for cattle (35), pili may also be necessary for *M. ovis* to attach to and colonize bovine cornea and conjunctiva. This hypothesis should be further addressed by using *M. ovis* field isolates from cattle. Although the results of this study indicate that *M. ovis* field isolates from cattle produce one or more exotoxins, further characterization of this suspected *M. ovis* toxin and studies that examine the piliation of field isolates and adherence of *M. ovis* to the bovine cornea are necessary to substantiate the potential role of *M. ovis* in the pathogenesis of IBK.

ACKNOWLEDGMENTS

This work was supported by the Nebraska Agricultural Experiment Station project NEB14-059.

We extend special thanks to L. George (University of California—Davis) for generously providing anti-*M. bovis* polyclonal antibodies.

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