Detection of cell detachment activity induced by *Moraxella bovis*

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Objective—To characterize the effect that filtrate obtained from cultures of *Moraxella bovis* has on cultured corneal epithelial cells and other types of cultured mammalian cells.

Sample Population—Cultured hamster corneal epithelial cells, bovine epithelial cells, and several transformed cell lines exposed to culture filtrate from a pathogenic isolate of *M bovis*.

Procedure—*Moraxella bovis* was cultured, and bacteria were removed by filtration. The resulting bacterial culture filtrate was incubated with various types of cultured cells, and effects of the filtrate on detachment of various mammalian cell types was quantified by the use of neutral red dye. Additionally, bacterial culture filtrate was treated with protease inhibitors as well as trypsin and heat prior to incubation with cultured mammalian cells.

Results—Bacterial culture filtrate of *M bovis* caused all types of cultured cells to detach from each other and from the substrate, with the maximal effect evident 2 hours after initiating incubation. Detached cells were alive, and detachment was reversible. Serine protease inhibitors (phenylmethylsulfonylfluoride and α_2-macroglobulin) inhibited cell detachment attributable to bacterial culture filtrate. Heating and treatment with trypsin also inhibited cell detachment.

Conclusions and Clinical Relevance—*Moraxella bovis* produces a soluble factor that causes reversible detachment of cultured cells. This activity may play a role in the pathogenesis of infectious bovine keratoconjunctivitis. (*Am J Vet Res* 2000;61:1145–1149)

Moraxella bovis is the causative agent of infectious bovine keratoconjunctivitis, the most common and economically important ocular disease of cattle in the world. Infection with *M bovis* results in corneal ulcers and, infrequently, rupture of the eye and blindness. Affected cattle have evidence of considerable amounts of ocular pain and visual impairment, resulting in inappetence and poor weight gain. The beef cattle industry throughout the world has tremendous economic losses from morbidity associated with infectious bovine keratoconjunctivitis.[1]

Bacterial flora of the eyes of cattle typically includes a number of commensal organisms as well as potential pathogens. The outermost layer of the cornea, the epithelium, acts as an effective barrier to the entry of almost all bacteria. *Moraxella bovis* is unusual, perhaps unique, among ocular pathogens in that it is able to disrupt the corneal epithelium and cause infection of the underlying stroma.[1,2] The mechanism by which these bacteria are able to invade the cornea has not been elucidated; however, a number of virulence factors have been described for *M bovis*. Pili can enhance the attachment of these bacteria to corneal epithelium.[3-6] Hemolysin[7,8] and leukocidin[9] activities have been described; these factors appear to be closely associated with each other and may be caused by the same moiety.[10] Expression of both pili and hemolysin are required for pathogenicity of this organism.[1,3]

Reports regarding the effect of *M bovis* on the corneal epithelium are inconclusive. Analysis of results of some studies suggests that these bacteria are cytotoxic for corneal epithelium,[9,11,12] whereas other studies did not reveal an epitheliotoxic effect of *M bovis*.[13,a] Thus, it is unclear whether *M bovis* produces cytotoxic activity. In the study reported here, our objective was to provide evidence for a factor produced by *M bovis* that causes cell detachment from, as well as rounding and decreased attachment of cultured corneal epithelial cells to, the substrate without causing cell death. This cell detachment may serve as an additional virulence determinant to enhance the pathogenicity of *M bovis*.

Materials and Methods

Organisms—*Moraxella bovis* strain 118 F[b] was isolated from affected cattle in a herd during an outbreak of severe keratoconjunctivitis. Strain 118 F produces hemolysin and leukocidin.[10] Bacteria were routinely passaged on **brain-heart infusion (BHI)** agar with 5% sheep blood (BHI-sheep blood)[c] at 37 C in an incubator with 5% CO_2.

Preparation of culture filtrates—Bacteria were harvested from BHI-sheep blood plates and diluted to an **optical density (OD)** measured at 600 nm (ie, OD 600) of 0.03 in **tryptic soy broth (TSB)**.[d] Flasks were incubated at 37 C on a shaker for 6 hours, at which time the cultures had achieved an OD 600 of 1.2 to 1.4. Bacteria were pelleted by centrifugation at 12,000 × g for 10 minutes, and the supernatant was filtered through a 0.45-µm filter to obtain culture filtrate. Concentrated filtrate was prepared by additional filtering of *M bovis* culture filtrate through a 30,000-molecular weight cutoff filter, using a CH2 concentrator.[e]

Cell lines—Simian virus 40-immortalized hamster corneal epithelial cells were developed and characterized in our laboratory.[14] The following cell lines were obtained from the **American Type Culture Collection (ATCC)**[f]: human embryonic intestinal cells (Henle, Intestine 407, ATCC CCL 6), 3T3 embryonic mouse fibroblast cells (ATCC CCL 92), human epithelioid cervical carcinoma (HeLa, ATCC CCL 2), mouse embryonic liver (BNL CL.2, ATCC TIB 73), and buffalo rat liver (BRL 3A, ATCC CRL 1442). Cells were

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cultured in **Dulbecco's modified Eagle medium with 5% fetal calf serum (DMEM-5% FCS)**[g] in an incubator at 37 C with 5% CO_2. Primary cultures of bovine corneal epithelial cells were prepared by digestion of corneal stroma with neutral protease and collection of sheets of epithelial cells, as described elsewhere.[15] Bovine corneal epithelial cells were cultured in medium composed of 1 part DMEM-5% FCS and 1 part 3T3 fibroblast-conditioned medium.[14] Cells were maintained at 37 C in an incubator with 5% CO_2 and were passaged once.

Cell detachment assays—Cultured mammalian cells were subjected to trypsin and plated in 96-well tissue culture plates[h] at a concentration of 3×10^4 cells/well in a total volume of 100 ml of medium. Unless specified otherwise, transformed hamster corneal epithelial cells were used in all experiments. After cells were allowed to attach for 2 hours, 100 µl of *M bovis* culture filtrate was added to each well.

After a 2-hour incubation in an incubator at 37 C with 5% CO_2, wells were washed 3 times to remove detached and loosely adhered cells by immersing the plate in an upright position into a container of PBS solution and then pouring the solution into a waste receptacle. After the third wash, 100 µl of DMEM-5% FCS, 100 µl of PBS solution, and 100 µl of 0.02% neutral red dye[i] were added to each well. Plates were incubated for 2 hours and then washed 3 times with PBS solution to remove excess stain. Incorporated stain was eluted with 200 µl of 50% ethanol-1% acetic acid, and the OD 570 of the eluant was determined on a microplate reader. All evaluations were performed in duplicate. Control wells were incubated with PBS solution or TSB, and percentage of cell loss was calculated by use of the following formula:

([OD 570 control sample–OD 570 experimental sample]/OD 570 control sample) × 100

A modification of the aforementioned experimental protocol was used to determine viability of detached cells. After incubating cultured cells with *M bovis* culture filtrate for 2 hours, an aliquot of cells was removed by use of a pipette; this aliquot was added to an equal volume of 0.4% trypan blue dye[j] in PBS solution. Cells were incubated with trypan blue dye for 3 minutes and then were microscopically observed on a hemocytometer to determine the percentage of viable cells. A minimum of 200 cells was evaluated in each assessment.

Protease inhibitors—To determine the effect of protease inhibitors on the cell detachment assay, *M bovis* culture filtrate was separated into aliquots. One aliquot was left untreated; a protease inhibitor was added to each of the other aliquots. Protease inhibitors were added as follows: antipain at a final concentration of 50 µg/ml of H_2O from a stock solution of 20 mg/ml, aprotinin at a concentration of 2 µg/ml of H_2O from a stock solution of 10 mg/ml, bestatin at 40 µg/ml of 150 m*M* NaCl from a stock solution of 1 mg/ml, chymostatin at 100 µg/ml of dimethyl sulfoxide from a stock solution of 20 mg/ml, E-64 at 10 µg/ml of ethanol from a stock solution of 20 mg/ml, leupeptin at 0.5 µg/ml of H_2O from a stock solution of 1 mg/ml, pefabloc at 0.1 mg/ml of H_2O from a stock solution of 40 mg/ml, pepstatin at 0.7 µg/ml of methanol from a stock solution of 1 mg/ml, phosphoramidon at 330 µg/ml of H_2O from a stock solution of 20 mg/ml, EDTA at 0.2 mg/ml of H_2O from a stock solution of 40 mg/ml, antithrombin III at 1 U/ml of H_2O from a stock solution of 100 U/ml, α_2-macroglobulin at 1 U/ml of H_2O from a stock solution of 100 U/ml, and phenylmethylsulfonylfluoride[k] at 400 µ*M* in methanol from a stock of 100 m*M*. All protease inhibitors except phenylmethylsulfonylfluoride were from a commercial kit.[l] A protease inhibitor was added to *M bovis* culture filtrate, and that solution then was added to transformed corneal epithelial cells. The cell detachment assay was performed as described previously. Protease inhibitors and protease inhibitor solubilization vehicles (ie, dimethyl sulfoxide, methanol, and ethanol) were added to TSB in place of bacterial culture filtrate and were included as control samples. Unless otherwise stated, results were reported as the mean of a minimum of 3 separate experiments.

Effect of trypsin or heat treatment on cell detachment—To determine protease sensitivity of *M bovis* culture filtrate activity, trypsin[m] at a concentration of 2 mg/ml, with and without soybean trypsin inhibitor[n] at a concentration of 5.4 mg/ml, was added to *M bovis* culture filtrate and allowed to incubate for 30 minutes at 37 C prior to use in cell detachment assays. To test heat sensitivity of *M bovis* culture filtrate, the latter was heated to 80 C for 30 minutes, then cooled to room temperature (22 C) prior to use in the cell detachment assay. Results were reported as the mean of 3 separate experiments.

Data analyses—For experiments that used protease inhibitors, trypsin, or heat treatment, percentage of cell detachment relative to that for the control culture supernatant was calculated as follows:

(OD 570 experimental sample/OD 570 culture supernatant control sample) × 100

Results for studies involving protease inhibitors were analyzed by use of a 1-way ANOVA, using a Tukey test as a secondary test. Data for studies involving heat or trypsin treatments were not normally distributed and were analyzed by use of a Kruskal-Wallis 1-way ANOVA on ranks, with the Dunn test as a secondary test. Statistical tests were performed, using a statistics program.[o] Values of $P < 0.05$ were considered significant.

Results

Effect of *M bovis* culture filtrate on corneal epithelial cells in vitro—Cultured corneal epithelial cells used in these experiments had typical epithelial morphologic characteristics (ie, flat and polygonal) with extensive attachments to the substrate. One to 2 hours after addition of *M bovis* culture filtrate, cells became rounded and appeared to have decreased attachment to the tissue culture well (**Fig 1**). Appearance of the cells did not change appreciably after overnight incubation. In contrast, cells incubated with PBS solution or TSB did not have altered morphology.

Analysis of results of tests to determine the proportion of cells treated with *M bovis* culture filtrate that detached indicated that approximately half the *M bovis* filtrate-treated cells detached from the plates, compared with cells in control wells incubated with PBS solution or TSB (**Fig 2**). The proportion of cells detached was almost maximal after incubation of cells with *M bovis* culture filtrate for 2 hours. Therefore, all subsequent experiments were performed with a 2-hour incubation.

Trypan blue dye was used to determine the viability of detached cells. Less than 5% of cultured corneal epithelial cells incubated with *M bovis* culture filtrate

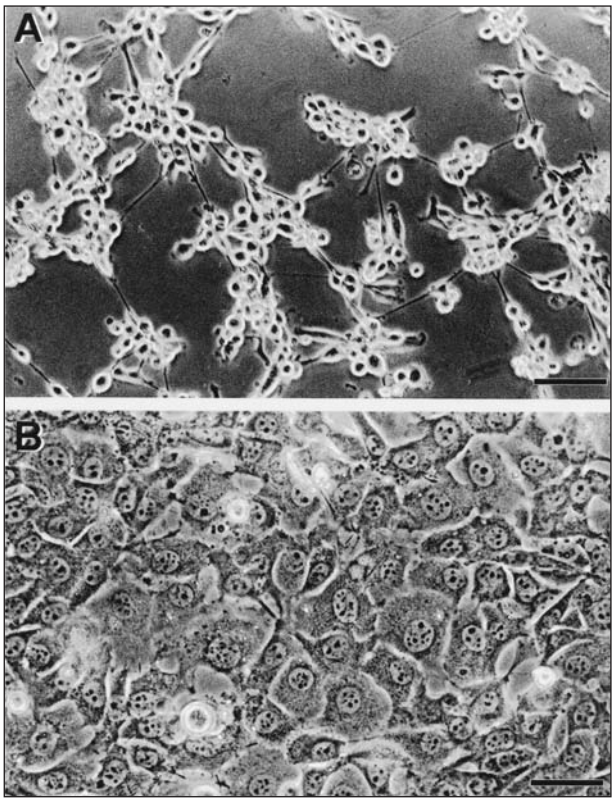

Figure 1—Morphologic appearance of transformed hamster corneal epithelial cells incubated for 2 hours with culture filtrate from *Moraxella bovis strain* 118 F (A) or with tryptic soy broth (B). Notice that cells incubated with bacterial culture filtrate become rounded and partially detached from wells. Unstained. Bar = 100 μm.

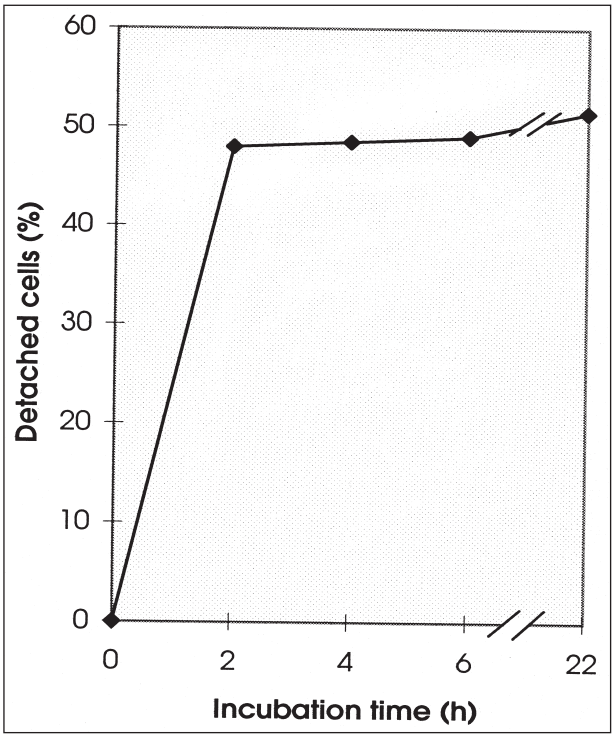

Figure 2—Representative graph depicting the time course for percentage of detached cultured corneal epithelial cells after initiating incubation with culture filtrate from *M bovis* strain 118 F.

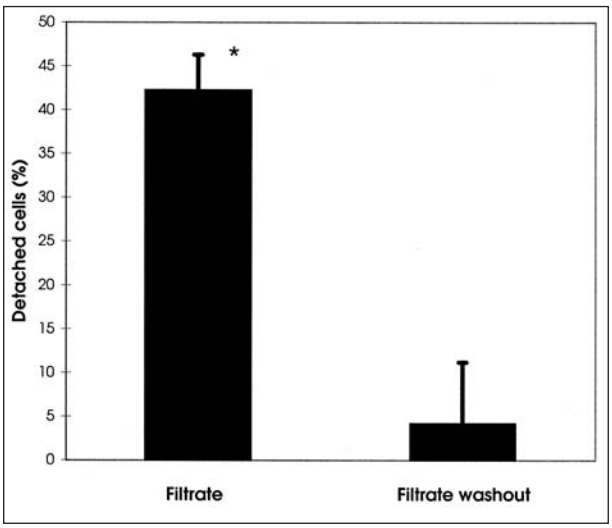

Figure 3—Graph of the percentage of detached cells as an indicator that the effect of *M bovis* culture filtrate on transformed hamster corneal epithelial cells is reversible. Filtrate represents cultured corneal epithelial cells incubated overnight with *M bovis* culture filtrate. Filtrate washout represents cultured corneal epithelial cells incubated overnight with *M bovis* culture filtrate, after which the bacterial culture filtrate was removed and replaced with mammalian cell culture medium, and cells then were incubated for an additional 2 hours. Results are mean ± SD from 3 experiments. *Differs significantly ($P = 0.001$) from value for filtrate washout group.

for 2 hours or overnight had trypan blue staining, indicating that the detached cells were alive.

On the basis of these results, we postulated that because the cells were alive after incubation with *M bovis* culture filtrate, the effect of bacterial filtrate on these cells should be reversible. To test this hypothesis, corneal epithelial cells were incubated overnight with *M bovis* culture filtrate. After incubation, the bacterial culture filtrate was removed from 1 group of wells and replaced with a 50:50 solution of DMEM-5% FCS:PBS solution (filtrate washout). Both groups of cells were incubated for an additional 2 hours, washed, and stained with neutral red, as described previously. Removal of bacterial culture filtrate followed by an additional 2-hour incubation resulted in increased cell attachment, compared with wells in which cells had been incubated continuously with culture filtrate (**Fig 3**).

The time course for cell detachment activity of *M bovis* culture filtrate was determined (**Fig 4**). Little cell detachment was observed after 2 hours of bacterial growth, but cell detachment was maximal by 6 hours of growth. Interestingly, cell detachment was less in cultures grown overnight. Therefore, 6 hours was chosen as the standard duration for culturing to obtain maximal cell detachment. Effects of culture filtrate on detachment of corneal epithelial cells was decreased with dilution; a 3-fold dilution of bacterial culture filtrate with TSB resulted in a 3-fold loss of cell detachment activity (data not shown).

Effect of *M bovis* culture filtrate on various cell types—The cell detachment assay was performed with epithelial and mesenchymal cells to determine whether *M bovis*-induced cell detachment activity was specific for corneal cells. All cell types tested were detached by

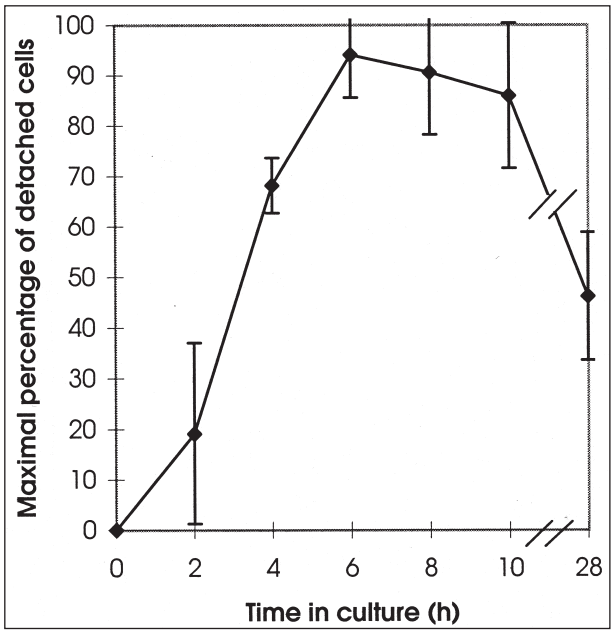

Figure 4—Graph of the time course for percentage of cells detached after initiating culture with *M bovis* culture filtrate. *Moraxella bovis* strain 118 F was grown in tryptic soy broth for varying periods, and culture filtrate was assayed for cell detachment activity. Results are mean ± SD from 3 experiments.

Table 1—Effect of protease inhibitors on cell detachment

Protease inhibitor	Percentage of control cell detachment*
Phenylmethylsulfonylfluoride	9.6 ± 10.3
α_2-Macroglobulin	1.6 ± 6.6
Antipain	98.0 ± 4.5
Aprotinin	87.8 ± 8.5
Bestatin	92.2 ± 9.0
Chymostatin	93.6 ± 11.0
E64	89.9 ± 6.2
Leupeptin	90.5 ± 3.7
Pepstatin	82.8 ± 3.6
Phosphoramidon	89.1 ± 10.1
Pefabloc	99.4 ± 13.7
EDTA	94.4 ± 5.7
Antithrombin III	93.7 ± 14.0

**Moraxella bovis* culture filtrate was separated into aliquots, and a protease inhibitor was added to each aliquot. Untreated aliquots served as control samples.

M bovis culture filtrate. The effect of the culture filtrate on various mammalian cell types was determined. Percentage of cells that were detached was as follows: human embryonic intestinal cells, 48%; embryonic mouse fibroblasts, 26%; mouse embryonic liver, 41%; buffalo rat liver, 59%; and bovine corneal epithelial cells, 26%.

Effect of protease inhibitors on cell detachment activity—The morphologic appearance of corneal cells exposed to *M bovis* culture filtrate was similar to that of cells treated with trypsin for passaging, suggesting that cell detachment activity may be attributable to a protease. Therefore, we added various protease inhibitors to culture filtrate prior to incubation with cultured corneal epithelial cells in cell detachment assays. The serine protease inhibitor phenylmethylsulfonylfluoride and the broad-specificity protease inhibitor α_2-macroglobulin significantly ($P < 0.001$) inhibited cell detachment activity (**Table 1**), but none of the other protease inhibitors tested significantly inhibited cell detachment activity. Incubation of cells with protease inhibitors alone or solubilization vehicles alone in TSB revealed that the protease inhibitors and vehicles themselves did not have an effect on cultured cells (data not shown).

Effect of trypsin or heat treatment—Treatment of *M bovis* culture filtrate with trypsin prior to incubation with cell cultures significantly decreased cell detachment activity to (mean ± SD) 14.8 ± 1.5% of that for untreated culture filtrate, indicating that cell detachment activity was attributable, at least in part, to a protein. Concurrent addition of soybean trypsin inhibitor along with trypsin neutralized the effect of trypsin on cell detachment activity, indicating that trypsin was abolishing cell detachment through enzymatic action. To define the heat stability of the factor responsible for cell detachment activity, heat treatment of *M bovis* culture filtrates significantly reduced cell detachment activity to 14.7 ± 1.5% that for untreated culture filtrate.

Discussion

The effect of *M bovis* infection on bovine corneal epithelial cells has been the subject of several reports. Electron microscopy has revealed in vivo intercellular separation and sloughing of corneal epithelium following infection of eyes of cattle with virulent strains of *M bovis*.[16-18] However, those studies did not establish the mechanism responsible for this phenomenon.

Several in vitro studies have also addressed the issue of whether *M bovis* is cytotoxic to corneal epithelial cells. Moore and Rutter[19] found that incubation with *M bovis* caused cultured calf corneal epithelial cells to detach from coverslips; similarly, Gray et al[12] reported that *M bovis* culture filtrates caused monolayers of bovine corneal epithelial cells to become rounded and to detach from each other. In both of those reports, the conclusion was that cytotoxic activity was documented; however, definitive data were not presented that could be used to argue for or against a cytotoxic effect. Schurig et al[13] studied the cytotoxicity of *M bovis* for several cell types by assaying trypan blue uptake. In that study, investigators reported that *M bovis* organisms were able to kill goat macrophages and bovine monocytes in vitro, but they did not affect the viability of bovine corneal epithelial cells. Analysis of results of the study reported here indicates that *M bovis* culture filtrates do affect corneal epithelial cells in vitro, but the effect is reversible for rounding and detachment, because cell viability is not altered. The previously reported in vivo[16,18] and in vitro data[12,19] documented rounding and intercellular separation of corneal epithelial cells, which are consistent with the cell detachment activity described in our report.

Several other bacteria can cause nonlethal detachment of mammalian cells in culture, including enteropathogenic *Escherichia coli*,[20] *Treponema denticola*,[21] and 2 species of staphylococci.[22-24] *Staphylococcus hyicus* and *S aureus* produce exfoliative toxins (exfoliatins) that cause exfoliation of epithelial cells in vivo through dis-

ruption of cell detachments in the stratum granulosum layer of the epidermis of their host species.[22-24] Similar to the cell detachment activity observed for the *M bovis* culture filtrate, exfoliatins cause rounding of cultured cells without apparent cytotoxic activity[22,23] and are inhibited by serine protease inhibitors.[25,26] On the basis of the aforementioned similarities, we postulate that the cell detachment activity observed in the experiments reported here may be the result of an exfoliatin. Studying the effect of purified *M bovis*-induced cell detachment on the morphologic characteristics of intact corneas as well as skin may help to elucidate whether the putative factor functions as an exfoliatin.

It is possible that *M bovis* produces a cytotoxin that was not detected in our experiments. The bacterial culture filtrate may have contained an inadequate concentration of cytotoxin to cause measurable cytotoxic activity. Additional studies to determine whether intact *M bovis* organisms possess cytotoxic activity are being conducted.

We believe that cell detachment activity induced by *M bovis* may play an important role in the pathogenesis of infectious bovine keratoconjunctivitis. A number of *M bovis* strains have been studied in our laboratory, and each apparently induces cell detachment activity. The action of the factor responsible for cell detachment may explain, at least in part, the unusual ability of *M bovis* to invade an intact cornea.

[a]Troutt HF, Pierson FW, Schurig GG. Scanning electron microscopy of *Moraxella bovis* on mouse corneal epithelium (abstr), in *Proceedings*. Conf Res Workers Anim Dis, 1981;10.
[b]Provided by Dr. Ricardo Rosenbusch, Veterinary Medical Research Institute, College of Veterinary Medicine, Iowa State University, Ames, Iowa, 50011.
[c]Brain-heart infusion sheep blood agar, Remel Laboratories, Lenexa, Kan.
[d]Tryptic soy broth, Remel Laboratories, Lenexa, Kan.
[e]S3 Y30, Amicon, Beverly, Mass.
[f]American Tissue Culture Collection, Rockville, Md.
[g]DMEM-5% FCS, Sigma Chemical Co, St Louis, Mo.
[h]96-well tissue culture plates, Becton Dickinson, Lincoln Park, NJ.
[i]0.02% neutral red dye, Sigma Chemical Co, St Louis, Mo.
[j]0.4% trypan blue dye, Sigma Chemical Co, St Louis, Mo.
[k]Phenylmethylsulfonylfluoride, Sigma Chemical Co, St Louis, Mo.
[l]Protease Inhibitor Set, Boehringer Mannheim, Indianapolis, Ind.
[m]Trypsin, Catalog No. S-8642, Sigma Chemical Co, St Louis, Mo.
[n]Soybean trypsin inhibitor, Sigma Chemical Co, St Louis, Mo.
[o]SigmaStat, Jandel Scientific, San Rafael, Calif.

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