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PATHOGENESIS OF CORNEAL AND CONJUNCTIVAL LESIONS CAUSED BY MORAXELLA BOVIS IN GNOTOBIOTIC CALVES

*Iowa State University* PH.D. 1987

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# Pathogenesis of corneal and conjunctival lesions caused by *Moraxella bovis* in gnotobiotic calves

by

Douglas Gress Rogers

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
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# GENERAL INTRODUCTION

Infectious bovine keratoconjunctivitis is the most important ocular disease of cattle. Moraxella bovis is the causative bacterium, although the suspected role of ultraviolet irradiation and other environmental factors in the pathogenesis is well-documented.[2,81] Clinically, the disease is characterized by varying degrees of blepharospasm, photophobia, lacrimation, conjunctivitis, keratitis, and corneal ulceration. Most epizootics of the disease occur during the summer months,[34,97] although a limited number of cases may occur during the winter months.[30,60] Highest incidence of the disease is in calves, but adult cattle can be severely affected in nonimmune herds.[7,34,59] In 1976, the United States Department of Agriculture estimated that 20% of the 48 million calves born annually in the United States and 10% of the 30 million feedlot cattle were afflicted with infectious bovine keratoconjunctivitis.[94] Economic losses in the United States due to decreased weight gain, drop in milk production, and treatment are currently estimated to exceed $200 million annually.[81]

Conventional calves do not always develop lesions after instillation of M. bovis into the conjunctival sac.[4] In addition, gnotobiotic calves do not develop lesions as readily as conventional calves after instillation of M. bovis.[15] This has led to the hypothesis that M. bovis can cause lesions readily only in the presence of predisposing factors such as ultraviolet irradiation,[36,37,38,52] Mycoplasma bovoculi,[84] or other infectious and physical agents[4,15,81]

Few papers describing the pathology of infectious bovine keratoconjunctivitis have been published.[12,81,83] There are no reports describing the pathogenesis of lesions caused by M. bovis in gnotobiotic calves protected from predisposing environmental factors.

The objectives of this study were i) to cause keratoconjunctivitis in gnotobiotic calves using a virulent strain of M. bovis, ii) to characterize the development of corneal and conjunctival lesions by light, scanning and transmission electron microscopy and, iii) to examine lacrimal tissues for evidence of histologic changes.

This dissertation is presented in the alternate format and consists of two manuscripts that have been submitted to Veterinary Pathology. The format used is that of Veterinary Pathology. The review of the literature precedes the first manuscript. A general summary and discussion follows the last manuscript. A list of references appears at the end of each manuscript. Literature cited in the introduction, literature review, and general summary and discussion appears at the end of the dissertation.

The Ph.D. candidate, Douglas G. Rogers, was the principal investigator for each study.

# LITERATURE REVIEW

Historical background Since Billings first described "keratitis contagiosa" in 1889,[5] infectious bovine keratoconjunctivitis (IBK) has been observed and studied throughout the world. The etiology often is considered to be a combination of physical factor and infectious agent. Today, IBK is regarded as a syndrome rather than a specific disease because of its obscure pathogenesis.[81]

During the past six decades, most research has been directed towards the etiology of IBK. Many infectious agents have been considered as etiologic agents, including bacteria, viruses, and rickettsia-like organisms. According to some investigators,[68] Mitter in India was the first to associate a Gram-negative diplobacillus with IBK. This bacterium resembled a human pathogen described earlier by Morax and Axenfeld (Moraxella lacunata). He was unable to reproduce the disease using this organism so he postulated that "an abraded surface" was probably necessary to produce the disease.[49]

In 1919, Allen isolated a Gram-negative diplobacillus from cattle with IBK. He was able to reproduce the disease in one calf by swabbing its eyes with exudate from infected animals. The disease was not produced in another calf after conjunctival instillation of the organism.[1]

In 1923, Jones and Little isolated a hemolytic, Gram-negative diplobacillus from cattle with a disease they termed "infectious ophthalmia." They reproduced the disease in four calves by conjunctival instillation of the organism. The disease produced was

milder than naturally occurring IBK. Mice, rabbits and guinea pigs failed to develop disease after conjunctival instillation of the organism.[41]

Hauduroy et al., in 1937, classified the organism isolated by Jones and Little as Hemophilus bovis.[27] Lwoff later placed this organism in the genus Moraxella.[47]

A bacteriologic and transmission study conducted by Baldwin in 1945 suggested an etiologic relationship between Moraxella bovis (M. bovis) and IBK. He cultured the eyes of twenty normal cattle but did not isolate M. bovis. M. bovis was isolated from cattle with IBK and produced the disease in healthy calves after conjunctival instillation. Calves used in these transmission experiments developed more severe disease during the summer months than during the winter months. Some calves were resistant to re-infection with M. bovis.[2]

Reid and Anigstein published findings similar to those of Baldwin in 1945. They transmitted the disease by direct transfer of ocular and nasal exudate from infected to normal cattle. Conjunctival instillation of the M. bovis culture also produced disease in eighteen calves, one sheep, and two goats. They concluded that this organism was the etiologic agent of IBK.[83]

In 1953, Jackson reported that M. bovis was isolated in most outbreaks of IBK observed in Texas, but it was never isolated from normal cattle. He was able to transmit the disease by direct transfer of ocular and nasal exudate from infected to normal cattle. The experimental disease was most severe during the summer months and the

Hereford breed was more severely affected. Cattle with pigmentation of the eyelids or around the eyes were as susceptible as those with less pigmentation. Rabbits were resistant to disease after conjunctival instillation of M. bovis, but developed the disease after subconjunctival and intracorneal injection.[40]

Henson and Grumbles reported on similar transmission experiments in Texas in 1960. Their findings suggested that M. bovis, and not a rickettsia-like organism, was the etiologic agent of IBK.[28]

Although some investigators succeeded in reproducing the disease with M. bovis, others were unsuccessful. Using strains of M. bovis obtained from Baldwin, Farley et al. instilled viable cultures into the conjunctival sac of calves but could not reproduce the disease. They concluded that M. bovis was not the primary etiologic agent.[20]

In 1957, investigators in India isolated M. bovis from cattle with IBK, but they could not reproduce the disease by conjunctival instillation despite numerous attempts. They suggested that a virus could be the etiologic agent.[82]

Sykes et al., in Texas, isolated infectious bovine rhinotracheitis virus from cattle with clinical signs of IBK. They reproduced the disease using the virus, but corneal lesions were not produced in all cattle. An etiologic role for infectious bovine rhinotracheitis virus was suggested.[93]

Pugh et al. demonstrated that infectious bovine rhinotracheitis virus inoculated concomitantly with M. bovis could cause keratoconjunctivitis typical of IBK. The virus by itself caused only

conjunctivitis. They concluded that infectious bovine rhinotracheitis virus was not the etiologic agent, but it could enhance the pathogenic effects of M. bovis.[69]

A five-year study of IBK in a cattle herd indicated a direct relationship between the intensity of solar ultraviolet irradiation, number of isolations of M. bovis, and the incidence of IBK.[34] The importance of ultraviolet irradiation in the etiology of IBK was first demonstrated experimentally by Hughes et al. Calves exposed to only ultraviolet irradiation (sunlamp) did not develop disease. Hemolytic M. bovis given alone occasionally produced mild disease. Exposure to ultraviolet irradiation followed by conjunctival instillation of hemolytic M. bovis produced lesions typical of IBK.[37,38] Since then, other investigators have confirmed the etiologic importance of ultraviolet irradiation.[52,56,64]

More recently, Chandler et al. reported that conventional calves develop lesions more readily than gnotobiotic calves after instillation of virulent M. bovis into the conjunctival sac. They suggest that environmental factors may influence lesion development in conventional calves.[15]

Characteristics of Moraxella bovis M. bovis is a non-motile, Gram-negative diplobacillus measuring 0.5 μm to 1.0 μm by 1.5 μm to 2.0 μm. The diplobacilli occur in pairs or in small chains. M. bovis is oxidase-positive and catalase-variable. It does not reduce nitrates or ferment carbohydrates. In litmus milk, the medium becomes alkaline

and progressively peptonized.[68] Freshly isolated strains are encapsulated.[2]

Virulent strains of M. bovis produce flat, friable, agar-corroding colonies with zones of β-hemolysis on blood agar. Initially the colonies are translucent and gray, measuring 1mm to 3mm in diameter. Most colonies develop a raised area in the center.[68]

M. bovis must have pili to adhere to ocular tissues.[56] The pili are elongate, unbranched, and originate from the outer wall of the bacterium. They have a peritrichous distribution and measure between 6.5nm and 8.5nm in diameter.[90] There are several pilus types,[88] but some types share common antigens.[45] Loss of pilus expression by M. bovis leads to dissociative changes in colony morphology. Some investigators describe colonies of virulent M. bovis as smooth that later dissociate to rough.[61] Others describe the virulent colonies as rough that later dissociate to smooth.[88] Crystal violet dye will stain virulent colonies. This staining technique should aid in the identification of M. bovis colonies.[89]

Piliated strains of M. bovis autoagglutinate when suspended in physiological saline and other solutions. Autoagglutination can be inhibited by the addition of 10% magnesium chloride to liquid media. This does not alter the virulence, viability, or morphology of M. bovis and can be used to estimate bacterial numbers.[63] Auto-agglutinating strains agglutinate red blood cells of the chicken, rabbit, sheep and pig. Hemagglutination is not inhibited by D-mannose or D-galactose.[26]

All virulent strains of M. bovis produce hemolysin.[29,64] The hemolysin is labile, cell-associated,[29,55] and is inactivated by heat, formalin and trypsin.[51,55,87] It is not a phospholipase.[21] M. bovis can change from hemolytic to nonhemolytic, or from nonhemolytic to hemolytic both in vivo and in vitro.[64] Reversion from hemolytic to nonhemolytic does not correlate with the loss of pili .[59,87] Nonhemolytic M. bovis is frequently isolated from cattle during the winter months, whereas hemolytic M. bovis is more commonly isolated during the summer months.[7,34] The reasons for this are unknown although the mutagenic effects of solar ultraviolet irradiation are suggested to cause avirulent nonhemolytic M. bovis to revert to virulent hemolytic M. bovis during the summer months.[64]

Virulent strains of M. bovis also produce lipases, proteases, and other enzymes, but they do not produce collagenase.[21] A cell-associated "dermonecrotic toxin" has been described by Henson and Grumbles.[29] The role of toxins and bacterial enzymes in the pathogenesis of IBK is unknown.

Plasmids from M. bovis have been partially characterized, but the relationship of these plasmids to virulence is unknown.[48,89] Plasmid profiles could be useful for identifying strains of M. bovis.[48]

Virulence Strains of M. bovis isolated from cattle with IBK differ in virulence.[9,62] Conjunctival instillation of two highly virulent strains, GS and Epp-63(300), can occasionally cause lesions in conventional calves.[9,12,36,37,64,69] However, gnotobiotic calves do not develop lesions as readily as conventional calves after

conjunctival instillation of the GS strain or some other virulent strains.[15] The GS strain can cause lesions in C57 Bl mice, particularly when the mice are pre-treated with a corticosteroid.[14] The lesions produced in mice resemble those produced in cattle.[13] Other laboratory animal species and other strains of mice may become infected with the GS strain, but they do not develop lesions.[14] The Epp-63(300) strain can occasionally cause lesions in mice,[62] particularly when the eyes of mice are exposed to ultraviolet irradiation prior to the instillation of the organism.[60,67] Sheep, rabbits, rats, and guinea pigs, even when exposed to ultraviolet irradiation, do not develop lesions after the conjunctival instillation of Epp-63(300) or some other virulent strains.[67]

Predisposing factors Most epizootics of IBK occur during the summer months[34,97] and correlate with the annual peak of solar ultraviolet irradiation.[34] Exposure to simulated ultraviolet irradiation alone, or to hemolytic M. bovis alone can occasionally cause a mild keratoconjunctivitis.[38] Exposure to ultraviolet irradiation followed by exposure to hemolytic M. bovis causes the severe lesions of IBK.[36,37,38,64] Irradiation damages the corneal epithelium and could facilitate the adherence by M. bovis.[95]

Mycoplasma bovoculi causes conjunctivitis[85] and is frequently isolated from cattle with IBK.[22,85] In one study, exposure to Mycoplasma bovoculi followed by exposure to hemolytic M. bovis caused keratitis. Exposure to ultraviolet irradiation followed by exposure to hemolytic M. bovis, or exposure to hemolytic M. bovis alone did not

cause keratitis. Mycoplasma bovoculi apparently extends ocular colonization by M. bovis and enhances the induction of keratitis.[84]

Although infectious bovine rhinotracheitis virus is not the primary etiologic agent of IBK, it could play a secondary role. Exposure to infectious bovine rhinotracheitis virus concomitantly with hemolytic M. bovis causes keratoconjunctivitis that is more severe than that caused by exposure to hemolytic M. bovis alone.[69]

Bos indicus cattle are more resistant to IBK than are Bos taurus cattle.[16,23,71,91,96] The secretory immune systems of these genetic lines are different and this may explain the differences in susceptibility.[3] There also are differences in susceptibility between breeds. Results of most surveys indicate that the Hereford breed is most susceptible.[96,97] These surveys also indicate that the amount of eyelid pigmentation can influence susceptibility within a breed; complete eyelid pigmentation reduces the incidence of IBK.[8,96] Genetic factors other than ocular pigmentation could be responsible for individual variation of susceptibility within a breed.[71,78]

Lacrimal secretions from European breeds of cattle reportedly lack lysozyme. It has been suggested that this deficiency may predispose cattle to IBK.[46]

There is an indirect relationship between the age of the animal and susceptibility to IBK.[7,65,74] Because IBK is enzootic in most herds, many investigators believe that most adult cattle are partially immune because of prior exposure to M. bovis.[34,35,53]

The face fly (Musca autumnalis), because of its preference for the area around the eye, is believed to be an important vector of M. bovis[6,25,92] and can harbor the organism for up to 3 days.[92] Feeding activity of the fly reportedly damages the conjunctiva which could predispose the eye to infection by M. bovis.[6] Other insects such as the house fly (Musca domestica), and the stable fly (Stomoxys calcitrans), could play a similar role.[34]

Other physical agents that could irritate ocular tissues such as dust, tall grass, grass seeds,[16] and plant pollen[24] have been suggested as predisposing factors in IBK.

Clinical signs The clinical signs of IBK vary from herd to herd and between individuals within the same herd. Initial signs are those of ocular pain, namely, blepharospasm, photophobia, and serous lacrimation. The ocular discharge soon becomes mucopurulent, causing hair on the face and eyelids to become matted. Conjunctivae of the eyelids, nictitating membrane, and globe are hyperemic and edematous. These signs are usually followed in 24 to 96 hours by the appearance of a central corneal vesicle, erosion, or ulcer. Corneal lesions may regress in the early stages or continue to progress. With progression, extensive stromal edema and vascularization extending from the limbus is usually evident within one week after onset of the disease; there may be complete opacity of the cornea. The majority of corneal ulcers heal in 4 to 6 weeks, but uncomplicated ulcers may heal in less time. A small opaque scar may remain after healing.

Sequelae of severe IBK can include keratoconus, descemetocele formation, iridocyclitis, hypopyon, synechia, staphyloma, glaucoma, and blindness. Although rare, corneal perforation may lead to phthsis bulbi, panophthalmitis, and occasionally meningitis.[4,42,81]

Pathology There are few published reports describing the pathology of IBK.[81] In 1945, Reid and Anigstein described the histologic changes in conjunctival biopsies from cattle with clinical signs of a disease they called IBK. In early stages, conjunctival epithelium of the eyelids was swollen and the "tissue" was hyperemic and edematous. The inflammatory cell infiltrate was primarily lymphocytic with small numbers of "mononuclear cells and plasma cells." More chronic lesions were characterized by hyperplasia of conjunctival epithelium, hyperemia, and edema. Neutrophils were present in conjunctival epithelium and "subacute inflammation" was present "beneath the epithelium and superficial connective tissue." Lymphoid follicles of the eyelids were hyperplastic. Rickettsia-like organisms were present in epithelial cells in conjunctival smear preparations.[83]

Chandler et al. produced corneal ulcers in conventional calves, but in only one gnotobiotic calf, by instilling virulent M. bovis into the conjunctival sac.[15] Early histologic changes in the conventional calves included loss of corneal epithelium and the presence of neutrophils at the base of the ulcers. Bacteria resembling M. bovis were associated with the base of the ulcers and were occasionally seen "within the lighter staining, altered epithelium bordering the ulcers." In later stages, bacteria resembling M. bovis were present near the

surface of ulcers. Neutrophils, lymphocytes, and increased numbers of fibroblasts were present in the vascularized stromas. Ultrastructural examination showed fibrin deposits, edema, neutrophils, and cellular debris in the stromas at all stages; macrophages were common at later stages. Bacteria were rarely seen although "structures resembling small microbial agents of various shapes" were present in the stromas. It was suggested that these could be "small forms of M. bovis or rickettsia-like organisms." The lesion in the gnotobiotic calf was similar to early lesions in the conventional calves, however, bacteria resembling M. bovis were more readily identified ultrastructurally at the base of the ulcer. These experimentally produced lesions were similar to lesions in calves with the naturally occurring disease.[12]

Scanning electron microscopic examination of corneas from calves exposed to ultraviolet irradiation and M. bovis showed ulceration and areas of swollen and sloughed surface epithelium. Fibrin, inflammatory cells, and debris were present on the corneal surface; small numbers of bacteria were seen only before the onset of ulceration.[95]

Virulent strains of M. bovis selectively colonize dark cells on the corneal surface in vitro.[10,11,39] "Pit-like depressions" on the surface of these cells are believed to result from activity of a "pitting factor" produced by M. bovis.[11]

Immunity and vaccination Secretory immunoglobulin A (SIgA) is the predominant immunoglobulin in bovine tears and is produced in lacrimal tissue. In addition to SIgA, smaller amounts of $IgG_1$, $IgG_2$ and IgM are present in tears. The bulk of these immunoglobulins is

derived from the serum, although some synthesis may occur in lacrimal tissue.[17,57]

Although SIgA predominates in bovine tears, its role in resistance to IBK is unclear. In one study, antibodies specific for M. bovis were monitored in the tears of calves with naturally occurring IBK. Antibodies of the IgG and IgM classes had the greatest specificity for M. bovis, but neither they nor SIgA were protective against the development of IBK.[43] Nayar and Saunders demonstrated an increase in SIgA levels in the tears of calves exposed to ultraviolet irradiation and challenged with M. bovis.[53] The calves were more resistant to further irradiation and challenge with homologous M. bovis.[52] These investigators suggest that secretory immunity is important in the resistance to re-infection with M. bovis.[53]

The role of humoral immunity in resistance to IBK is unclear. Cattle that develop lesions of IBK produce serum antibody specific for M. bovis;[34,98] however, this antibody does not always prevent the development of lesions after re-exposure to M. bovis.[34] Cattle exposed to naturally occurring IBK or those experimentally infected with M. bovis also produce serum antibody specific for M. bovis hemolysin.[54] Some investigators have shown that resistance to re-infection with M. bovis is due, at least in part, to humoral immunity. They suggest that vaccination be directed at stimulating humoral (general) immunity.[44]

Early attempts to vaccinate against IBK included the use of both viable and nonviable M. bovis cultures. After exposure to ultraviolet

irradiation and challenge with M. bovis, calves vaccinated intramuscularly with a viable M. bovis culture develop fewer lesions than nonvaccinated calves.[33] A formalin-killed culture, given intramuscularly, is also effective.[32]

Although the formalin-killed vaccine affords partial protection against homologous strains of M. bovis, it provides little or no protection against heterologous strains.[65,70] Intramuscular vaccination with whole cells, disrupted cells, and pili fractions of M. bovis do not protect against challenge with heterologous strains, although pili afford partial protection against homologous strains.[66] Vaccines made from nonhemolytic strains of M. bovis are as effective as vaccines made from hemolytic strains.[72]

Attempts to improve the immunogenicity of M. bovis vaccines have had variable results. M. bovis pili have been incorporated with both Freund's incomplete adjuvant and diphtheria-tetanus toxoids and pertusis (DPT) vaccine. Both vaccine preparations enhance immunoresponsiveness, but they afford only partial protection against homologous challenge.[66,73] The pilus-DPT vaccine appears to be more effective when injected subconjunctivally.[75] Combining M. bovis pili with a Mycobacterium paratuberculosis bacterin does not enhance immunoresponsiveness.[79] Vaccines composed of M. bovis ribosomes are not protective.[80]

Calves fed colostrum from dams vaccinated with M. bovis pili or formalin-killed vaccines are more resistant to IBK.[71,77]

Carrier animals are believed to be an important source of M. bovis.[76] However, attempts to control or eliminate the carrier state by vaccination have had variable results.[71,76] Good management, isolation of infected animals, and treatment with antibiotics have been suggested as alternatives to vaccination.[31,71]

# PATHOGENESIS OF CORNEAL LESIONS CAUSED BY *MORAXELLA BOVIS* IN GNOTOBIOTIC CALVES

# PATHOGENESIS OF CORNEAL LESIONS CAUSED BY *MORAXELLA BOVIS* IN GNOTOBIOTIC CALVES

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## ABSTRACT

Moraxella bovis was instilled into the conjunctival sac of gnotobiotic calves and corneas were sampled serially after infection. Lesions developed in seven of eight infected calves, but were absent in a noninfected control calf. Histologically, M. bovis was first seen in foci of swollen epithelium and within basal epithelial cells adjacent to ulcers. Corneal ulcers were severe in later stages of infection; fibrin deposits, neutrophils, and bacteria were present in the stromas. Examination of early lesions by scanning electron microscopy showed M. bovis in pits on the surfaces of dark epithelial cells, enmeshed in degenerate epithelial cells and within erosions and an ulcer; in later samples, bacteria were rare. Ultrastructurally, M. bovis was seen in surface pits in superficial epithelial cell processes and within swollen epithelial cells. In stroma, M. bovis was frequently seen among collagen fibrils, within neutrophil phagosomes, and associated with cellular debris. This study demonstrates that a virulent strain of M. bovis can invade bovine corneal epithelial cells and can cause keratitis in the absence of injurious ultraviolet irradiation or other known predisposing environmental factors.

# INTRODUCTION

Piliated, hemolytic strains of the gram-negative bacterium, Moraxella bovis (M. bovis), are the principal etiologic agents of infectious bovine keratoconjunctivitis,[13,20,25] although the suspected role of other factors in the pathogenesis is well-documented.[3] Ultraviolet irradiation,[14,20,22,25] Mycoplasma bovoculi,[28] and infectious bovine rhinotracheitis virus[26] have been used in conjunction with M. bovis to cause lesions. Gnotobiotic calves do not develop lesions as readily as conventional calves after inoculation with M. bovis leading to the hypothesis that environmental factors are important in the initiation/progression of lesions in conventional calves.[10]

The primary step in the pathogenesis of infectious bovine keratoconjunctivitis is the adherence by M. bovis to corneal epithelium. By scanning electron microscopy, surface epithelium of the bovine cornea is composed of light and dark cells.[6] Light cells are younger and possess surface microplicae (ridges); dark cells are older and relatively devoid of microplicae.[6,21] Virulent strains of M. bovis selectively colonize the dark cells in vitro.[6,7,15] "Pit-like depressions" on the surface of these cells are believed to result from activity of a "pitting factor" produced by M. bovis.[7] Piliation and elaboration of this "pitting factor" appear to be necessary for M. bovis to damage corneal epithelium early in infection,[7] but adhesins other than pili may contribute to pathogenicity.[1,15]

The objectives of this study were to determine if a virulent strain of M. bovis could cause keratitis in gnotobiotic calves protected from known predisposing environmental factors and, if so, to characterize the development of corneal lesions by light, scanning and transmission electron microscopy.

## MATERIALS AND METHODS

Calves Nine gnotobiotic calves derived by open Cesarean methods were placed in portable isolator units[18] and fed three quarts of Similac (Ross Laboratories, Columbus, OH) daily until killed (Table 1). Calves were Angus, Pinzgauer, Holstein, Angus and Hereford crossbreeds of both sexes. Rooms housing isolator units were illuminated by fluorescent lights. Block-glass windows covered with white paint admitted outside light and were located approximately three feet above the calves.

Organism The M. bovis strain Epp-63(300) was serially passed four times on 5% bovine blood agar and used as the inoculum. This strain was isolated from a calf with naturally occurring infectious bovine keratoconjunctivitis and has been used in other studies.[14,23,25] The stock culture (serially passed two times on 5% bovine blood agar) had been stored at -60C. Samples were thawed, streaked onto 5% bovine blood agar plates, and incubated for 24 hours at 37C. A typical hemolytic colony was selected to inoculate each plate of a series of 5% bovine blood agar plates. After incubation for 24 hours at 37C, the surface growth was scraped from plates with an inoculating loop, suspended in tubes containing 10 ml of trypticase soy broth (TSB), and stored at -60C until used.

Inoculation and bacterial quantitation Cultures of M. bovis were thawed approximately 15 minutes prior to the inoculation into calves. Eight calves were inoculated bilaterally by instillation of 1 ml of M. bovis culture into the ventral conjunctival sac. The eyelids

were held closed for 4-5 seconds after inoculation. Aliquots of M. bovis cultures were then serially diluted in 10% magnesium chloride,[24] grown on 5% bovine blood agar for 24 hours at 37C, and the number of colony-forming units/ml were determined (Table I). One calf (normal control) received a bilateral instillation of 1 ml of TSB into the ventral conjunctival sac.

Necropsy and tissue collection Calves were killed by sodium pentobarbital overdosage followed by exsanguination. Corneas were then irrigated with 2.5% glutaraldehyde and 1.5% paraformaldehyde in .1M cacodylate buffer (pH 7.4). After removal of eyes together with eyelids and attached conjunctival sacs, corneas were dissected posterior to the limbus, immersed in the glutaraldehyde-paraformaldehyde fixative and examined for lesions with a dissecting microscope.

Preparation of tissues For light microscopy, full-diameter samples were taken from each cornea and transferred to formalin, processed routinely, and embedded in paraffin. Sections were cut at 4 μm and stained with hematoxylin and eosin (HE), phosphotungstic acid-hematoxylin, and Brown-Brenn modified Gram stain.[31]

Samples for scanning and transmission electron microscopy were fixed for 4 hours with 2.5% glutaraldehyde and 1.5% paraformaldehyde in .1M cacodylate buffer (pH 7.4) containing 0.05% ruthenium red.[12] Samples for scanning electron microscopy were then rinsed in cacodylate buffer, post-fixed in 1% osmium tetroxide, rinsed in distilled water, and dehydrated in graded acetones. After critical point drying with

Freon 13, preparations were mounted on stubs with silver paint, sputter coated with gold, and examined with a scanning electron microscope at 15-25 kv. When present, one or more macroscopic lesions (or portions) from each cornea were examined. Samples (approximately 0.5 cm x 0.5 cm) from the central and peripheral cornea were examined when there were no macroscopic lesions. Some preparations were passed through two changes of propylene oxide (2 hours each) and flat embedded in epoxy resin for correlative transmission electron microscopy.

Samples for transmission electron microscopy were embedded in molten 2% agarose, rinsed overnight in cacodylate buffer, post-fixed in 1% osmium, rinsed in cacodylate buffer, dehydrated in graded ethanols, infiltrated with propylene oxide, and flat embedded in epoxy resin. Two to four samples were taken from each macroscopic lesion. Ten to twelve samples were taken from the peripheral cornea and two samples were taken from the central cornea when there were no macroscopic lesions. One micron thick sections were cut from each block and stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope at 60 kv.

Contaminant monitoring Nasal[27] and fecal swabs (Culturette, American Scientific Products, McGaw Park, IL) were taken from calves in the isolator units prior to necropsy and analyzed for contamination (ocular swabs were not taken to avoid damaging ocular tissues). Fecal swabs were processed for aerobic and anaerobic bacteriologic culture as previously described.[18] Nasal swabs were streaked onto 5% bovine blood

agar and incubated for 24 hours at 37C. Bacterial contaminants isolated from nasal and fecal swabs were identified by biochemical methods. Nasal swabs stored at -60C were later processed[16] and eluted onto bovine kidney cells[17] and examined for 5 days for infectious bovine rhinotracheitis virus. Nasal swabs stored at -60C were later processed to detect Mycoplasma bovoculi by modification of a previously described method.[29] Briefly, swabs were moistened in 1 ml of phosphate buffered saline for 1 hour at 4C. The absorbed fluid was expressed and then centrifuged at 800 rpm for 5 minutes. Serial ten-fold dilutions ($10^{-2}$ and $10^{-3}$) of the fluid were prepared in Friis broth and incubated at 37C. Tubes were examined for acidification or turbidity in the medium for 21 days.

# RESULTS

Corneal lesions and microbial status of calves Seven of eight calves infected with M. bovis developed macroscopic or microscopic corneal lesions (Table 1). Large numbers of hemolytic M. bovis were isolated from the nares of calves 1 through 7. Small numbers of saprophytic bacteria were isolated from the nares and/or feces of calves 4, 5, 7, 8, and 9. Blood agar plates streaked with nasal swabs had 2-3 contaminant bacterial colonies. Calf 8 had mild lacrimation following inoculation, but failed to develop further clinical signs. Large numbers of nonhemolytic M. bovis and small numbers of hemolytic M. bovis were isolated from the nares of this calf. Mycoplasma bovoculi and infectious bovine rhinotracheitis virus were not isolated from any of the nine calves.

Clinical signs Calves 3 through 7 had bilateral mucopurulent conjunctivitis with varying degrees of lacrimation, chemosis, photophobia, and blepharospasm. Calf 2 had bilateral conjunctival hyperemia. The noninfected control calf 9 remained clinically normal throughout the observation period.

Light microscopy Early histologic lesions were restricted to epithelium; M. bovis was associated with foci of swollen epithelium (Fig. 1), vesicles (Fig. 2), and erosions.

A shallow ulcer was present at 10 hours post-inoculation. Epithelial cells adjacent to the ulcer were swollen; M. bovis organisms were seen within swollen epithelial cells of the basal layer and in superficial collagen lamellae of the stroma. Neutrophils, fibrin, and

necrotic debris were present at the base of the ulcer; mild perivascular accumulations of neutrophils were present in the limbus.

Corneal ulceration was severe at 24, 45, and 72 hours post-inoculation. Epithelium adjacent to ulcers was degenerate and detached. Other epithelial changes included extensive intercellular edema with foci of rounded, detached cells. Necrotic epithelial cells were common in areas of neutrophilic infiltrate. Moderate perivascular accumulations of neutrophils were present in the limbus and stromas were characterized by edema, swollen fibroblasts, and moderate to severe diffuse neutrophilic infiltrates. Moraxella bovis organisms were seen in superficial (Fig. 3, 4) and middle collagen lamellae. Vascularization of superficial collagen lamellae was present at 72 hours post-inoculation. Corneal endothelium was detached or absent from most sections at these later stages, however, neutrophils were present between Descemet's membrane and endothelium in areas where it remained intact. Intact endothelium was swollen or necrotic in areas associated with adherent neutrophils at 72 hours post-inoculation and correlated with the presence of hypopyon.

The macula from calf 8 (from which a majority of nonhemolytic M. bovis was recovered) was characterized by increased mitotic figures in the basal epithelial layer, increased numbers of fibroblasts and small numbers of chronic inflammatory cells in superficial collagen lamellae of the stroma.

Scanning electron microscopy Surface epithelial cells of the control calf's cornea were flat and polygonal with well-defined cell

borders. Mucus obscured surface detail in some areas, but epithelial cells could be classified as light, dark, or intermediate in density. Similar cell types were often grouped together. Light cells had more surface microplicae than intermediate cells. Dark cells had few or no microplicae. Microvilli were interspersed between microplicae of light and intermediate cell types. Short, blunt microvilli were present on the surface of dark cells. Round holes with raised edges were commonly seen on the surface of all cell types. Small numbers of desquamated cells were present on the surface; bacteria were not seen.

Examination of corneas from infected calves showed large numbers of M. bovis adhered to the dark cells at 1 and 2 hours post-inoculation. Fewer M. bovis were associated with other cell types. Diplobacilli occurred singly, in chains, and in clusters. Moraxella bovis, often in large numbers, was seen in pits on the surface of dark cells (Fig. 5). Fewer M. bovis were in pits between dark cells and in pits between dark and lighter cell types. Pits were more frequent at 2 hours post-inoculation. Pili were not seen on the surface of M. bovis.

At 6 hours post-inoculation, a 115 by 70 μm focus of degenerate epithelium was partially detached from the peripheral cornea. Moraxella bovis was enmeshed in and beneath degenerate epithelial cells (Fig. 6). At 4 and 6-hours post-inoculation the corneas had multiple peripheral, punctate erosions that were approximately 100 μm in diameter. Moraxella bovis, cellular debris (Fig. 7) and leukocytes were present on the surface of epithelial cells at the base of the

erosions. Bacteria were often seen in pits on the surface of these epithelial cells.

At 10 hours post-inoculation, one cornea had a 1 mm peripheral ulcer (Fig. 8) that was characterized by large numbers of bacteria, fibrin, cellular debris, mucus, and leukocytes.

Fibrin, cellular debris, mucus, and leukocytes filled the ulcers at 24, 45, and 72 hours post-inoculation. Many desquamated epithelial cells were present peripheral to the ulcers; Moraxella bovis was rarely seen at these later stages.

Transmission electron microscopy At 1 and 2 hours post-inoculation, M. bovis was seen within superficial epithelial cells, in pits in cell processes (Fig. 9), beneath cell processes, and associated with cell remnants on the corneal surface. Bacterial cells were pleomorphic, measured 0.5-1.0 μm by 1.5-2.0 μm, and had rugose cell walls. Many had electron-lucent nuclear regions with either fibrillar or condensed nuclear material. Some M. bovis had one or more electron-dense granular inclusions, but bacterial capsules and pili were not seen. Superficial epithelial cells with pits and intracellular bacteria had few surface structures and contained cytoplasmic vacuoles. Intracellular M. bovis was surrounded by electron-lucent halos. Moraxella bovis organisms beneath cell processes were not always surrounded by halos. When present, these halos often extended past disrupted plasma membranes into the cytoplasm of adjacent cells.

At 2-6 hours post-inoculation, epithelial cells of deeper layers were characterized by rarefied cytoplasm, aggregated tonofilaments, swollen mitochondria, dilated rough endoplasmic reticulum, vacuoles, clumped chromatin and karyolysis. Most swollen cells contained M. bovis and replicative bacteria were seen. Halos surrounding M. bovis often coalesced and formed lucent areas of varying size within the cells. Plasma membranes of apposing cells were disrupted in areas traversed by bacteria; many epithelial cells had undergone cytolysis. Bacteria within the basal layer were often seen in depressions in Bowman's membrane (Fig. 10). The basement membrane was often missing in areas where bacteria made direct contact.

At 10 hours post-inoculation, bacteria in the stroma occurred singly, or in small chains, and were surrounded by electron-lucent halos. The long axes of M. bovis were parallel to structurally intact collagen fibrils. Varying amounts of flocculent electron-dense material were seen in close association with some M. bovis and were most prevalent near bacterial poles (Fig. 11). Stromal fibroblasts, Descemet's membrane, and endothelium were structurally normal.

At 24, 45, and 72 hours post-inoculation, stromal collagen fibrils were displaced by precipitated plasma proteins and electron-lucent areas of edema. Fibroblasts in edematous areas were swollen and some had membranous cytoplasmic inclusions. Viable and degenerate bacteria were associated with cellular debris (Fig. 12), and were in phagosomes of viable, degranulate, and degenerate neutrophils. Small (110-270 nm) electron-dense granules were present in areas of dense neutrophilic

infiltrates. Fibrin deposits and fragments of aggregated, electron-dense collagen fibrils were seen near the surface of ulcers. Descemet's membrane was structurally normal.

The endothelium was absent from most blocks at 24,45, and 72-hours post-inoculation. Only one block from corneas sampled at 72 hours could be examined for endothelial changes. Neutrophils were adhered to and present within the endothelial layer which was partially detached from Descemet's membrane. Endothelial cells had markedly swollen mitochondria, dilated rough endoplasmic reticulum, and vacuoles; some endothelial cells had undergone cytolysis.

Fig. 1: Bacteria associated with swollen epithelial cells in superficial and polyhedral layers; 2 hours post-inoculation. Toluidine blue

Fig. 2: Focus of swollen epithelial cells surround a vesicle (*), bacteria superficial to Bowman's membrane (arrow); 4 hours post-inoculation. Toluidine blue

Fig. 3: Corneal ulcer; 24 hours post-inoculation. Stromal edema, bacteria concentrated subjacent to Bowman's membrane (arrow). Neutrophils, necrotic cells on surface. Brown-Brenn Gram stain

Fig. 4: Peripheral to corneal ulcer; 45 hours post-inoculation. Bacteria diffusely distributed in edematous superficial collagen lamellae. Neutrophils not evident in this field. Brown-Brenn Gram stain

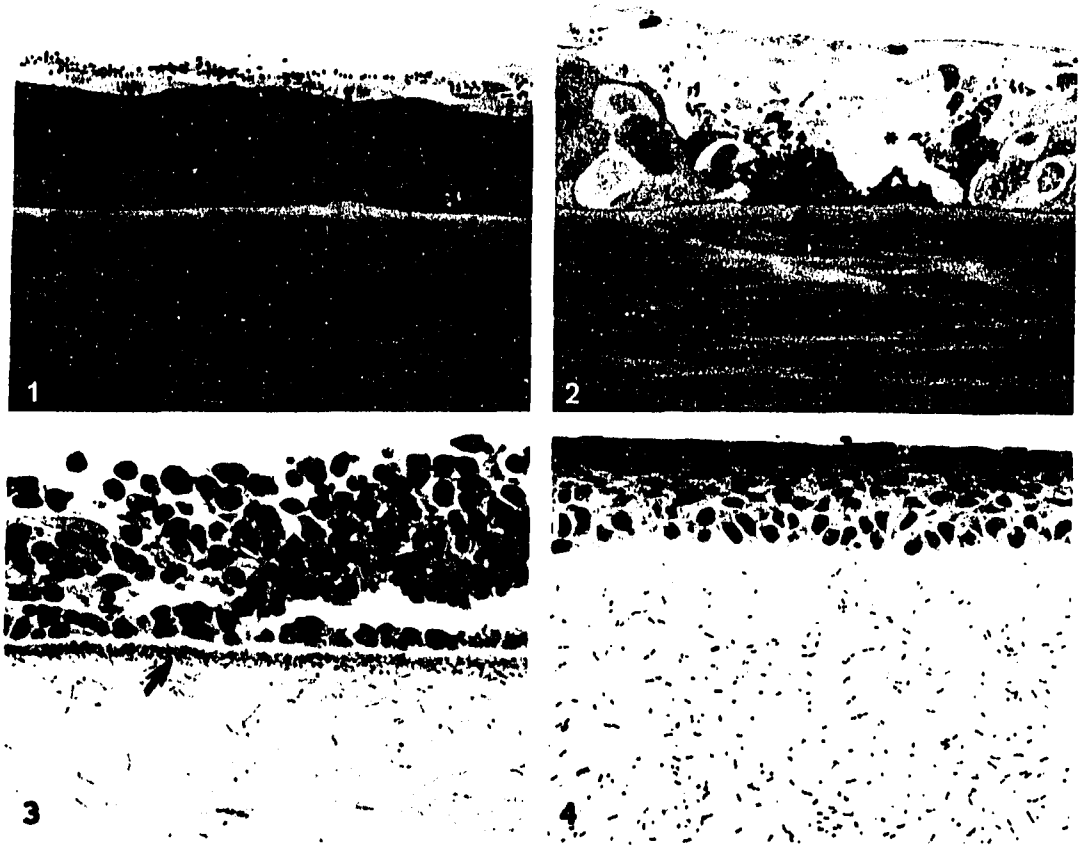
1
2
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Fig. 5: Corneal surface; 2 hours post-inoculation.

*Moraxella bovis* in a pit on surface of a dark cell.

Microvilli (Mv). Bar = 1 μm

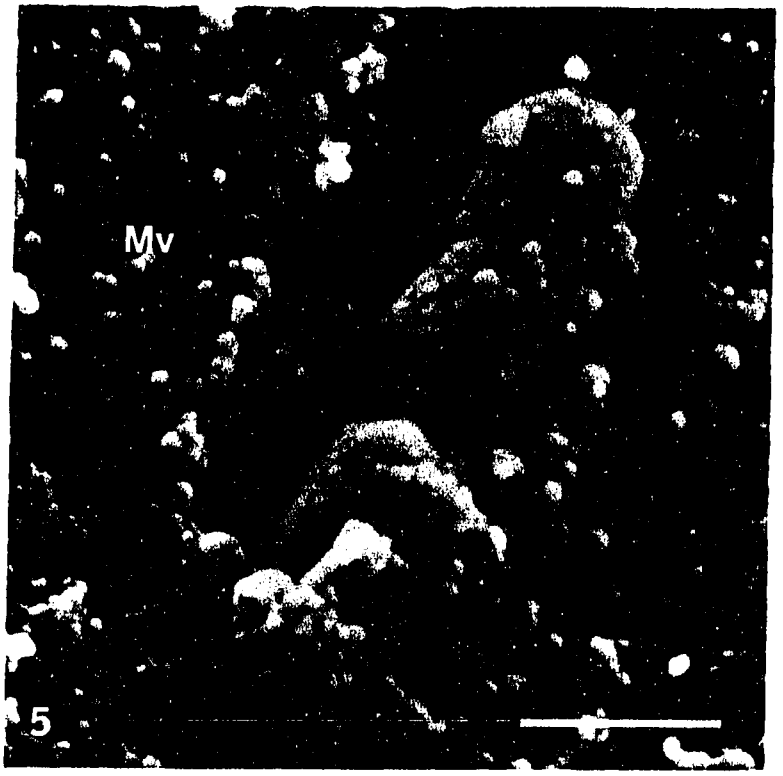
Mv
5

Fig. 6: *Moraxella bovis* enmeshed in and beneath (arrow) degenerate epithelial cells; 6 hours post-inoculation. Bar = 5 µm

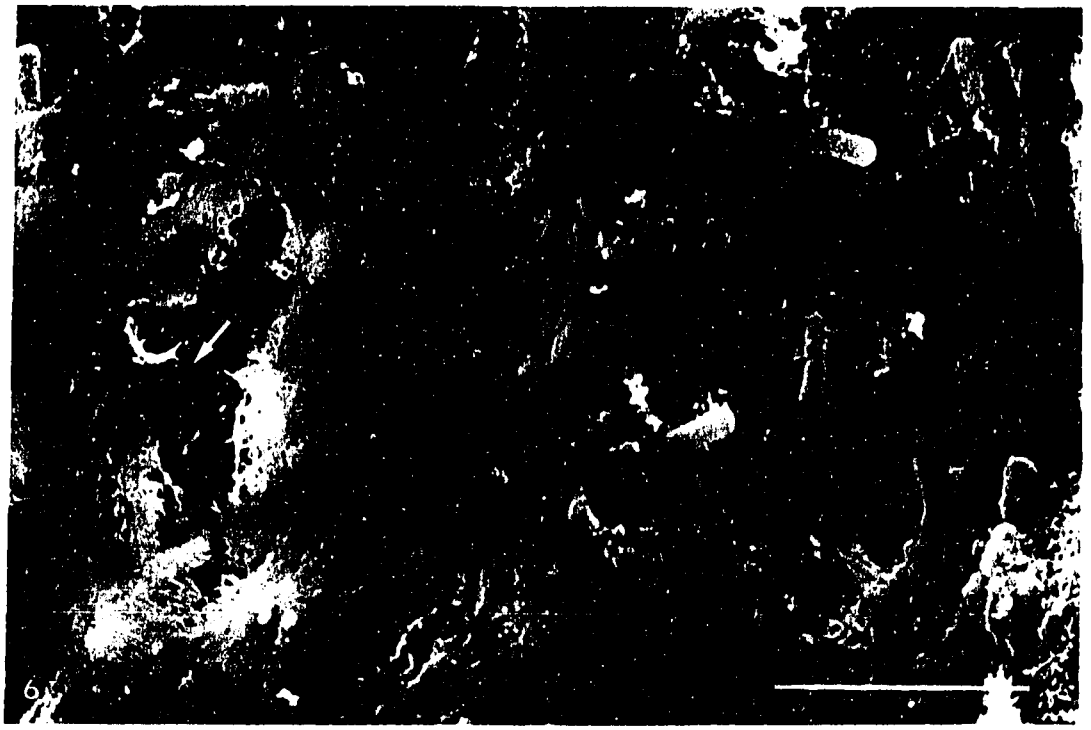

Fig. 7: Corneal erosion; 6 hours post-inoculation. Moraxella bovis (arrow) and cellular debris on surface of epithelial cells at base of erosion. Bar = 10 μm

Fig. 8: a) Corneal ulcer; 10 hours post-inoculation. Bar = 25 μm. b) Large numbers of Moraxella bovis, fibrin (F), and cellular debris at base of ulcer. Bar = 10 μm

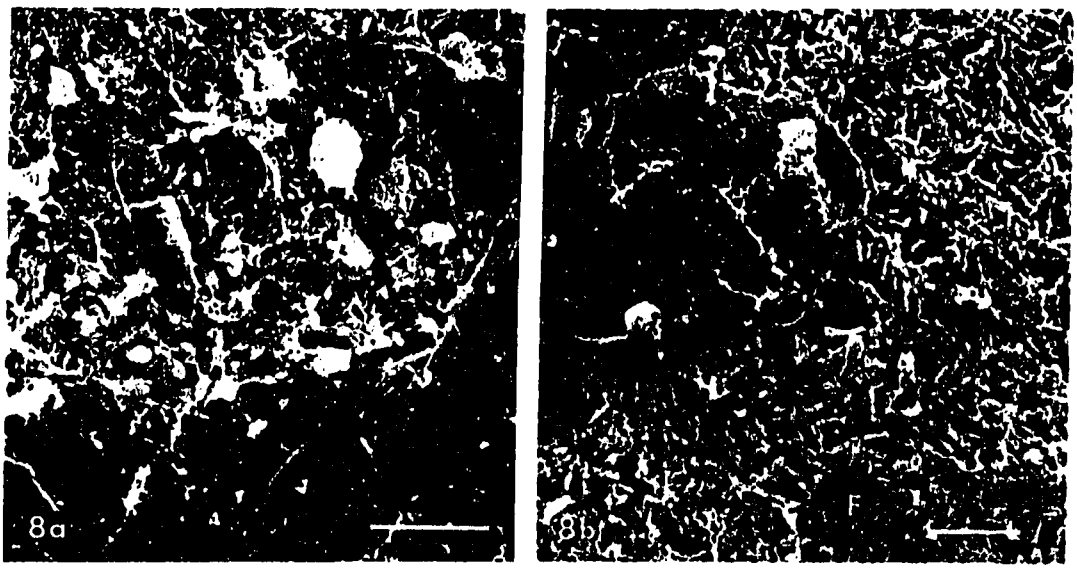
8a
8b
F

Fig. 9: Corneal surface; 2 hours post-inoculation. Bacterium with prominent nucleoid in pit in epithelial cell process. Cell beneath has intact plasma membrane, rarified cytoplasm, ribosomes, tonofilaments, and an intracellular bacterium that is not visible. Bar = 0.5 μm

Fig. 10: Cell lysis, basal epithelial layer; 4 hours post-inoculation. Moraxella bovis surrounded by electron-lucent halos in cellular debris and in depressions in Bowman's membrane (arrows). Basement membrane (BM). Plasma membrane (PM). Bar = 1 μm

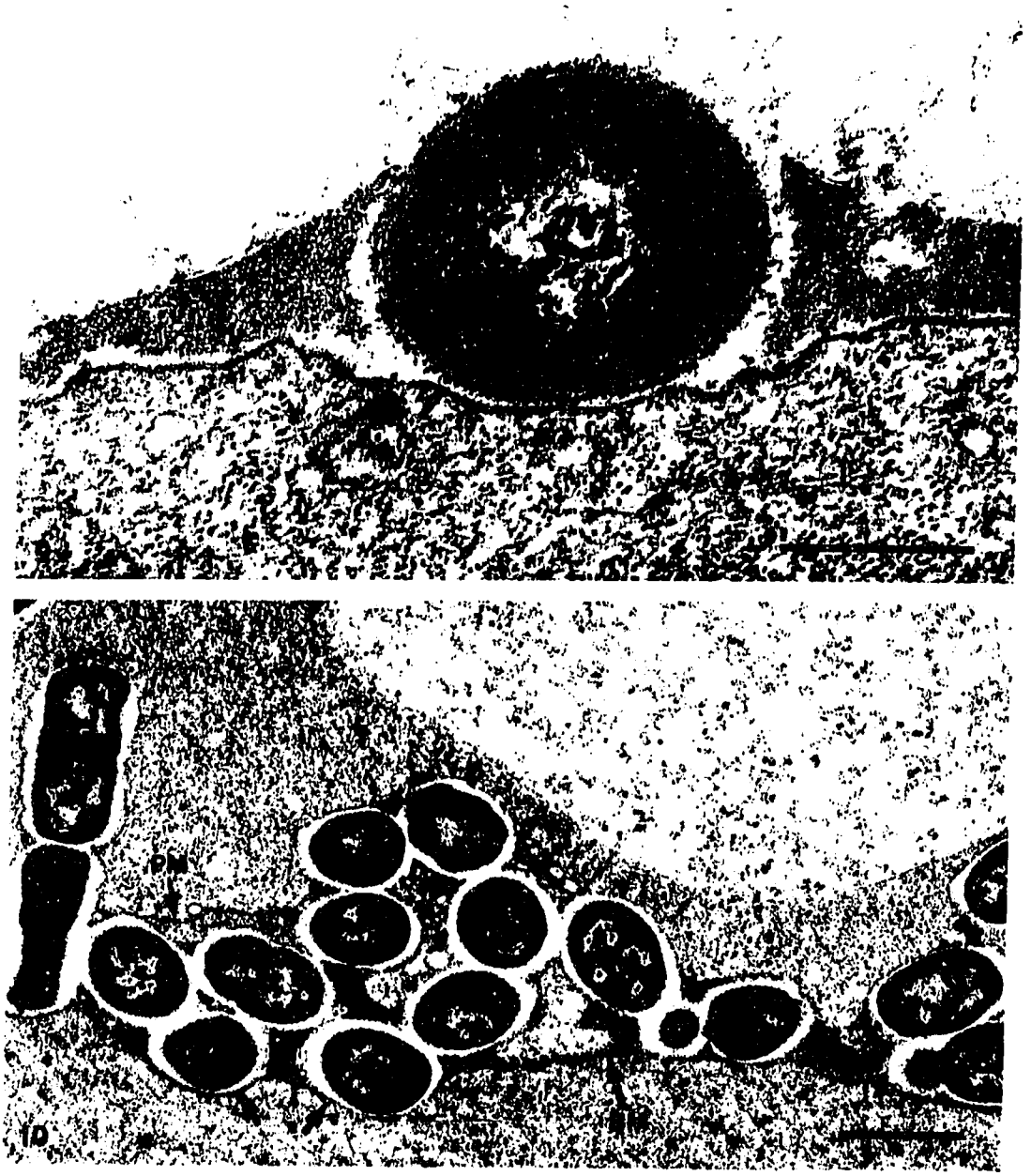
PM
ID

Fig. 11: Corneal stroma; 10 hours post-inoculation. *Moraxella bovis* surrounded by electron-lucent halos with flocculent electron-dense material near bacterial poles. Collagen fibrils are structurally intact. Bar = 1 μm

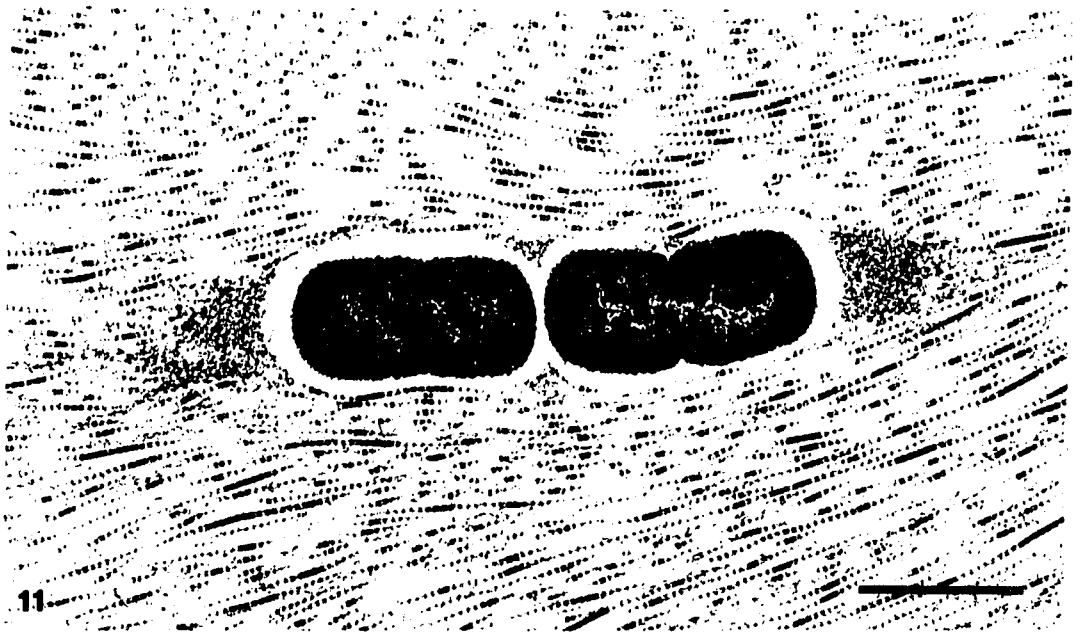
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Table 1. Protocol, type, and distribution of lesions

| Calf | Dose of Inoculum | Sampling Time | Type and Distribution of Lesions | Age at Inoculation (Days) |
|---|---|---|---|---|
| 1 | $2.0 \times 10^{10}$ | 1h | Microscopic, peripheral cornea* | 3 |
| | $1.0 \times 10^{10}$ | 2h | Microscopic, peripheral cornea† | |
| 2 | $1.0 \times 10^{10}$ | 4h | Microscopic, peripheral cornea* | 3 |
| | $1.9 \times 10^{10}$ | 6h | Microscopic, peripheral cornea† | |
| 3 | $2.0 \times 10^{9}$ | 10h | 1 mm ulcer, peripheral cornea, right | 7 |
| 4 | $1.0 \times 10^{9}$ | 16h | None | 14 |
| 5 | $2.8 \times 10^{11}$ | 24h | 7,7,4,2 mm leukomas and ulcers, some coalescing, peripheral cornea, right<br>3,2 mm leukomas and ulcers, peripheral corneal, left | 6 |
| 6 | $1.0 \times 10^{9}$ | 45h | 8,3 mm leukomas and ulcers, peripheral cornea, left | 4 |
| 7 | $1.6 \times 10^{10}$ | 72h | 1.5 cm leukoma and ulcer, central cornea, neovascularization; hypopyon, left | 7 |
| 8§ | $2.8 \times 10^{10}$ | 13d | 3 mm macula, peripheral cornea, right | 14 |
| 9 | Control | 5d | None | 14 |

*=left eye inoculated.
†=right eye inoculated.
§=large numbers of nonhemolytic M. bovis isolated from nares.

## DISCUSSION

This morphologic study demonstrates that a virulent strain of M. bovis can invade the intact bovine cornea in the absence of injurious ultraviolet irradiation or other known predisposing environmental factors. It also indicates that M. bovis is cytotoxic for and invades corneal epithelial cells, in contrast to earlier suggestions that M. bovis disrupts epithelial cell junctions without damaging the cell.[30] The toxic factor(s) associated with M. bovis are unknown although virulent strains produce a "dermonecrotic toxin," a hemolysin, and enzymes such as lipases and proteases which could damage the cornea.[11,13,25] Pits on the surface of dark cells are believed to result from activity of a "pitting factor" produced by M. bovis.[7] This pitting phenomenon may correlate with the agarolytic activity of virulent strains grown in vitro.

The reasons why bacterial adherence is selective for dark cells of the cornea[6,7,15] are unclear. Because dark cells are relatively devoid of surface microplicae,[6,21] receptors for M. bovis adhesins could be more accessible on the cell surface. Age-related degenerative changes in dark cells[21] may predispose them to invasion by M. bovis, resulting in initiation of lesions. Our data also suggest that intense colonization on dark cells may be necessary for M. bovis to invade the corneal epithelium and induce ulceration.

A β-hemolysin produced by M. bovis could play a central role in pathogenesis (only hemolytic strains are virulent[13,25]). The relationship between virulence and hemolytic activity was evident in an

infected calf that only had lacrimation of short duration and from which a majority of nonhemolytic M. bovis was recovered. The ability of hemolytic strains to revert to nonhemolytic in vivo has been reported previously.[25]

Virulent strains of M. bovis do not produce collagenase[11] and we found no evidence of stromal collagen damage in the early stages of infection. Fragmentation and aggregation of collagen fibrils in later stages may have resulted from the action of neutrophil hydrolases.[19] Similarly, electron-dense granules seen in later stages are believed to represent neutrophil-mediated breakdown products of collagen, proteoglycan, or both.[32]

Electron-lucent halos surrounding M. bovis[9] and depressions in the surface of Bowman's membrane in electron micrographs may represent areas digested by bacterial enzymes. Although bacterial capsules were not seen, it is possible that halos represent collapsed bacterial capsules[4] or shrinkage artifacts from tissue processing. Fresh cultures of M. bovis are reported to be encapsulated,[2] however, we did not examine the Epp-63(300) strain separately for presence of a capsule.

In previous studies using different strains of M. bovis, only one gnotobiotic calf developed a corneal lesion.[10,28] Lesions from that gnotobiotic calf and conventional calves infected with M. bovis[8] were similar to the later stages of ulceration reported here. Differences in pathogenicity of M. bovis strains[5,23] may account for our findings and those of others. Because in vitro growth can alter pathogenicity

of M. bovis[5] and the dose of inoculum can influence the course of experimental disease,[22] the strain of M. bovis used received few blood agar passages and large doses of inoculum were used. Although one calf did not have corneal lesions, it did have conjunctivitis and may have been killed before corneal lesions could develop. In addition, the immune status of calves was not known, but immune mechanisms could have influenced lesion development. Further work is required to determine whether age, sex, or breed influence susceptibility in gnotobiotic calves.

Most lesions in our study involved the peripheral cornea whereas lesions of naturally occurring and experimental infectious bovine keratoconjunctivitis in conventional calves usually begin in the central cornea.[3] Much of our study focused on the early stages of pathogenesis. It is possible that many of these lesions would have progressed to involve the central cornea and thus represent stages of the disease reported by others. Environmental factors (natural and simulated) could also influence lesion development at different sites.

The strain of M. bovis used in this study is a corneal pathogen in gnotobiotic calves. The pathogenesis of corneal lesions probably can be attributed to one or more invasive factors produced by M. bovis and to the acute inflammatory response. Corneal cells damaged by bacterial factors and neutrophils may provide additional sources of degradating enzymes.

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# PATHOGENESIS OF CONJUNCTIVAL LESIONS CAUSED BY *MORAXELLA BOVIS* IN GNOTOBIOTIC CALVES

# PATHOGENESIS OF CONJUNCTIVAL LESIONS CAUSED BY *MORAXELLA BOVIS* IN GNOTOBIOTIC CALVES

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## ABSTRACT

A hemolytic strain of Moraxella bovis was instilled into the conjunctival sac of gnotobiotic calves and conjunctivae were sampled serially after infection. Bilateral lesions developed in seven of eight infected calves, but were absent in a noninfected control calf. Histologically, M. bovis was first seen within swollen epithelial cells near the lid margins and occasionally within superficial epithelium in other areas. Conjunctival erosions and ulcers were seen in later stages. Scanning electron microscopy showed M. bovis in pits on the surfaces of epithelial cells and within erosions on palpebral conjunctivae; lesions were prominent near lid margins. Ultrastructurally, M. bovis was seen within swollen epithelial cells near lid margins and many epithelial cells had undergone cytolysis. This study demonstrates that a virulent strain of M. bovis can invade bovine conjunctival epithelial cells and can cause conjunctivitis in the absence of injurious ultraviolet irradiation or other known predisposing environmental factors.

# INTRODUCTION

Piliated, hemolytic strains of the gram-negative bacterium, Moraxella bovis (M. bovis), are the principal etiologic agents of infectious bovine keratoconjunctivitis,[7,10,13] although the suspected role of other factors in the pathogenesis is well-documented.[1] Ultraviolet irradiation,[8,11,13] Mycoplasma bovoculi,[17] and infectious bovine rhinotracheitis virus[14] have been used in conjunction with M. bovis to cause lesions. Gnotobiotic calves do not develop lesions as readily as conventional calves after inoculation with M. bovis, leading to the hypothesis that environmental factors are important in the initiation/progression of lesions in conventional calves.[5]

Other investigators have described the histologic changes in conjunctival biopsies from acute cases of naturally occurring infectious bovine keratoconjunctivitis.[15] These changes include swollen conjunctival epithelium, hyperemia, edema and a mononuclear cell infiltrate. Studies combining light, scanning and transmission electron microscopy, which would clarify the role of M. bovis in the pathogenesis of the conjunctival lesion, have not been reported.

We have reported on the corneal lesions caused by M. bovis in gnotobiotic calves.[16] The conjunctival lesions from these calves are described in this paper. The objectives were to: (1) determine whether a virulent strain of M. bovis could cause conjunctivitis in gnotobiotic calves and, if so, (2) to characterize the development of conjunctival lesions by light, scanning and transmission electron

microscopy, and (3) to study lacrimal tissues for evidence of histologic changes.

# MATERIALS AND METHODS

Reference to materials and methods Information pertaining to the nine gnotobiotic calves, housing, maintenance, preparation of the M. bovis inoculum, determination of dosage, inoculation, sampling intervals, contaminant monitoring, and clinical signs present in the calves has been reported previously.[16]

Necropsy and tissue collection Calves were killed by sodium pentobarbital overdosage followed by exsanguination. After removal of eyes, the eyelids, nictitating membranes (and associated glands), bulbar conjunctivae and lacrimal glands were dissected away and immersed in 2.5% glutaraldehyde and 1.5% paraformaldehyde in .1M cacodylate buffer (pH 7.4). Skin was later dissected away from the eyelids.

Preparation of tissues Samples for light, scanning and transmission electron microscopy were processed and examined as previously described.[16] Only tissues from the 1- and 2-hour sampling periods and from the control calf were examined by scanning electron microscopy. Tissues included bulbar conjunctivae, nictitating membranes, dorsal and ventral palpebral conjunctivae, conjunctival fornices and lid margins. Eight samples from each of these tissues were taken at all sampling times and from the control calf for transmission electron microscopy.

Bacteriologic culture Right and left parotid and lateral retropharyngeal lymph nodes[18] were aseptically collected from each calf at necropsy and stored at -70 C. For bacteriologic culture, each node

was thawed and minced in 3 ml of trypticase soy broth in a Ten Broeck tissue grinder. Homogenate was spread onto 5% bovine blood agar plates (1 ml per plate), incubated at 37 C for 24 hours, and examined for hemolytic M. bovis.

# RESULTS

Microbial status of calves Hemolytic M. bovis was isolated in large numbers from the nares of calves from 1 to 72 hours post-inoculation. Large numbers of nonhemolytic M. bovis and small numbers of hemolytic M. bovis were isolated from the nares of the calf killed at 13 days post-inoculation. Hemolytic M. bovis was not isolated from the control calf. Small numbers of saprophytic bacteria were isolated from the nares and/or feces of the control calf and calves killed at 16, 24, and 72 hours and 13 days post-inoculation. Mycoplasma bovoculi and infectious bovine rhinotracheitis virus were not isolated from any of the nine calves. Bacteria were not isolated from parotid or lateral retropharyngeal lymph nodes.

Light microscopy Bilateral lesions developed in seven of eight infected calves, but were absent in the noninfected control calf. At 1 and 2 hours post-inoculation, bacteria were seen within swollen epithelial cells and associated with cells that had undergone cytolysis near the lid margins (Fig.1). Bacteria were occasionally seen within superficial epithelium of the nictitating membranes, palpebral conjunctivae and conjunctival fornices; desquamated cells, often with intracellular bacteria were common in these areas. Bacteria were not seen within epithelium of the bulbar conjunctivae.

Neutrophils were present in conjunctival epithelium at 4 and 6 hours post-inoculation. Nictitating membranes, palpebral conjunctivae and lid margins had erosions. Lamina propriae had mild neutrophilic infiltrates, hyperemia and patchy areas of edema. Bacteria were seen

within epithelium near lid margins and occasionally free and within desquamated cells in other areas.

Nictitating membranes, palpebral conjunctivae and lid margins had pustules and erosions between 10 and 72 hours post-inoculation. However, lesions varied in severity and often varied between right and left conjunctivae within the same animal. Lamina propriae had hyperemia, edema and neutrophilic infiltrates that varied from mild to severe, except in the bulbar conjunctivae, where the infiltrates were always mild. Fibrin deposits were seen in areas of severe neutrophilic infiltrates in the palpebral conjunctivae. The calf killed at 10 hours post-inoculation had ulcers of the right dorsal lid margin and palpebral conjunctiva (Fig. 2); bacteria were seen within epithelium and within desquamated cells near the lid margin. Ulcers of the right and left dorsal lid margins were seen at 16 and 24 hours post-inoculation respectively. However, bacteria were not seen in any animal after 10 hours post-inoculation.

No lesion was found in the calf from which a majority of nonhemolytic M. bovis was isolated (13 days post-inoculation). Lacrimal glands, adnexal glands and glands of the nictitating membranes were normal morphologically in all infected calves.

Scanning electron microscopy The surfaces of conjunctival fornices and the bulbar surface of the nictitating membrane from the noninfected control calf were rugose and mucus obscured surface detail in some areas. Surfaces of the bulbar and palpebral conjunctivae were comparatively smooth, which facilitated the classification of surface

cells on the basis of electron density. Lighter cells, with surface microplicae (ridges) and microvilli, were numerous in these areas. Dark cells had few surface structures and were rare. Small numbers of desquamated cells were present on palpebral conjunctivae near the lid margins. Bacteria were not seen on any of the samples.

Palpebral conjunctivae sampled at 1 and 2 hours post-inoculation had M. bovis, often in large numbers, adhered to and in pits on the surfaces of both dark (Fig. 3) and lighter cell types. Many lighter cells had lost surface structures in the area of pits (Fig. 4). Both pits and erosions were prominent near lid margins; many of the erosions contained large numbers of M. bovis (Fig. 5). Pits were seen in one area of the nictitating membrane at 1 hour post-inoculation; however, this tissue and the conjunctival fornices could not be examined in detail due to the rugose surfaces and the presence of mucus. Bacteria were not seen on the bulbar conjunctiva.

Transmission electron microscopy At 1 and 2 hours post-inoculation, M. bovis was seen in pits in superficial epithelial cell processes and within epithelial cells of the palpebral conjunctivae near lid margins. Epithelial cells with intracellular bacteria were characterized by rarefied cytoplasm, vacuoles, aggregated tonofilaments and karyolysis. Plasma membranes were often disrupted (Fig. 6) and many cells had undergone cytolysis. Intracellular M. bovis often was surrounded by electron-lucent halos of varying size and replicative bacteria were occasionally seen. Moraxella bovis was

occasionally seen within sloughed degenerate epithelial cells of the nictitating membranes, palpebral conjunctivae and conjunctival fornices; however, it was not seen within epithelial layers in these areas.

Ultrastructural changes correlated with histologic changes between 4 and 72 hours post-inoculation. Bacteria were occasionally seen free and within degenerate epithelial cells of eroded and ulcerated lid margins. They were not seen after 10 hours post-inoculation and were never seen in lamina propriae.

Fig. 1: Bacteria associated with conjunctival epithelial cells that have undergone cytolysis near dorsal lid margin; 1 hr post-inoculation. HE

Fig. 2: Ulcer in dorsal palpebral conjunctiva; 10 hours post-inoculation. Lamina propria has severe neutrophilic infiltrate, hyperemia and edema. HE

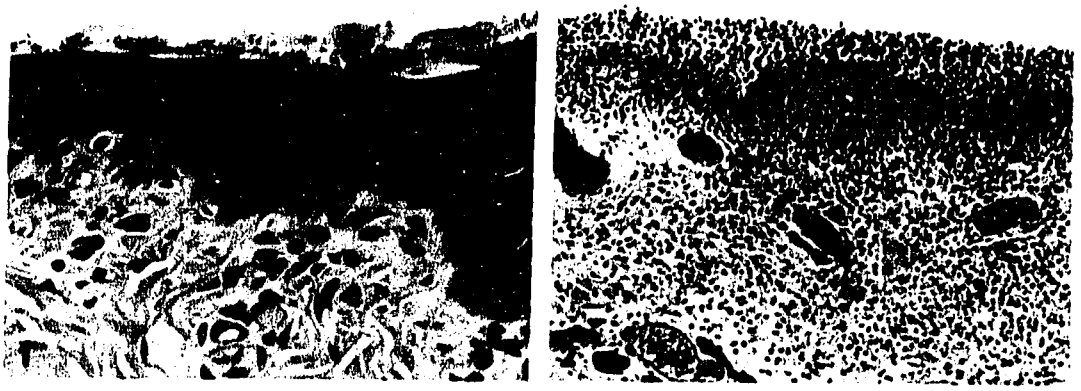

Fig. 3: Intense colonization by Moraxella bovis on surface of a dark cell. Palpebral conjunctiva; 1 hr post-inoculation. Note polar attachment by Moraxella bovis and pits without bacteria (arrows). Bar = 10 μm

Fig. 4: Moraxella bovis in pits on surfaces of lighter cell types. Palpebral conjunctiva; 1 hr post-inoculation. Note loss of surface structures in area of pits. Cell border (arrows). Bar = 5 μm

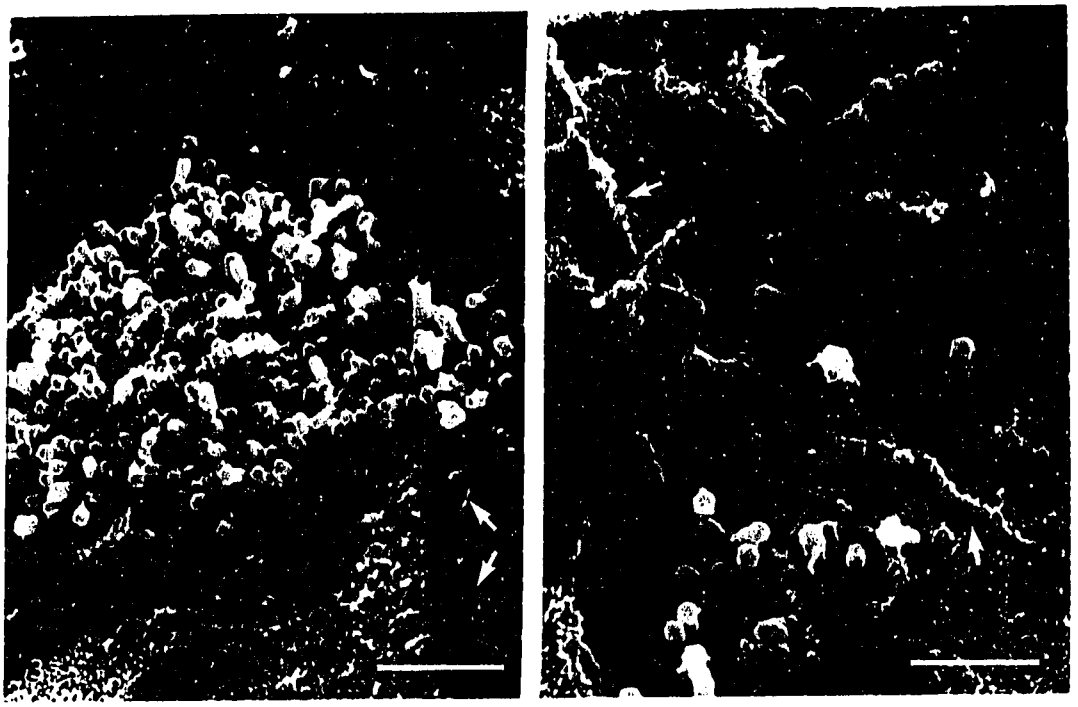

Fig. 5: Large numbers of Moraxella bovis within erosions near dorsal lid margin; 1 hr post-inoculation. Bar = 10 μm


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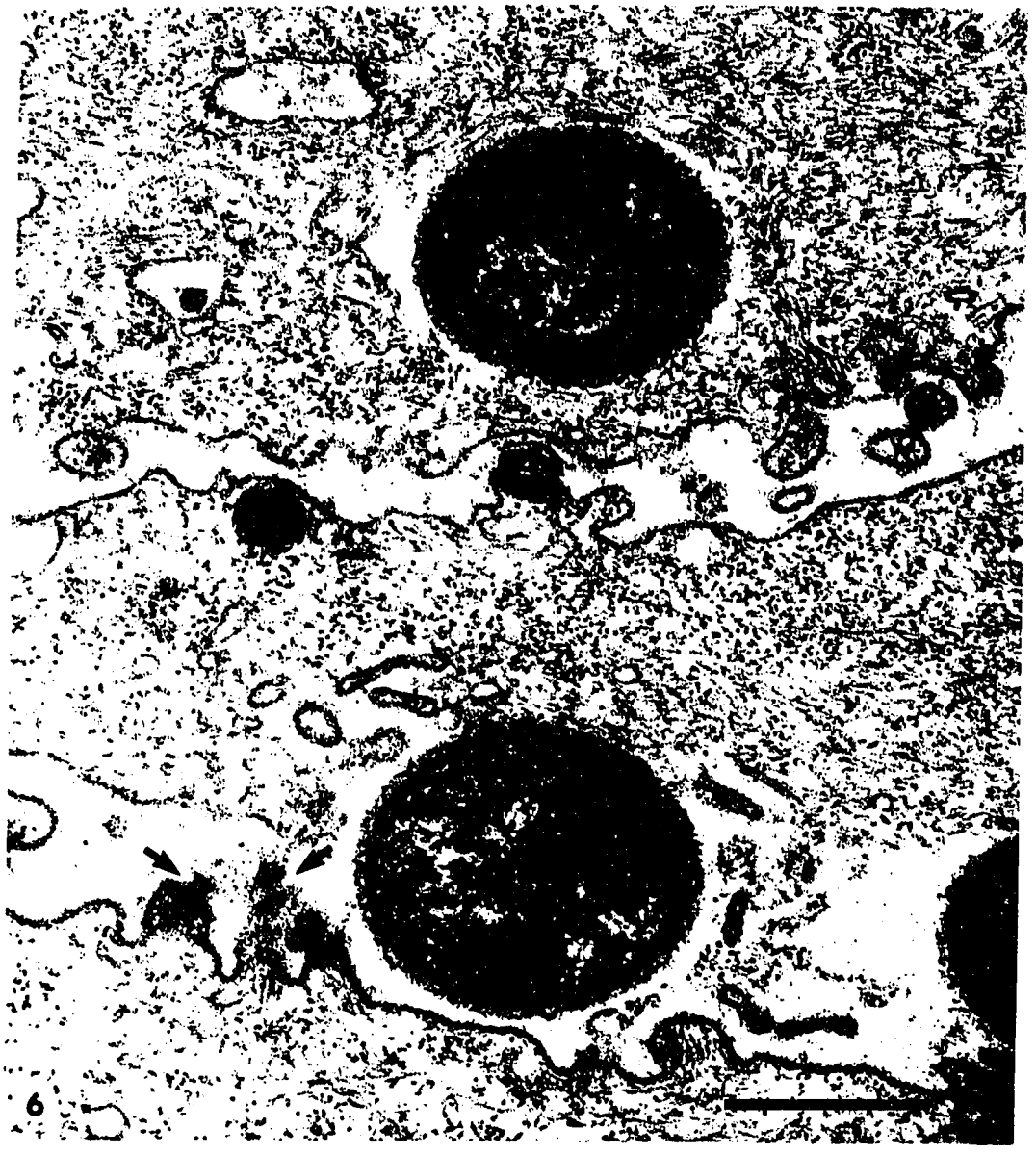
Fig. 6: Moraxella bovis within epithelial cell and associated with epithelial cell with disrupted plasma membrane. Broken desmosomes (arrows). Epithelial cells have rarefied cytoplasm, vacuoles, numerous tonofilaments and ribosomes. Dorsal palpebral conjunctiva near lid margin; 1 hr post-inoculation. Bar = .5 μm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## DISCUSSION

This morphologic study demonstrates that a virulent strain of M. bovis is cytotoxic for and can invade bovine conjunctival epithelial cells in the absence of injurious ultraviolet irradiation or other known predisposing environmental factors. The toxic factor(s) associated with M. bovis are unknown although virulent strains produce a "pitting factor," a "dermonecrotic toxin," a hemolysin, and enzymes such as lipases and proteases which could damage the conjunctiva.[4,6,7,13]

In vitro and in vivo studies have shown that virulent strains of M. bovis selectively adhere to and produce pits on the surfaces of dark epithelial cells of the cornea.[3,4,9,16] In the present study, selective adherence was not evident and pits were seen on the surfaces of both dark and lighter cell types of the palpebral conjunctivae. As with corneal infection,[16] there was intense surface colonization of some cells and large numbers of M. bovis often were seen within early lesions. Intense colonization by M. bovis may be an important pathogenetic mechanism.[16] In addition, lesions were prominent near lid margins which suggests that M. bovis is not effectively cleared from these areas. Based on our histologic findings, M. bovis can invade areas other than palpebral conjunctivae near lid margins early in infection, however, invasion is infrequent and limited to superficial epithelial layers.

Mononuclear cells were not components of the lesions which differs from results of an earlier study.[15] However, cattle in that study were

exposed to environmental factors which could have influenced the inflammatory response.

A β-hemolysin produced by M. bovis could play a central role in pathogenesis (only hemolytic strains are virulent[7,13]). The relationship between virulence and hemolytic activity was evident in an infected calf that did not develop conjunctival lesions and from which a majority of nonhemolytic M. bovis was recovered. The ability of hemolytic strains to revert to nonhemolytic in vivo has been reported previously.[13]

In a previous study using different strains of M. bovis and gnotobiotic calves, only one calf developed a lesion.[5] Differences in pathogenicity of M. bovis strains[2,12] may account for our findings and those of others.

The strain of M. bovis used in this study is a pathogen in gnotobiotic calves. The pathogenesis of conjunctival lesions probably can be attributed to one or more invasive factors produced by M. bovis and to the acute inflammatory response. Conjunctival epithelial cells damaged by bacterial factors and neutrophils may provide additional sources of degradating enzymes.

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## GENERAL DISCUSSION AND SUMMARY

This study demonstrates that a virulent strain of M. bovis can invade the intact bovine cornea in the absence of predisposing environmental factors. It also indicates that M. bovis is cytotoxic for and can invade corneal and conjunctival epithelial cells.

By scanning electron microscopy, M. bovis was first seen in pits on the surfaces of dark epithelial cells of the cornea. This correlates with previous in vitro studies.[10,11,39] In addition to damaging corneal epithelium, exposure to ultraviolet irradiation increases the number of dark cells on the corneal surface;[95] this could facilitate adherence by M. bovis in naturally occurring IBK.[10,95]

There is no evidence that M. bovis selectively adheres to and/or initiates lesions on a specific conjunctival epithelial cell type. Large numbers of M. bovis were seen near lid margins early in infection and lesions were prominent in these areas; this suggests that M. bovis is not effectively cleared from lid margins. Based on our histologic findings, M. bovis can invade areas other than palpebral conjunctivae near lid margins early in infection, however, invasion is infrequent and limited to superficial epithelial layers.

Large numbers of M. bovis often were seen within early corneal and conjunctival lesions. This suggests that intense surface colonization may be necessary for M. bovis to invade corneal and conjunctival epithelial cells. Following the invasion of epithelial cells, bacterial replication can occur.

At least three mechanisms probably can be proposed for pathogenesis of the corneal lesion. The first mechanism, which may be acting alone, in sequence, or concurrently with the second and third mechanisms, is that M. bovis produces one or more invasive factors that damage or kill corneal epithelial cells. Virulent strains of M. bovis do not damage collagen,[21] but it is not clear whether M. bovis degrades stromal proteoglycan and is cytotoxic for stromal fibroblasts and corneal endothelium. The second mechanism is that neutrophil hydrolases damage corneal epithelium,[19] endothelium,[18] and stroma.[50,86] Histologic and/or ultrastructural changes suggestive of neutrophil-mediated damage were seen in corneal stroma and endothelium, but were less evident in corneal epithelium. The third mechanism is that corneal cells damaged by bacterial factors and neutrophils may provide additional sources of degradating enzymes. These mechanisms also may be applicable to the pathogenesis of the conjunctival lesion.

Because this was primarily a morphologic study with emphasis on the early stages of pathogenesis, several important questions remain to be answered. Is the M. bovis hemolysin an invasive factor? Are the lipases, proteases, and other enzymes produced by M. bovis[21] important in the pathogenesis? What is the relationship between M. bovis plasmids[89] and virulence? Does M. bovis produce nonpilus adhesins? Is antigenic variation a feature of M. bovis pilus expression? Although other studies have shown that M. bovis produces several types of pili,[45,88] further work is needed to characterize and classify pili

from field isolates to enable better understanding of pilus expression and prophylaxis.[45]

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