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CHARACTERIZATION OF MORAXELLA BOVIS AND
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CHARACTERIZATION OF MORAXELLA BOVIS AND ITS
RELATIONSHIP TO BOVINE INFECTIOUS KERATOCONJUNCTIVITIS

by

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INTRODUCTION

Infectious keratoconjunctivitis or pinkeye is an important contagious disease of cattle. It was first recognized in North America approximately 80 years ago. Its occurrence has since been reported in Africa, Asia, Australia, Europe and South America. Bovine infectious keratoconjunctivitis (IBK) occurs in epizootic proportions in cattle herds, particularly during the summer months. It affects cattle of all ages, sexes and breeds. The disease is thought to be transmitted through direct contact, fomites, and mechanical vectors such as flies.

Many workers have attempted to determine the etiologic agent of infectious keratoconjunctivitis. Although numerous microbes have been implicated in the disease, Moraxella bovis (M. bovis) has been incriminated more often and is generally considered to be the causative agent. However, there is a lack of specific information on M. bovis pertaining to its characteristics as they relate to the pathogenesis of IBK. Some of the areas where there is insufficient knowledge are: (1) the identity of the organism is unclear since certain other bacterial species are very similar in general characteristics, (2) attempts by other workers to experimentally reproduce IBK consistently with M. bovis have been unsuccessful, (3) infection with or vaccine prepared from the organism apparently does not confer immunity against the disease, (4) little is known about the relative virulence of different strains of M. bovis,

and (5) what role other agents, such as infectious bovine rhinotracheitis virus, play in the evolution of the disease.

The purposes of this research were to characterize M. bovis and to determine its relationship to IBK. This treatise consists of a general introduction, a joint literature review, and an overall discussion and summation. However, for simplicity and clarity the experimental data are divided into eight sections, each one dealing with a different aspect of the subject.

LITERATURE REVIEW

Bovine Infectious Keratoconjunctivitis
and Moraxella Bovis

The first report of a contagious keratitis was made by Billings (16) in 1889 who described the disease as it had occurred in dairy cattle in the vicinity of Lincoln, Nebraska during the summer and fall of the year 1888. He stated that this was not a new disease and that it probably occurred throughout the United States. He noted the presence of thin, short bacilli with rounded ends in sections of the cornea but was unsuccessful in reproducing the disease by transferring the organisms from the eyes of diseased cattle to healthy ones. He was also unable to reproduce the disease in the eyes of rabbits. Billings did not further characterize these organisms.

Poels (101) in 1917 reported in an article, translated by Kappeyney and Ward (76), that the etiologic agent of bovine keratitis in Holland was Bacillus pyogenes. He was unable to reproduce the disease by inoculating the cornea with B. pyogenes, but he reproduced a typical case of keratitis by injecting the organism between the layers of the cornea. This organism has not been incriminated by any other investigator as being the etiologic agent of IBK.

Probably the first report of IBK attributable in part to M. bovis, was made by Mitter (91) who in 1915 reported the isolation of a diplobacillus from an outbreak of keratitis in

Bengal. He identified the organism as the bacillus of Morax-Axenfeld (9, 92).

Allen (5) in 1919 reported an outbreak of infectious keratitis in Canada. By staining, he demonstrated short, thick Gram-negative diplobacilli in direct smears from eyes of infected animals. He was able to transfer material from a diseased eye to normal eyes of healthy animals and thereby reproduce the disease. His attempts at isolation of the bacilli on agar failed, but in subsequent work Loeffler's blood serum agar was employed and pleomorphic bacilli, nearly always occurring in pairs were isolated. He failed to reproduce IBK with a pure culture of the newly-isolated organism.

In 1923 Jones and Little (73) reported the isolation of a Gram-negative diplobacillus from cattle with infectious keratitis. They reproduced the disease by dropping a pure culture of the bacillus into eyes of susceptible animals. In characterizing the disease produced, Jones and Little reported that it was characterized by sudden onset, marked photophobia and impairment of vision. Lacrimation was excessive and accompanied by considerable thick yellow discharge, especially at the medial canthus extending down over the face. The eyelids were thickened, and the membrana nictitans was often deeply reddened and swollen. The conjunctiva was bright red in color. There was injection of blood vessels of the eyeball and conjunctivae. In a few cases, irregular ulcers were observed on the cornea. In some cases there was extensive corneal opacity.

Jones and Little (73) also described the cultural and biochemical characteristics of their organism. The organism grew and produced hemolysis on horse blood agar plates; it also grew on plain and serum agar slants, coagulated serum, agar and serum agar stabs and gelatin which was liquified in 10 days when incubated at 22 C. There was slow growth in plain bouillon which became slightly turbid with considerable sediment. There was no fermentation in dextrose bouillon or other carbohydrate mediums; in fact, all these mediums became more alkaline. No growth was obtained on potato. Litmus milk was described as becoming more alkaline and, after 10 days, 3 zones were visible: an upper deep blue liquid zone, an intermediate zone containing soft flocculi of casein which was lighter in color, and a still lighter coagulated casein zone at the bottom.

The individual elements were described as being short, plump bacilli with rounded ends, the length being fairly uniform in 24- or 48-hour cultures, varying from 1.5 to 2 microns, and the width as 0.5 micron. Longer forms occurred commonly in the exudate. The bacilli usually occurred in pairs and short chains both in the exudate and culture mediums. They were nonmotile, nonsporulating and possessed well-developed capsules. The organism stained well by ordinary methods and was Gram-negative. Growth was better on mediums containing blood or serum.

The colonies produced on blood agar plates containing 8% defibrinated horse blood were characteristic. After 24 hours the surface colonies were round, translucent, and grayish white. They were surrounded by a narrow, clear zone of hemolysis. Also deep colonies were barely perceptible to the naked eye at 24 hours in sharp contrast to their clear hemolytic zone, usually 1.5 mm in diameter. After 48 hours the surface colonies were more flattened in appearance and reached a diameter of 3.5 to 4 mm. The deep colonies were ovoid and bioconvex in shape, and the hemolytic zone had increased in diameter from 2.5 to 3 mm.

Jones and Little (74) in 1924 reported on the treatment and transmission of infectious ophthalmia. In their experiments they failed to establish that the disease could be transmitted by flies, but they suggested that the methods employed were at fault. They found that the characteristic diplobacillus was rapidly destroyed in the digestive tract of flies and that the organism lived only 3 hours on the external surfaces. It is interesting to note that Steve and Lilly (121) in 1965 found that the bacterium M. bovis: (a) may remain viable in the environs of the fly (Musca autumnalis) up to 3 days; (b) M. bovis was readily recovered from the exudate from infected eyes; (c) M. bovis was readily recovered from wings and legs of face flies up to 3 days after exposure to laboratory cultures; (d) the bacterium apparently is rapidly destroyed in the digestive tract of the face fly; and (e) M.

bovis was recovered from laboratory-reared flies exposed to lacrimal exudates on infected cattle in the field. Steve and Lilly, like Jones and Little, suggested that since the fly is a persistent feeder around eyes of cattle, their findings are further evidence tending to incriminate the fly as a potential carrier and therefore is a contributing factor in the spread of IBK.

Reid and Anigstein (112) investigated keratitis along the Gulf Coast of Texas in the summer of 1944. They demonstrated the contagious nature of the disease. No difficulty was encountered in transmitting the disease from an infected to a susceptible animal by rubbing the exudate from the infected eye into the conjunctivae of another calf. They also found that nasal exudate was as virulent for susceptible animals as the eye exudate. They isolated an organism identified as M. bovis from cases of keratoconjunctivitis using blood agar and on the basis of morphology, as well as cultural and pathogenic characteristics, they concluded it was identical with the diplobacillus of Jones and Little.

Keratoconjunctivitis was produced in susceptible animals by simple instillation of a pure culture of M. bovis into the eye. They also produced keratoconjunctivitis experimentally in sheep and goats. Moraxella bovis was found to be nonpathogenic for small laboratory animals except for white mice which died after intra-abdominal injection of young cultures. From the results of their experimental data they concluded that M. bovis

was the etiologic agent of infectious keratoconjunctivitis in cattle.

Baldwin (13) reported in 1945 that he found M. bovis in 93 out of 112 infected eyes of cattle and was able to induce an infection indistinguishable from infectious keratitis in one or both eyes of 12 of 15 animals using cultures of M. bovis inoculated into the conjunctival sac. Injuring the cornea and conjunctivae did not seem to be necessary to induce an infection. He found that mice, sheep, guinea pigs, and rabbits were not susceptible to M. bovis.

Farley et al. (47) in 1950 reported an investigation where they used 3 isolants of M. bovis supplied by Baldwin (13) as well as M. bovis isolated from active field cases of IBK. Using broth cultures of the organism they failed to produce disease in susceptible cattle (34 head exposed to Baldwin supplied strains and 5 head exposed to field herds isolants). Rabbits and guinea pigs also failed to develop diseased eyes following numerous exposures with M. bovis.

Watt (127) in 1951 described an outbreak of bovine keratitis in Scotland in which he isolated an organism identical to M. bovis from 4 animals. This was the first report of M. bovis in Great Britain. However, there had been several reports of outbreaks of infectious keratitis. These include Brown (32) who in 1934 described a "contagious ophthalmia" corresponding clinically to the keratitis described by Allen in Canada; no etiologic agent was identified in Brown's report. Blakemore

(17) in 1947 demonstrated inclusion bodies in two outbreaks of keratoconjunctivitis in cattle. The inclusion bodies were similar to the rickettsial bodies described by Coles (40) in South Africa. Pook (102) in 1951 reported 33 cases of conjunctivitis and keratitis in a group of 44 crossbred Friesian calves. The conjunctivae of several were curetted and stained inclusion bodies were seen in the epithelial cells. This and other laboratory findings were identical to those reported by Blakemore (17) and the outbreak was therefore diagnosed as one of inclusion cell conjunctivitis but M. bovis was not recovered.

Barner (14) in 1952 reported the isolation of M. bovis from field cases of infectious keratitis. He grew the organism in pure culture and with it reproduced the disease in 4 calves. Two of 4 normal cows placed in contact with the 4 calves developed the disease. Neither pleuropneumonia-like organisms nor rickettsia-like organisms were demonstrated in conjunctival scrapings of 36 normal and 23 diseased cattle. He was able to isolate M. bovis from a calf 147 days after infection. Barner concluded from his studies that keratoconjunctivitis was caused by M. bovis.

For the most part Barner's findings on the morphological and cultural characteristics of M. bovis were comparable to those of other workers. In addition, he described colony dissociation of the organism from the typical smooth forms into rough, intermediate and dwarf forms following 4 months of multiple transfer on solid mediums. The culture phases were

not stable. Rough phases reversed into smooth forms and vice versa. Intermediate and dwarf forms changed into smooth and rough forms. Old or senescent cultures (5 to 7 days) showed evidence of the formation of minute protuberances or papillae. Daughter colonies were also observed.

Barner was unable to establish M. bovis infection in the eyes of 2 rabbits, 2 guinea pigs and 8 sheep by the instillation of M. bovis into their conjunctival sacs. Neither was he able to recover M. bovis from the eyes of these animals.

Jackson (71) in 1953 reported the characteristics of M. bovis and the clinical syndrome produced by M. bovis isolated from cattle in Texas resembled those described by other workers, notwithstanding the fact that he reported that M. bovis was very exacting in its growth requirements and must have the X and V factors for maximum growth. Significantly, he reported that because M. bovis will readily dissociate from the smooth virulent to the rough avirulent types indicates that a close observation must be made on all cultures before experimental inoculation or antigen production is attempted.

Gallagher (51) in 1954 reported the isolation of M. bovis from diseased eyes of cattle in Australia. The cultural and morphological characteristics of his organism did not differ from those described by earlier workers. He used recently isolated M. bovis which was encapsulated and in the smooth phase to inoculate 4 calves and reproduced the disease in 3 days. He reported that cattle may harbor M. bovis in their

eyes for at least 139 days. He suggested that animals that have recovered from ophthalmia, but which still harbor the organism, are probably the greatest source of contagion for susceptible cattle.

Gallagher also reported that M. bovis was found to be pathogenic to mice, in which it produced keratoconjunctivitis when instilled into the conjunctival sac, whereas it was non-pathogenic to sheep, rabbits and guinea pigs.

Faull and Hawksley (48) in 1954 reported seven outbreaks of infectious keratitis in cattle in Great Britain. Moraxella bovis was isolated from 16 of the 24 cases examined. The organism isolated did not differ from those strains isolated by other workers. In addition, Faull and Hawksley reported that their M. bovis was catalase positive.

Formston (50) in 1954 reported the occurrence of five outbreaks of infectious keratitis in cattle in Great Britain during 1952 and 1953 in which an organism identified as M. bovis was isolated. They noted that the disease appeared to be most active during the summer months, particularly if the weather was warm and humid.

Hoffman (65) in Argentina in 1956 reported that he isolated M. bovis from cattle having infectious keratitis and instilled the material into the eyes of 19 calves. Twelve developed clinical signs but the infection did not develop to the severe stage. He also succeeded in transmitting the disease in three or four attempts by direct transfer of the

infective material from the eyes of diseased cattle to those of healthy calves. He tried to infect calves with filtrates, but was unsuccessful.

Ahmed and Rao (3) in 1956 reported on the clinical manifestation of infectious keratoconjunctivitis in 134 cases in Parbhani District of India involving both cattle and buffalo. Moraxella bovis was recovered from the eyes of cattle. This, according to these authors, represented the first reported cases of infectious keratoconjunctivitis in India (note Mitter (91) notwithstanding).

In 1957 Seth and Chandrasekariah (119) in India reported studies on IBK where they described the morphological, cultural and biochemical characteristics of M. bovis as well as its pathogenicity in experimental animals and cattle. Their description of M. bovis did not differ from that of other investigators (13, 14, 73, 112). However, differing from Formston (50), they reported M. bovis as being catalase negative. They were able to produce the disease in the eyes of 2 calves with pure cultures of M. bovis. Broth cultures injected intravenously killed mice in 3 to 5 days; the organism was recovered from heart blood and spleen. Instillation of virulent cultures into the eyes of rabbits failed to produce symptoms or lesions of the disease. Intraocular injection of guinea pigs and rabbits, however, produced severe conjunctivitis in less than 24 hours; swelling of the eyelids, conjunctivitis and circum-corneal infection were observed after

48 hours. No corneal lesion was observed and resolution was complete within 5 days. The injection of broth cultures of M. bovis into the scrotal sac of rabbits produced severe hemorrhagic necrosis of the scrotal skin and adjacent tissue.

Raghavachari and Reddy (108) also in India in 1957 reported a study on infectious keratitis carried out by them using the organism originally isolated by Ahmed and Rao (3) and used for further studies by Seth and Chandrasekariah (119). To ascertain host specificity of the M. bovis strain, Raghavachari and Reddy exposed 2 buffalo calves, rabbits, guinea pigs, goats, sheep, and a dog with material from a diseased bovine eye. A control calf developed the disease but the other animals did not, even after repeated exposure. They also recovered M. bovis from a calf with infectious keratitis. This organism was also used in transmission studies. Two calves, sheep, goats, guinea pigs, rabbits and a dog were exposed by the instillation of growing 24-hour cultures of the freshly isolated M. bovis. All the experimental animals used (including calves) proved refractory to infection in spite of repeated attempts. This failure led these investigators to suggest that, besides M. bovis, there is some other agent responsible for the disease, possibly a virus which was present in the normal discharges but not in the pure culture.

Henson and Grumbles (60) in 1960 reported on a study in which M. bovis was isolated from lacrimal secretions of 90% (66 isolations were made from 73 animals in 15 herds) of cattle

with clinical cases of infectious keratoconjunctivitis.

Moraxella bovis was also recovered from nasal secretions of 3 animals. Infectious keratoconjunctivitis was experimentally produced in 43 of 59 calves inoculated by three methods.

Henson and Grumbles (61) in 1960 also reported an investigation on the susceptibility of laboratory animals to M. bovis. They exposed embryonating chicken eggs, day-old chicks, weanling rats, rabbits, guinea pigs and mice by injecting by various routes using organisms grown on agar in chicken embryos and liquid mediums. Chicken embryos were killed by intravenous, intraperitoneal, and intracerebral inoculations of the cultures. Dermonecrosis and ophthalmitis were observed in rabbits when they were given intradermal and intraocular injection of viable cultures.

Henson and Grumbles (62) also in 1960 reported the demonstration of toxins in cultures of M. bovis. They reported that M. bovis produced 2 toxins, a hemolytic toxin and a dermonecrotic toxin. The hemolytic toxin appeared to be a very labile substance which was thought to be closely tied to the bacterial cell, dependent upon the viability of the cell and inseparable from it. The dermonecrotic toxin was stable to heat at 56 C for 24 hours and to other treatments. They suggested that the dermonecrotxin has a nonspecific nature and that its stability and location indicate a similarity to toxins of other Gram-negative bacteria.

Cooper (41) in 1960 reported on a study on IBK in Great Britain where disease was produced in young calves and yarded bullocks by the instillation of a M. bovis suspension into the conjunctival sac. Some eyes harbored M. bovis up to $5\frac{1}{2}$ months. He also exposed the eyes and/or conjunctival sacs of mice, guinea pigs, pigs, a rabbit, 2 sheep and 2 cockerels to saline suspension of M. bovis in the smooth phase. Neither conjunctivitis nor keratitis was produced and M. bovis was not recovered.

Cooper suggested that variation in response to experimental infection may be due to factors concerning the animal or the inoculum. In the latter case he reported that certain strains of M. bovis exhibited colony differences, e.g., being flat topped, rough and smooth surfaced, and more or less mucoid. These variations indicate that there may be fundamental strain differences apart from virulence variation.

Adinarayanan and Singh (2) reported in 1961 that they isolated 40 strains of M. bovis from clinical cases of IBK which conformed to the description of M. bovis in Bergey's Manual (31). Two cows and 3 buffalo were found to be healthy carriers of the organism. These investigators were able to produce the disease in 1 of 4 calves using a pure culture of M. bovis.

In 1962 Calcarami and Witt (34) reported on a study carried out in Argentina. They isolated an organism from a majority of the cases of infectious keratoconjunctivitis which

was identical in characteristics to those prescribed for M. bovis.

Not all the cases of infectious keratoconjunctivitis with the clinical manifestations and symptoms so characteristic of M. bovis pinkeye have been attributed to it. Citing what is probably the most important example, Sykes (123) in 1962 of Texas isolated a virus from cases of infectious keratoconjunctivitis of cattle which produced a cytopathic effect in monolayers of bovine embryonic lung, which by serum neutralization test was shown not to be IBR virus. Later Sykes et al. (124) in 1964 reproduced infectious keratoconjunctivitis in a large number of cattle using this isolate, viruses isolated from bovine "cancer eye" lesions and various strains of IBR virus. The experimental disease was indistinguishable from the naturally-occurring disease under study, but corneal lesions were not observed in all animals. The predominant feature of the experimental condition was lacrimation.

Reference literature on the role of IBR virus and other etiologic agents of eye diseases has been extensively reviewed recently by Wilcox (130) and we feel that further discussion here is not warranted because of the nature of our studies.

Griffin et al. (55) as well as Gleeson (53) in 1965 reported an outbreak of infectious keratoconjunctivitis in cattle in Nigeria, West Africa where the role of M. bovis was inconclusive. Among 55 cases of the disease they observed 29 cases of keratitis and 26 of keratoconjunctivitis. Both

hemolytic and nonhemolytic diplobacilli were isolated; the hemolytic organism was later identified as M. bovis. Attempts at transmitting infection to 3-month-old calves by instillation of lacrimal washing or broth cultures of M. bovis failed. Attempts to produce the disease by ocular instillation in rabbits, guinea pigs and mice also failed.

Griffin et al. (55) also cultured conjunctival secretions from 24 normal animals and 6 animals which had recovered from infectious keratoconjunctivitis. Moraxella bovis was isolated from one of the previously infected animals and from 6 normal animals; one of the 6 normal animals subsequently developed clinical disease. Repeated instillation of saline washed-off plate cultures of M. bovis failed to cause disease. These investigators therefore concluded that M. bovis was not the primary etiologic agent of IBK.

Hughes et al. (67) in 1965 reported on the experimental production of IBK using M. bovis inoculation along with daily mercury sunlamp irradiation. The authors proposed that the ultraviolet radiation of the sunlamp has an enhancing effect on M. bovis infection in the bovine eye. The disease produced by mercury sunlamp irradiation and M. bovis was indistinguishable from field cases of IBK.

Pugh et al. (106) in 1966 reported on the isolation and characterization of M. bovis. They made a cultural comparison between laboratory and field strains of M. bovis as well as among Moraxella liquefaciens, Moraxella nonliquefaciens, Mima

polymorpha, and Mima polymorpha var. oxidans. Also they compared M. bovis with unidentified diplococci isolated from cattle with infectious keratoconjunctivitis. Although it was found that these organisms were very similar in general characteristics, they could be distinguished from each other without difficulty. For example, only M. bovis and the hemolytic diplococci had been reported occurring in cases of IBK.

Moraxella bovis differed from the hemolytic diplococcus in that the former was a diplobacillus and did not reduce nitrates to nitrites (which also distinguished it from M. liquefaciens) while the latter was a diplococcus and reduced nitrates to nitrites. All but the genus Mima produced similar characteristic changes in litmus milk. Moraxella liquefaciens and M. nonliquefaciens differed from each other in that the former liquefied gelatin and the latter did not. They described both catalase positive and negative strains of M. bovis. Later Hughes et al. (68, 69) could not find any difference in the pathogenicity of these strains for cattle. Pugh et al. (106) also reported the occurrence of both hemolytic and nonhemolytic strains of M. bovis. Later Pugh and Hughes (105) in 1968 were able to make a correlation between the hemolytic ability of M. bovis and its ability to produce disease. They also described instances where nonhemolytic M. bovis changed into hemolytic M. bovis and vice versa.

More recently Pugh et al. (107) in 1968 exposed sheep, rabbits, rats, guinea pigs, and mice to M. bovis by

conjunctival instillation. Moraxella bovis became established in the eyes of sheep and mice, but not in other animals. Thirteen of 18 mice exposed to M. bovis developed conjunctivitis, and 10 of the 13 developed keratitis. The disease in mice was grossly similar to IBK. The organisms did not produce disease in the eyes of sheep.

Immunologic Aspects of Moraxella Bovis Infection

Reid and Anigstein (112) investigated IBK in the summer of 1944 and reported that they found agglutinins specific for M. bovis in the serums of convalescent animals from both natural and experimental infections. They suggested that their findings indicated that prophylactic measures against IBK would be possible.

Baldwin (13) in 1945 conducted tests on the serum of 11 experimental calves after they had developed signs of IBK. Antibodies were not detected before or after infection. He used the precipitin and complement-fixation tests in his study. Agglutination tests were not conducted because no method was found whereby the spontaneous agglutination of M. bovis could be inhibited.

Barner (14) in 1952 reported that 6 cattle known to have had an acute attack of keratitis were not susceptible to reinoculation of M. bovis one year following experimental infection. It was suggested that these animals had enhanced resistance to a second infection.

Jackson (71) in 1953 reported that agglutination test serums from animals experimentally infected showed an agglutinin titer as high as 1:320 after the 14th to 20th day of infection. These results indicated that circulating antibodies were produced as a result of infection.

Gallagher (51) in 1954 was able to demonstrate an increase in serum agglutinins in four cases of experimental IBK. A titer of 1:280 was reached in 2 cattle between the 25th and 33rd days after infection, and 1:640 in the other 2 between the 18th and 31st days. The titers of the first 2 animals had fallen to 1:80 and 1:40, respectively, at the 110th day. He described the flocculum formed by M. bovis in the presence of immune serums as being slow in settling and at 24 hours appeared mostly a feathery suspension with a clear liquid phase. Gallagher was unable to reinfect recovered animals.

Formston (50) in 1954 reported on an outbreak of IBK where some animals had as many as three attacks in the same eye. He suggested that animals which had passed through all stages of the disease acquired some immunity and those which had had a transient attack are prone to subsequent attacks.

Seth and Chandrasekariah (119) in 1957 reported that they used a formalinized antigen of M. bovis for a tube agglutination test on serums of infected animals. Serum titers increased to 1:320 20 days postinfection.

Henson et al. (63) in 1960 reported on the use of experimental formalized bacterins for the immunization of cattle

against IBK using smooth cultures of M. bovis. A total of 1,837 cattle were injected with the bacterins while 1,777 served as controls. These bacterins did not stimulate an immunity sufficient to protect cattle from artificial or natural exposure to the disease. However, calves that recovered from the disease caused by the first challenge did not become reinfected when exposed a second time.

Adinarayanan and Singh (2) in 1961 reported that serums of experimental animals as well as natural cases gave a positive agglutination titer varying between 1:80 to 1:640, when taken 2 to 3 weeks after infection. They used a tube test.

Chowdhury (39) in 1963 in a study on the nutrition, antigenicity and serological characteristics of different strains of M. bovis, showed that the different strains had different abilities to stimulate antibody formation in rabbits. He also found nutrients in the mediums influence the antigenicity of the bacterins used to produce antibodies. Heat-killed organisms were used because live cultures of M. bovis were highly lethal to experimental animals.

Hughes et al. (69) in 1968 exposed animals to different strains of M. bovis and later reexposed them to homologous and heterologous strains. After the initial exposure all of 42 eyes became infected and 28 developed keratitis; after the second and third reexposures, 32 of 58 eyes became infected and only 2 developed keratitis. Both instances of keratitis that occurred in the eyes after reexposures were caused by the

heterologous strain of M. bovis. The reduction in the rate of infection and the number of eyes developing keratitis was considered a reflection of enhanced resistance.

History of the Species Within the Genus Moraxella

Moraxella bovis, historically, has its origin intimately related to that of similar Moraxellae recovered from human sources. The first species of Moraxella described was the Morax-Axenfeld bacillus which is responsible for subacute or acute angular conjunctivitis in man, and was described independently by Morax (92) in 1896 and Axenfeld (9) in 1897. In 1900 Eyre (46) conducted a clinical and bacteriological study on the conjunctivitis and its cause and suggested the name Bacillus lacunatus for the organism. He suggested this name because of the pit-like depression produced upon the surface of inspissated blood serum agar. Later in 1942 Oag (94) discussed the biological properties of this organism.

In 1898 Petit (10) described an organism, that was very similar to the Morax-Axenfeld bacillus, isolated from the eye of a patient suffering from ulcus serpens. This organism became known first as Petit's bacillus, and later as Diplobacillus liquefaciens Petit (10) because in addition to serum it also liquefied gelatin. More recently Oeding (95) has reported a study of Diplobacillus liquefaciens Petit, isolated from a patient with an ulcus serpen corneae, in which he gave

a description of the microbe, along with a historical survey and discussion on the classification of the diplobacilli.

In 1916 Scarlett (115) described 2 organisms; one he described as Bacillus duplex nonliquefaciens which was identical to Bacillus liquefaciens but was nonproteolytic and the other Bacillus duplex Josephi which was isolated from a patient (Joseph) with a corneal ulcer. This latter organism was similar in all respects to the bacillus of Morax-Axenfeld, apart from being Gram positive. No antigenic relationship between Diplobacillus nonliquefaciens Scarlett and the diplobacillus of Morax-Axenfeld or Petit's bacillus could be demonstrated.

The generic position of these nonmotile diplobacilli isolated from human eye disease remained obscure until in 1917 when Bacillus lacunatus was included in the genus Haemophilus, which had been newly created by a committee of American Bacteriologists as part of a sweeping nomenclatorial revision of the bacteria (131). The type species selected for the genus Haemophilus was Haemophilus influenzae. This taxonomic treatment was adopted and maintained through the first four editions of Bergey's Manual of Determinative Bacteriology.

Allen (5) in 1919, reported the findings of a Gram-negative diplobacillus in the ocular discharges of cattle affected with ophthalmia which resembled Hemophilus liquefaciens in its cultural characteristics. Later Jones and Little (73) isolated an organism from diseased cattle eyes in 1923. The organism

was Gram-negative hemolytic and similar to the Morax-Axenfeld bacillus as well as the Petit's bacillus in its cultural characteristics. This organism isolated by Jones and Little was classified by Hauduroy et al. (56) as Haemophilus bovis, and later placed in the genus Moraxella by Lwoff (83).

Lwoff (83) in 1939 created a new genus for some of the Gram-negative species of diplobacilli previously assigned to the genus Haemophilus. Lwoff thought that these organisms (Moraxellae) should be separated from the Haemophileae, as its species neither resembled the Haemophileae morphologically nor required haematin or phosphopyridinenucleotide as growth factors. The genus created by Lwoff included the organisms Morax-Axenfeld bacillus (Moraxella lacunata), Moraxella duplex liquefaciens (Petit, 1900), Moraxella duplex nonliquefaciens (Scarlett, 1916), and Moraxella duplex des Bovides (Jones and Little, 1923).

Audureau (8) in 1940 described 3 diplobacilli isolated from human eyes with corneal ulcers. She added Moraxella lacunata atypica and Moraxella lwoffii to the list of Lwoff. It could be differentiated from both M. lacunata and M. duplex by its ability to grow in a mineral medium with ethyl alcohol as the sole carbon source. Piéchaud et al. (100) in 1950 proposed one additional species, M. glucidolytica, for strains nutritionally nonexacting Moraxellae that were able to produce acid from glucose and other monosaccharides.

The work of Lwoff (84), Audureau (8), and Piéchaud et al. (99, 100) led to the recognition of three species in the genus Moraxella: M. lacunata and M. duplex, both apparently nutritionally exacting but to different degrees; and M. glucidolytica, which requires no organic growth factors and can be distinguished from one another by action on sugars. An additional important difference between the lacunata-duplex and lwoffii-glucidolytica subgroups was reported by Henriksen (57) and Piéchaud et al. (100), who discovered that the former are oxidase-positive, whereas the latter are oxidase-negative. They also found that the oxidase-positive subgroup is much more sensitive to penicillin than is the oxidase-negative one.

Independently and at about the same time, De Bord (44), in a study on organisms from cases of conjunctivitis and vaginitis, described two organisms under the names Herellea vaginicola and Mima polymorpha which correspond in their properties to M. glucidolytica and M. lwoffii respectively. He also described oxidase-positive strains equivalent to Lwoff's M. duplex which he classified as M. polymorpha var. oxidans.

It should be noted here that the oxidase-positive Moraxellae are parasitic upon the mucous membranes as opposed to the oxidase-negative Moraxellae that are common inhabitants of soil and water. Many of them are very versatile with respect to the range of utilizable carbon sources, as first reported by Lwoff and Audureau (84) for a strain of M. lwoffii. A similar

study has been reported by Hodgson and McGarry (64) as well as Rampon (109).

Bövre and Henriksen (29) in 1967 described a new Moraxella species, Moraxella osloensis, and presented a revised description of Moraxella nonliquefaciens. This report was based on studies by one of the authors (20, 24, 27) on a collection of Moraxella strains by transformation experiments, by analysis of the DNA base composition and by more conventional methods.

Pande and Sekariah (97) in 1960 described a new species of Moraxella, Moraxella caprae, from an outbreak of infectious keratoconjunctivitis in goats but this organism has not been reported by other workers. Still more recently a new species of Moraxella from human sources has been reported by Henriksen and Bövre (58). This species, Moraxella kingii, is characterized by beta haemolysis, acid production from glucose and maltose and lack of catalase activity.

Recent Classification of Moraxella

Earlier taxonomists have classified Moraxellae on the basis of morphology and nutritional requirement and/or growth in biochemical mediums and sensitivity to antibiotics (93). More recently Baumann et al. (15), Bövre (18, 19, 20, 21, 22, 23, 24, 25, 26, 27), Bövre and Henriksen (28) and Henriksen and Bövre (59) have proposed a new approach to Moraxella classification based on transformation experiments and determination of

DNA base composition along with nucleic acid hybridization experiments.

Henriksen and Bövre (59) as well as Baumann et al. (15) point out that one of the main problems still associated with the classification of Moraxella is the fact that the Moraxellae mimic two other groups of Gram-negative bacteria: morphologically, many of them are difficult to distinguish from the Gram-negative cocci of the genus Neisseria; physiologically and biochemically, the more nutritionally versatile Moraxellae resemble in many ways members of the genus Pseudomonas.

Baumann et al. (15), on the basis of their study, have proposed the following definition for the genus Moraxella:

"Genus Moraxella lwoff (emend). Nonspore-forming, nonflagellated, rod-shaped bacteria, occurring characteristically in pairs, or sometimes in short chains; Gram-negative, although some strains are relatively resistant to decolorization with alcohol by comparison with such frankly Gram-negative species as Escherichia coli. The G+C content of the DNA is in the neighborhood of 40 to 46 moles per cent. Oxidase positive, possessing cytochromes of the B and C types as the major components of the cytochrome systems. Catalase positive. Obligately aerobic chemoorganotrophs, incapable of denitrification. Incapable of using carbohydrate, polyols, or aromatic compounds as carbon sources. Do not produce acid from glucose aerobically. Penicillin-sensitive: None can grow in the presence of 1 unit of penicillin G per milliliter."

Henriksen and Bövre (59) disagree with this definition of the genus Moraxella. In their revision of the genus Lwoff, 1939 they place the genus under the family Neisseriaceae defined by the following characteristics:

"Organisms coccial or rod-shaped. Tendency toward pleomorphism. No pigment. Growth on standard media may be poor or fail. Biochemical activities limited. Most species do not attack carbohydrates. Indole is not produced. Nitrates may or may not be reduced. Catalase may or may not be produced. G+C content of DNA in the range of 40-50 moles per cent. Parasites of mammals."

The type species is Moraxella lacunata.

The species under the genus Moraxella according to Henriksen and Bövre would be as follows:

Species 1. M. lacunata (includes the biotype M. liquefaciens).

Species 2. M. nonliquefaciens (Scarlett, 1915),
Murray 1948.

Species 3. M. bovis (Hauduroy et al., 1937), Murray,
1948.

Species 4. M. osloensis (Bövre and Henriksen, 1967).

Species 5. M. phenylpyrouvica (Bövre and Henriksen,
1967).

Species 6. M. kingii (Henriksen and Bövre, 1967).

Species 7. M. catarrhalis (Frosch and Kolle, 1896),
Henriksen and Bövre, 1967.

Species 8. M. ovis (Lindquist, 1960), Henriksen and
Bövre, 1967.

Species 9. M. caviae (Pelczar, 1953), Henriksen and
Bövre, 1967.

The classification of the genus Moraxella has also been discussed by Mitchell and Burrell (89) as well as by Cary (37)

on the basis of antigenic relationship between the Moraxellae. Mitchell and Burrell in 1964 demonstrated strong serological cross reactions between strains of Herellea vaginicola, Mima polymorpha var. oxidans, Moraxella nonliquefaciens, Moraxella lwoffii (Moraxella bovis listed under this species), Bacterium anitratum (Herellea species) and Moraxella liquefaciens. They suggested that the species Moraxella nonliquefaciens should contain both M. nonliquefaciens and Mima polymorpha var. oxidans and distinguished on the basis of distinct serotypes. Mima lwoffii would include the organism previously designated as Moraxella lwoffii and Mima polymorpha and Mima anitratum would include the designation Herellea vaginicola. Bacterium anitratum and other glycolytic organisms would be given synonymous status with H. vaginicola.

SECTION I. STUDIES ON THE MORPHOLOGICAL, CULTURAL AND
PHYSIOLOGICAL CHARACTERISTICS OF MORAXELLA BOVIS

Introduction

Probably the greatest impediment to a better understanding and appreciation of the disease known as IBK has been the lack of information on the etiologic agent, namely, M. bovis. Few investigators have studied the morphological, cultural, and physiological characteristics of M. bovis as they relate to the ability of the organism to cause IBK and neither have they postulated such a relationship. However, as with other organisms, it would not be surprising if the virulence of different strains of M. bovis would vary irrespective of whether those characteristics of the organism are detectable by routine laboratory methods. Nevertheless, a prudent approach to the resolution of the confusion associated with M. bovis infection in the bovine eye would be to characterize the infectious agent and to relate these characteristics to the ability of a specific strain of the organism to cause IBK.

Also many confusing similarities exist between the group of organisms that Henriksen and Bøvre (59) and Baumann et al. (15) would classify under the genus Moraxella. Therefore, there is a question of whether organisms assumed to be M. bovis by some investigators are actually strains of M. bovis or are they members of other species of the genus Moraxella. This question emanates from the fact that variations exist in the descriptions of the cultural characteristics of M. bovis

reported by many investigators (5, 8, 31, 47, 113). Some workers (5, 47, 53, 54, 55) have failed to produce IBK using pure cultures of the organism they described. Obviously the first criterion of an effective exposure organism, the importance of the host animal notwithstanding, is that it must be virulent. It should be pointed out, however, that a number of these reports (8, 113) were concerned mainly with the cultural characteristics of other members of the genus Moraxella or Moraxella-like organisms and did not consider M. bovis in any detail.

Recently some investigators (41, 105, 106, 127) have demonstrated or suggested differences between cultures of M. bovis which is thought to relate to their ability to produce IBK. The main differences noted were colony variation, catalase activity, and hemolytic ability.

The purpose of this study was to characterize isolates from cattle suspected of having IBK and to compare them with reference strains of M. bovis and with other Gram-negative organisms with which they might be confused.

Materials and Methods

Organism

The field strains of M. bovis were isolated from the eyes of cattle in herds affected with IBK. Eye secretions were collected from the conjunctiva with sterile cotton-tipped applicators and the applicators placed in screw-capped tubes

containing sterile trypticase soy broth (TSB). The capped tubes containing the applicators were placed in an insulated box (ice chest maintained at temperatures of less than 15 C) and transported to the laboratory. The applicators containing the secretions were streaked on blood agar plates immediately or they were stored at -60 C until used (from 1 week to 5 years). In order to examine the stored secretions, the applicators were allowed to stand at room temperature until the secretions thawed and then streaked on blood agar plates.

Two laboratory strains of M. bovis (ATCC, 10900¹ and Beltsville 8613²) were used as reference strains in the investigation of the field isolants. Colonies from blood agar plates streaked with eye secretions from the affected cattle herds which resembled those of the reference strains were picked and stained by the Gram method to determine the morphologic features of the cells.

Those organisms which proved to be Gram-negative, non-motile diplobacilli were then tested with M. bovis (ATCC 10,900) rabbit antiserum with an agar gel double-diffusion technique which will be discussed in Section II. Those organisms which had one or more lines of identity with the reference strains

¹American type culture collection, Washington, D.C.

²Courtesy of Mr. K. Heddleston, National Animal Disease Laboratory, Ames, Iowa.

were included in this study as field strains¹ and identified as: KGD-63 (1 culture²), SWN-66 (2 cultures), IBH-68 (2 cultures), NTN-63 (3 cultures), KTy-65 (3 cultures), NDL-67 (3 cultures), HIM-63 (4 cultures), GLN-63 (4 cultures), NDL-68 (4 cultures), NDL-63 (6 cultures), IVI-64 (7 cultures), WSE-64 (8 cultures), FLA-64 (8 cultures), EPP-63 (9 cultures), IBH-66 (19 cultures), IBH-65 (22 cultures), IBH-64 (24 cultures), IBH-63 (26 cultures), and IBH-67 (33 cultures).

Organisms which produced colonies similar to those of M. bovis after 24 hours incubation on blood agar were isolated frequently from the various herds. Although they were diplococci, they were included in the study because they were similar to M. bovis and designated as: GLD-63 (2 cultures), EPD-63 (2 cultures), IBD-65 (2 cultures), IBD-66 (2 cultures), IBD-63 (4 cultures), and WSD-64 (5 cultures).

For comparison, several organisms which are likely to be confused with M. bovis were studied. These organisms were obtained from a culture collection³ and were identified as follows: Mima polymorpha, A-130 and A-188; Mima polymorpha var. oxidans, 9561 and 9714; Moraxella liquefaciens, 9833 and

¹All organisms isolated from the same herd during a specific epizootic are considered to be the same strain.

²Culture is considered to mean the organisms isolated from a single animal.

³Courtesy of Miss Elizabeth O. King, Communicable Disease Center, U.S. Public Health Service, Atlanta, Georgia.

9985; Moraxella nonliquefaciens 9893 and 9944; and Herellea vaginicola A-259, 255, and 173.

Culture mediums

Carbohydrate fermentation mediums were prepared in phenol red broth base¹. The following carbohydrates were incorporated individually at the 1.0% level: arabinose, dextrose, dulcitol, galactose, inositol, inulin, lactose, maltose, mannitol, mannose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose. Other liquid mediums included urea broth, methyl red and Voges-Proskauer test medium, thiol medium, nitrate broth, potassium cyanide medium (45), lysine and ornithine decarboxylase test mediums (35, 45), trypticase soy broth² (TSB), and enriched TSB containing 5.0% by volume of rabbit serum. Litmus milk medium was prepared from Bacto³ skim milk and contained 0.16% litmus. Liquid mediums were inoculated in duplicate for each test with 0.1 ml. of 24-hour TSB subculture of a typical colony picked from a 24-hour blood agar plate.

Several semisolid and solid culture mediums from commercial sources were used. The mediums were prepared according to the manufacturer's directions. Bacto mediums included blood

¹Phenol red broth base, Difco Laboratories, Detroit, Michigan.

²Trypticase soy broth, Baltimore Biological Laboratories, Baltimore, Maryland.

³Bacto mediums, Difco Laboratories, Detroit, Michigan.

agar base containing 5.0% by volume defibrinated bovine blood (blood agar plates, hereafter referred to as BAP) nutrient gelatin, Simmons citrate agar, and sulfide-indole-motility (SIM) medium. Trypticase soy agar¹ (TSA) was also used.

Potato infusion agar (PIA) was prepared from the infusion of 250 Gm. of potatoes to which were added 1.5% agar, 1.0% proteose peptone, 0.5% beef extract, 0.5% NaCl, and 0.5% glycerin. The pH was adjusted to 6.8, and the medium was sterilized by autoclaving at 121 C for 15 minutes. Oxidation-fermentation test medium (45) utilizing the 16 carbohydrates was also used.

The semisolid mediums were inoculated by streaking a typical colony from a 24-hour blood agar plate culture. However, 6 to 8 colonies were used to inoculate the PIA plates. All cultures were incubated at 37 C; in addition to this, the nutrient gelatin also was incubated at 25 C.

Oxidase test

Twenty-four hour isolated colonies on TSA were flooded with 0.1% distilled water solution of N, N, dimethyl-p-phenylene diamine². Colonies were observed for 2 to 4 minutes for the development of a dark purple color indicating oxidase activity.

¹Trypticase soy agar, Baltimore Biological Laboratories, Baltimore, Maryland.

²Eastman Organic Chemical Distillation Products, Inc., Rochester, New York.

Catalase test

Twenty-four hour cultures were used. One drop of 3% hydrogen peroxide was added to each of the test colonies on TSA plates, and 0.1 ml of 3% hydrogen peroxide was added to each tube of thiol medium. The presence of the enzyme catalase was evidenced by effervescence.

Results

Staining and morphologic features

All strains of M. bovis were Gram-negative, bipolar staining, short, plump rods with rounded ends usually occurring in pairs or in short chains. The morphologic features of characteristic strains of M. bovis are represented in electron photomicrographs (Figures 1-4). The typical cells were encapsulated and from 0.5 to 1.0 micron in width and 1.0 to 2.0 microns in length. As the cultures grown on agar plates aged, the organism became progressively more pleomorphic. Strains of Mima polymorpha var. oxidans, Mima polymorpha, Moraxella liquefaciens, Moraxella nonliquefaciens were similar to the pleomorphic M. bovis but had more coccoid forms. The strains of Herellea vaginicola occurred in pairs mostly but were more coccoid than rod shaped. The unidentified diplococcus differed from these in that they were always spherical and occurred in pairs.

Figure 1. Depicts Moraxella bovis (hemolytic strain) illustrating typical surface convolutions and clean borders using negative staining with phosphotungstic acid (PTA). Note the wide and prominent division planes in dividing cells - 60,000X

Figure 2. Depicts Moraxella bovis (hemolytic strain) with PTA staining. Note that the surface bears numerous short fibrillar extrusions - 120,000X

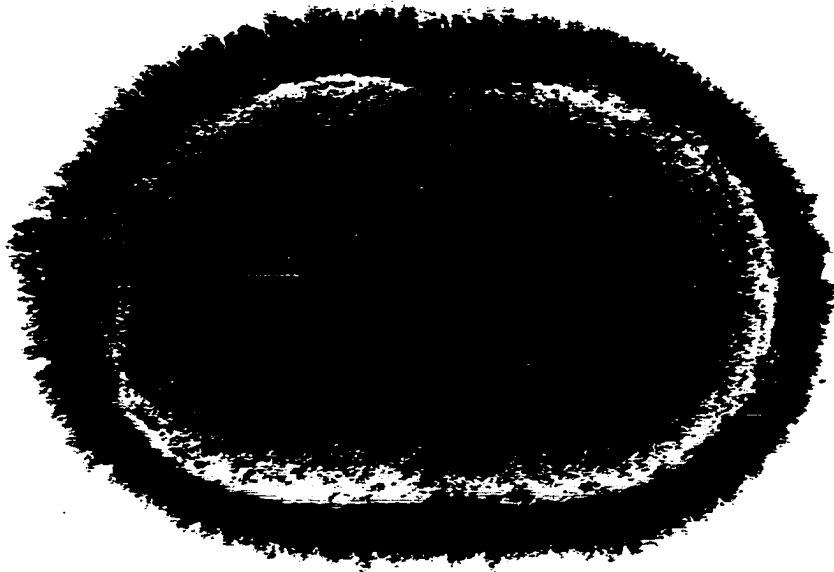


Figure 3. Thin section of Moraxella bovis (hemolytic strain) dividing cell illustrating its typical scalloped outer membrane and underlying thick cytoplasmic membrane with PTA staining. Note the wide septum at the division plane and a pool of amorphous mass in the space between the cells - 105,000X

Figure 4. Thin section of Moraxella bovis (nonhemolytic strain) dividing cell illustrating a narrow septum of the division plane with PTA staining - 105,000X



Growth on blood agar

After 24 hours incubation at 37 C, typical colonies of M. bovis were 1.0 to 3.0 mm. in diameter, with a zone of beta hemolysis about 0.5 to 1.0 mm. in width. Colonies were circular with an entire edge, convex to umbonate, glistening, translucent, grayish white, and slightly indented into the medium. A small pit was evident when the colony was displaced with an inoculating needle. Typical colonies were firm and adherent but tended to fragment when picked with an inoculating needle but atypical colonies, to be described in a later paragraph, did not conform to all of these features.

After 48 hours, the typical colonies (Figures 5 and 6) were 3.0 to 4.0 mm. in diameter and appeared flat, with the zone of hemolysis increased to 1.0 to 1.5 mm. in width. The zone of hemolysis increased twofold in width when the cultures were first incubated at 37 C for 24 hours and then incubated 24 hours at 25 C. Some cultures (Table 1) did not produce hemolysis on blood agar plates. Others that are to be discussed below formed dissociated and atypical forms (Figures 5 to 11).

The rough and dwarf forms of colony variation were the most frequent types of atypical colonies seen on blood agar plates. The dwarf forms occurred most frequently in low blood agar passaged material (1-5 passages) along with the normal size colony (Figure 5) and intermediate size colonies. The dwarf colonies usually gained their greatest size (1.0 to

Table 1. Cultural reactions of reference and field strains of Moraxella bovis and unidentified diplococci

Strains	No. of cultures	Hemolysis		Catalase activity		Nitrate reduction		Gelatin liquefaction	
		pos.	neg.	pos.	neg.	pos.	neg.	pos.	neg.
diplococci (6 strains)	17	17	--	17	--	17	--	17	--
8613	1	1	--	--	1	--	1	1	--
10900	1	1	--	--	1	--	1	1	--
KGD-63	1	1	--	1	--	--	1	1	--
SWN-66	2	2	--	--	2	--	2	2	--
IBH-68	2	2	--	2	-- ^a	--	2	1	1
NTN-63	3	2	1	2	1 ^a	--	3	3	--
NDL-67	3	3	--	3	--	--	3	3	--
KTY-65	3	3	--	3	--	--	3	3	--
HIM-63	4	4	--	4	--	--	4	4	--
GLN-63	4	4	--	4	--	--	4	4	--
NDL-68	4	2	2	4	--	--	4	4	--
NDL-63	6	6	--	--	6	--	6	6	--
IVI-64	7	7	--	7	--	--	7	7	--
WSE-64	8	8	--	8	--	--	8	8	--
FLA-64	8	8	--	8	--	--	8	8	--
EPP-63	9	8	1	9	--	--	9	9	--
IBH-66	19	6	13	10	9	--	19	19	--
IBH-65	22	16	6	20	2	--	22	22	--
IBH-64	24	24	--	--	24	--	24	24	--
IBH-63	26	23	3	22	4	--	26	26	--
IBH-67	33	27	6	--	33	--	33	33	--
Totals	207	175	32	124	83	17	190	206	1

^aThe catalase negative culture was also nonhemolytic.

- Figure 5. (Upper left). Depicts a typical colony of smooth Moraxella bovis showing the characteristic morphology. Note the concentric rings and dense center and round even borders (48 hours incubation) - 40X
- Figure 6. (Upper right). Depicts two contrasting colonies of M. bovis, one rough (left) and one smooth (right). Note the unevenness and flatness of the border as well as the granular texture of the rough colony. The smooth colony lacks the outstanding detail of the smooth colony in Figure 5 (48 hours incubation) - 40X
- Figure 7. (Lower left). Depicts a dwarf colony of M. bovis after 48 hours incubation; note difference in size of this colony as compared to those in Figures 6 and 7 - 40X
- Figure 8. (Lower right). Depicts a typical colony of M. bovis with a dwarf colony at its upper border (48 hours incubation) - 40X

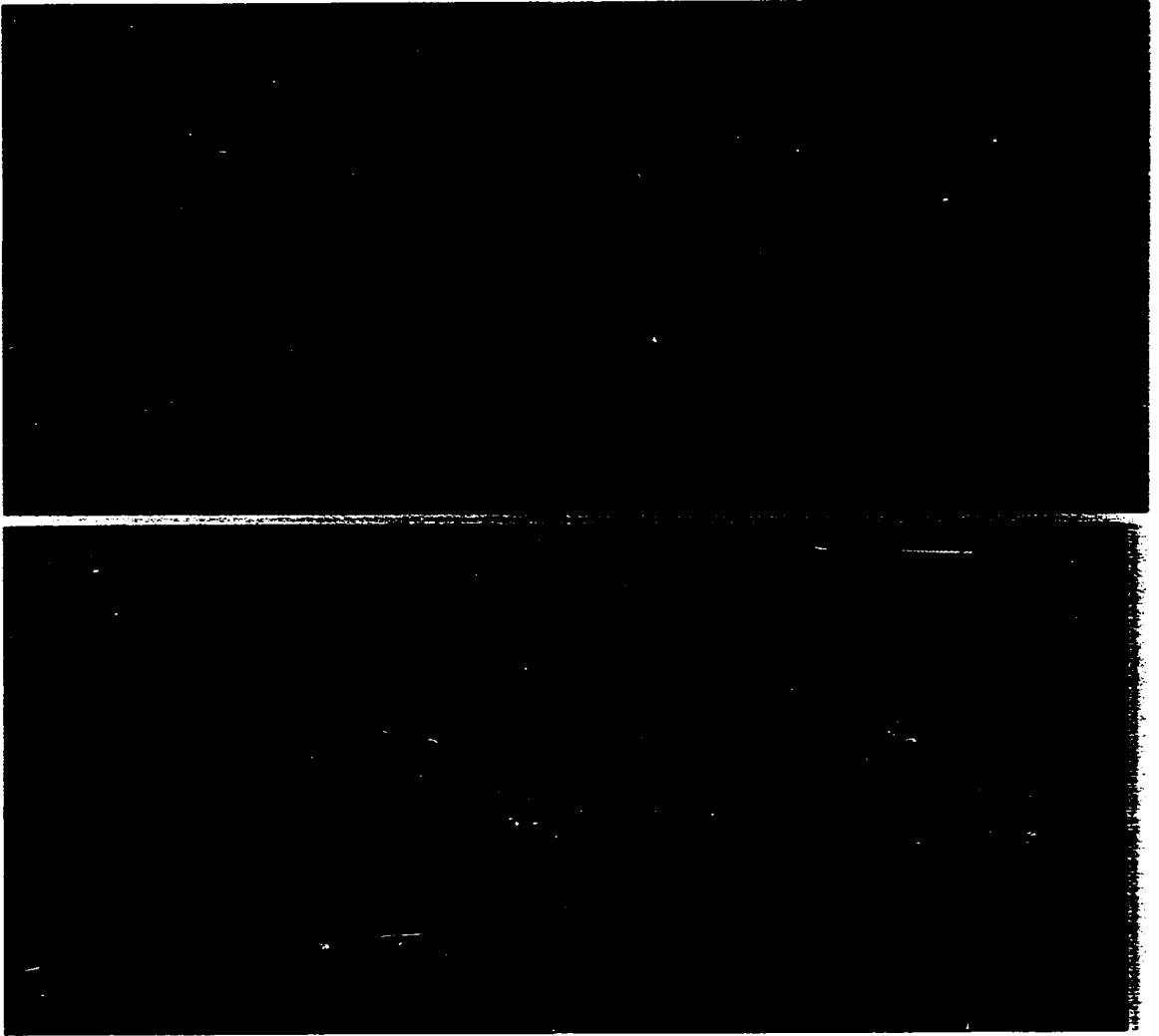
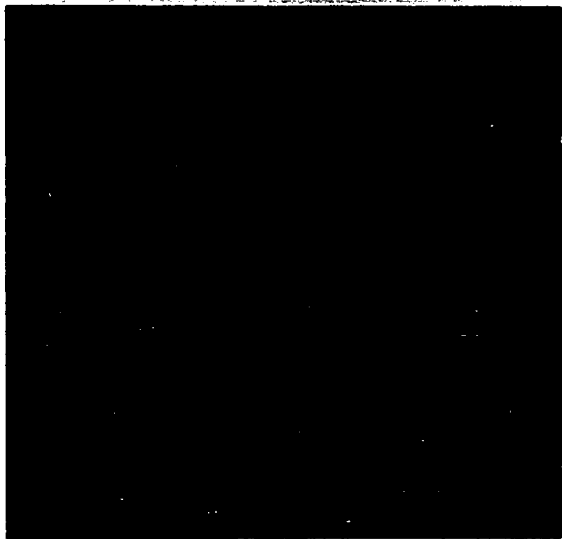


Figure 9. (Upper left). Depicts an intermediate stage in the smooth variation. Note the extension of the uppermost border of the colony (36 hours incubation) - 40X

Figure 10. (Upper right). Depicts a typical rough M. bovis colony after 48 hours incubation. Note the unevenness and flatness of the border as well as the granular texture of the colony's periphery. The center of the colony is butyrous - 40X

Figure 11. (Lower left). Depicts a typical rough M. bovis colony after 72 hours incubation. Note the increased granularity of the colony's texture - 40X



2.0 mm. in diameter) within 48 hours and failed to increase in size thereafter. Subcultures of the dwarf colonies produced only dwarf colonies as contrasted with the normal sized M. bovis which gave rise to both normal and dwarf size colonies. There was a tendency to eliminate the ability to produce dwarf colonies as the colonies were subcultured until fifth and sixth blood agar passage when the typical colonies rarely gave rise to dwarf colonies. The cultures producing dwarf colonies did not differ from the typical M. bovis in any other way.

Usually the rough colonies were seen when cultures of M. bovis were subcultured from liquid medium onto blood agar plates or isolated from convalescent cattle. Many times there were intermediate stages of roughness (Figures 9-11), where one-eighth to seven-eighths of the colony was typically rough, and the other one-eighth being like the typically smooth colonies or vice versa. If a subculture was taken from the rough portion of the colony, usually the progeny was rough. When the smooth portion of the colony was subcultured, both smooth and rough colonies were produced with the former predominating. The rough colonies were usually larger, flatter, had irregular borders, sometimes granular and were more butyrous in texture than the other colonial forms. The rough type readily suspended uniformly in TSB, distilled water, and physiological saline solution (PSS) while the other types of colonies autoagglutinated in these mediums.

Another significant variation encountered was the spontaneous change of hemolytic M. bovis to the nonhemolytic type. On several occasions hemolytic M. bovis gave rise to nonhemolytic progeny, and in two instances nonhemolytic M. bovis gave rise to hemolytic progeny under normal incubation and growth on blood agar plates. The nonhemolytic M. bovis also showed dissociation similar to that of the hemolytic cultures mentioned earlier.

Prolonged subculturing on blood agar (100 passages) did not cause one culture (EPP-63(300) of a smooth hemolytic M. bovis to dissociate to rough forms but the organism suspended to a greater extent in liquid mediums. Minute protuberances or papillae were not seen in old or senescent cultures of any of the colonial forms.

The colonies of the unidentified hemolytic diplococcus were similar to those of M. bovis after 24 hours incubation. However, after 48 to 72 hours, the colonies were larger than those of M. bovis and developed minute protuberances which became more prominent with age. The colonies also had concentric rings which were wider and more prominent than those of M. bovis colonies (Figure 5). The colonies of the unidentified diplococci suspended uniformly in TSB, distilled water and PSS.

Colonies of M. liquefaciens were similar to those of M. bovis except that they were smaller (the maximum size obtained up to 72 hours incubation was 2 mm in diameter).

Colonies of M. nonliquefaciens were similar to those of M. bovis except that they were nonhemolytic. Strains of M. liquefaciens and M. nonliquefaciens suspended uniformly in the liquid mediums.

The colonies of Mima polymorpha and Mima polymorpha var. oxidans resembled grossly those of M. bovis after 72 hours incubation; however, they were nonhemolytic and of different consistency than M. bovis. Colonies of Mima polymorpha were firmly adherent to the surface of the agar and tended to be membranous while those of Mima polymorpha var. oxidans differed from M. bovis by being more butyrous in consistency. Strains of both species of Mima failed to suspend in the liquid mediums.

After 24 hours incubation the colonies of Herellea vaginicola were 4 to 7 mm. in diameter, and two strains (255 and A-173) were nonhemolytic. Colonies were circular with entire borders, convex, glossy with a greenish tinge, and butyrous. Strain A-259 produced hemolysis characterized by lack of hemolysis immediately surrounding the colony (the first 3.0 mm.) but a wide band of beta hemolysis (4 mm) adjacent to it. Otherwise, colonies of this strain were similar to the other two strains. Colonies from plates suspended uniformly in water, TSB and PSS.

Growth on trypticase soy agar

Colonial morphologic features of all organisms in the study were similar whether they were grown on TSB or on blood agar plates.

Growth in trypticase soy broth

After 48 to 72 hours incubation at 37 C, scant growth was observed with all strains of M. bovis; turbidity was slight, but considerable sediment was formed, which, when agitated, broke into coarse particles. By incubating some cultures on a rotary shaker, the broth became turbid (comparable to McFarland nephelometer No. 5) within 24 hours, with only a small amount of sedimentation. Growth was further enhanced by the addition of 5% by volume of rabbit serum. Spontaneous agglutination (autoagglutination) was marked in newly isolated cultures, but this characteristic lessened upon continuous subculturing and upon selection of the most stable colony type (rough) for inoculation. In all instances where there was pronounced turbidity the cultures produced rough colonies when subcultured on blood agar plates, and the colonies from the latter suspended well in distilled water, TSB and physiological saline. The turbid cultures also resuspended uniformly in these mediums after being centrifuged and washed twice.

The growth of M. liquefaciens was similar to the smooth phase M. bovis. Moraxella nonliquefaciens produced a slight to moderate finely granular turbidity with a moderate amount

of flocculent sediment and a slight ring formation at the surface of the liquid.

The unidentified diplococci produced a uniform growth and the medium became progressively more turbid with continued incubation.

Mima polymorpha produced abundant growth in TSB. The growth appeared as a thick viscid band at the surface and an extremely viscid deposit at the bottom. Mima polymorpha var. oxidans produced a membrane-like pellicle, a surface ring, and a slight amount of flocculent turbidity and sediment.

Herellea vaginicola produced abundant growth in TSB. All strains except A-173 showed uniform turbidity. Strain A-173 produced a flaky ring at the surface.

Growth on potato infusion agar

All of the hemolytic cultures of M. bovis produced moderate growth on PIA but the nonhemolytic M. bovis cultures either failed to produce growth or produced only scant growth. After 24 hours incubation at 37 C, M. bovis growth appeared as a grayish, moist, confluent, irregular layer on the surface of the medium. The few isolated colonies which appeared were circular, smooth, grayish, umbonate, and about 2 mm. in diameter. Colonies older than 24 hours became progressively larger and flatter, and adjacent colonies tended to coalesce.

All strains of the unidentified diplococcus, Mima polymorpha, Mima polymorpha var. oxidans, M. liquefaciens, M.

nonliquefaciens and Herellea vaginicola grew abundantly on PIA and their growth was not unlike that on blood agar plates.

Growth in nutrient gelatin

All strains of M. bovis, M. liquefaciens, diplococci, and Herellea vaginicola strain A-259 produced a stratiform type of liquefaction which progressed slowly to the bottom of the tube with prolonged incubation at room temperature. Liquefaction progressed more rapidly when the tubes were incubated at 37 C. Subcultures of M. bovis from gelatin onto blood agar plates produced typical smooth, rough, intermediate and dwarf colonies with the intermediate types predominating. Strains of Mima polymorpha, Mima polymorpha var. oxidans, Moraxella nonliquefaciens, and strains 255 and A-173 of Herellea vaginicola produced abundant surface growth, but liquefaction was not observed.

Growth in litmus milk

All strains of M. bovis and M. liquefaciens gave similar reactions when grown in litmus milk. After 24 hours of incubation, a dark blue band developed at the top part of the litmus milk; after further incubation, the medium became progressively more alkaline. Within 6 days, three distinct zones were discernible: an upper zone of dark blue fluid, a middle zone of lavender soft curd, and a bottom layer of pale lavender, coagulated casein. Continued incubation resulted in complete peptonization of the litmus milk and a homogenous purple color.

All strains of diplococci produced a uniform reaction. After 7 to 10 days incubation, there was a purple, opaque, partially coagulated zone at the top of the litmus milk, with a violet translucent zone beneath and a heavy white casein deposit at the bottom. Complete peptonization did not occur even after 30 days incubation.

Moraxella nonliquefaciens, Mima polymorpha, Mima polymorpha var. oxidans changed the litmus milk slightly alkaline. Strains of Herellea vaginicola produced acidity, coagulation and reduction in litmus milk.

Carbohydrate fermentation

The carbohydrates tested were not fermented by M. bovis. The pH of the carbohydrate mediums increased during incubation from 7.2 to an average pH of 8.2 with all strains of M. bovis studied. The cultures of M. liquefaciens, M. nonliquefaciens, Mima polymorpha, Mima polymorpha var. oxidans and the hemolytic diplococci produced similar changes in carbohydrates. The type of growth produced by the organisms was similar to that produced in TSB. Strains of Herellea vaginicola produced acid in xylose, dextrose, galactose, arabinose, and mannose but the other carbohydrate mediums turned slightly alkaline.

Oxidation-fermentation medium

Moraxella bovis, M. liquefaciens, M. nonliquefaciens, Mima polymorpha, Mima polymorpha var. oxidans and the nonhemolytic diplococci did not oxidize or ferment the carbohydrates

but the mediums became more blue, indicating increased alkalinity. Cultures of Herellea vaginicola oxidized arabinose, dextrose, galactose, mannose, and xylose but failed to ferment any of the mediums.

Potassium cyanide medium and urea broth

All the test organisms with the exception of one strain (255) of Herellea vaginicola failed to grow in these mediums. Strain 255 grew in potassium cyanide medium but not in urea broth.

Methyl red and Voges-Proskauer test

Growth occurred in the MR-VP medium, but neither acid nor acetyl-methyl-carbinol was produced by any of the organisms.

SIM medium

All organisms were indole-negative, hydrogen sulfide-negative, and nonmotile.

Ornithine and lysine decarboxylase test mediums

Scant growth was obtained with these mediums, and the organisms were negative.

Simmons citrate medium

Moraxella bovis, M. liquefaciens, and M. nonliquefaciens did not grow on this medium. The growth of Mima polymorpha and Mima polymorpha var. oxidans was variable; one strain of each organism grew in this medium, (Mima polymorpha-188 and

Mima polymorpha var. oxidans-9714). Two strains (A-259 and 255) of Herellea vaginicola grew on the medium but the other strain (A-173) did not.

Growth on other mediums

The results of the test for hemolytic activity, catalase, nitrate-reducing activity, and liquefaction of gelatin are summarized for M. bovis and the hemolytic diplococci (Table 1). The results of tests for nitrate-reducing activity, oxidase, hemolytic activity, gelatin liquefaction, and growth on Herellea agar are summarized for 21 strains of M. bovis (Table 2), 6 strains of diplococci, 3 strains of Herellea vaginicola, and 2 strains each of Mima polymorpha, Mima polymorpha var. oxidans, M. liquefaciens, and M. nonliquefaciens.

Discussion

The results of the present study suggest that the following features characterize most strains of M. bovis associated with IBK: (1) Gram-negative, nonmotile diplobacillus; (2) usually hemolytic, smooth, circular colonies with an entire edge, convex to umbonate, grayish white, and slightly indented into the medium; (3) do not reduce nitrates to nitrites or ferment carbohydrates; (4) are proteolytic, oxidase positive, and produce a typical three-zone reaction when grown in litmus milk; (5) produce no surface growth in liquid medium but develop a coarse, flocculent sediment with little turbidity;

Table 2. Cultural reactions of Moraxella bovis and related organisms

Organism	No. of strains	Reduction of nitrate	Oxidase activity	Hemolysis	Gelatin liquefaction	Growth on Herellea agar
<u>Moraxella bovis</u>	21	neg.	pos.	var.	pos. ^a	neg.
Hemolytic diplococci	6	pos.	pos.	pos.	pos.	neg.
<u>Herellea vaginicola</u>	3	neg.	neg.	var.	var.	pos.
<u>Mima polymorpha</u>	2	neg.	neg.	neg.	neg.	pos.
<u>Mima polymorpha</u> var. <u>oxidans</u>	2	neg.	pos.	neg.	neg.	pos.
<u>Moraxella liquefaciens</u>	2	pos.	pos.	var.	pos.	neg.
<u>Moraxella non-liquefaciens</u>	2	pos.	pos.	neg.	neg.	neg.

^aOne culture (748) from one strain (IBH-68) failed to liquefy gelatin but this was not considered justification for stating that M. bovis is variable in this characteristic.

(6) do not grow on Herellea agar; and (?) produce firm easily fragmented colonies which autoagglutinate when placed in most liquid mediums.

Many microorganisms such as Mima polymorpha, Mima polymorpha var. oxidans, M. liquefaciens, M. nonliquefaciens, an unidentified diplococcus, and Herellea vaginicola are similar to M. bovis in general characteristics, but most investigators should be able to differentiate M. bovis from these microbes by routine laboratory means and a consideration of their source.

The hemolytic diplococcus is found in IBK and produces colonies similar to those of M. bovis. Usually it can be distinguished from M. bovis on the basis of its cellular morphologic features and its colonial morphology as well as the fact it reduces nitrates to nitrites. Moraxella liquefaciens, M. nonliquefaciens, Mima polymorpha, and Mima polymorpha var. oxidans are not reported as occurring in cases of IBK. It is interesting to note that in one study¹ of naturally occurring IBK, an organism very similar to Mima polymorpha var. oxidans which, unlike hemolytic and nonhemolytic M. bovis, did not give a line of identity (96) when used with antiserum against M. bovis in the agar gel double-diffusion technique.

¹Hughes, David E. and Pugh, George W., Jr., National Animal Disease Laboratory, ADP Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010: Unpublished data, 1963-1968.

Some of the data reported in the present study that differ from that of other workers are concerned with catalase activity, changes in litmus milk, and growth on potato medium. In the present study an equal number of catalase-positive and negative M. bovis was found, whereas some investigators (48, 58, 59, 113) had reported that M. bovis was catalase-positive while others (2, 3) had described it as being catalase-negative. The changes in litmus milk resembled those observed by most other investigators, but a heavy casein deposit was not observed in any tubes after prolonged incubation as reported by some workers (2, 14, 106). However, the hemolytic diplococcus did give a heavy casein deposit which did not disappear after 30 days incubation.

All the organisms studied produced growth on PIA. Reports were not found in the literature on the growth of these organisms on this medium, but some (8, 31, 73) have indicated that M. bovis does not grow on potato. In the present instance, growth may have been due to the extra nutrients in the PIA.

Probably the most significant finding of this study was the changing of nonhemolytic M. bovis to hemolytic and from hemolytic to nonhemolytic type. This phenomenon was reported earlier (105) and was thought to be related to the erratic epizootiological nature of the disease. Prior to that report, Pugh et al. (106), as well as Griffin et al. (55) had reported the occurrence of nonhemolytic diplobacilli in cattle eyes affected with IBK. Pugh et al. (106) reported the nonhemolytic

organism as being a variant of the hemolytic M. bovis. Perhaps some of the confusion of the past has been due to the failure of some investigators to identify and recognize the role of the nonhemolytic M. bovis in IBK.

In addition to the variation in hemolytic ability of M. bovis there are several others that may be important. For example, the ability of M. bovis to dissociate into various forms may be a very significant characteristic. Cooper (41) suggested that the dissociation forms of M. bovis vary in their virulence. Supporting this is the finding of Jackson (71) who reported that one dissociated form (rough type of colony) was avirulent while the smooth form (the typical type) of M. bovis culture readily induced IBK. The relative virulence of the other dissociated forms such as intermediate and dwarf types has not been reported. Also it is interesting to note that the results of the present study indicate that liquid mediums such as nutrient gelatin, litmus milk, embryonating chicken eggs (Section VIII), and TSB tend to cause smooth cultures of M. bovis to change into the rough type. Therefore, it is axiomatic that liquid medium not be used in attempts to grow cultures of M. bovis to be used for the experimental production of IBK. Possibly many of the investigators (3, 13, 47, 53, 127) who encountered difficulty producing IBK experimentally with pure cultures of M. bovis were using dissociated forms (rough) of the organism.

Summary and Conclusion

The morphological, cultural and physiological characteristics of 21 strains of Moraxella bovis, 6 strains of a hemolytic diplococcus, 3 strains of Herellea vaginicola, 2 strains each of Mima polymorpha, Mima polymorpha var. oxidans, Moraxella nonliquefaciens and Moraxella liquefaciens were investigated using routine bacteriologic laboratory procedures. The characteristics of M. bovis were compared with those of the other organisms and the distinguishing features of each organism detailed.

Although it was found that these organisms are similar in general characteristics, they can be distinguished from each other without difficulty. For example, only M. bovis and the hemolytic diplococcus have been reported as occurring in cases of IBK. Moraxella bovis differs from the hemolytic diplococcus in morphologic features as well as the fact that the diplococcus reduces nitrates to nitrites while M. bovis does not. Mima polymorpha and Mima polymorpha var. oxidans do not reduce nitrates to nitrites and they do not act on litmus milk appreciably while Moraxella produces a marked and characteristic reaction in litmus milk. Moraxella liquefaciens is very similar to M. bovis but the former reduces nitrates to nitrites. Moraxella liquefaciens liquefied gelatin while Moraxella nonliquefaciens did not. The Mima spp. also differed from the Moraxella spp. in that they grew on Herellea agar. Strains of Herellea vaginicola differed from the other organisms in that

they oxidized arabinose, dextrose, galactose, mannose and xylose. Herellea vaginicola also grew on Herellea agar.

The general characteristics of M. bovis can be summarized as follows: (1) Gram-negative, nonmotile diplobacillus; (2) usually hemolytic smooth circular colonies with an entire edge, convex to umbonate, grayish white, and slightly indented into the medium; (3) does not reduce nitrates to nitrites or ferment carbohydrates; (4) are proteolytic, oxidase positive, and produce a typical three-zone reaction when grown in litmus milk; (5) produces no surface growth in liquid medium but develops a coarse, flocculent sediment with little turbidity; (6) does not grow on Herellea agar; (7) tendency to dissociate; and (8) produces firm easily fragmented colonies which auto-agglutinate when placed in most liquid mediums.

SECTION II. IMMUNOLOGIC STUDIES ON MORAXELLA BOVIS:
DETECTION, IDENTIFICATION, AND ANTIGENICITY

Introduction

Although M. bovis is considered to be the etiologic agent of IBK, no one has been able to immunize cattle using cultures of this organism. This suggests that M. bovis is not very antigenic or at least that antibodies formed do not protect cattle. Also there is the possibility that the different strains of M. bovis differ so much from each other antigenically that immunity against one strain or culture does not protect against another. Supporting this, Chowdhury (39) has reported that different strains of M. bovis varied in their ability to stimulate antibody formation in rabbits.

The available literature does not contain reports of a suitable serologic procedure for either the detection of M. bovis or the occurrence of antibody induced as a consequence of infection or vaccination. The plate and tube agglutination tests have been used in studies by others (13, 14, 50, 51, 71, 112, 119) but are considered unreliable because of the peculiar characteristic of M. bovis which causes it to autoagglutinate when suspended in various liquids.

The purposes of this portion of the study were to perfect serological tests for use in the detection, identification and antigenic determination of M. bovis and to study the antibody response in cattle affected with IBK.

Materials and Methods

Experimental design

The experiment was conducted in two parts. Part 1 involved the development of serologic tests. Hyperimmune serums were produced in rabbits against field and laboratory strains of M. bovis. These hyperimmune serums were later used to develop serologic tests for the detection and identification of M. bovis and antibodies against it.

Part 2 involved the application of the agar gel double-diffusion technique (96) for the antigenic comparison of laboratory and field strains of M. bovis as well as strains of bacteria which are similar to M. bovis. This test was also used to identify M. bovis isolated from cattle naturally affected with IBK and to detect antibodies against M. bovis in experimentally produced and naturally occurring cases of IBK.

Part 1. Production of Hyperimmune Serums and Development of Serologic Tests

Production of bacterins

Organisms Ten strains (12 cultures) of M. bovis, identified as ATCC 10900, 8613, NTN-63 (8033), KGD-63(1), HIM-63(5), IBH-63(15), NDL-63(5077), GLN-63(3), EPP-63(6 and 300) and WSE-64(4 and 7) were used as representative strains for this study. Strains ATCC 10900 and 8613 were laboratory strains and have been used as reference strains in other studies (105). The other strains were isolated from cattle naturally affected with IBK and are considered to be

representative of field strains of M. bovis. Cultures of the different strains had been stored in litmus milk at -60 C. The organisms were divided into two groups depending on whether they were grown on solid plating medium or in liquid medium. Group A organisms were used to produce bacterins of the A series (1 through 10) and Group B organisms were used to produce bacterins of the B series (1 through 3).

For the production of the A series bacterins, frozen cultures of EPP-63(300), WSE-64(4), WSE-64(7), ATCC 10900, NTN-63(8033), IBH-63(15), NDL-63(5077), HIM-63(5), KGD-63(1) and GLN-63(3) were thawed at room temperature and streaked on blood agar plates. After 24 hours incubation at 37 C isolated colonies of each culture were used to inoculate two Erlenmeyer flasks containing trypticase soy broth (TSB) with the addition of 5% by volume of rabbit serum. The inoculated mediums were incubated at 37 C for 48 hours. Later the flasks were placed in a hot water bath which was maintained at 56 C for 1 hour. These heat-killed organisms were considered to be bacterins. Colonies were not seen on plates with subcultures from these bacterins.

In order to prepare the bacterins of the B series, frozen cultures of EPP-63(300), 8613 and EPP-63(6) were thawed at room temperature and streaked on blood agar plates. After 24 hours incubation at 37 C smooth hemolytic colonies from the plates were streaked on a second group of plates; two plates per cultures were used. After 24 hours the growth from the

plate for each culture was harvested and suspended in PSS. Tubes containing the different cultures were placed in a 56 C water bath for 1 hour.

Standardization of the bacterins for immunization Bac-
terins of Group A series were suspended in distilled water to give a density comparable with that of a MacFarland Nephelometer number 2; this suspension was injected into rabbits.

Bacterins of Group B series were diluted with distilled water to give a density which approximated that of a MacFarland Nephelometer number 4. These bacterins tended to clump; therefore the density determination is not thought to be very meaningful. Once diluted, aliquots of incomplete freund adjuvant¹ and bacterin were mixed and used for subcutaneous injection of rabbits.

Immunization of rabbits

Animals Twenty-six adult healthy albino rabbits were used. The rabbits were equally allotted to 13 groups (I-XIII) based on the bacterin received. The backs of the 6 rabbits in Groups XI, XII and XIII were shaved to provide sites for injection of antigens.

Exposure The rabbits in Groups I through X were exposed to the bacterins of the A series: the 2 rabbits in Group I received A1 bacterin, Group II rabbits received A2

¹Bacto-Incomplete Freund adjuvant. Difco Laboratories, Detroit, Michigan.

bacterin, and so on until rabbits in Group X received A-10 bacterin. Each rabbit was injected with 0.5 ml of the respective bacterin via its ear vein on day 1 (first inoculation). Each rabbit was reinoculated on day 8, day 15, day 22, and day 30 with 1.0 ml, 2.0 ml, 4.0 ml and 6.0 ml of bacterin respectively.

The rabbits in Group XI received bacterin B1, Group XII rabbits received B2 bacterin and rabbits in Group XIII received B3 bacterin. To immunize the rabbits 0.2 ml of the bacterin and adjuvant mixture was injected into each of five sites on the shaved back of each rabbit. These rabbits were not reinoculated.

Serologic examination

Fifteen ml of blood were taken from each rabbit on days 1 and 21. On the last day of the experiment (day 40) all the animals were exsanguinated and the maximum amount of blood was taken. The blood was processed and the specific serums collected, labeled and stored at -60 C for later use. The serums were designated by number as follows: A-1 through A-10, and B1 through B3 based on their specific antigen (Table 3).

Preparation of antigens for serologic tests

Agar gel double-diffusion antigens Trypticase soy agar plates and/or blood agar plates were inoculated with each culture of M. bovis or those organisms similar to M. bovis which were being studied and incubated at 37 C for 24 hours

at which time the plates usually contained abundant growth. The growth was scraped from the plates with an inoculating needle and placed in tubes containing cold distilled water. The tubes containing the organisms were then frozen and thawed at least three times with alternating temperatures of -60 C and 25 C. The bacterial suspensions (antigen) were then clarified by centrifugation or used after allowing the organism to settle out by gravity. The antigen was usually stored at -60 C without a preservative, but when it was stored at a higher temperature (i.e. 4 C) Merthiolate¹ was added (final conc. 1:10,000) as a preservative.

Tube and plate agglutination test antigens Blood agar and/or TSA plates were inoculated with a heavy suspension of each culture. After 24 hours incubation at 37 C the growth was scraped from the plates and suspended in PSS. Since the cells autoagglutinated, an attempt was made to disperse them by autoclaving with flowing steam for 1 hour. The bacterins used to hyperimmunize the rabbits were also used as antigens for the plate and tube tests. Because of the poor results with the typical smooth autoagglutinable M. bovis culture, rough variants were sometimes used.

¹Merthiolate - Eli Lilly and Co., Indianapolis, Indiana.

Preparation of the agar gel double-diffusion medium

The agar-gel diffusion medium contained the following constituents: Agar,¹ 1.0 Gm; NaH_2PO_4 , 0.3 Gm; Na_2HPO_4 , 0.06 Gm; and Merthiolate, 0.01 Gm. In some cases 0.01 Gm of trypan blue was added to aid in the observation of the precipitin lines but was not a necessary component of the medium.

The agar was added to 100 ml of distilled water in an Erlenmeyer flask and completely dissolved by heating the flask in a beaker of boiling water. The other constituents were then added to the melted agar and mixed. After mixing, the medium was autoclaved at 121 C for 5 minutes at 15 pounds of pressure. The solution was cooled to 45 C and dispensed into glass Petri dishes to a thickness of 6-8 mm. When Petri plates of a volume of 65 ml (15 X 100) were used, approximately 15 to 20 ml of the medium were placed in each plate. The plates were then stored in an air-tight container at 5 C until used.

Procedures for serologic tests

Agar gel double-diffusion The diffusion plates were prepared using a standard template which was adopted after a preliminary study. It was determined that two templates were adequate for detection of antibodies; those templates used different well dimensions and distances between wells. Well dimensions were of two types, (1) approximately 0.8 cm in

¹Ionagar no. 2, Consolidated Laboratories Inc., Chicago Heights, Illinois.

diameter (used primarily for antigen) and (2) which was approximately 0.6 cm in diameter and usually used for the serum. The distances between wells were taken as the center to center measurement and were of two types. The center to center distance was 0.8 cm in one type. This type was used in weakly positive or diluted serum, usually for the initial screening of a large number of serum samples. The distance measurement in the other type was 1.2 cm from center to center of each well. The latter type of well spacing was used in more definitive tests because it gave better separation of precipitation lines. Each template was designed to give 16 determinations based on four equally spaced center wells, each circumscribed by four additional wells.

The following method was found to give the best results and was used as the standard procedure in this study. A central well and four circumferential wells were cut equal distances apart so that the distance from the center of the central wells to that of each surrounding well was 1.2 cm, and adjacent peripheral wells were 1.2 cm apart. Four patterns such as this were placed on each diffusion plate using a standardized template. All wells (0.6 cm in diameter) were cut with a cork borer and the agar was removed with a pipette attached to a vacuum pump.

When one serum was to be tested against numerous antigens, the serum was placed in the central wells, and the antigens in the four surrounding wells. When one antigen was being tested

against numerous antiserums, the antigen was placed in the center well. Usually the wells were filled only once with the test materials using Pasteur pipettes. The plates were incubated at 25 C for 72 hours at which time they were examined for precipitation lines. Positive and negative controls were also handled in this way.

This procedure was used on all the organisms and serums discussed in this report, but the following will be given special consideration (Tables 3, 4, and 5): (1) the serums taken from cattle herds naturally affected with IBK (IBH-65, IBH-66, and IBH-67) and cattle affected with experimentally-induced IBK (cattle (NDL-68) used in thesis research)); (2) hyperimmune serums produced in rabbits against strains of M. bovis; and (3) to study the antigenic relationship between field and laboratory strains of M. bovis as well as organisms which resemble M. bovis.

Slide agglutination test The slide agglutination test was conducted according to the method described by Chowdhury (39). The test was conducted using the standard Minnesota Brucellosis testing box. Diluted and/or undiluted serum was used with varying concentrations of antigen. One drop each of the serum and antigen was mixed and allowed to incubate while undergoing a rotational rocking movement. Negative and positive controls as determined by the agar gel double-diffusion technique were carried out also for each serum or antigen tested.

Table 3. Antigens used to hyperimmunize rabbits and/or test the specificity of their antiserums

Series A and B - antiserums were produced in rabbits against these cultures of M. bovis. The antigens and the homologous antiserums are given the same designation in Table 4.

A1 - EPP-63(300)	A8 - HIM-63(5)
A2 - WSE-64(4)	A9 - KGD-63(1)
A3 - WSE-64(7)	A10 - GLN-63(3)
A4 - ATCC 10900	B1 - EPP-63(300)
A5 - NTN-63(8033)	B2 - 8613
A6 - IBH-63(15)	B3 - EPP-63(6)
A7 - NDL-63(5077)	

Series C antigens represent strains of organisms which resembled M. bovis and were used to test the reaction of the antiserums.

C1 - EPD-63(300) hemolytic diplococcus	C10-CDC(9714) <u>Mima polymorpha</u> var. <u>oxidans</u>
C2 - CDC(9833) <u>Moraxella</u> <u>liquefaciens</u>	C11-CDC(9561) <u>Mima polymorpha</u> var. <u>oxidans</u>
C3 - CDC(9967) <u>Moraxella</u> <u>nonliquefaciens</u>	C12-CDC(9985) <u>Moraxella lique-</u> <u>faciens</u>
C4 - CDC(9893) "	C13-ATCC(11748) <u>Moraxella</u> <u>lacunata</u>
C5 - CDC(9944) "	
C6 - CDC(A259) <u>Herellea</u> <u>vaginicola</u>	
C7 - CDC(173) "	
C8 - CDC(A130) <u>Mima polymorpha</u>	
C9 - CDC(188) <u>Mima polymorpha</u>	

Table 4. Results^a of agar gel double-diffusion technique on antiserums against homologous and heterologous antigens

Antigen	Antiserums ^b												
	Antibodies produced in rabbits												
	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	B-1	B-2	B-3
A-1	5	3	1	4	5	2	4	2	4	5	5	5	0
A-2	3	4	1	2	2	3	2	2	1	3	3	3	5
A-3	2	2	3	2	3	2	3	1	3	2	2	3	3
A-4	4	4	2	2	4	4	3	3	2	3	4	5	3
A-5	2	1	1	2	4	2	2	4	1	3	4	3	3
A-6	3	3	2	3	2	2	1	2	3	4	5	5	3
A-7	4	2	3	4	2	1	3	2	1	2	4	2	3
A-8	2	2	1	3	4	2	2	4	0	2	2	2	4
A-9	4	1	3	3	1	3	0	3	3	3	5	2	2
A-10	5	3	2	3	3	4	3	2	1	5	4	3	3
B-1	5	3	2	3	2	3	4	3	1	3	5	4	3
B-2	5	4	3	4	2	3	2	2	2	3	5	2	3
B-3	4	3	3	4	4	5	2	4	3	4	4	1	3
C-1	2	-	-	-	-	2	-	2	1	-	1	1	-
C-2	4	-	-	-	-	3	-	3	1	-	3	2	-
C-3	2	-	-	-	-	2	-	0	0	-	1	1	-

^aThe results are given as the number of precipitation lines on a diffusion plate.

^bAntiserums produced in rabbits against antigens of same designation listed in Table 3.

Table 4. (Continued)

Antigen	Antiserums ^b												
	Antibodies produced in rabbits												
	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	B-1	B-2	B-3
C-4	1	-	-	-	-	1	-	1	1	-	1	1	-
C-5	0	-	-	-	-	0	-	0	0	-	0	0	-
C-6	1	-	-	-	-	1	-	1	1	-	1	1	-
C-7	1	-	-	-	-	0	-	0	0	-	0	0	-
C-8	1	-	-	-	-	0	-	0	0	-	1	1	-
C-9	1	-	-	-	-	0	-	0	0	-	1	0	-
C-10	1	-	-	-	-	0	-	0	0	-	1	0	-
C-11	1	-	-	-	-	0	-	0	0	-	1	0	-

Table 5. Results-detection of antibodies for Moraxella bovis in the serums of cattle affected with infectious keratoconjunctivitis using the agar gel double-diffusion technique

Source	No. of animals		No. of animals with serum antibodies				No. diseased animals with antibodies			
			<u>Preinfection</u>		<u>Postinfection</u>		<u>Present</u>		<u>Absent</u>	
	Cows	Calves	Cows	Calves	Cows	Calves	Cows	Calves	Cows	Calves
IBH-65	21	20	0	0	9	4	1	4	5	0
IBH-66	26	19	5	0	5	0	1	0	0	8
IBH-67	24	23	12	0	9	9	3	5	4	12
Sub-total	71	62	17	0	23	13	5	9	9	20
IBH-65										
66										
67	133		17		36		14 ^a		29	
NDL-68	64		0		19		16		32	
Total	197		17		55 ^b		30		61	

^aAntibodies were detected in serums of 11 animals which had not been affected with clinical IBK and M. bovis was not recovered from their eyes.

^bOnly 38 of these represented changes in antibodies status.

Table 5. (Continued)

Source	No. of animals in which <u>M. bovis</u> was recovered							
	With disease				With antibodies			
	Present		Absent		Present		Absent	
	Cows	Calves	Cows	Calves	Cows	Calves	Cows	Calves
IBH-65	6	15	7	3	4	4	9	14
IBH-66	1	7	11	3	2	0	10	13
IBH-67	6	16	10	3	8	7	7	13
Sub- total	13	38	28	9	14	11	26	40
IBH-65 66 67	51		37		25		66	
NDL-68	48		13		18		43	
Total	99		50		43		109	

Tube agglutination test The various experimentally produced antiserums and serums from cattle naturally and experimentally affected with IBK as well as normal cattle were tested against the antigens of the various strains of M. bovis. The antigen was standardized to give a density comparable to that of a MacFarland Nephelometer number 4. Because of the difficulty encountered in getting meaningful results, some tests were carried out using densities greater than those of a MacFarland Nephelometer number 4 and other used densities which were less than this. Usually 0.5 ml of the antigen was mixed with each 0.5 ml of diluted or undiluted serum to be tested as well as negative and positive controls. The antigen was added to undiluted serum, and to two-fold dilutions of the serum from 1:2 through 1:1024 dilutions. The antigen and serum were mixed using a pipette and then incubated at 37 C for 24 hours, then they were allowed to sit at room temperature until observed for agglutination under artificial light. Tests were also carried out using ten-fold dilutions of serum.

Fluorescent antibody technique In the fluorescent antibody staining of M. bovis and M. bovis-like organisms, the procedure outlined by Cherry et al. (38) was followed for the most part and is given in summarized form in the following paragraphs.

Specific hyperimmune serum The hyperimmune serums represent three strains of M. bovis, namely, 10900, 8613 and EPP-63(300) and were discussed earlier. Each serum was handled

in exactly the same manner; therefore only one of them will be discussed here. The agar gel double-diffusion technique was used to verify the presence of antibodies in the serums. All the mediums used were maintained at 4 C and the procedures were carried out using the cooled (4 C) liquids.

Fractionation and clarification

The first two steps were the fractionation of the serum with saturated ammonium sulfate and clarification with chloroform. For fractionation, 18 ml of the serum were added to each of two 50-ml centrifuge tubes. Then 18 ml of saturated $(\text{NH}_4)_2 \text{SO}_4$ were added to each tube while gently rotating the tube. The tubes were placed in a refrigerator (maintained at 4 C) for 12 hours, at which time they were centrifuged at 2,000 R.P.M. for 1 hour using a Model U International Centrifuge¹ with a bucket head (cat. no. 229). The supernatant fluid was discarded and the precipitate (in situ) dissolved in 5 ml of cold distilled water. Five ml of $(\text{NH}_4)_2 \text{SO}_4$ were used to reprecipitate the fractions (globulins) in each tube. The tubes were immediately centrifuged at 2,000 R.P.M. for 20 minutes, the supernatant fluid discarded and the precipitate dissolved in water (4 ml per tube). This latter fractionation, centrifugation and decanting procedure was repeated for two additional times; after the last centrifugation the precipitate in each tube was dissolved in 9 ml (1/2 of the serum original volume) of cold

¹International Model U Centrifuge - New York Laboratory Co., Inc., New York.

distilled water. The serum fractions (globulins) were pooled to give a volume of 37 ml and then dialyzed against 0.85% saline at 4 C to eliminate the $(\text{NH}_4)_2 \text{SO}_4$. To do this the globulins were placed in dialysis tubing¹ which was suspended in a 1-liter glass cylinder and dialyzed against 0.85% saline maintained at 4 C. A magnetic bar was placed in the cylinder and the latter placed on a magnetic mixing apparatus (Mag-Mix²) which resulted in constant stirring of the saline solution and facilitated the dialysis process. The saline solution was frequently changed until all the $(\text{NH}_4)_2 \text{SO}_4$ had been eliminated. Saturated barium chloride was used to determine whether or not $(\text{NH}_4)_2 \text{SO}_4$ remained in the fractions in the following manner. Equal volumes of BaCl_2 and dialysate were mixed; when $(\text{NH}_4)_2 \text{SO}_4$ was present the mixture became cloudy or opalescent. It required 20 liters of 0.85% saline solution to eliminate the $(\text{NH}_4)_2 \text{SO}_4$ from the globulin solution.

The globulin solution was opalescent before as well as after dialysis; therefore it was treated in the following manner with cold (4 C) chloroform in order to clarify it. One part of cold chloroform was added to the 5 parts of the cold globulin solution and the mixture was shaken vigorously by hand for 5-10 minutes. After agitation the mixture was placed in centrifuge tubes and centrifuged for 15 minutes at 2,000 R.P.M.,

¹Dialysis tubing - Union Carbide Corporation, Food Products Division, Chicago, Illinois.

²Mag-Mix - Precision Scientific Co., Chicago, Illinois.

after which the supernate (globulin) was transferred to other tubes. The thick sediment containing the chloroform and lipids was discarded. The clarification process had to be repeated three times in order to eliminate the opalescence. Once clarified the globulin was dialysed against 1 liter of 0.85% saline for 24 hours and checked for antibodies against M. bovis using the agar gel double-diffusion technique. The protein content of the globulin solution was determined by a Biuret reaction.¹ The fractions contained antibodies as evidenced by lines of precipitation and contained 2.87% protein. The globulin fractions were diluted with sufficient 0.85% saline to give a 1% protein solution and stained with a fluorescent dye.

Labeling of the globulins The globulin fractions were labeled with fluorescein isothiocyanate² in the following manner. Carbonate buffer of pH 9.0 was added to the chilled globulin solution in a volume equal to 10% of that of the globulin solution. Then 0.025 mg of fluorescein isothiocyanate powder was added for each mg of protein present (approximately 25 mg of dye were required), the mixture was placed on a Mag-Mix and stirred overnight at 4 C at which time the labeled globulin solution was placed in dialysis tubing and dialyzed

¹Biuret Reaction. Methods and Calibrations, Bausch and Lomb Spectronic 20 Colorimeter. Bausch and Lomb Optical Co., Rochester, New York.

²Fluorescein isothiocyanate (FIC) - Nutritional Biochemical Corporation, Cleveland, Ohio.

against phosphate buffered saline (pH 7.2) with stirring and frequent saline change. Dialysis was continued until the overnight dialysate did not show fluorescence when exposed to U.V. light. Eighteen liters of phosphate buffered saline were used.

Sorption of labeled globulin with liver powder In order to eliminate nonspecific staining by the labeled fractions, they were absorbed with rabbit and/or dog liver powder. The liver powders were prepared according to the procedure of Cherry et al. (38). Once prepared rabbit or dog liver powder was added to aliquots of the labeled globulin solution and the mixture allowed to stand at room temperature for 2 hours while being stirred constantly. The mixture was then clarified by centrifugation and allotted into tubes for storage or further sorption. Aliquots of globulin previously absorbed with rabbit liver powder were again absorbed with rabbit and/or dog liver powders. The globulin fractions which were initially similarly absorbed with canine liver powder were reabsorbed with canine and laprine liver powder. After sorption, Merthiolate was added to the aliquots of absorbed and unabsorbed globulin. Five ml aliquots of each were then placed in plastic tubes. Some of the tubes were kept at 4 C for immediate use and the other stored at -60 C for later use.

Preparation of smears Smears were made on clean slides from the organisms listed in Table 3, other organisms listed in Section I, as well as from eye secretions taken from cattle affected with IBK. The individual smear on a slide was

air dried and fixed for 1 minute in 95% ethanol, the slide was then drained and dipped in phosphate buffered saline (pH 7.2) and blotted dry. The dry fixed smear was flooded with labeled globulin and allowed to stain in a moist chamber at 37 C for 30 minutes after which it was rinsed by being placed in phosphate buffered saline for two 5-minute periods and blotted dry. One drop of phosphate buffered glycerol (pH 7.2) was added to the smear and the smear covered with a cover slip. The smear was then examined for specific fluorescence using a microscope with an ultraviolet light source.

Part 2. Application of the Agar Gel Double-Diffusion Technique

Comparison of field and laboratory strains of *Moraxella bovis*

An attempt was made to use the agar gel double-diffusion technique in order to detect the presence of homologous and heterologous antibodies against strains of *M. bovis*, *Moraxella liquefaciens*, *Moraxella nonliquefaciens*, *Mima polymorpha*, *Mima polymorpha* var. *oxidans*, a hemolytic diplococcus and *Herellea vaginicola* (Tables 3 and 4). The whole cell antigen of each culture was tested against each of the antiserums prepared in Part 1. The appropriate antiserum was placed in the center well and the antigens were placed in the peripheral wells. The plates were then incubated and observed in the usual manner.

Detection of antibodies in cattle serums

Serums from animals affected with IBK both experimentally and naturally were checked for antibodies against M. bovis (Table 5). The antigen was placed in the center well and the serums were placed in the peripheral wells.

Identification of M. bovis isolated from cattle with IBK

The different strains and cultures of M. bovis isolated from cattle herds affected with IBK were checked for the presence or absence of common antigens using the agar gel double-diffusion technique (Section I). Each culture was tested against the antiserum produced in rabbit against M. bovis strain ATCC-10900.

Results

Antiserums were produced in rabbits against 13 cultures of M. bovis (Tables 3 and 4). The serums taken from the rabbits on day 1 of the experiment did not contain antibodies as evidenced by the lack of lines of precipitation against homologous and heterologous antigens, but all of the serums taken on the other days contained antibodies against their homologous antigen and most of them had antibodies against the heterologous antigens. The precipitation lines produced by the antiserums taken on day 21 were faint and occurred singly, while those representing the antiserums taken on day 40 were usually dense, distinct and multiple (Tables 3 and 4).

The tube and plate agglutination tests also indicated an increased antibody response from day 1 through day 40. Slight clumping attributed to autoagglutination occurred in the negative control serum, a water suspension of the organism, and in the tubes and on plates representing the serums taken on day 1. Marked clumping occurred in the tubes and on plates representing the low dilution of serums taken on days 21 and 40.

The results of the fluorescent antibody staining technique indicated the antigenicity of M. bovis and the potency of three of the antiserums. All of the organisms fluoresced with the unabsorbed globulin fractions but only members of the genus Moraxella fluoresced with the serums after sorption with canine and lapine liver powders. Those strains giving specific fluorescence included 21 strains of M. bovis, 6 strains of hemolytic diplococci, 3 strains of Moraxella nonliquefaciens, 2 strains of Moraxella liquefaciens and 1 strain of Moraxella lacunata. Strains of Herellea vaginicola, Mima polymorpha, and Mima polymorpha var. oxidans did not show any specific fluorescence. There was also specific fluorescence of M. bovis and hemolytic diplococci in smears made from the eye secretions of cattle affected with IBK.

The agar gel double-diffusion technique proved useful in the antigenic comparison of field and laboratory strains of M. bovis as well as M. bovis-like organisms (Tables 3 and 4). There were reactions between all antiserums and their homologous antigen as well as with most of the heterologous antigens.

Only three antiserums (A-7, A-9, and B-3) failed to react with all 13 M. bovis antigens. One strain (Moraxella nonliquefaciens 9944) of the M. bovis-like organisms failed to react with any of the antiserums (Tables 3 and 4). The strongest reaction (largest number of lines) occurred between the different strains of M. bovis and the M. bovis antiserums. The next strongest reaction occurred between M. bovis antiserums and the diplococcus and Moraxella liquefaciens. The weakest reaction occurred between the antiserums and Mima polymorpha, Mima polymorpha var. oxidans, Herellea vaginicola and Moraxella nonliquefaciens.

Antibodies were detected in the serums of 55 of 197 cattle either exposed naturally or experimentally to M. bovis using the agar gel double-diffusion technique (Table 5). The agglutination tests proved inadequate for the determination of antibodies because of the tendency of the test organisms to auto-agglutinate in liquids.

Discussion

The results indicate that the subcutaneous or intravenous injection of M. bovis antigens into rabbits induces the production of specific antibodies. Previously Chowdhury (39) reported that he was able to induce antibodies against M. bovis using cultures grown on a variety of laboratory mediums. Therefore it appears that M. bovis is very antigenic but the role of the antibodies in immunity remains to be confirmed.

The antibodies can be detected readily with the agar gel double-diffusion technique (ADDT) but less readily with the tube and plate agglutination tests. Although the ADDT test seems to be adequate for the qualitative determination of antibodies, it does not give the concentration of the antibody present. It should be pointed out, however, that the concentration of M. bovis antibodies in the antiserums produced in this experiment was adequate for the conjugation procedures.

Possibly the tests developed in this experiment could be used in more definitive studies on M. bovis such as those involved with identification, antigenicity (antigenic analysis) and the serologic responses of cattle affected with IBK. While the ADDT appears to offer the most promise for such studies, more work is needed. The tube and plate agglutination tests could offer a quantitative method for such studies if one could eliminate autoagglutination in liquid suspensions of M. bovis. It is interesting to note that although many workers have used the tube agglutination test in their studies on the serologic response in M. bovis infection, only one has described the reaction in any detail. He (51) described the appearance of the flocculum formed by M. bovis in the presence of immune serums as being slow in settling and at 24 hours appeared mostly as a feathery suspension with a clear supernatant fluid. In the present experiment a similar reaction was seen in tubes with immune as well as negative control serums, therefore it is thought that the results of the tube agglutination are not

very significant. Similarly the plate agglutination test gives inconclusive results because of autoagglutination of M. bovis in liquids.

The use of the fluorescent antibody staining method could possibly facilitate the rapid detection of M. bovis in smears made from infectious eye secretions and augment other cultural methods. There have not been any reports of this method being used in studies involving M. bovis or its infections but it seems to offer promise as a tool for further studies. For example, it is thought that M. bovis is involved in all cases of IBK and is usually present in the eye secretions, but many workers have had difficulty finding the organism and they have concluded that M. bovis was not the etiologic agent. Therefore, there is a need to identify and detect M. bovis in such obscure cases of IBK. Even in cases where the organism cannot be isolated, if one detects specific fluorescence of diplobacilli in smears from cases of IBK this in itself would be very suggestive of M. bovis presence and thereby encourage a more vigorous search for this organism.

The results indicate that there are antigenic relationship between M. bovis, Moraxella lacunata, Moraxella liquefaciens, Moraxella nonliquefaciens, Mima polymorpha, Mima polymorpha var. oxidans and Herellea vaginicola as well as with hemolytic diplococci, thus confirming the work of Mitchell and Burrell (89). It is interesting to note that the M. bovis antigens reacted more strongly with M. bovis antiserum than did the

antigens of the other organisms with the ADDT, nevertheless all but one of the organisms reacted with the antiserums. However, only the diplococci and members of the genus Moraxella fluoresced. Herellea vaginicola, Mima polymorpha, and Mima polymorpha var. oxidans also gave the smallest number of lines of precipitation, probably indicating further that they are not closely related to the other organisms.

The fact that the serums of cattle affected with infectious keratoconjunctivitis contained antibodies suggests that the disease may be prevented by vaccination. Admittedly, there may or may not be any relationship between the production of antibodies and the immune response, but such correlation seems probable. The ADDT seems adequate for the detection of these antibodies in most severe cases of IBK but is not sensitive enough to indicate all states of infection. For example, M. bovis became established in the eyes of 149 animals but only 55 of these developed antibodies detectable by this method.

Summary and Conclusion

Antiserums were produced in rabbits against 13 cultures of Moraxella bovis. Serologic tests were used and/or developed to determine the qualitative antibody response in animals exposed to M. bovis. The four tests included an agar gel double-diffusion technique, a tube agglutination test, plate agglutination test, and a fluorescent antibody staining technique.

The agar gel double-diffusion technique was used to determine the antigenic relationship between strains of M. bovis, Moraxella liquefaciens, Moraxella nonliquefaciens, Mima polymorpha, Mima polymorpha var. oxidans, Herellea vaginicola and unidentified hemolytic diplococci isolated from cattle affected with IBK. All these were found to be related as was demonstrated by a precipitation reaction when antigen and antiserums were tested against each other. The test was also used to study the qualitative determinations of antibodies against M. bovis in the serums of 197 cattle naturally and experimentally exposed to M. bovis. Thirty-eight animals developed antibodies as a result of infection but 91 animals developed disease and of these only 30 developed antibodies. Moraxella bovis was recovered from the eyes of 149 animals, 99 of which had disease and antibodies were detected in the serums of 43.

The tube and plate agglutination tests were found inadequate for the detection of antibodies against M. bovis because of autoagglutination of M. bovis in normal as well as immune serums. A fluorescent antibody staining technique was used to selectively identify M. bovis, Moraxella lacunata, Moraxella liquefaciens, Moraxella nonliquefaciens and unidentified hemolytic diplococci in smears with antibodies stained with fluorescein isothiocyanate. Herellea vaginicola, Mima polymorpha and Mima polymorpha var. oxidans could not be detected.

Although much more work is needed in order to make these tests more reliable, they appear adequate for preliminary

studies such as this one. Possibly the agar gel double-diffusion technique could be used in a study for a complete antigenic analysis of M. bovis and organisms similar to it. The fluorescent antibody staining technique probably could prove of value in the detection of M. bovis in smears made of eye secretions from animals affected with IBK.

SECTION III. PRELIMINARY STUDIES ON THE EXPERIMENTAL PRODUCTION OF BOVINE INFECTIOUS KERATOCONJUNCTIVITIS USING DIFFERENT EXPOSURE MATERIALS AND METHODS

Introduction

Many investigators have attempted to produce IBK experimentally with varying degrees of success. In their studies many different exposure materials and methods were used. Because the exposure materials and procedures used by these investigators varied so much, it was deemed necessary to conduct some preliminary experiments to test materials and methods before initiating definitive studies on IBK.

Materials and Methods

Experimental design

These studies were divided into four experiments (I, II, III, IV) based upon their purposes. Experiment I was designed to determine whether M. bovis would become established and produce keratoconjunctivitis in bovine eyes as well as to establish a routine procedure for recovery of M. bovis. An eye of 1 calf was exposed to a laboratory strain of M. bovis.

Experiment II was designed to develop a rapid screening method for obtaining a virulent strain of M. bovis for use in later studies on experimental IBK. The eyes of 3 cattle were exposed to a mixed culture of two strains of M. bovis isolated from naturally occurring IBK. Experiment III was designed as a screening method for testing the effects of different exposure materials to be used in later studies. This experiment

consisted of 6 animals whose eyes were exposed to a series of materials from eyes affected with IBK. Experiment IV was designed in order to establish the efficacy of different exposure methods and materials in producing IBK when used in different combinations. The eyes of 3 cattle were used in this experiment; the eyes of two were exposed to cultures of M. bovis and infectious bovine rhinotracheitis (IBR) virus simultaneously and the eyes of the other exposed naturally.

Experiment I

Organism The organism used was a culture of a hemolytic laboratory strain of M. bovis (strain 8613) which was used in other studies (106, 107). Cultures of this strain were stored at -60 C in TSB.

Inoculum for exposing the animal was prepared from the frozen culture as follows: the latter was thawed and streaked on 5% bovine blood agar; the agar plate culture was incubated at 37 C for 24 hours. A typical smooth hemolytic colony was streaked on a second plate. After the second plate culture was incubated 24 hours, the growth was scraped from the surface of the medium and suspended in TSB; 0.5 ml of the suspension was instilled in the eye.

Animal The animal used was a specific-pathogen-free (SPF) calf of the Holstein-Friesian breed. The calf had been kept in isolation units since birth and had never had clinical

signs of IBK. The eyes and nostrils of the calf were culturally negative for M. bovis.

Animal quarters The animal quarters were indoor isolation units which excluded sunlight and flies. These quarters were air conditioned and mechanically ventilated with filtered air. Each room had two separate stalls for housing individual animals. The rooms were illuminated with fluorescent lamps, recessed back of an impact-resisting prismatic glass lens.

Bacteriologic examination Secretions from the eyes and internal nares were collected with sterilized cotton-tipped applicators before exposure and periodically throughout the observation period. The applicators were placed in TSB immediately after collection to prevent drying. They were streaked on the surface of 5% bovine blood agar plates and the plates were incubated at 37 C for 24 hours.

Exposure On day 1 of the experiment, 0.5 ml of a TSB suspension of the M. bovis culture was instilled in the calf's left eye. On day 83 an antibiotic ointment¹ was used to free the infected eye of M. bovis. On day 96 the eye was re-exposed to the homologous M. bovis strain recovered from the infected eye before treatment.

Experiment II

Organisms Three isolants representing two strains of M. bovis identified as Gln-63 (1 and 2) and EPP-63 (300) were

¹Cortef-F Ointment, Upjohn Company, Kalamazoo, Michigan.

used. These isolants were recovered from 3 different cattle naturally affected with IBK. Cultures of the three isolants had been stored at -60 C in TSB.

Inoculums for exposing animals were prepared from the frozen cultures. The latter were thawed and streaked on 5% bovine blood agar; the agar plate culture was incubated at 37 C for 24 hours. Typical smooth hemolytic colonies were streaked on the second plate. After the second plate culture had incubated for 24 hours, the growth was scraped from the surface of the medium and suspended in TSB. For exposure of the animal's eyes, equal amounts of TSB suspension of the three isolants were mixed.

Animals The 3 cattle used were of the Holstein-Friesian breed and were from NADL's clean animal herd. They had never had clinical signs of IBK. Two were adult cows and the other a 2-week-old calf, the offspring of one of the cows. Their eyes were culturally negative for M. bovis.

Animal quarters The animal quarters were similar to those used in Experiment I.

Bacteriologic examination Secretions from the eyes were collected with sterilized cotton-tipped applicators before exposure and periodically throughout the observation period. The applicators were placed in TSB immediately after collection to prevent drying. They were streaked on the surface of 5% bovine blood agar plates, and the plates were incubated at 37 C

for 24 hours at which time they were observed for M. bovis colonies.

Exposure The right eye of each animal was injected with 0.5 ml of the mixture of the three isolants of M. bovis into the upper conjunctivae. The left eye of each animal was exposed by instilling 0.5 ml of the mixture in the ventral conjunctival sac.

Experiment III

Organisms and eye-secretion filtrate The eye-secretion filtrate, the diplococcus cultures, and the M. bovis cultures were obtained from the eye secretions of 2 cattle (540 and 575) affected with naturally occurring acute bilateral IBK. The secretions from the four eyes were pooled and filtered through a Millipore filter¹ using a 0.45-micron filter pad with positive pressure; the filtrate was instilled in the eyes of 6 experimental animals.

A hemolytic Gram-negative hemolytic diplococcus isolated from the eye secretions of animal number 575 was grown on the surface of a blood agar plate for 24 hours at 37 C. The growth was scraped from the plate and suspended in TSB; the suspension was instilled in the eyes of the experimental cattle.

The nonhemolytic M. bovis was isolated from the eye secretions of cow number 540. The second BAP of this organism was

¹Millipore Filter Corporation, Bedford, Massachusetts.

scraped from the surface of the plate, suspended in TSB and used to expose cattle eyes.

The hemolytic M. bovis was isolated from the eye secretions of one of the cows (575) mentioned previously. The second BAP culture was scraped from the surface of the plate, suspended in TSB and used to expose cattle eyes.

Animals The cattle used were SPF calves of the Holstein-Friesian breed and had never had clinical signs of IBK. They ranged from 6 to 12 months of age.

Animal quarters The animal quarters were similar to those used in Experiments I and II.

Bacteriologic and virologic examinations Secretions from each eye were collected with two sterilized cotton-tipped applicators for two days before exposure and each day during the observation period. One applicator was used for bacteriologic examination and the other for virologic examination. The applicator for bacteriologic examination was placed in TSB immediately after collection to prevent drying; later it was used to streak the surface of a 5% bovine blood agar plate, and the plate was incubated at 37 C for 24 hours. The applicator for virologic examination was placed in Earle's medium immediately after collection to prevent drying and to suspend any viral particles that might be present. Later 0.1 ml of the suspension was used to inoculate each of two tubes of primary embryonic bovine kidney (PEBK) cells. The inoculated

PEBK cells were incubated at 37 C and observed daily for cytopathic changes (CPE) for 7 days.

Exposure On day 1 of the experiment each eye was exposed to 0.25 ml of eye-secretions filtrate (ESF) by instillation into the ventral conjunctival sac. On day 20 each eye was exposed to 0.5 ml of TSB suspension of the hemolytic diplococcus by instillation into the ventral conjunctival sac. On day 30 each eye was exposed to 0.5 ml of a nonhemolytic M. bovis culture suspended in TSB by instillation into the ventral conjunctival sac. On day 50 each eye was exposed to 0.5 ml of a hemolytic M. bovis culture suspended in TSB by instillation into the ventral conjunctival sac.

Irradiation A mercury sunlamp (67) was used as the source of ultraviolet radiation. The sunlamp was a combination mercury discharge element and a tungsten-filament resistance ballast incorporated within an ultraviolet transmitting, reflector-type bulb.

The eyes of each animal were irradiated individually for 10 minutes at a distance of 20 inches. Irradiation was made immediately prior to exposure to cultures or eye-secretions filtrate and once daily throughout the observation period. Each animal was secured in a stanchion and tied with a rope halter to restrict head movements. The lamp was positioned so that the flat surface of the lamp was approximately parallel to the plane of the cornea so that the eye was in the approximate center of the beam.

Experiment IV

Organisms The IBR virus (Himstra 6) used was a cytopathogenic agent isolated from a cattle herd affected with clinical IBR and IBK; it is described in Section V of this report. The inoculum was a tenth tissue culture passaged (TCP) virus in a concentration of $10^{6.5}$ TCID₅₀ per ml and was antibiotic-free. The virus suspension was freed of antibiotic in the following manner: 0.1 ml of the sixth TCP virus suspension (10^7 TCID₅₀ per ml) containing antibiotics was diluted with antibiotic-free Earle's medium to give a virus dilution of 10^{-7} . One-tenth ml of the diluted virus was inoculated into cultures of PEBK cells which were also free of antibiotics. The PEBK cells were started in antibiotic-containing medium but were washed five times in antibiotic-free medium before being inoculated.

The M. bovis cultures (EPP-63(300) and GLN-63(1)) were hemolytic strains isolated from the eyes of cattle affected with acute naturally occurring IBK. Second blood agar passaged cultures of these organisms were scraped from the surfaces of the plates and suspended in physiological saline solution. Equal amounts of each strain were mixed and used as exposure material.

Animals The 3 cattle were of the Holstein-Friesian breed and had never had clinical signs of IBK or IBR. The 2 animals exposed experimentally were a cow (5004) obtained from NADL's clean animal herd and her 2-week-old calf (5004C). The

other cow (4998) was obtained from the same herd. The eyes of the animals were culturally negative for M. bovis and IBR virus prior to exposure.

Animal quarters The animal quarters were similar to those used in Experiments I, II and III except that the calf, because of its size, could move from one stall to another.

Bacteriologic and virologic examinations The same method was followed in Experiment IV as in Experiment III.

Exposure One-half ml of the IBR virus suspension was injected into the upper conjunctiva of the right eye of animals 5004 and 5004C; this caused a small bleb to form. Adjacent to the bleb, 0.5 ml of the mixed cultural suspension of the M. bovis strains was injected into the conjunctiva.

The left eyes of the cow (5004) and calf (5004C) were exposed to IBR virus and the mixed suspension of the two strains of M. bovis by instillation. Five-tenths ml of the IBR virus suspension along with 0.5 ml of the mixed suspension of M. bovis was instilled into the open eye. The other cow was used as a contact exposure control.

Results

Experiment I

After exposure of a single calf to a culture of M. bovis by conjunctival instillation, the organism was recovered consistently from the exposed eye as well as the left nostril. Moraxella bovis was also recovered from the right nostril from

the 8th through the 15th day of the experiment but it was never recovered from the right eye. The first clinical signs of disease were observed on day 7 when the left eye appeared moist and slightly inflamed. Each day thereafter the inflammation became progressively more severe until day 20, at which time the animal manifested the signs of a mild case of keratoconjunctivitis. The most prominent features of the disease were photophobia (manifested by blinking and turning of affected eye from the light), swollen and erythematous conjunctivae and third eyelid. The iris was spastic. There were slight scleral injection and a moderate amount of serous discharge but the cornea remained normal. After day 20 the clinical signs began to abate and on day 25 the left eye was nearly normal. The right eye remained normal throughout the observation period.

Moraxella bovis was recovered from the left eye up to day 83, at which time it was treated with the antibiotic ointment. After treatment, however, M. bovis could not be recovered and the eyes remained normal.

Experiment II

After exposure of the left eyes of 3 cattle by simple instillation and their right eyes by injection of the conjunctivae with M. bovis cultures, M. bovis became established in the left eyes but not in the right eyes of the 3 animals. Two animals developed typical moderately severe unilateral IBK in their left eyes; the left eye of the other animal remained

normal. The right eyes of the 3 cattle became inflamed; the upper eyelids and conjunctivae were red and swollen and there was a slight serous discharge. One animal developed corneal opacity in its right eye but it was very transient (disappeared within 24 hours). All of the eyes returned to normal after 6 days with the exception of the two which had developed keratoconjunctivitis; the latter two left eyes (2 animals) returned to normal within 20 days, except for a small scar which remained in the center of each cornea.

Experiment III

The following results were obtained after sequential exposure of the eyes of 6 cattle to eye-secretion filtrate, a hemolytic diplococcus, nonhemolytic M. bovis and hemolytic M. bovis. Neither M. bovis nor a virus was isolated from the eyes of the cattle after their exposure to eye-secretion filtrate, but various nondescript bacteria were isolated before and after exposure. After exposure of the eyes to the hemolytic diplococcus, it became established. With the exception of a mild inflammation in each eye, which was attributed to the effects of ultraviolet irradiation, all the eyes remained normal. Similarly, the nonhemolytic M. bovis also became established in the eyes of the 6 cattle and they remained normal.

Hemolytic M. bovis became established in all eyes after exposure. Two animals developed severe unilateral IBK; another

one developed severe bilateral IBK. The eyes not affected with keratoconjunctivitis showed signs of conjunctivitis (erythematous, edematous conjunctivae and third eyelids), blepharitis (swollen eyelids and erosions especially at the lateral canthi) and scant serous discharge.

It is interesting to note that the hemolytic diplococcus was present in the eyes when they were exposed to the nonhemolytic M. bovis and both of these organisms were present in the eyes at the time the hemolytic M. bovis was introduced. It is thought that the presence of the hemolytic diplococcus and the nonhemolytic M. bovis did not interfere with or enhance the outcome of the hemolytic M. bovis infection.

Experiment IV

The following results were obtained after simultaneous exposure of the eyes of 3 cattle to IBR virus and M. bovis. Infectious bovine rhinotracheitis virus became established in the eyes of the 2 cattle exposed experimentally as well as in those of the contact control cow. Moraxella bovis became established only in the left eyes of the 2 animals that were exposed experimentally.

Initially, all of the animals developed bilateral conjunctivitis characterized by a mucopurulent discharge, erythematous and edematous conjunctivae and third eyelid. Blepharitis was also marked. Later, however, the symptoms became more severe, evidenced by necrosis and sloughing of the lids and

conjunctivae, protrusion of the third eyelid and copious amounts of mucopurulent eye discharge which wetted and matted the hair on the face. Coincident to these severe eye lesions were mild respiratory symptoms which are characteristic of IBR (1).

The right eye of one animal (calf 5004C) developed very severe lesions (panophthalmia) which resulted in permanent blindness in this eye. The right eye of the control cow developed keratitis in addition to severe conjunctivitis and blepharitis. The keratitis was very mild (faint haze seen on day 6 postexposure) and transient. After reaching a peak around the 7th day after exposure, the symptoms rapidly receded in all eyes except the eye with panophthalmia, and had disappeared on day 11. The eye with severe panophthalmia healed gradually, and, after 3 months, was free of an active inflammatory process. However, a cicatrix which encompassed the cornea remained and rendered the animal permanently blind.

Discussion

The results of Experiment I indicate that M. bovis readily becomes established in the bovine eye and can be recovered from it with routine laboratory methods; these results are substantiated by those of the other three experiments. It is also interesting to note that the antibiotic treatment freed the eye of M. bovis. This latter finding is in agreement with the results of other investigators

(34, 78, 79, 111) who have reported that M. bovis is very susceptible to antibiotics. The establishment of M. bovis in the nostrils of cattle is thought to be significant because this may be an important mechanism for the maintenance of occult M. bovis infection in cattle herds. Possibly the sporadic occurrence and erratic nature of the disease may be due to this type of carrier state. Of course, this experiment was too elementary to prove this contention; therefore additional work in this area is indicated.

The results of Experiment II indicated that one or more of the isolants in the mixture of M. bovis cultures was pathogenic and could produce IBK. It is interesting to note that one of the isolants (EPP-63-300) contained in the mixture was later shown to be highly virulent and has been used in other studies (67, 68, 69, 105, 106, 107). The results also indicated that M. bovis does not have to be injected into the tissue in order to induce IBK; in fact, they indicate that conjunctival instillation is a better method of exposure.

The results of Experiment III indicate that filtrable agent (eye-secretion filtrate), hemolytic diplococci, and non-hemolytic M. bovis do not cause IBK, but the hemolytic M. bovis is probably the etiologic agent. However, the results did not preclude a role for these other organisms in naturally occurring IBK or other eye diseases. Augmentation in the establishment of infection is thought to be the main role of

ultraviolet irradiation in this study. Possibly it influences M. bovis in other ways but this remains to be confirmed.

The results of Experiment IV indicate that IBR virus and M. bovis readily become established and produce disease in cattle eyes instilled with a mixture of IBR virus and M. bovis. They also indicate that severe conjunctivitis and blepharitis are produced when these organisms are injected into the conjunctivae. However, it is interesting to note that the disease produced by mixed IBR virus and M. bovis infection is different from the disease caused by M. bovis alone as was observed in Experiment III; the main differences were the severity of the conjunctivitis and blepharitis and the copious amounts of purulent eye discharge. The results also tend to indicate that the instillation of IBR virus and M. bovis in the conjunctival sac is sufficient to establish infection.

Summary and Conclusion

The eyes of 13 cattle were exposed to different inoculums using various exposure methods as follows: one calf was exposed to a pure culture of a laboratory strain of M. bovis by conjunctival sac instillation. Moraxella bovis became established and produced a case of mild keratoconjunctivitis. The test organism was readily recovered from the exposed eye as well as both nostrils. The organism persisted in the eye and one nostril for 83 days at which time antibiotic treatment was used to free the organs of M. bovis.

The eyes of 3 cattle were exposed to a mixed culture of two strains of M. bovis by instillation of the conjunctival sac of one eye of each animal and the other eye of each was exposed by injecting the mixture of M. bovis into the upper conjunctivae. Moraxella bovis became established and was recovered readily from the eyes that were exposed by conjunctival instillation. Two of these eyes developed moderately severe keratoconjunctivitis; the other remained normal. The three eyes exposed by injections into the conjunctivae developed severe inflammation at the site of the injection, but otherwise they remained nearly normal. Moraxella bovis was not recovered from these eyes.

The eyes of 6 cattle were exposed to infective eye-secretion filtrate, hemolytic diplococcus, nonhemolytic M. bovis and hemolytic M. bovis in sequential order with periods between exposure of 20 days, 10 days, and 20 days respectively. Viruses and M. bovis were not recovered from any of the eyes after exposure to eye-secretion filtrate and the eyes remained normal. The hemolytic diplococcus, the nonhemolytic M. bovis and the hemolytic M. bovis became established in all eyes after experimental exposure. Of the three organisms which became established, only M. bovis induced keratoconjunctivitis. Two animals developed unilateral IBK and another developed bilateral IBK. The eyes not affected with keratoconjunctivitis developed moderately severe cases of conjunctivitis and blepharitis.

The eyes of the three remaining animals were exposed to infectious rhinotracheitis virus and a hemolytic M. bovis. Two animals were exposed by instilling both IBR virus and M. bovis cultures into their left eyes; the other eye of each of these animals was exposed by injection of the cultures into their conjunctivae. The other animal was exposed by contact with one of the experimentally exposed cattle. Moraxella bovis became established only in the left eyes of the experimentally exposed animals but IBR virus became established in all eyes. All the cattle developed severe bilateral conjunctivitis and blepharitis with copious amounts of mucopurulent discharge from the eyes. One of these animals subsequently developed unilateral panophthalmia which resulted in blindness. Another animal developed an opacity which disappeared within a day.

In conclusion it can be stated that M. bovis and IBR virus readily become established in cattle eyes when instilled and are easily recovered by using routine laboratory methods. There is little doubt that M. bovis produces IBK but the role of IBR virus remains to be elucidated. There is no doubt that IBR virus produces a severe conjunctivitis with a transient corneal opacity, but these symptoms are different from those seen in typical IBK. Finally it should be noted that the fact that IBK was produced by M. bovis infection in cattle eyes after they had been resistant to eye-secretion filtrates and bacteria further substantiate its role as a primary etiologic agent.

SECTION IV. EXPERIMENTAL PRODUCTION OF BOVINE
INFECTIOUS KERATOCONJUNCTIVITIS: SELECTED
FACTORS PERTAINING TO THE INOCULUM

Introduction

Moraxella bovis is considered the etiologic agent of IBK but little is known about the number of organisms required or the appropriate exposure method needed to establish infection.

Heretofore, studies on the dosage of M. bovis needed to establish infection were not carried out because of the lack of an adequate vehicle for suspension and subsequent plating out of the organism so that viable plate counts (VPC) or microscopic counts could be made. This is due to the fact M. bovis agglutinates spontaneously in most laboratory mediums commonly used to suspend bacteria. After many different vehicles were used in an attempt to obtain a diluent in which M. bovis would suspend uniformly, 10% magnesium chloride was found to give the best results.¹

The purposes of this experiment were to determine (1) whether a specific number of M. bovis suspended in 10% $MgCl_2$ was an effective means of exposing cattle eyes, (2) whether cattle would develop keratoconjunctivitis within a limited time after exposure to secretions from eyes acutely affected with

¹Pugh, George W., Jr., National Animal Disease Laboratory, ADP Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa 50010: Unpublished data, 1966.

disease, and (3) whether the virulence of the eye secretions or M. bovis could be enhanced by rapid passage in cattle eyes.

Materials and Methods

Experimental design

Moraxella bovis and eye secretions were carried through rapid cattle passages in an attempt to increase their virulence in the following manner. Fifteen cattle were divided into eight groups on a random basis and exposed sequentially. The animal in Group I was exposed to a pathogenic culture of M. bovis and after the infection was established the eye secretions from the acutely affected eye¹ in this group were used to expose the cattle in Group II. The eye secretions from an affected eye in Group II were transferred to the cattle in Group III, and this procedure continued serially through Group VI. Groups VII and VIII were exposed to M. bovis cultures only. Approximately one week after the cattle in each group were exposed to eye secretions, they were challenged with virulent homologous² cultures of M. bovis.

Organism

Moraxella bovis strain EPP-63(300) was used in this experiment as the primary organism. It was originally isolated from

¹Eye secretions were taken from the first eye within a group to develop IBK and used to expose cattle in the succeeding group.

²Homologous culture means the organism isolated from the eye secretions used to expose the cattle in the group initially.

an animal naturally affected with IBK and, on the basis of other studies, was found to be pathogenic for cattle (67, 68, 69, 105). Cultures of the strain had been stored at -60 C in litmus milk for 5 years.

The original inoculum of M. bovis for exposing animal number 5611 was prepared from a frozen stored culture. The latter was thawed and streaked on 5% bovine blood; the agar plate culture was incubated at 37 C for 24 hours. Typical smooth hemolytic colonies were streaked on plates and carried through second and third passages. After the third plate culture was incubated 24 hours, the growth was scraped from the surface of the medium and suspended in 10% MgCl₂ solution; the suspension was instilled in the eyes.

Determining the exposure dose To determine the number of organisms used, the following procedure was carried out in each case: an aliquot of inoculum was placed in the appropriate amount of 10% magnesium chloride. Seven ten-fold dilutions of the organisms were made and 0.2 ml of each dilution used to inoculate blood agar plates in duplicate. This was done by placing 0.2 ml of the dilution in the center of the plate and then rocking the plate in order to spread the suspension uniformly over the surface of the agar. After this, the plates were incubated for 24 hours at 37 C and then for 24 hours at room temperature at which time the colonies of M. bovis were enumerated. An appropriate calculation for each dilution was

conducted to determine the VPC which was extrapolated to deduce the number of organisms used as the exposure dose.

Animals

Fifteen healthy male animals, ranging in age from 10 months to 2 years of age were used in this experiment. They were of the Holstein-Friesian breed and did not have a history of prior eye disease; they were obtained from NADL's clean animal herd and were considered specific pathogen free (SPF). It should be noted, however, that they had had access to natural sunlight and flies during the preceding summer. The animals were placed in NADL isolation units for two weeks prior to use. These isolation units excluded sunlight, were air conditioned and mechanically ventilated with filtered air. Two cattle were kept in the same room but in separate stalls. The rooms were illuminated with fluorescent lamps, recessed back of an impact-resisting prismatic glass lens.

Bacteriologic examination

Sterilized cotton-tipped applicators were used to collect eye secretions immediately before exposure and daily during the observation period. The applicators were used to transport the eye secretions to expose the other eyes and/or they were placed in TSB immediately to prevent drying. Later they were streaked on the surface of blood agar plates. The plates were incubated at 37 C for 24 hours, and then for 24 additional hours at room

temperature at which time they were examined for M. bovis colonies.

Irradiation

A sunlamp (67), used as the source of ultraviolet radiation, was a combination Mercury discharge element and a tungsten-filament resistance ballast incorporated within an ultraviolet transmitting, reflector-type bulb. Each animal was irradiated for 10 minutes at 24 inches beginning the day of its exposure to eye secretions or M. bovis and daily throughout the first 10 days of the experiment. Ultraviolet irradiation was used to enhance the establishment of M. bovis in the cattle eyes (105).

Exposure

Collection and exposure to eye secretions were carried out in the following manner: at the first sign of IBK in an animal within each group, four cotton-tipped applicators were placed in the affected eye, and passed across the third eyelids, conjunctivae, sclera, and cornea. The applicators became wet due to absorbed eye secretions. These applicators were used to expose the next group of animals. In order to expose the eye to eye secretions, the animal head was held in a stanchion and immobilized by a rope halter; the upper and lower eyelids were held apart and an applicator containing the eye secretions from the diseased eye was placed in the eye and gently passed over the conjunctivae, third eyelid, sclera and cornea. After

exposure of the eye, the applicator was used to streak a blood agar plate. The plate was incubated for 24 hours at 37 C and for 24 hours at room temperature at which time it was examined for the presence of M. bovis colonies.

Exposure to M. bovis was made by the instillation of 0.15 to 0.25 ml of a 10% MgCl_2 suspension of the test organism into the lower conjunctival sac. The upper and lower eyelids were held together for 30 to 60 seconds after the instillation was made.

The animals in the eight groups were exposed under the following regimentation. Group I consisted of 1 animal (5611), each eye of which was exposed to a third blood agar passage (BAP) culture of M. bovis strain EPP-63(300) with an exposure dose of 10^3 organisms determined by a viable plate count (VPC) Group II consisted of 2 animals (5663 and 5653) which were exposed to secretions from an affected eye of animal number 5611. After 7 days, each eye was exposed to a second BAP culture of M. bovis (6×10^3 organisms VPC) recovered from eye secretions (5611L). Group III consisted of 2 animals (5674 and 5662) which were exposed to secretions from an affected eye of animal number 5653. Seven days later these eyes were exposed to a second BAP culture of M. bovis (6×10^3 organisms VPC) recovered from eye secretions (5653R). Group IV consisted of 2 animals (5678 and 5680) which were exposed to secretions from an affected eye of animal number 5662. After 7 days each eye was exposed to a second BAP culture of M. bovis (2×10^4

organisms VPC) recovered from eye secretions (5662R). Group V consisted of 2 animals (5673 and 5524) which were exposed to secretions from an affected eye of animal number 5680. Eight days later each eye was exposed to a second BAP culture of M. bovis (4×10^4 organisms VPC) recovered from eye secretions (5678L). Group VI consisted of 2 animals (5751 and 5752) which were exposed to secretions from an affected eye of animal number 5673. Seven days later each eye was exposed to a third BAP culture of M. bovis (2×10^5 organisms VPC) recovered from eye secretions (5673R). Group VII consisted of 2 animals (5748 and 5756) which were exposed to a third BAP culture of M. bovis (2×10^5 organisms VPC) recovered from eye secretions (5751R). Group VIII consisted of 2 animals (5754 and 5759) which were exposed to a third BAP culture of M. bovis (2×10^4 organisms VPC) recovered from eye secretions (5748R).

Serologic examination

Blood samples were taken from the 15 animals prior to their exposure to eye secretions or M. bovis and again at the termination of the experiment. The serums were checked for precipitating antibodies for M. bovis using the agar gel double-diffusion technique described in Section II.

Results

Group I

Forty-eight hours after exposure to a culture of M. bovis both eyes were moist and congested. After 3 days, one eye

developed severe keratoconjunctivitis but the other eye was normal (Table 6). Moraxella bovis was recovered from both eyes. The pre- and post-infection serum samples did not contain precipitating antibodies against M. bovis.

Group II

All eyes remained normal after exposure to eye secretions from the animal (5611) in Group I and M. bovis was not recovered from them (Table 6). However, M. bovis was recovered from the eye secretions.¹ Both animals developed IBK after exposure to M. bovis; one animal (5653) developed severe bilateral and the other (5663) severe unilateral IBK. Moraxella bovis was recovered from all the eyes. The pre- and post-infection serum samples from the animal developing bilateral IBK did not contain precipitating antibodies against M. bovis (Table 7). Antibodies were not detected in the pre-infection serum of the other animal but its post-infection serum was positive for antibodies (four lines of precipitation were observed).

Group III

All eyes remained normal after exposure to eye secretions transferred from animal (5653) in Group II and M. bovis was not recovered from them (Table 6). However, M. bovis was recovered

¹Not more than ten colonies of M. bovis were observed on any plate streaked with eye secretions which were used to expose cattle.

Table 6. Results of exposure of cattle eyes to serially passaged Moraxella bovis

Group	Animal number	Source of exposure material		Number of animals developing keratoconjunctivitis			
		Eye secretions	<u>M. bovis</u> culture	After exposure to eye secretions		After exposure to <u>M. bovis</u> culture	
				Unilateral	Bilateral	Unilateral	Bilateral
I	5611	- ^a	EPP-63 (300)	-	-	0 ^b	1
II	5663 5653	5611L	5611L	0	0	1	1
III	5662 5674	5653R	5653R	0	0	0	2
IV	5678 5680	5662R	5662R	0	0	0	2
V	5673 5524	5680R	5678L	0	0	1	1
VI	5751 5752	5673R	5673R	0	0	1	0
VII	5748 5756	-	5751R	-	-	1	1
VIII	5754 5759	-	5748R	-	-	2	0

^a- = Not done.

^b0 = None.

Table 6. (Continued)

Group	Animal number	Number of eyes <u>M. bovis</u> recovered	
		Eye secretions	<u>M. bovis</u> exposure
I	5611	-	2
II	5663 5653	0	4
III	5662 5674	0	4
IV	5678 5680	0	4
V	5673 5524	0	3
VI	5751 5752	0	2 ^c
VII	5748 5756	-	4
VIII	5754 5759	-	2 ^d

^cRecovered from only 1 animal.

^dRecovered from 2 animals.

Table 7. Antibody response of cattle exposed to serially passaged Moraxella bovis

Group	Animal number	Developed keratoconjunctivitis	<u>M. bovis</u> established	Antibodies demonstrated at time of exposure to secretion sample	Period between bleeding	Antibodies demonstrated at the end of experiment
I	5611	pos.	pos.	neg.	20 days	neg.
II	5663	pos.	pos.	neg.	17 days	neg.
	5653	pos.	pos.	neg.	17 days	pos.
III	5662	pos.	pos.	neg.	25 days	pos.
	5674	pos.	pos.	neg.	25 days	neg.
IV	5678	pos.	pos.	neg.	34 days	neg.
	5680	pos.	pos.	neg.	23 days	neg.
V	5673	pos.	pos.	neg.	33 days	pos.
	5524	pos.	pos.	neg.	33 days	neg.
VI	5751	pos.	pos.	neg.	29 days	neg.
	5752	neg.	neg.	neg.	29 days	pos.
VII	5748	pos.	pos.	neg.	21 days	neg.
	5756	pos.	pos.	neg.	14 days	pos.
VIII	5754	pos.	pos.	neg.	19 days	pos.
	5759	pos.	pos.	neg.	21 days	pos.

from the eye secretions. Both animals developed mild bilateral IBK after exposure to M. bovis culture. Moraxella bovis was recovered from the eyes of both animals. The pre-infection serum samples of both animals and the post-infection of one (5674) did not contain precipitating antibodies against M. bovis (Table 7).

Group IV

All eyes remained normal after exposure to eye secretion transferred from the animal (5662) in Group III and M. bovis was not recovered from them (Table 6). However, M. bovis was recovered from the eye secretions. After exposure to M. bovis culture, both animals developed mild bilateral IBK and M. bovis was recovered from their eyes. The pre- and post-infection serum samples did not contain precipitating antibodies against M. bovis (Table 7).

Group V

All eyes remained normal after exposure to eye secretions from the animal (5678) in Group IV and M. bovis was not recovered from them (Table 6). However, M. bovis was recovered from the eye secretions. After exposure to M. bovis culture, one animal developed mild bilateral IBK and the other one mild unilateral IBK. Erosion of the eyelids was a prominent feature of the disease (IBK) in both animals. Moraxella bovis was recovered from the affected eyes only. The pre-infection serum samples of both animals and the post-infection serum sample of

one animal (5524) did not contain precipitating antibodies against M. bovis (Table 7).

Group VI

All eyes remained normal after exposure to eye secretions from the animal (5673) in Group V and M. bovis was not recovered from them (Table 6). However, M. bovis was recovered from the eye secretions. After exposure to M. bovis culture, one animal (5751) developed mild unilateral IBK but the eyes of the other animal (5752) remained normal. Moraxella bovis was recovered from the eyes of the animal with unilateral IBK only. The pre-infection serum samples of both animals as well as the post-infection serum sample of the animal (5751) developing IBK did not contain precipitating antibodies against M. bovis (Table 7).

Group VII

Moraxella bovis was recovered from all the eyes of the animals in Group VII after exposure to a culture of M. bovis isolated from an animal (5751) in Group VI (Table 6). One animal (5756) developed severe unilateral IBK and the other (5748) mild bilateral IBK. The pre-infection serum samples of both animals and the post-infection samples of one animal (5748) did not contain precipitating antibodies against M. bovis (Table 7). The post-infection serum sample of the animal with unilateral IBK contained antibodies.

Group VIII

Moraxella bovis was recovered from the diseased eye of each animal in Group VIII (Table 6) when exposed to a culture from (5748). One animal (5754) developed severe unilateral IBK while the other (5759) developed mild unilateral IBK. The pre-infection serum samples of both animals did not contain precipitating antibodies against M. bovis while their post-infection serum samples did contain them (Table 7).

Discussion

The results indicate that M. bovis suspended in 10% $MgCl_2$ is an effective inoculum for exposing cattle eyes. Previously, most investigators used indeterminate numbers of M. bovis such as that found in eye secretions or spontaneously agglutinated cultures harvested from the surface of plating mediums. Because M. bovis forms a uniform suspension in $MgCl_2$ but not in other mediums, $MgCl_2$ could serve as a useful tool for enumerating M. bovis in studies such as those on dose response. Admittedly, the present study did not establish a minimum exposure dose, but it is interesting to note that as few as 1,000 organisms (Group II) did induce IBK in 2 animals.

Apparently, the number of M. bovis in secretion from infected eyes is not sufficient to establish infection or cause disease after short incubation periods. This is partially substantiated by the fact that the animals became infected and developed disease within 3 days when exposed to

cultures of M. bovis which represented larger doses. Supporting this is the work of Barner (14) who indicated that it takes M. bovis approximately 20 days to cause IBK when eye secretions are used as exposure material. Possibly the longer incubation period allows the development of a larger and more effective population of organisms which can produce disease. It is interesting to note that other investigators (3, 6, 13, 47, 55) who have had poor results in reproducing IBK have used eye secretions as exposure material.

Apparently direct serial passage of M. bovis in eye secretion through six passages did not enhance its virulence. Nor was there evidence that the virulence of M. bovis cultures was enhanced when serially passed from eyes affected with IBK. However, it should be noted that there were two blood agar passages of the organism between each group of cattle.

The results of the serological study indicate that M. bovis infection may cause cattle to develop antibodies, but whether these antibodies render the animal immune remains to be elucidated. It is interesting to note that animals which had severe unilateral IBK developed antibodies and those with mild bilateral IBK did not. Also one animal which did not develop IBK, and M. bovis did not become established in its eyes, developed antibodies against M. bovis. A possible explanation for this latter result is that M. bovis became established in the eye and/or nasal cavity and induced the

production of antibodies, but the method of detection was not adequate.

Summary and Conclusion

The eyes of 15 bovine animals were serially exposed to M. bovis cultures and/or eye secretions containing M. bovis. The eyes of 10 of these animals were exposed to eye secretions from eyes acutely affected with keratoconjunctivitis but did not develop disease. Moraxella bovis could not be recovered from these eyes during the test period (7 days). However, when the eyes were exposed to cultures of M. bovis later, 9 developed keratoconjunctivitis. Five other animals exposed only to cultures of M. bovis developed IBK. Moraxella bovis became established in the eyes of 14 of the 15 cattle and 7 animals developed antibodies against M. bovis.

It is concluded from the results that eye secretions are inadequate for the experimental production of IBK even when ultraviolet irradiation and rapid-passaged inoculum are used. These findings also substantiate the fact that M. bovis is the primary etiologic agent of IBK and that cattle develop antibodies against M. bovis as a consequence of severe IBK.

SECTION V. THE ROLE OF INFECTIOUS BOVINE RHINOTRACHEITIS
VIRUS IN BOVINE INFECTIOUS KERATOCONJUNCTIVITIS

Introduction

Moraxella bovis is generally considered the etiologic agent of infectious bovine keratoconjunctivitis (IBK) or pink-eye of cattle (2, 14, 15, 67, 69, 71, 73, 105, 108, 110, 112, 119). Some workers (17, 53, 124), however, doubt M. bovis is the primary agent and have incriminated other organisms. Probably the strongest evidence put forth for another organism is that of Sykes et al. (123, 124) who incriminated infectious bovine rhinotracheitis (IBR) virus as the cause of IBK.

Although IBR virus produces lesions predominantly in the upper respiratory tract (87), it may also cause conjunctivitis (1, 86, 87, 122) and in some cases keratoconjunctivitis (70). In two reports, experimental IBR virus infections produced by conjunctival inoculation were studied (1, 122). The lesions described were those of simple conjunctivitis which resembled lesions of IBK (2, 6, 60, 73, 108, 112, 119) except that there were no corneal ulcers or opacities. But in two other reports (70, 124) where similar exposure methods were used, keratoconjunctivitis was a prominent feature of eye disease caused by IBR virus. From the preceding reports, one can deduce that there is a possible relationship between M. bovis and IBR virus infection in eyes of cattle.

The present study was an attempt to establish the role of IBR virus in M. bovis infection in the eyes of cattle. It was

assumed that M. bovis is the primary agent of IBK and hypothesized the role of IBR virus to be secondary. It was also assumed that the keratoconjunctivitis observed by some investigators (123, 124) was due to occult M. bovis infection.

The study was conducted in four parts (Experiments Ia, Ib, Ic, and Id); each part had a specific function. Experiment Ia served as an M. bovis infection control for the other three parts. Experiment Ib was designed to determine whether an eye previously affected with IBK caused by M. bovis would develop a similar disease when subsequently exposed to IBR virus. Experiment Ic was used to determine the type of eye disease produced with concomitant M. bovis and IBR infections. Experiment Id investigated the influence of previous IBR-induced conjunctivitis on subsequent infection and disease by M. bovis.

Materials and Methods

Cultures

Moraxella bovis strain (EPP-63-300) was used throughout the experiment. It was isolated from a bovine animal naturally affected with IBK and, on the basis of other studies, was found to be pathogenic for cattle (67, 68, 69). Methods of storage and preparation of inoculums are discussed in Section IV. A fourth blood agar passage (BAP) culture of the organism suspended in trypticase soy broth (TSB) was instilled in the eye.

The virus culture was an IBR virus (Himstra 6) isolated from a cattle herd with acute and chronic IBK accompanied by

clinical signs of IBR. It was isolated from a "normal" bovine eye along with M. bovis. A report on the isolation and identification of the IBR virus is given in the following paragraphs.

Isolation of Himstra 6 cytopathogenic agent (CPA) Eye secretions were obtained from both normal and diseased eyes using sterilized cotton-tipped applicators in the following manner. Once the head of the animal was restrained properly by the use of a stanchion and a rope halter, one hand was used to hold the eyelids apart and the other to pass two applicators over the conjunctivae, Membrana nictitans and the surface of the eyeball. The applicators were then removed from the eye without coming in contact with any other surfaces. Immediately after the secretions were taken, the applicators were placed in screw-cap tubes containing storage medium; either stock tissue culture medium (Earle's medium containing antibiotics) or TSB. The capped tubes containing the applicators were placed in an insulated box (ice chest maintained at temperatures less than 15 C) and transported to the laboratory.

Procedures and mediums used Once at the laboratory the secretions were used to inoculate 6-day-old primary embryonic bovine kidney (PEBK) cells or stored at -60 C for later use. Hanks' and Earle's mediums as described by Cunningham (43) were used as culture mediums for the PEBK cells. The PEBK cells were started in Hanks' medium containing 0.25% lactalbumin hydrolysate (LAH) plus 10% SPF calf serum. After the appropriate period of incubation (usually 3 days) Earle's

medium containing 0.25% LAH, 10% SPF calf serum and antibiotics (dihydrostreptomycin 0.1 mgm/ml, Kanamycin 0.1 mgm/ml, and penicillin - 100 units/ml) was used as the first change medium. Some tubes also contained an antifungal agent (fungizone, 2 micrograms/ml). Earle's medium containing antibiotics was also used throughout the study as stock medium (ESM) and as a diluent for the virus, serums and ether. Phenol red was used as the pH indicator for all the mediums.

Using the materials and methods described above, the eyes of 10 cattle were cultured for viral agents. Four tubes of 6-day-old PEBK cells were each inoculated with a 0.1 ml sample representing each eye. Two tubes of PEBK cells contained the antifungal agent and two did not. All tubes were incubated at 37 C and observed daily for cytopathic changes (CPE). Subcultures were made to new PEBK cells after 7 days incubation and at each 7-day interval through four subcultures. Each subculture was made from a pool of tissue culture from four tubes (representing one original sample).

Cytopathic changes were observed in four tubes (representing one eye sampled - Himstra 6 R) after 48 hours incubation of the first subculture. Complete destruction of monolayer of the PEBK cell was evident after 96 hours incubation. Subsequent passages with undiluted material resulted in cytopathic changes, characterized by rounding and shrinking of the cells and increased granularity and clumping usually within 24 to 48 hours.

Identification of the cytopathogenic agent

The fact

that the CPA was isolated from a herd with respiratory symptoms and clinical signs similar to those of IBR (1, 85, 86, 87, 122) and the type of CPE produced in PEBK cells, which corresponds to that observed by other workers (85, 86, 122) intimated that the CPA might be IBR virus. Using the methods outlined by Cunningham (43) and others (7, 123, 132), the following attempt was made to identify the organism.

Determination of size

The size of the CPA was

determined by a filtration method (43). The agent was cultivated in PEBK cells for 72 hours and the culture fluid harvested and either filtered through a Seitz filter (sterilizing filter pad) and negative pressure or a Millipore filter (filter pads of sizes HA-0.45 micron, PH-0.3 micron, GS-0.22 micron, VC-100 millimicrons, and UM-50 millimicrons) using positive pressure.

The CPA did not pass through the Seitz filter pad, or the Millipore filter pads of sizes VM-50 millimicrons, VC-100 millimicrons, and GS-0.22 micron; but passed sizes HA-0.45 micron and PH-0.3 micron. The size of the CPA was thought to be directly correlated with the filterability of the virus and CPE occurred in tubes of PEBK cells that were inoculated with filtrate containing the CPA. It was concluded from this that the CPA size less than 300 millimicrons but greater than 100 millimicrons was within the general size range of the

herpesvirus group when the virus envelope and the method of size determination were considered (54, 82, 126).

The size and shape of the CPA were also determined by the use of the electron microscope; a photomicrograph of the CPA is given in Figures 12-15.

Pathogenicity for chicken embryos The agent did not survive, multiply or produce death in chicken embryos when inoculated via the yolk sac, allantoic sac or the amniotic cavity.

Hemagglutinating and hemadsorbing ability It failed to hemagglutinate a 0.5% suspension of washed chicken red blood cells. It also failed to hemadsorb chicken red blood cells.

Neutralization by known serums Attempts to neutralize the virus was made using IBR virus antiserum, negative SPF calf serum, mucosal disease virus antiserum, and shipping fever 4 (SF₄) virus antiserum. The CPA was neutralized by IBR virus antiserum, giving a titer of 1:16 against 10² TCID₅₀ CPA per ml (110). The same concentration of the CPA was not neutralized by mucosal disease virus antiserum, SF₄ virus antiserum or the negative SPF calf serum.

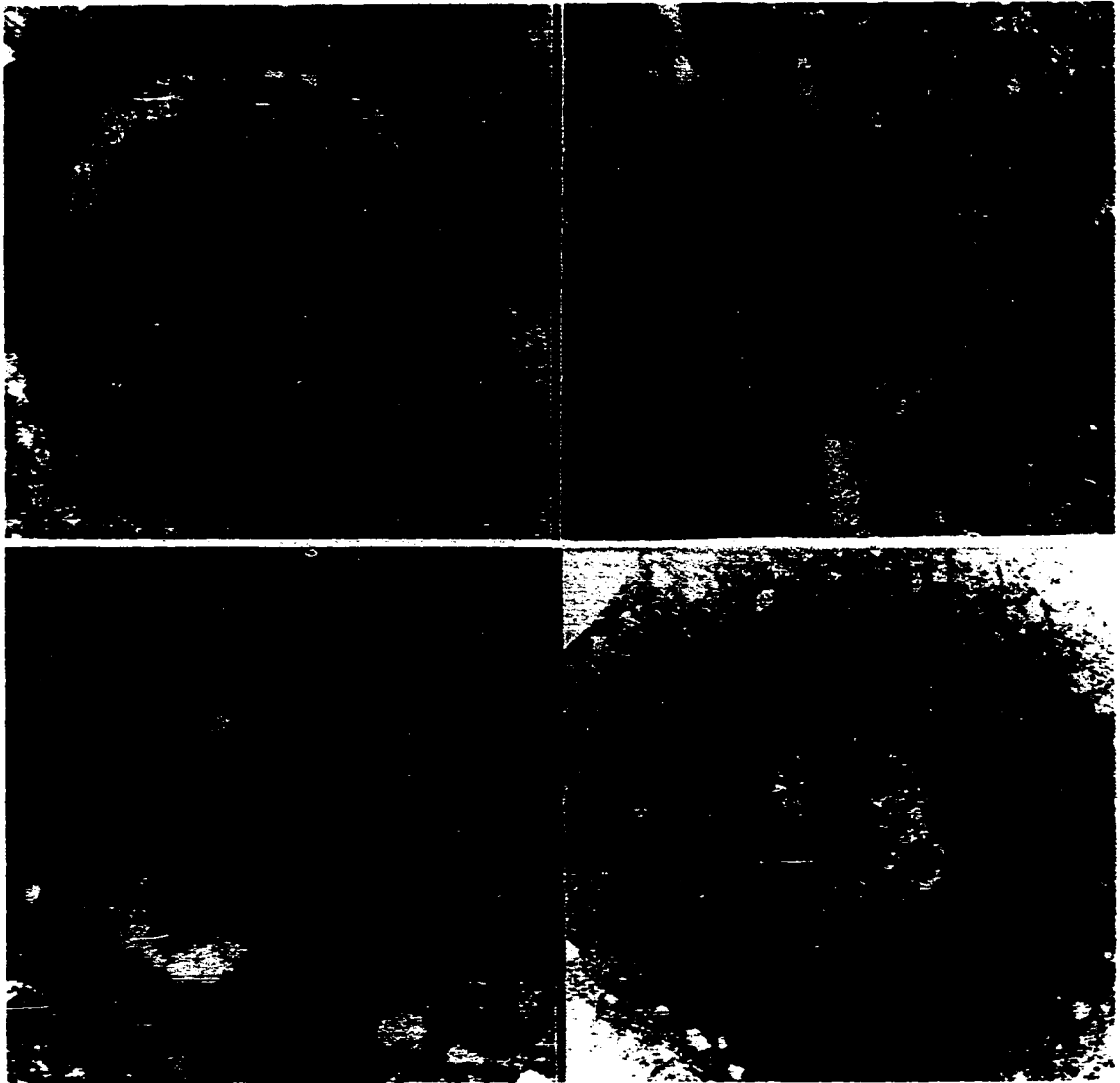
Ether sensitivity Ether sensitivity was used as a criterion for determining whether the CPA contained an envelope, a structure which is characteristic of the herpesvirus group (54). The following procedure was followed in determining whether the CPA was ether sensitive: 0.5 ml of

Figure 12. (Upper left). Depicts Himstra 6 cytopathogenic agent (CPA) with PTA staining. Note the complete virus particle - 400,000X

Figure 13. (Upper right). Depicts Himstra 6 CPA with PTA staining. Note the broken capsid showing the hollow capsomeres - 400,000X

Figure 14. (Lower left). Depicts Himstra 6 CPA with PTA staining. Note the capsid with a bleb of its surrounding envelope which is characteristic of the herpesvirus group - 400,000X

Figure 15. (Lower right). Depicts Himstra 6 CPA with PTA staining. The shape of the virus particle resembles that of the herpesvirus group - 320,000X



undiluted 5-day-old PEBK culture of the CPA ($10^{6.5}$ TCID₅₀ per ml) was added to each of the following dilutions of diethyl ether: 1:2, 1:5, 1:10, 1:20, 1:40 and 1:80. The ether was diluted with Earle's medium only. Undiluted ether and CPA were also mixed together in a 1:1 ratio. Controls were set up using (1) Earle's medium and ether, in a 1:1 ratio without CPA, (2) ether only, (3) CPA only, and (4) Earle's medium only. All the mixtures and controls were then incubated at 37 C for 30 minutes. After the incubation period, 0.1 ml of each ether mixture and control was each inoculated into four tubes of PEBK cells. The tubes were then incubated at 37 C and observed daily for CPE for 5 days.

Cytopathic changes were seen in two tubes representing the 1:10 ether dilution mixture. Also there was CPE in all tubes with mixtures representing higher dilutions of ether. No CPE was observed in tubes with mixtures of less than 1:10 dilution or in the control tubes not inoculated with the CPE. From these results it was concluded that the CPA was ether sensitive and therefore probably contained an envelope.

Pathogenicity of Himstra 6 CPA Two Holstein cattle, one a yearling heifer (5158) and the other a 9-year-old cow (3571) were used in this study. They were culturally negative for M. bovis and IBR virus. Also their serums did not contain precipitating or neutralizing antibodies against M. bovis and IBR respectively. The animals were quartered in indoor isolation units which excluded sunlight. These quarters

were air conditioned and mechanically ventilated with filtered air. The cattle were kept in the same room but in separate stalls. The rooms were illuminated with fluorescent lamps, recessed in back of an impact-resisting prismatic glass lens.

The virus culture used was a fourth PEBK cell passage of the Himstra 6 CPA with a concentration of $10^{6.5}$ TCID₅₀ per ml. The cattle eyes were exposed to the CPA culture in the following manner: the left eye of each animal was exposed to 0.5 ml of the culture by holding the eyelids apart and flooding the eye with culture. After flooding the eye, the eyelids were held together and massaged for approximately 1 minute. The right eye of each animal was first irritated mechanically by dropping sterilized sand into the eye and gently rubbing the closed eyelids and then immediately exposed to the CPA in the same manner as the left eye.

Twenty-four hours (day 2) after exposure, clinical changes were seen in all eyes. The major changes were reddening of the Membrana nictitans and the conjunctivae accompanied by slight increased lacrimation. On subsequent days the symptoms became more severe manifesting the general changes described as being characteristic of IBR virus eye infection (70, 87, 122, 124). However, the disease in the eyes of one animal (3571) became very severe; the eyelids and the conjunctivae were swollen to such an extent that they had to be forced apart in order to observe internal structures of the eyes. The corneas of both eyes developed opacities which enveloped the ventral one-half

of each cornea. No corneal lesions were observed in the eye of animal number 5158, otherwise the clinical disease was similar to that of cow number 3571. It is interesting to note that the eyes were most severely affected about 4 days after exposure and thereafter the disease began to abate, and after 7 days the eyes were normal.

The CPA was recovered from both eyes of 5158 for the first 4 days and for 3 days from the eyes of 3571. Neutralizing antibodies against the CPA were found in the serums of both animals 7 days after exposure.

Conclusion as to the identity of Himstra 6 CPA On the bases of the CPE produced, the structure and size of the agent, its sensitivity to ether, its susceptibility to neutralization by IBR virus antiserum, its pathogenicity for cattle eyes, and the clinical lesion produced, it was concluded that the Himstra 6 CPA was an IBR virus.

Animals

Thirty-six healthy Holstein cattle with no history of IBK or IBR were used in Experiment I. All were apparently culturally negative for M. bovis and IBR virus and their serums did not contain precipitating and neutralizing antibodies respectively. Experiment Ia consisted of 6 yearling SPF calves, both males and females, which had been maintained since birth in indoor stalls. These animals were obtained from the NADL clean animal herd. Experiment Ib consisted of ten 2- to

3-month-old calves, both males and females, obtained from a local dairy herd. They were born during the winter months and had not been exposed to flies. Experiment Ic consisted of 10 calves, 2 to 6 months old, both males and females born after November 1 and before April 1. These calves were obtained from two local dairy herds. They were maintained in isolation units for one month prior to use. Experiment Id consisted of 10 male calves with the same history as those in Experiment Ic.

Animal quarters

The animal quarters were indoor isolation units which excluded sunlight and flies. These quarters were air conditioned and mechanically ventilated with filtered air. Each room housed two of the cattle in separate stalls. The rooms were illuminated with fluorescent lamps, recessed in back of an impact-resisting glass lens.

Bacteriologic and virologic examination

Secretion from each eye was collected with two sterilized cotton-tipped applicators for 5 days before exposure and each day during the observation period. One applicator was used for bacteriologic examination and the other for virologic examination. The applicator for bacteriologic examination was placed in TSB immediately after collection to prevent drying; later it was streaked on the surface of 5% bovine blood agar plate, and the plate was incubated at 37 C for 24 hours. The applicator for virologic examination was placed in ESM immediately

after collection to prevent drying and to suspend the viral particles. Later 0.1 ml of the suspension was used to inoculate each of two tubes of PEBK cells. The inoculated PEBK cells were incubated at 37 C and observed daily for CPE for 7 days.

Serologic examination

Blood samples were obtained from the 6 cattle in Experiment Ia on the day of their exposure to M. bovis (day 1) and again 38 days later (day 39). Blood samples were obtained from the 10 animals in Experiment Ib on the day of their exposure to M. bovis (day 1), on the day they were exposed to IBR virus (day 41) and finally at the end of the experiment (day 71). Blood samples were obtained from the 20 animals in Experiment Ic and Id on the day of their exposure to IBR virus (day 1), on the day the animals in Experiment Id were exposed to M. bovis (day 25), on the day all the animals were re-exposed to M. bovis (day 49) and on the final day of the experiment (day 69). The serums were checked for neutralizing antibodies against IBR virus and for precipitins against M. bovis using methods previously described.

Exposure methods and experimental design

Exposure to M. bovis was made by the instillation of 0.5 ml of the TSB suspension of M. bovis culture into the ventral conjunctival sac. Exposure to IBR virus was made by instilling 0.5 ml of a sixth TCP virus culture in the ventral conjunctival

sac. The exposure virus culture did not contain antibiotic since the medium used as the diluent and the last two tissue culture passages of PEBK cells were free of antibiotics. The upper and lower eyelids were held together for 30 to 60 seconds after the instillation was made. The sequence of exposure, number of organisms used and other pertinent data are given in Table 8.

Results

Symptoms and course of IBK due to *M. bovis*

Moraxella bovis-caused IBK was characterized by serous lacrimation, photophobia, spasms and constriction of the iris, conjunctivitis, blepharitis, and keratitis. Usually lacrimation was seen first; it became evident as the eyes appeared unusually moist. Later a few tears streaked the face and the amount of lacrimation increased until the side of the face with the affected eye became wet. At that time, copious amounts of serous lacrimal fluids (eye discharge) containing few bacteria were prominent; later the discharge became slightly mucopurulent and numerous micrococcus-like organisms could be isolated. This period usually lasted from 1 to 2 weeks, but did not occur in mild cases.

Photophobia was usually manifested by the animal turning the affected eye away from the light, blinking involuntarily or closing its affected eye. The degree of photophobia seemed to have been a function of the severity of other lesions. Usually

Table 8. Experimental design for exposure of bovine eyes to Moraxella bovis (M. bovis) and infectious bovine rhinotracheitis (IBR) virus

Experiment no.	Exposure sequence	<u>No. exposed</u>		First exposure to <u>M. bovis</u>	Exposure to virus
		Animals	Eyes	No. organism used ^a	No. organism used ^b
Ia	<u>M. bovis</u> only	6	12	2×10^8	--
Ib	<u>M. bovis</u> 1st IBR virus later	10	20	1×10^5	$1 \times 10^{6.5}$
Ic	<u>M. bovis</u> and IBR virus concomi- tantly	10	20	7.25×10^8	$1 \times 10^{6.5}$
Id	IBR virus 1st and <u>M. bovis</u> later	10	20	2.5×10^8	$1 \times 10^{6.5}$ {

^aViable plate count.

^bTCID₅₀ per ml.

Table 8. (Continued)

Experiment no.	Exposure sequence	No. of days between virus and <u>M. bovis</u> exposure	<u>Re-exposure</u> <u>No. of organism used</u>		No. of days between exposure
			<u>M. bovis</u>	IBR virus	
Ia	<u>M. bovis</u> only	--	--	--	--
Ib	<u>M. bovis</u> 1st IBR virus later	41	--	--	--
Ic	<u>M. bovis</u> and IBR virus concomi- tantly	0	1×10^6	--	49
Id	IBR virus 1st and <u>M. bovis</u> later	24	1×10^6	--	49

photophobia became evident about the same time as the animal started tearing, blinking, or closing its affected eye or eyes. Spasms and/or constriction of the iris were noted about this stage of the disease. The conjunctivae, third eyelid and eyelids were not markedly affected, but they were sometimes slightly edematous and/or reddened. As the disease became more severe and the lesions more accentuated, it was more difficult to assess the role of photophobia in the behavior of the animal.

Conjunctivitis usually occurred early in the disease. There was increased reddening of the mucous membranes and the eyes became moist with involuntary blinking. As the disease progressed, there were increased redness and swelling (edema) of the conjunctivae as well as congestion of the conjunctivae and scleral blood vessels; in some cases there were congestion and protrusion of the Membrana nictitans. It is interesting to note that this stage of conjunctivitis corresponds to the period of severest photophobia and greatest amount of serous lacrimation. About this stage of the infection (IBK), blepharitis usually became evident; its most conspicuous features were swelling and erosion, especially in the lateral canthi. Significantly, if the disease (IBK) became abated at this stage, erosion of the lids accompanied by moist eyes, and the presence of M. bovis in pure culture many times was the only signs of persistent IBK.

Keratitis usually appeared, first, at the height of the photophobic stage when there were marked blepharitis and conjunctivitis. The eyes were held shut by the animals and when they were forced open, the iris was observed to be either constricted or spastic and, usually, on careful examination, a small area of the cornea was opaque. The opacity was usually located in the center of the cornea but corneal opacities were also found in other places in some eyes. In some cases, the opacity was a flat spot; in others it appeared as a vesicle which in many severe cases developed into ulcers. Many times when the ulcer developed, there was a haze surrounding it. In a few cases there were multiple flat spots or vesicles which either remained static for a while and then receded or they progressed individually into ulcers. Still others coalesced to form larger ulcers which in some cases covered the entire cornea. It should be pointed out, however, that some of these flat spots, vesicles or ulcers were barely visible. Usually the large ulcers were surrounded by an opacity ranging from a haze to grayish-white to a yellowish-white color. These ulcers in some cases deepened until the internal structures and media of the eye protruded through the perforation. These ulcers were surrounded by red bands of blood vessels. At this stage of the disease, the lacrimation occurred in copious amounts and was mucopurulent. Bacteriological examination revealed few M. bovis isolants, but micrococcus-like organisms, diplococci, Neisseria-like organisms, Escherichia coli, Proteus spp. as

well as other nondescript bacteria were in abundance. Prior to this stage of the disease, only M. bovis, and sometimes micrococci and other nondescript bacteria, were recovered from the eye secretions.

The stage of the perforated ulcer was the most severe stage observed. From this stage, corneal healing took place rapidly and characteristically through adventitious vascularization. Vascularization usually began at the corneal scleral junction (limbus) as circumcorneal blood vessels became more prominent. These vessels either circumscribed the cornea or they were limited to a specific area on the limbus immediately adjacent to the ulcer or opacity. As healing progressed, more and more blood vessels became evident which formed a meshwork of vessels which is commonly referred to as a vascular band. This band gradually widened and advanced toward the center of the cornea (if the ulcer was located here). In the most severely affected eyes, vascularization many times completely covered the cornea. It is interesting to note also that as the vascularization reached the center of the cornea, there was a tendency for the corneal opacity to clear from the periphery inward. As other symptoms of the disease disappeared, the vascularization gradually receded leaving an area (previously ulcerated) with a few blood vessels along with a scar, or only a scar surrounded by hazy opacity in severe cases of IBK. In mild cases, no signs of IBK remained. It should be pointed

out, however, that some of the most severe cases left the animal blind in the affected eyes.

Symptoms and course of IBR virus eye disease

The most outstanding characteristics observed in IBR virus eye disease were copious amounts of mucopurulent discharge, severe conjunctivitis, blepharitis and the conspicuous lack of keratitis. The earliest changes observed, however, were similar to those caused by M. bovis. Usually they were seen in the first 24 hours after exposure; the eyes became moist; the third eyelid and conjunctivae became pink to red due to congestion and the animal was slightly photophobic. Later, around 48 hours, the conjunctivae were intensely reddened and the periscleral blood vessels injected. A profuse serous to mucopurulent lacrimal discharge accumulated at the medial canthus which ran down the face wetting it and causing matting of the hair. At this stage, the third eyelid and conjunctivae were swollen and bulged out, and in some cases the surfaces of the eyelids and conjunctivae became granular and bled when a cotton-tipped applicator was passed over them in the collection of samples for bacteriological and virological examination. The surface of the conjunctivae ulcerated and sloughed in some cases. In many cases, the mucopurulent discharge was replaced by a thick viscid, creamy mucopurulent discharge which also further matted the hair; on drying, this discharge appeared as flaky and crusty yellowish material around the eyes and down

the face. None of the animals in Experiment I developed keratitis attributable to IBR virus.

The IBR virus eye disease reached its severest stage from the 7th to the 10th day after which the symptoms subsided rapidly and the eyes returned to normal in about 15 days, except for presence of dried exudate around them. It is also interesting to note that the virus was recovered from the eye from the time of exposure through its most severely affected stage but not afterward.

Symptoms and course of IBK due to simultaneous *M. bovis* and IBR virus infections

For the most part, this type of IBK was similar to the disease produced by IBR virus alone but in addition to the severe conjunctivitis, blepharitis and copious amounts of mucopurulent discharge, there was keratitis. Usually these symptoms occurred within 48 hours after exposure. The first symptoms observed were profuse discharge and marked photophobia. Later, swelling of the eyelids and conjunctivae, injection of the sclera, and iridospasms or constriction became prominent. Keratitis was first observed in many cases during this later stage. Following this stage, the conjunctivitis and blepharitis became increasingly more severe, simulating the disease produced by IBR virus alone. The severest stage was usually attained about the 8th day after exposure, and thereafter the disease symptoms subsided. The keratitis, once

developed, progressed and healed with the same consequences as previously described for M. bovis infection.

Results for the specific parts of Experiment I

Experiment Ia Five of the 6 animals exposed to M. bovis only developed typical keratoconjunctivitis (Table 9). Two animals developed unilateral and three, bilateral cases of IBK. Moraxella bovis became established in 11 of the 12 eyes exposed. The eye from which M. bovis was not recovered remained normal throughout the experiment.

The serum samples taken from the calves on day 1 as well as those taken on day 38 did not contain precipitating or neutralizing antibodies against M. bovis and IBR virus respectively.

Experiment Ib Ten animals were exposed to M. bovis initially and later (41 days) exposed to IBR virus. Five of the 10 animals exposed to M. bovis developed unilateral IBK within 14 days after exposure (Table 9). Moraxella bovis was recovered from all affected eyes as well as eight eyes which were not affected. Cultures from six eyes (5 animals) were negative for M. bovis. It is interesting to note that M. bovis was not recovered from any of the eyes at the time of their exposure to IBR virus nor was it recovered during the IBR virus infection. All of the animals exposed to IBR virus developed typical bilateral conjunctivitis, but in no case was keratitis observed.

Table 9. Results of exposing cattle eyes to Moraxella bovis and/or infectious bovine rhinotracheitis virus

Parts of Experiment I ^a	No. of cattle	No. of cattle with IBR virus conjunctivitis ^b	No. of cattle with <u>M. bovis</u> keratoconjunctivitis ^c	
			Unilateral	Bilateral
Ia - Exposed to <u>M. bovis</u> only	6	0	2	3
Ib - Exposed to <u>M. bovis</u> first and later exposed to IBR virus	10	10	5	0
Ic - Exposed to <u>M. bovis</u> and IBR virus simultaneously	10	10	6	4
Id - Exposed to IBR virus first and later exposed to <u>M. bovis</u>	10	10	4	3

^aRefers only to first exposure results.

^bAll the exposed cattle developed bilateral conjunctivitis.

^cNone of the cattle developed keratoconjunctivitis attributable to IBR virus.

Table 9. (Continued)

Parts of Experiment I	No. of eyes organism(s) recovered		No. of animals with serological antibodies against	
	IBR virus	<u>M. bovis</u>	<u>M. bovis</u>	IBR virus
Ia - Exposed to <u>M. bovis</u> only	0	11	0	0
Ib - Exposed to <u>M. bovis</u> first and later exposed to IBR virus	20	11	1	10
Ic - Exposed to <u>M. bovis</u> and IBR virus simultaneously	20	20	4	10
Id - Exposed to IBR virus first and later exposed to <u>M. bovis</u>	20	19	1	10

Serum samples taken on days 1 and 41 were free of detectable neutralizing or precipitating antibodies against organisms used. However, all the serums taken on day 71 contained neutralizing antibodies against IBR virus with titers as high as 1:16, but precipitating antibodies were detected in the serum of only one animal (an animal which had developed IBK after exposure to M. bovis).

Experiment Ic Ten animals were exposed to M. bovis and IBR virus simultaneously. Six of the animals developed unilateral IBK similar to that produced by M. bovis and IBR virus combined, and four other animals developed bilateral cases (Table 9). Both M. bovis and IBR virus were recovered from all eyes. After re-exposure, M. bovis was recovered from four eyes in addition to the four that were still carrying M. bovis from the first exposure. It is interesting to note that nonhemolytic M. bovis was recovered from one eye 7 days after re-exposure to hemolytic M. bovis.

The serum samples taken on day 1 did not contain neutralizing or precipitating antibodies. Serum samples taken from 3 animals on day 25 contained antibodies against IBR virus (these 3 animals had developed severe IBK), but none of the 10 samples contained precipitating antibodies against M. bovis. On day 49 all of the serum samples contained neutralizing antibodies against IBR virus, with titers ranging from 1:8 to 1:32. Four animals also developed antibodies against M. bovis, three of which had severe unilateral IBK. On day 71, the serums of

9 animals (one had died earlier from acute bloat) had neutralizing antibodies against IBR virus. The serums of 2 animals also contained precipitating antibodies. The serums of these had contained precipitating antibodies on day 49.

Experiment 1d Ten animals were exposed to IBR virus only and 24 days later were exposed to M. bovis. Twenty-four hours after exposure, IBR virus was recovered from all eyes (Table 9) and all animals developed bilateral conjunctivitis within 72 hours. Five days after exposure to IBR virus a non-hemolytic M. bovis was recovered from one eye, and on day 6 a nonhemolytic M. bovis was recovered from one eye of each of 3 other animals. Later hemolytic M. bovis was also recovered from the four eyes (4 animals) originally containing spontaneously occurring nonhemolytic M. bovis; three of these eyes subsequently developed typical M. bovis IBK attributable to it.

Forty-eight hours after exposure to M. bovis (EPP-63(300), 4 animals developed typical unilateral M. bovis IBK. Later 3 other animals developed unilateral IBK; one of the affected eyes had previously developed keratoconjunctivitis due to the spontaneously occurring hemolytic M. bovis. Moraxella bovis was recovered from the affected eyes as well as from three others (2 animals) for the first 72 hours after exposure. After a second exposure to M. bovis, M. bovis was recovered from nine eyes (9 animals) in addition to the five eyes carrying M. bovis from the first exposure. Only one eye developed acute IBK after the second exposure to M. bovis. It is

interesting to note that this affected eye had had two previous episodes of IBK.

The serum samples taken on day 1 did not contain IBR virus neutralizing antibodies or precipitating antibodies for M. bovis. On day 25 the serums of 5 animals contained neutralizing antibodies but none contained precipitating antibodies. On day 49 all the serums contained neutralizing antibodies (titers from 1:4 to 1:32) but only one contained precipitating antibodies. The animal whose serum contained antibodies against M. bovis had not been affected with IBK but spontaneously occurring nonhemolytic and hemolytic M. bovis had been isolated from one of its eyes prior to day 24. On day 71, the serums of 9 animals (one had died earlier from acute bloat) contained neutralizing antibodies. None of the serum samples contained precipitating antibodies; the animal whose serum contained antibodies on day 49 was the one which died from bloat.

Discussion

Experiment Ia indicates that M. bovis alone is sufficient to cause severe IBK and other factors such as sunlight (5, 6, 13, 16, 67, 68, 69, 101, 105), dust (6, 13, 71) and wind (4, 13), which may play an important role in natural infections, are probably only enhancing influences. This is substantiated by the fact that the animals in this part of Experiment I were kept free of these influences but still the

keratoconjunctivitis produced was as severe as those occurring naturally or experimentally where many of these factors were present (60, 67, 68, 69, 71, 105). Apparently no special factors are needed for the establishment of M. bovis in the bovine eye except a virulent strain and a susceptible animal.

The results of Experiment Ib support the finding in Ia that no special factor is needed to produce IBK other than a virulent culture of M. bovis. It also demonstrates the difference between M. bovis-produced IBK and IBR virus-induced eye disease. Furthermore, the results indicate that if an infected eye becomes free of M. bovis it will not develop IBK if subsequently exposed to IBR virus. It is also interesting to note that IBR virus alone did not cause keratitis, an observation reported previously by others (1, 70, 86, 87, 122, 132).

The results of Experiment Ic possibly explain some of the most important questions associated with the etiology of IBK under natural conditions, namely the role IBR virus may play in IBK as an exciting agent in eyes already infected with M. bovis. Significantly, it is therefore interesting to note that the clinical manifestations of combined M. bovis and IBR virus infection (Experiment Ic) are similar to those described by others (3, 16, 53, 66, 73, 108, 112, 119, 123, 125) who investigated the etiology of IBK. It is therefore suggested that the disease described by these workers was caused by simultaneous M. bovis and IBR virus infections; furthermore, some of these workers (53, 55, 108) proposed that a virus in

addition to M. bovis might have been a factor in the disease observed by them. It may also be possible that some of these workers (123, 124) used animals whose eyes were infected with M. bovis for their studies with IBR virus. However, one group of workers (122) treated the experimental animals to eliminate M. bovis before exposing them to IBR virus.

The results in Experiment Id give further support to the concept that IBK produced by M. bovis alone is different from the eye disease produced solely by IBR virus. It should be noted, however, that the keratoconjunctivitis caused by M. bovis is similar to that which is produced by combined IBR virus and M. bovis infection. The results also show that the sequence of introduction of IBR virus or M. bovis does not influence the disease produced unless they are introduced simultaneously. Nevertheless, there is no doubt that the presence of IBR virus exacerbates the condition caused by M. bovis as pointed out earlier.

The presence of IBR virus possibly influences M. bovis infection in other ways. For example, the occurrence of hemolytic M. bovis in eyes previously infected solely by nonhemolytic M. bovis is thought to be related to the presence of the virus. Although the most obvious explanation for the occurrence of both the nonhemolytic and hemolytic M. bovis is accidental exposures, the overall management of the experiment precludes such adventitious introductions.

A more tenable explanation was the existence of an occult carrier state in the eyes of some of the experimental animals involving the nonhemolytic organism. Possibly the nonhemolytic M. bovis was present in the eye in very low numbers and with the advent of IBR virus infection, the organism began to multiply and thereby became detectable; later it changed into the hemolytic type and produced disease. Supporting this latter contention is the finding of Pugh and Hughes (105), who described the change of an avirulent nonhemolytic M. bovis into a virulent hemolytic type under the influence of ultraviolet irradiation. In the present study a similar mechanism is probably involved but the precipitating factor is the IBR virus. The fact that the presence of neutralizing antibodies against IBR virus did not protect cattle against IBK is additional evidence that this virus is not the primary agent in this disease.

It should be pointed out also that there is a question of whether the presence of precipitating antibodies per se protects cattle against IBK. The information gathered here bears upon this question when the results of Experiment Id are taken in conjunction with those of an earlier study (Section IV). The results of these two studies suggest that there is a relationship between the presence of antibodies against M. bovis and the state of resistance. For example, in the earlier study cattle which developed severe IBK as a consequence of M. bovis infection developed precipitating antibodies against the organism, and in the present study those animals which had severe

disease developed antibodies. Also those animals which had severe IBK in one eye seldom were affected in both eyes, while those with mild disease were frequently affected bilaterally. It is interesting to note that one animal which did not develop precipitating antibodies against M. bovis had three mild episodes of IBK in the same eye within a 71-day period. Similar observations have been made by Formston (50).

Summary and Conclusion

Cattle eyes were exposed to M. bovis and IBR virus under different schedules. In one experiment the eyes of 10 cattle were exposed to M. bovis and to IBR virus 41 days later. Five of the 10 animals developed unilateral keratoconjunctivitis, prior to exposure to IBR virus, but none of the animals developed keratitis upon the introduction of IBR virus, in spite of the fact that all 10 animals developed bilateral conjunctivitis. The eyes of 10 other cattle were exposed to M. bovis and IBR virus concomitantly; 6 animals developed severe unilateral and 4, severe bilateral cases of keratoconjunctivitis. The other eye of each of the 6 animals with unilateral IBK developed severe conjunctivitis. The eyes of still another group of 10 cattle were exposed to IBR virus initially and exposed to M. bovis 24 days later. All animals developed severe bilateral conjunctivitis attributable to IBR virus; keratitis, however, was not produced but 4 animals developed unilateral and 3, bilateral IBK attributable to M. bovis.

When 6 control cattle were exposed to M. bovis only, 2 developed unilateral and 3, bilateral IBK.

In conclusion it can be stated that the results of the study indicate that M. bovis is the primary etiologic agent in IBK and that the role of infectious rhinotracheitis virus is secondary in nature. There is the possibility, however, of IBR virus facilitating or enhancing the establishment of M. bovis infection as well as causing M. bovis disease to be more severe, or at least to make the clinical manifestations more pronounced. The results also substantiate the contention that IBR virus produces a severe conjunctivitis as well as a blepharitis, but this disease is distinct from M. bovis-caused IBK.

SECTION VI. KERATOCONJUNCTIVITIS PRODUCED BY
MORAXELLA BOVIS IN LABORATORY ANIMALS

Introduction

Although keratoconjunctivitis is readily produced by M. bovis in cattle, certain studies on this disease or its causative agent are prohibitive because of economic and other factors. Also there is a shortage of nonexposed susceptible cattle because of the high incidence of IBK, and the M. bovis carrier state in cattle herds. Likewise, the expense of producing SPF cattle and the cost of providing space to maintain them make their use prohibitive.

Attempts to infect certain laboratory animals with M. bovis have been reported. Usually the results were negative or inconclusive (5, 13, 47, 61, 71, 127). However, some workers (51, 112) have described transmission experiments in which pure cultures of M. bovis instilled in the conjunctival sac of sheep and mice (as is commonly done in cattle) resulted in keratoconjunctivitis. There do not seem to be any reports of animals other than cattle being naturally infected by M. bovis. However, other species of *Moraxella* have been recovered from sheep and guinea pigs (12, 113).

The present experiment was done to determine whether animals other than cattle could be used in the laboratory to determine the pathogenicity and virulence of M. bovis.

Materials and Methods

Cultures

Three strains of M. bovis, identified as EPP-63(300), 8613, and ATCC 10900, were used. Strain EPP-63(300) was isolated from a bovine animal naturally affected with IBK and has been used to produce IBK in cattle experimentally. Strains 8613 and ATCC 10900 were laboratory strains and have been used as reference strains (106). Cultures of the three strains were stored at -60 C in TSB.

Inoculums for exposing animals were prepared from the frozen cultures. The latter were thawed and streaked on 5% bovine blood agar. Following 24-hour incubation at 37 C, typical smooth hemolytic colonies were streaked on the second plate. The second plate culture was incubated 24 hours; the growth was scraped from the surface of the medium and suspended in TSB and used as the inoculum. Since the typical smooth colony of the organism did not form a stable uniform suspension, the TSB suspension had to be shaken immediately before instillation was made.

Animals

Sheep Nine healthy adult sheep were used; their eyes were normal and culturally negative for M. bovis. They were housed in indoor isolation units which excluded sunlight for 4 months prior to exposure and throughout the observation

period. At the time of exposure they were separated into units of two per stall according to treatment received.

Rabbits Fifteen apparently healthy adult albino rabbits, whose eyes were normal and culturally negative for M. bovis were used. All rabbits were maintained in individual cages in indoor isolation units which excluded sunlight.

Rats Six apparently healthy adult white rats whose eyes were normal and culturally negative for M. bovis were used. They were maintained in rat cages in units of two per cage according to the treatment received. The cages were kept in a laboratory room which was free of natural light.

Guinea pigs Sixteen healthy guinea pigs whose eyes were normal and culturally negative for M. bovis were used. They were about 4-1/2 months of age and weighed from 400-450 Gms. All were housed in individual cages in an indoor laboratory room.

Mice Twenty-two healthy albino Swiss mice whose eyes were normal and culturally negative for M. bovis were used. The mice were separated according to treatment received into separate mouse cages and kept in an indoor laboratory room.

Bacteriologic examination

Secretions from the eyes were collected with sterilized cotton-tipped applicators before exposure and at the end of the observation period. Immediately after collection the secretions were streaked on the surface of 5% bovine blood agar

plates and the plates were incubated at 37 C for 24 hours. After an additional 24 hours incubation at room temperature they were observed for colonies of M. bovis.

Experimental design and exposure

Sheep Exposure was made by instilling in each eye 0.5 ml of a suspension of strain EPP-63(300) into the ventral conjunctival sac. The eyelids were held together manually for 1 to 2 minutes after the instillation. Irradiation (67) was done with a sunlamp placed at eye level and approximately 60 cm in front of the standing sheep. The sheep was allowed free vertical movement of the head, but horizontal movement of the head was restricted so that both eyes were given the same amount of irradiation.

The sheep were allotted to three groups on the basis of the treatment given. Group I comprised 2 sheep which were used as controls; their eyes were neither exposed to M. bovis nor irradiated. Group II comprised 3 sheep whose eyes were exposed to M. bovis but were not irradiated. Eyes of Group II sheep were re-exposed 11 days after the first exposure. Group III comprised 4 sheep whose eyes were exposed to M. bovis once and irradiated for 20 minutes each day throughout the observation period (25 days).

Rabbits Exposure was made by instilling 0.25 ml of TSB suspension of either strain EPP-63(300) or ATCC 10900 into the conjunctival sac. The eyelids were closed manually and

held for 1 to 2 minutes. Irradiation was made by placing the sunlamp slightly above eye level at 60 cm from the rabbit, which was confined in a holding box with the head protruding.

The rabbits were allotted to three groups on the basis of the treatment given. Group I comprised 6 rabbits whose eyes were irradiated for 20 minutes (initial; first day), then exposed to strain EPP-63(300). Irradiation was repeated each day throughout the observation period (25 days). Group II comprised 6 rabbits whose eyes were exposed to strain EPP-63(300) but were not irradiated. Group III comprised 3 rabbits whose eyes were exposed to strain ATCC 10900 and were not irradiated.

Rats Exposure was made by instilling 0.25 ml of suspension of strain EPP-63(300) in the open eye. The eyelids were held closed for 1 to 2 minutes. Irradiation was made in a manner similar to that used for the rabbits.

The rats were allotted to three groups on the basis of treatment given. Group I comprised 2 rats whose eyes were irradiated for 5 successive days but were not exposed to M. bovis. Group II comprised 2 rats whose eyes were irradiated and then exposed once to M. bovis. The eyes were irradiated each day for 25 days. Irradiation was made by placing the sunlamp directly in front of the animal at a distance of 60 cm for 20 minutes. Group III comprised 2 rats whose eyes were exposed to M. bovis but were not irradiated.

Guinea pigs The guinea pigs were allotted to two groups of 8 each. Group I guinea pigs were exposed to strain 8613 and Group II guinea pigs were exposed to strain ATCC 10900; 0.25 ml of TSB suspension of either strain was instilled in the open eye. Immediately before exposure, one eye of each of 2 guinea pigs in Group I was irritated mechanically by dropping sterilized sand into the eye and by gently rubbing the closed eyelids. Guinea pigs were not irradiated.

Mice Exposure was done by flooding the open eye with 0.25 ml of suspension of a strain of M. bovis. The eyes were irradiated for 20 minutes immediately before exposure and each day throughout the observation period unless otherwise specified. Mice were irradiated in their cages (with cage top removed). The lamp was placed 60 cm above the mice, which were allowed to move around freely but were not allowed to hide their faces from the direct light of the sunlamp.

The mice were allotted to four groups. Group I comprised 8 mice whose eyes were exposed to strain ATCC 10900 but were not irradiated. Four of the mice (Group Ia) were re-exposed to strain ATCC 10900 (homologous re-exposure) 15 days after the first exposure was done. The four other mice (Group Ib) were exposed (re-exposure) to strain EPP-63 (300) (heterologous re-exposure) 15 days after the exposure to strain ATCC 10900. Group II consisted of 7 mice whose eyes were exposed to strain EPP-63(300) but were not irradiated. These mice were exposed (re-exposure) to M. bovis strain ATCC 10900 10 days after the

exposure to strain EPP-63(300). Group III comprised 3 mice whose eyes were irradiated each day for 20 minutes for 3 days and then each day for 30 minutes for 9 days. This group of mice were used as irradiation controls for the first part of the experiment with mice but were exposed to strain EPP-63(300) on day 12 of the experiment when the irradiation was discontinued. Group IV consisted of 4 mice whose eyes were neither irradiated nor exposed to M. bovis.

Results

Sheep

The eyes of the exposed sheep as well as of the controls remained normal throughout the observation period. Moraxella bovis was not isolated from the eyes of the 2 sheep in Group I (control) but became established in six of the eight eyes of the 4 sheep in Group III (exposed and irradiated) and was recovered each day for 20 consecutive days. The organism was not recovered from one eye of each of 2 sheep of Group III. Moraxella bovis was not recovered from the eyes of the sheep in Group II (exposed, but not irradiated) after the first exposure, but after re-exposure, the organism became established in both eyes of 1 sheep and in one eye of another. Moraxella bovis was recovered from one eye of the third sheep in Group II but was not considered established.

Rabbits

The eyes of the 15 rabbits in the three experimental groups remained normal throughout the observation period. Moraxella bovis was not recovered.

Rats

The eyes of the 6 rats remained normal. Moraxella bovis was recovered for 4 days from the eyes of the rats (2) in Group II only.

Guinea pigs

The guinea pigs in which one eye was irritated with sand before exposure to M. bovis developed mild inflammation in the irritated eyes, but M. bovis was not recovered from them. All other eyes (total of 16 guinea pigs) remained normal and M. bovis was not recovered.

Mice

The results of exposure of mice to M. bovis are summarized (Table 10). After their first exposure, 4 of the 8 mice in Group I remained normal; one developed severe unilateral keratoconjunctivitis¹ and three developed only conjunctivitis. Moraxella bovis was recovered for 6 to 8 days from the affected eyes.

¹Characterized by swelling, profuse lacrimation, severe conjunctivitis, ulceration of the cornea, and crusty material around the eyes.

Table 10. Results of exposure of mice to Moraxella bovis

Group no.	Exposure to <u>M. bovis</u>	No. of mice	Number of mice		Number of mice		<u>M. bovis</u> recovered	
			Conjunctivitis		Kerato-conjunctivitis		(No. of eyes)	
			Unilat- eral	Bilat- eral	Unilat- eral	Bilat- eral	Diseased	Normal
I	Initial, to ATCC 10900	8	4	0	1	0	4	0
	Ia Re-exposure ^a to ATCC 10900	4	0	1	0	1	2	0
	Ib Exposure to EPP-63 (300)	4	2	0	2	0	2	2
II	Initial, to EPP-63(300)	7	0	3	0	3	6	0
	Exposure to ATCC 10900	7	0	1	0	1	2	6
III	Controls--irradiated	3						
	Nonexposed	3	0	0	0	0	0	0
	Subsequent initial exposure to EPP-63 (300)	3	1	1	1	1	4	0
IV	Controls (nonexposed)	4	0	0	0	0	0	0
	Total	22						

^aThe mice were re-exposed 15 days after the initial exposure.

After homologous re-exposure of the mice constituting Group Ia, 1 mouse developed severe bilateral keratoconjunctivitis, and the other 3 mice remained normal. Moraxella bovis was recovered for 8 days from the affected eyes.

After heterologous re-exposure of the 4 mice constituting Group Ib, 2 mice developed severe unilateral keratoconjunctivitis and two remained normal. Moraxella bovis was recovered from the two diseased mice only. One isolation was made from a normal eye and the other from an affected eye.

After the 7 mice constituting Group II were exposed, 3 mice developed severe bilateral keratoconjunctivitis, and 4 mice remained normal. Moraxella bovis was recovered from the affected eyes only. After heterologous re-exposure of all Group II mice, 1 mouse which had remained normal after the first exposure developed bilateral keratoconjunctivitis. The eyes of the other mice remained normal. Moraxella bovis was recovered from the two affected eyes as well as from six others (4 mice) for 8 days after re-exposure.

The eyes of the 3 mice constituting Group III remained normal during the time they were used as irradiation controls. After their exposure to M. bovis, 1 mouse developed bilateral and the other, unilateral keratoconjunctivitis. Moraxella bovis was recovered from both eyes of the two affected mice. Both eyes of the third mouse remained normal, and M. bovis was not recovered. The eyes of the 4 mice constituting Group IV

remained normal throughout the experiment, and M. bovis was not recovered.

Discussion

The results indicate that mice and sheep, in addition to cattle, can be used to study the pathogenicity and virulence of M. bovis. Furthermore, as with cattle, a simple exposure method can be used, and M. bovis can become established in the eyes of mice and sheep after simple instillation, with or without ultraviolet irradiation.

The finding in the present experiment that pure cultures of a strain of M. bovis produced keratoconjunctivitis in mice similar to that produced in cattle under comparable conditions indicates that the mouse may be substituted for cattle in some studies on M. bovis infection (especially studies on virulence and virulence factors). Also, since similarities of M. bovis infection exist in mice and cattle, many aspects of M. bovis infection can be studied in mice and extrapolated for cattle. Using mice also could alleviate the shortage of bovine animals suitable for experiment, reduce the expense for doing research, and ensure the ready availability of susceptible animals (mice) for use in the laboratory.

Moraxella bovis became established in the eyes of sheep but did not produce disease. Apparently, either the organism was not able to elaborate its disease-causing principle or the sheep is resistant to its effect. Moraxella bovis did not

become established in the eyes of guinea pigs, rats, or rabbits; therefore, these animals were considered to be non-susceptible to M. bovis infection. Possibly, the use of gastric mucin, ultraviolet irradiation, and other enhancing factors (128) would make them suitable experimental animals in the study of M. bovis infection.

Summary and Conclusion

Sheep, rabbits, guinea pigs, and mice were exposed to Moraxella bovis by conjunctival instillation. Moraxella bovis became established in the eyes of sheep and mice but not in the eyes of the other animals. Thirteen of 18 mice exposed to M. bovis developed conjunctivitis, and 10 of the 13 developed keratitis. The disease in mice was grossly similar to IBK.

Possibly mice could be used as an inexpensive, readily available test animal for determining the pathogenicity and virulence of M. bovis. It should be emphasized, however, that the effects of M. bovis infection in the mouse are incompletely understood, especially the histopathologic changes and the immunologic responses. Information necessary for understanding the disease in cattle might be gained by complete characterization of the keratoconjunctivitis produced in the mouse. This would be a prudent approach, because M. bovis infection in the mouse simulates the gross changes and the immune responses seen in the bovine animal (69, 105).

SECTION VII. COMPARISON OF THE VIRULENCE OF DIFFERENT
STRAINS OF MORAXELLA BOVIS USING THE EYES OF MICE

Introduction

While Moraxella bovis is generally considered to be the etiologic agent of infectious bovine keratoconjunctivitis (IBK) or pinkeye of cattle, attempts to produce experimental IBK with this organism have sometimes failed or been inconclusive (5, 47, 53). This suggested to some workers (41, 105) that there might be differences in the virulence of different strains of M. bovis for the bovine eye. Because of this and other inconsistencies associated with M. bovis infection, additional work was needed to determine whether or not virulence variation existed.

The cost of such study using cattle was prohibitive, therefore the mouse was used as the experimental host. A previous study (107) had confirmed that keratoconjunctivitis was an important manifestation of M. bovis infection in murine eyes. Since the keratoconjunctivitis produced in mice and cattle was grossly similar, it was thought that M. bovis infection by different strains could be studied in mice, and the results extrapolated for cattle. In addition to this, the virulence of various strains of Moraxella and Moraxella-like organisms could be studied in the mouse and used as a criterion of characterization.

Materials and Methods

Cultures

Twenty-one strains of M. bovis (46 smooth hemolytic, two rough hemolytic, and seven smooth nonhemolytic isolants) and one strain each of Mima polymorpha (A-130), Mima polymorpha var. oxidans (5961), Moraxella liquefaciens (9985) and Moraxella nonliquefaciens (9893) were used. A summary of the strains of M. bovis used is given in Table 11. The eyes of 6 mice were exposed to TSB only and used as controls.

Eighteen of the M. bovis strains were isolated from bovine animals naturally affected with IBK. Two strains (8613 and 10900) were laboratory strains used in a previous study (106). The other strain (Hug-68) was recovered from a horse with acute conjunctivitis and blepharitis. Mima polymorpha, Mima polymorpha var. oxidans, Moraxella liquefaciens and Moraxella nonliquefaciens were laboratory strains and have also been used in another study (106).

Inoculums for exposing animals were prepared from frozen cultures. Cultures of the 26 strains had been stored at -60 C in TSB. They were thawed and streaked on 5% bovine blood agar; the agar plate culture was incubated at 37 C for 24 hours. Typical colonies were streaked on the second plate. After the second plate culture was incubated 24 hours, the growth was scraped from the surface of the medium and suspended in TSB; the suspension was used to flood the eyes.

Table 11. Summary of the strains of M. bovis and the number of isolants used to expose mice eyes

Strains	Hemolytic isolants		No. of isolants with catalase activity		Nonhemolytic isolants	
	No. of smooth	No. of rough	Positive	Negative	No. of smooth	No. of rough
KGD-63	1	-	1	-	-	-
NTN-63	1	-	1	-	-	-
NDL-63	1	-	1	-	-	-
NDL-68	2	1 ^a	3	-	1	-
NDL-67	3	-	3	-	-	-
EPP-63	3	1 ^a	5	-	1	-
HIM-63	3	-	3	-	-	-
GLN-63	3	-	3	-	-	-
WSE-63	3	-	3	-	-	-
FLA-64	3	-	-	3	-	-
VRI-64	3	-	3	-	-	-
KTy-65	3	-	3	-	-	-
IBH-63	3	-	4	-	1 ^a	-
IBH-64	3	-	-	3	-	-
IBH-65	3	-	3	-	-	-
IBH-66	3	-	4	-	2	-
IBH-67	3	-	4	-	1	-
IBH-68	1	-	1	-	-	-
8613	1	-	-	1	-	-
10900	1	-	-	1	-	-
Hug(68)	-	-	1	-	1	-
Total	46	2	46	8	7	0

^a Was derived from one of the smooth hemolytic isolants of its strain.

Animals

Five hundred and ninety-six mice (*Mus musculus*) were used. They were adult females with weights ranging from 26 to 30 Gms. All were healthy, culturally negative for *M. bovis* and Moraxella-like organisms, and were housed in mouse cages (10 per cage) in rooms free of sunlight.

Bacteriologic examination

Secretions from the eyes were collected with sterilized cotton-tipped applicators before exposure and at the end of the experiment (12 days). The eye secretions were collected and examined for bacterial agents in the same way as in previous studies.

Serological examination

The mice were bled at the end of the experiment only and each serum examined for antibodies against an aliquot of the same exposure culture using the agar gel double-diffusion technique described in Section II.

Exposure

Exposure was made by flooding the open eye with a TSB suspension of the test organisms. To expose, each mouse head was held so that the eyes bulged and a cotton-tipped applicator soaked with bacterial suspension was placed over the eye and pressed slightly. The eyes of 10 mice were exposed to each test organism.

Results

At least one isolant of each of the 20 strains of smooth hemolytic M. bovis produced keratoconjunctivitis and at least one isolant of each of 16 strains was recovered from the exposed eyes of mice (Table 12). Strains NDL-68 and EPP-63 infected the largest percent of eyes (30%); the infection rate for the other strains was considerably lower. Strains FLA-64, EPP-63, KTy-65, WSE-64, IBH-65 and NDL-63 produced the largest number of cases of keratoconjunctivitis. Strain FLA-64 produced keratoconjunctivitis in 53% of the mice and 45% of the eyes; these were the highest percentage of cases produced by any strain. The next most effective inducer was strain EPP-63 which caused keratoconjunctivitis in 43% of the mice and 27% of the eyes.

Keratoconjunctivitis was produced by at least one isolant of four of the six strains of smooth nonhemolytic M. bovis but M. bovis was recovered post inoculation from one eye only. A hemolytic variant was also recovered from this eye (Table 13). Strains NDL-68 caused the highest percent of disease (20% of the eyes) while the other three strains produced keratoconjunctivitis in approximately 5% of the eyes.

Only one (EPP-63) of the strains of rough hemolytic M. bovis caused keratoconjunctivitis but the organism was not recovered during the post-inoculation period (Table 13). The other strain (NDL-68) was recovered from 4 mice (five eyes) but did not cause disease. Strains of M. bovis with catalase

Table 12. Results of the exposure of mice to hemolytic Moraxella bovis strains

<u>M. bovis</u> strain	No. of iso- lants	No. exposed		No. with keratoconjunctivitis		No. infected with M. bovis		No. infected with M. bovis affected with keratoconjunctivitis		No. of mice with keratoconjunctivitis	
		Mice	Eyes	Mice	Eyes	Mice	Eyes	Mice	Eyes	Unilat- eral	Bilat- eral
KGD-63	1	10	20	3	4	0	0	0	0	2	1
NTN-63	1	10	20	2	3	0	0	0	0	1	1
NDL-63	1	10	20	3	5	0	0	0	0	1	2
NDL-68	2	20	40	4	7	10	12	2	3	0	3
NDL-67	3	30	60	5	9	1	1	0	0	1	4
EPP-63	3	30	60	13	16	14	18	6	7	10	3
HIM-63	3	30	60	10	14	3	3	0	0	6	4
GLN-63	3	30	60	6	10	6	6	1	1	3	2
WSE-64	3	30	60	11	14	5	5	0	0	6	4
FLA-64	3	30	60	16	27	3	4	1	2	5	11
VRI-64	3	30	60	4	6	2	2	0	0	5	2
KTy-65	3	30	60	11	16	3	3	1	1	5	6
IBH-63	3	30	60	6	8	6	6	2	2	4	2
IBH-64	3	30	60	7	9	3	4	0	0	5	2
IBH-65	3	30	60	8	13	7	9	4	5	5	3
IBH-66	2	20	40	2	3	2	2	0	0	1	1
IBH-67	3	30	60	7	10	6	6	1	1	4	3
IBH-68	1	10	20	1	2	0	0	0	0	0	1
8613	1	10	20	1	2	1	1	0	0	0	1
10900	1	10	20	2	3	1	1	0	0	1	1

Table 13. Summary of results using smooth nonhemolytic M. bovis, rough hemolytic M. bovis and Moraxella-like organisms to produce keratoconjunctivitis in mice

Strains	No. of isolants	No. exposed		No. with kerato- conjunctivitis		No. infected with isolants	
		Mice	Eyes	Mice	Eyes	Mice	Eyes
Nonhemolytic							
<u>M. bovis</u>							
EPP-63	1	10	20	0	0	0	0
NDL-68	1	10	20	3	4	0	0
IBH-63	1	10	20	1	1	1 ^a	1
IBH-66	2	20	40	2	2	0	0
IBH-67	1	10	20	0	0	0	0
Hug-68	1	10	20	1	1	0	0
Rough hemolytic							
<u>M. bovis</u>							
EPP-63	1	10	20	4	6	0	0
NDL-68	1	10	20	0	0	4	5
<u>Mima polymorpha</u>							
var. <u>oxidans</u>							
5961	1	10	20	0	0	4	7
<u>Mima polymorpha</u>							
A-130	1	10	20	0	0	3	3
<u>Moraxella</u>							
<u>liquefaciens</u>							
9985	1	10	20	0	0	2	2
<u>Moraxella non-</u>							
<u>liquefaciens</u>							
9893	1	10	20	0	0	6	11

^aHemolytic M. bovis also recovered.

Table 13. (Continued)

Strains	No. infected with isolant affected with <u>keratoconjunctivitis</u>		No. of mice with <u>keratoconjunctivitis</u>	
	Mice	Eyes	Unilateral	Bilateral
Nonhemolytic				
<u>M. bovis</u>				
EPP-63	0	0	0	0
NDL-68	0	0	2	1
IBH-63	1	1	0	1
IBH-66	0	0	1	1
IBH-67	0	0	0	0
Hug-68	0	0	1	0
Rough hemolytic				
<u>M. bovis</u>				
EPP-63	0	0	2	2
NDL-68	0	0	0	0
<u>Mima polymorpha</u>				
<u>var. oxidans</u>				
5961	0	0	0	0
<u>Mima polymorpha</u>				
A-130	0	0	0	0
<u>Moraxella</u>				
<u>liquefaciens</u>				
9985	0	0	0	0
<u>Moraxella non-</u>				
<u>liquefaciens</u>				
9893	0	0	0	0

activity (i.e. EPP-63, NDL-68, KTy-63) as well as those without (FLA-64, IBH-64, 10900) induced cases of keratoconjunctivitis. Strain Hug-68 caused keratoconjunctivitis but was not recovered during post-inoculation periods.

The type of keratoconjunctivitis induced by the strains of M. bovis in this experiment was similar to that previously described (107) but there was less lacrimation. The clinical signs of keratoconjunctivitis usually occurred within the first 24 to 48 hours after exposure; only a few cases occurred later.

Moraxella liquefaciens, Moraxella nonliquefaciens, Mima polymorpha and Mima polymorpha var. oxidans became established in the eyes of mice but did not cause keratoconjunctivitis.

None of the serums contained precipitating antibodies against the organism to which the respective mouse was exposed.

Discussion

The results indicate that mice can be used to study the virulence of M. bovis. They also indicate that M. bovis strains vary in their ability to cause keratoconjunctivitis. Whether these results can be extrapolated for cattle remains to be elucidated. It is interesting to note, however, that an isolant (300) of strain EPP-63 which has been a very effective inducer of IBK in cattle in other studies produced disease in 40% of the mice.

The number of eyes developing disease was less than that anticipated, but many factors could explain this. Possibly

many of the eyes would have developed keratoconjunctivitis if the experimental period had been lengthened. Also, if younger mice had been used, possibly a larger proportion of the eyes would have become infected and/or developed disease; in a previous study (107) where younger mice (16-20 Gms) were used, a large proportion of them developed disease. It should be pointed out also that the results are "composite" results; therefore one isolant might have caused a high disease rate but another a much lower, but when they were taken as one strain, the overall rate dropped drastically. For example, certain isolants of strains FLA-64 and EPP-63 induced keratoconjunctivitis in 70% and 50% of the mice respectively, but the percent for each strain was lower.

Apparently, nonhemolytic M. bovis strains behave in murine eyes as they do in those of cattle (105). Even though some of the eyes developed keratoconjunctivitis there is a question of whether this was due to hemolytic or nonhemolytic varieties. Supporting this is the fact that nonhemolytic M. bovis was only recovered from an eye containing hemolytic M. bovis as well.

The rough hemolytic isolants of M. bovis apparently can become established in murine eyes as well as produce disease. Mima polymorpha, Mima polymorpha var. oxidans, Moraxella liquefaciens and Moraxella nonliquefaciens also can become established in murine eyes but their failure to produce disease can not be explained. Possibly if different strains of these

organisms, or more and younger mice had been used, disease would have been produced. Since these were laboratory strains, they might have lost virulence because of excessive passage on laboratory mediums.

The most important idea discerned from this study is the fact that the variation in colony type of M. bovis is related to the ability of an isolant or strain to become established and produce disease in murine eyes. Possibly the nonhemolytic-hemolytic variation is the most important.

There may or may not be any relationship between establishment of infection and the production of keratitis by M. bovis within 24 hours after exposure. It is difficult to concur with the idea that M. bovis could have multiplied and/or produced a disease factor to such an extent in so short a time. Therefore, possibly some factor associated with the exposure material (at time of exposure) had the toxic effect in some cases.

The fact that antibodies could not be detected within 12 days after exposure is in agreement with the results of the other studies.

Summary and Conclusion

Of 460 mice whose eyes were exposed to 46 isolants (20 strains) of smooth hemolytic M. bovis, 57 developed bilateral and 63, unilateral cases of keratoconjunctivitis. Some strains caused a higher percentage of disease than others; for example,

one strain induced keratoconjunctivitis in 13% of the eyes while another produced disease in 53% (45% of the eyes) of the mice. Sixteen strains became established in the eyes of mice, and organisms representing eight strains were recovered from eyes with clinical keratoconjunctivitis.

Smooth nonhemolytic M. bovis became established in the eyes of five of the 70 (140 eyes) mice exposed to the strains. Four mice developed unilateral and three, bilateral cases of keratoconjunctivitis. Nonhemolytic as well as hemolytic M. bovis was recovered from an eye of one of the mice. One of the two rough hemolytic M. bovis strains induced keratoconjunctivitis, but was not recovered from the eye. The other strain was recovered from the eye but did not cause keratoconjunctivitis. Mima polymorpha, Mima polymorpha var. oxidans, Moraxella liquefaciens and Moraxella nonliquefaciens became established in the eyes of mice but did not cause keratoconjunctivitis.

It appears that different strains of M. bovis vary in their ability to produce keratoconjunctivitis in the mouse. This variation is not necessarily related to the ability of different strains to produce hemolysis on BAP because some of the nonhemolytic strains caused keratoconjunctivitis in some of the animals. Also there was no relationship between the recovery of the organism from an eye and whether the eye became affected with keratoconjunctivitis.

SECTION VIII. THE TOXIC EFFECTS OF MORAXELLA BOVIS AND ITS
GROWTH PRODUCTS IN CATTLE AND LABORATORY ANIMALS

Introduction

The results of previous studies indicated that M. bovis caused keratoconjunctivitis in both cattle and mice. The factors responsible for the production of keratitis were thought to be related to the products of the organism. In those studies cattle and mice developed keratoconjunctivitis, many times within 24 hours after exposure, even though in some cases M. bovis could not be recovered from the eye secretions. This suggested that at least some of the changes observed were due to toxic substances in the exposure material not necessarily the result of a state of infection. Moreover, it is very difficult to perceive how an organism such as M. bovis could have established itself and produced such changes in such a short period of time.

Supporting the explanation offered in the preceding paragraph are the results of a previous study in which rabbits that were given intramuscular and intradermal injections of M. bovis in an attempt to hyperimmunize them developed severe dermal and muscular necrosis at the site of injections (Section II). These findings intimated the possibility of a toxin associated with M. bovis. Other investigators (61, 62, 71, 119) also reported that M. bovis produces toxins, and one of them (62) suggested that the toxin or toxins probably account for the

severe ocular lesions seen in IBK. These workers (62) injected viable M. bovis cultures into mice, rabbits and chicken embryos and produced changes which they considered consistent with the presence of one or more toxins.

The purpose of the present study is to study the toxic effect of M. bovis in cattle and laboratory animals.

Materials and Methods

Exposure materials

Two strains of M. bovis identified as EPP-63(300) and 8613 were used. Strain EPP-63(300) was recovered from a bovine animal naturally affected with IBK and has been used in other studies (Sections III, IV, V and VI). Strain 8613 was a laboratory strain which had been carried through one cattle passage (Section III).

Experiment I The inoculum for exposing cattle was prepared in the following manner: a fourth blood agar passage (BAP) culture of strain EPP-63(300) was scraped from two plates (24-hour cultures) and suspended in PSS; the cells were washed two times and finally resuspended in 10 ml of PSS.

Experiment II The inoculum for exposing rabbits was prepared from M. bovis strain 8613. A colony from a BAP culture was added to TSB containing 10% rabbit serum. The inoculated TSB was placed on a rotary shaker and incubated at 37 C for 48 hours. Different quantities of the TSB cultures were injected into the posterior ear veins of 3 rabbits.

Ten ml of the culture were filtered through a Millipore filter pad having a porosity of 450 millimicrons.

Experiment III To prepare the inoculum for exposure of guinea pigs, a colony from a 24-hour BAP culture of strain 8613 was inoculated into TSB containing 5% rabbit serum. The TSB was then incubated for 20 hours at which time it was used to expose guinea pigs by different methods.

Experiment IV Inoculum for exposure of guinea pigs to egg-passaged culture of M. bovis was prepared in the following manner: a colony of a 24-hour BAP culture of strain 8613 was suspended in 1 ml of TSB; 0.1 ml of the suspension was used to inoculate each of four 5-day-old chicken embryos via the chorioallantoic cavity (CA). The CA fluid was recovered from four chicken embryos after 24 hours incubation and pooled (first passage); 0.1 ml of the fluid was used to inoculate additional embryonating chicken eggs. This process was continued through 18 passages. The CA fluid representing first, fourth, and 18th passage cultures of M. bovis was used to expose 16 guinea pigs.

Experiment V A 24-hour TSB culture of M. bovis strain 8613 was used to expose embryonating chicken eggs.

Animals and embryonating chicken eggs

Experiment I Two Holstein yearling bulls were used. They had been used in a previous experiment and had developed

experimental IBK but had recovered from the disease. Both weighed approximately 500 lbs and were in excellent health.

Experiment II Four healthy male rabbits (Oryctolagus cuniculus), identified as #1, #2, #3, and #4 were used; their weight was 11, 9, 10, and 11 lbs respectively.

Experiment III Twelve healthy guinea pigs (Cavia porcellus) were used. They were approximately 4-1/2 months of age and weighed from 400-450 Gm. Their eyes were normal and culturally negative for M. bovis.

Experiment IV Eighteen guinea pigs with the same history as those in Experiment III were used.

Experiment V Three dozen embryonating chicken eggs were used. Their stage of incubation (embryonating age) ranged from 5 through 17 days. They were incubated at approximately 99 F (dry thermometer) or 84-86 F (wet bulb thermometer) reading.

Experimental design and exposure

Experiment I Five ml of the M. bovis suspension were injected into the jugular vein of each of 2 cattle.

Experiment II Five-tenths ml of the M. bovis culture was injected into the posterior ear vein of rabbit #1, 1.00 ml was injected into rabbit #2, and 2 ml were injected into rabbit #3. Rabbit #4 was exposed by injecting 1 ml of sterile filtrate of the M. bovis culture into the ear vein.

Experiment III Twelve guinea pigs were exposed to TSB cultures of M. bovis in the following manner (Table 14).

Table 14. Method of exposure guinea pigs to TSB cultures of Moraxella bovis

Route of exposure	No. of animals	Amount of inoculum given ^a
Intracardial injection	2	0.5 ml
Intraperitoneal injection	2	0.5 ml
Subcutaneous injection	2	0.5 ml
Intraocular injection (anterior chamber)	2	0.2 ml
Intranasal inoculation ^b	2	0.2 ml
Oral inoculation ^b	2	0.5 ml

^a Amount of M. bovis culture used for each animal.

^b They were inoculated using a medicine dropper.

Experiment IV Eighteen guinea pigs were exposed to egg-passaged M. bovis as given in Table 15.

Experiment V Three dozen embryonating chicken eggs were allotted to 12 groups of three. A group was inoculated each day so that embryos from 6 to 17 days of age were exposed. Each egg was inoculated with 0.2 ml of a culture of M. bovis.

Table 15. Inoculum and exposure method for 18 guinea pigs exposed to egg-passaged Moraxella bovis

Route of exposure	No. of guinea pigs	Amount of inoculum ^a	Egg passaged
Subcutaneous injection	2	0.5 ml	1st
" "	2	0.5 ml	4th
" "	2	0.5 ml	18th
Intraperitoneal injection	2	0.5 ml	1st
" "	2	0.5 ml	4th
" "	2	0.5 ml	18th
Intracardial injection	2	0.5 ml	1st
" "	2	0.5 ml	4th
" "	2	0.5 ml	18th

^aThis represents the amount of exposure material used for each animal (unit).

Results

Experiment I

Immediately after injection of the washed M. bovis cells into cattle, the following manifestations were observed: rapid shallow breathing, dilation of pupils of the eyes, increased serous lacrimation, slight frothing from the mouth, hacking cough and micturition. Within 20-30 minutes respiratory distress had increased, there was red frothing from the nostrils and mouth and the animals began to bloat. At this time epinephrine was administered but the bloating became more

pronounced and a serous discharge exuded from the animals' mouths and the animals became recumbent. Subsequent intramuscular injection of epinephrine did not ameliorate the condition. Both animals became moribund and were dead within 10 hours.

Experiment II

The results of the inoculation of M. bovis and a filtrate into rabbits are given in Table 16.

Experiment III

The results obtained by exposing guinea pigs by different routes to TSB cultures of M. bovis are given in Table 17.

Experiment IV

The egg-passaged M. bovis cultures caused varying degrees of disease in guinea pigs when given by different routes (Table 18).

Experiment V

All the chicken embryos exposed to 24-hour TSB cultures of M. bovis were dead after 24 hours and M. bovis was recovered from all of them.

Table 16. Results of injection of Moraxella bovis or its growth products into rabbits

Rabbit no.	Approximate weight	Clinical symptoms after specific periods of observation				
		16 hours	24 hours	36 hours	54 hours	96 hours
1	11 lbs	Malaise injected sclera dyspnea	No change	Improved	Nearly recovered	Completely recovered
2	9 lbs	Dead	-	-	-	-
3	10 lbs	Moribund	No change	Dead	-	-
4	11 lbs	Moribund	No change	Dead	-	-

Table 17. Guinea pigs inoculated with TSB cultures of M. bovis

Method of exposure	No. of animals	Disease produced	<u>M. bovis</u> recovered
Intracardially	2	Septicemia and death	Yes
Intraperitoneally	2	Malaise, fever, ruffled hair coat but recovered in 72 hours	No
Subcutaneously	2	Severe inflammation at site of injection	No
Intranasally	2	Remained normal	No
Intraocular	2	Mild inflammation of eye with corneal opacity	No
Orally	2	Remained normal	No

Table 18. Results of inoculating guinea pigs by various routes using different egg-passaged M. bovis culture

Method of exposure	No. of guinea pigs	Egg-passaged	Observation
Subcutaneous	2	1	Slight inflammation site of injection.
"	2	4	Slight inflammation.
"	2	18	Severe local inflammation, rough hair coat
Intraperitoneal	2	1	Mild anaphylaxis
"	2	4	Immediately anaphylaxis death within 24 hours.
"	2	18	Death within 20 min.
Intracardial	2	1	1 died and other developed severe anaphylaxis but recovered.
"	2	4	1 guinea pig died other mild anaphylaxis.
"	2	18	Both died, 1 within 10 minutes, other within 24 hours.

Discussion

The results indicate that M. bovis produces a potent toxin. The nature of which and whether it is related to the keratitis seen in infectious keratoconjunctivitis remain to be explicated. Nevertheless, results from previous studies give reason to assume that the toxin production is related to keratitis. For example, in the study on the virulence (88) of different strains of M. bovis, mice developed keratitis within 24 hours after exposure. Similarly, in other studies involving cattle, keratitis was produced much earlier than would be expected if a toxin would not have been present.

No attempt was made to characterize the toxin, but the results from this study as well as others tend to suggest that it is a potent endotoxin. Notwithstanding is the fact that 1 rabbit in Experiment II died from the effects of a filtrate--possibly the filter pad allowed cell fragments to pass through containing the endotoxins. The other results seen in the experiments were in accord with those of Henson and Grumbles (62).

Summary and Conclusion

Cattle, rabbits, guinea pigs and embryonating chicken eggs were exposed to cultures of M. bovis or its growth products by various methods. The 2 cattle died when exposed to 5 ml of M. bovis suspension intravenously, 3 of 4 rabbits died when exposed to M. bovis cultures or filtrate. Guinea pigs which

were exposed by subcutaneous injection of M. bovis developed severe inflammation with subsequent dermal necrosis at the site of injection; those exposed to M. bovis nasally and orally remained normal; but those exposed intracardially and intraperitoneally died subsequent to showing severe systemic anaphylactoid-like reactions. Two guinea pigs which were given intraocular injection of M. bovis cultures developed inflammation and corneal opacity in both eyes. Chicken embryos of different ages also succumbed to the effect of M. bovis culture within 24 hours after exposure via the chorio-allantoic cavity.

The only conclusion that can be made is the fact that M. bovis and its growth products have a toxic effect on cattle, rabbits, guinea pigs and chicken embryos.

DISCUSSION

The results of the present research indicate that Moraxella bovis is the etiologic agent of bovine infectious keratoconjunctivitis. However, they also point out that apart from the host, there are many other factors, both intrinsic and extrinsic which may influence the pathogenesis of the disease. These results thereby substantiate the contention of others such as Hughes et al. (67, 68, 69), Pugh and Hughes (105), Jackson (71), Watt (127), Cooper (41), and Gallagher (51) who suggested that the characteristics of a particular culture of M. bovis as well as environmental influences play an important role in M. bovis infections. While most of these factors are involved in various disease processes caused by other microorganisms, they appear to have a special significance in the evolution of IBK.

Of all the discernible intrinsic factors pertaining to M. bovis, those concerned with its hemolytic-nonhemolytic properties and the smooth-rough dissociations appear to be of the greatest importance in the production of IBK. Apparently the hemolytic M. bovis is necessary for the production of the clinical disease but the nonhemolytic variety, although it does not cause disease, can establish a state of infection. Also it can change, under the influence of the proper stimulant, into the hemolytic type and induce disease. This notion was first presented by Pugh and Hughes (105) and is supported by

the results found in Sections I, II, III, V and VII of this report. Probably of paramount importance is the fact that the results found in Sections I, II and VII demonstrate that the hemolytic M. bovis and the nonhemolytic M. bovis represent the same organism, and either variety can change into the other. The findings of Pugh and Hughes (105) along with that found in Sections III and VII indicate that the nonhemolytic variety of M. bovis does not cause clinical disease in cattle and mice under experimental conditions. Significantly, if these results can be extrapolated for the naturally occurring disease, which is thought to be the case, they would explain the erratic epizootiological picture associated with the evolution of IBK. They would also help to elucidate some of the confusion surrounding the etiology of the disease. For example, they would explicate the spontaneous occurrence of IBK in cattle herds in which hemolytic M. bovis was not detected prior to the outbreak or in cases where other agents such as IBR virus were thought to be the cause of the disease (120, 124).

The ability of M. bovis to dissociate into different forms as suggested by several workers (41, 51, 71, 127) appears to be related to its ability to cause IBK. Jackson (71) reported that smooth cultures of M. bovis are virulent while the rough cultures are avirulent. Watt (127) attributed his failure to experimentally produce keratoconjunctivitis in calves to the fact that old cultures (pleomorphic organisms) which represented dissociated forms of M. bovis were used as exposure

materials. It is also interesting to note that Farley et al. (47) failed to produce disease in cattle using cultures of M. bovis grown in liquid medium. Their failure is thought to have been due to their use of rough organisms as exposure material. This is substantiated by the results in Section I which indicate that smooth cultures of M. bovis change into the rough form when they are grown in liquid mediums. However, the results given in Section VII indicate that the rough culture can cause keratoconjunctivitis in mice. Moreover, the results of Section VIII indicate that M. bovis representing either the smooth or rough phases of M. bovis can cause a toxic effect. Therefore, it seems logical to assume that both phases should induce IBK if a common toxin is the mechanism through which M. bovis elaborates its effect; of course this is possible only if the rough type can become established in the bovine eye or if the keratoconjunctivitis is due to a state of infection.

Other intrinsic factors are also no doubt involved in IBK but they are not so easily discerned. For example, there appears to be differences in the virulence of different strains of M. bovis as indicated by the results of Section VII. Also the fact that an attack of IBK does not protect animals against subsequent attacks and an animal can have a series of episodes in the same eye (Section V) indicates the occurrence of a complex antigenic makeup of M. bovis or at least a confused immunologic picture. Of course this peculiar immunologic phenomenon may be due to the anatomical makeup of the eye which

prohibits systemic and/or other factors of resistance from reacting with agents invading the mucous membranes. In this regard, it is interesting to note that infection by certain members of the genus Niesseria, which resemble M. bovis in general characteristics and involve diseases of mucous membranes, are also not amenable to vaccination, and repeated episodes are common.

The results given in Sections III, IV, and V indicate that the extrinsic factors associated with M. bovis infection are nearly as important as the intrinsic factors. For example, they suggest that such factors as method of exposure, exposure dose, interactions of other organisms and enhancing factors (i.e. ultraviolet irradiation and mechanical injuries) definitely influence the evolution of IBK. Furthermore, they indicate that the route of entry, the dose, the inoculum and the vehicle used determine whether the animal becomes infected, the incubation period, and the type of disease produced. Of these, the dose appears to be the most critical; for example, the results in Section IV indicate that the number of M. bovis in eye secretions from cattle acutely affected with IBK is not sufficient to cause clinical IBK in a short period of time (10 days) even when an enhancing agent such as ultraviolet irradiation was used. Although under natural conditions, eye secretions are thought to be the vehicle through which the organism is transmitted. However, there is a possibility that under natural conditions the organism is disseminated from

chronic cases by flies where a larger number of organisms are involved. The latter hypothesis is supported by the findings in Section V which indicate that chronic cases of IBK may contain an excessive number of M. bovis as evidenced by the recovery of numerous organisms from eye swabs taken from chronically diseased eyes while very few organisms were recovered from acute cases. It also should be pointed out that, under natural conditions, there may be repeated exposures and the disease usually has a longer incubation period. Barner (14) has indicated that it takes approximately 20 days for IBK to develop after exposure to infective eye secretions.

The route of exposure is only important in that the culture of M. bovis should be deposited onto the mucous membranes of the eye rather than injected into its tissues. Under natural conditions the organism is probably not injected into the tissue. It is thought that factors such as ultraviolet irradiation and traumatic influences may facilitate the establishment of M. bovis in the eye. Hughes et al. (67) have reported that ultraviolet irradiation augments M. bovis infections. Other investigators have suggested that dust (6, 13), wind (4, 13), and sunlight (5, 6, 13, 16, 51, 77, 116, 117) are contributive factors in IBK.

The presence of other microorganisms can also facilitate the establishment of M. bovis in the bovine eye as well as accentuate the accompanying clinical manifestations. A noteworthy illustration of this is described in Section V where

IBR virus was used to enhance and intensify the effects of M. bovis. The results also suggest that in spite of the fact that the IBR virus infection and M. bovis-induced IBK are separate entities, the IBR virus can augment the evolution of M. bovis infections. Presumably the IBR virus influence is mediated through enhancement of the multiplication of M. bovis and an intensification of its pathogenic effects. Therefore an animal which harbors an occult M. bovis infection could develop IBK under the stimulus of an eye infection by IBR virus, and the accompanying clinical manifestation would be accentuated.

Finally, it should be noted that there are probably many other intrinsic and extrinsic factors pertaining to M. bovis that are relevant to its ability to induce IBK but the aforementioned ones are deemed more important, the resistance of the host animal obviously notwithstanding.

SUMMARY AND CONCLUSION

A pragmatic approach was used to characterize M. bovis and to determine its relationship to IBK. The investigation was carried out in eight sections and each section dealt with a different aspect of the organism.

In Section I the morphological, cultural and physiological characteristics of strains of M. bovis, hemolytic diplococci, Herellea vaginicola, Mima polymorpha, Mima polymorpha var. oxidans, Moraxella liquefaciens, and Moraxella nonliquefaciens were investigated. The characteristics of M. bovis were compared to those of the other organisms and the distinguishing features detailed. Although it was found that these organisms are similar in general characteristics, they can usually be distinguished from each other without difficulty.

Section II dealt with the antigenicity of M. bovis which pertained to IBK. Moraxella bovis stimulated the formation of antibodies in rabbits inoculated with bacterins and in cattle exposed to M. bovis naturally or experimentally. A fluorescent antibody staining method and an agar gel double-diffusion technique were developed to detect and demonstrate M. bovis as well as the antibodies formed against this organism. Antigenic relationships were demonstrated among the various strains of M. bovis, Moraxella lacunata, Moraxella liquefaciens, Herellea vaginicola, Moraxella nonliquefaciens, Mima polymorpha, Mima

polymorpha var. oxidans, and hemolytic diplococci isolated from IBK.

The fact that M. bovis stimulates the formation of antibodies in cattle may indicate that IBK can be prevented by vaccination but much more work is needed in this area.

Sections III and IV dealt with the experimental production of IBK using different exposure methods and materials. In Section III, groups of cattle were exposed sequentially to infective eye-secretion filtrate, unidentified hemolytic diplococci, nonhemolytic M. bovis, and hemolytic M. bovis. All the microorganisms became established in cattle eyes but only hemolytic M. bovis induced IBK. Other cattle were exposed to mixtures of strains of M. bovis as well as a mixture of M. bovis and IBR virus by instillation into the conjunctival sac and/or injections into the conjunctivae. Exposure by instillation appears to be the method of choice and IBR virus accentuates the pathogenic effects of M. bovis. In Section IV, 15 cattle were allotted to eight groups and serially exposed to M. bovis cultures and/or eye secretions containing M. bovis. Ten animals exposed to eye secretions from eyes acutely affected with keratoconjunctivitis did not develop disease. However, when their eyes were subsequently exposed to hemolytic cultures of M. bovis, nine developed IBK. It was concluded from these results that eye secretions were not adequate for the experimental production of IBK.

Section V involved an investigation of the role of IBR virus in IBK. Cattle eyes were exposed to M. bovis and IBR virus under different schedules. In one experiment 10 cattle were exposed to M. bovis and to IBR virus 41 days later. Five of the animals developed IBK prior to exposure to IBR virus. Following exposure to IBR virus, all 10 animals developed bilateral conjunctivitis but none developed keratitis. Ten other cattle were exposed to M. bovis and IBR virus concomitantly, and all of them developed IBK. Still another group of 10 cattle was exposed to IBR virus initially and exposed to M. bovis 24 days later. An occult nonhemolytic M. bovis infection was exacerbated in 3 animals by the introduction of the IBR virus. Seven other animals developed conjunctivitis but none developed keratitis until the M. bovis culture was introduced after which all 7 animals developed IBK. It is concluded that although the diseases caused by M. bovis and IBR virus are similar in many respects they are separate entities, but may be reciprocally influenced.

Sections VI and VII are concerned with the experimental production of keratoconjunctivitis in laboratory animals. In Section VI rabbits, sheep, rats, guinea pigs and mice were exposed to cultures of M. bovis. Moraxella bovis became established in the eyes of sheep and mice but only mice developed keratoconjunctivitis. The keratoconjunctivitis was grossly similar to the disease produced in cattle. In Section VII the virulence of different strains was studied using mice as hosts.

The virulence of different cultures and strains of M. bovis varied. From the results of these two sections it was concluded that the mouse would be a suitable animal for the study of M. bovis and/or the pathogenesis of IBK.

Section VIII dealt with the toxic effects of M. bovis for cattle, rabbits, guinea pigs and embryonating chicken eggs. Viable cultures, killed organisms and cultural filtrate were toxic for the test animals.

This report can be concluded by stating that M. bovis is the etiologic agent of IBK and that many intrinsic and extrinsic factors influence the evolution of this disease.

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