

STUDIES OF THE MORAXELLA BOVIS ORGANISM

by

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INTRODUCTION

Infectious keratitis, an acute infectious disease of cattle and sheep, is frequently referred to as pinkeye, infectious keratitis and infectious keratoconjunctivitis. The disease was first described in cattle in 1869 by Billings. Some of the organisms named as the causative agents are Bacillus pyogenes, Micrococcus lanceolatus, Moraxella bovis and a rickettsial-like organism. Nearly every country has reported infectious keratitis in their cattle. A majority of the workers in the United States are of the opinion that Moraxella bovis is the causative organism (Anthony, 1957).

Pinkeye causes a great economic loss each year to the cattle industry in weight and milk production. There are no death losses reported directly caused by pinkeye. Farley, Foote, et al. (1950) stated that infectious keratitis is one of the most costly beef cattle diseases in Oklahoma. Serious loss in milk production is recorded among dairy cattle.

Symptoms first seen usually are swelling of the eyelids and photophobia. The discharge is watery at first and later becomes mucopurulent. The conjunctiva is inflamed and turns pink giving rise to the term "pinkeye". The affected animal shows evidence of pain especially when exposed to direct sunlight. Ulcers of the eye are frequently observed in severe cases. These occur on the anterior portion of the eye near the pupil. In severe, acute attacks, the eye may be completely destroyed and the animal becomes blind. This disease may occur any time of the year but most frequently appears during the summer months. The explanation for this seasonal occurrence is that insect vectors are more abundant during these months. Mild cases may not be noticed during the winter months when there is less intense sunlight. Some of the subclinical cases may show marked symptoms in the summer with bright sun.

Various attempts have been made to demonstrate the presence of antibodies against Moraxella bovis (Jackson, 1954 and Anthony, 1957). The results obtained by these workers are largely inconclusive. It is impossible to reinfect an animal after it has been infected with infectious keratitis. This information would tend to indicate that some type of antibodies are formed against Moraxella bovis. One of the large barriers to creative research on this condition has been the complete lack of a test for the detection of antibodies of Moraxella bovis. Resistance to reinfection may be assumed to be a local tissue immunity if no systemic antibodies are demonstrated.

It is the intent of this investigation to use the so-called soluble antigens of Moraxella bovis in an immunological procedure to demonstrate antibodies. A live and a dead Moraxella bovis culture were injected between the conjunctiva and cornea to determine the possible existence of a local tissue immunity.

REVIEW OF LITERATURE

Billings (1889) reported a disease in cattle which he termed contagious inflammation of the cornea or keratitis contagiosa. He reported the pathological lesions of the disease. Transmission attempts were unsuccessful. His recommended treatment was to isolate all infected animals in dark stalls and to apply wet bandages to the eyes. It was the opinion of the author that other species of animals were not susceptible to the disease.

The organism that was observed in the lesion was described (Billings, 1889), and the morphology of the organism was similar to that given for the causative agent by later workers (Baldwin, 1945).

Allen (1919) was able to transmit infectious keratitis by instilling lachrymal secretions from an active case to susceptible calves. He observed a

Gram negative diplobacillus in the lachrymal secretions from active cases, and was unable to reproduce the disease by instillation of pure cultures of the Gram negative diplobacillus into the eye of susceptible animals.

He also observed that an abrasion of the eye was not necessary for the development of a case of infectious keratitis. The symptoms of the disease were described. The cornea developed a smoky haze which began at a point just below the center of the cornea. In the later stages of the disease, the smoky haze spread over the entire surface of the eye, resulting in impaired vision. As the animal recovered, the corneal haze disappeared beginning at the periphery and working in towards the center. It was found that an acute attack of the disease caused very little systemic disturbance. It was concluded that flies may play an important part in the dissemination of the infection.

Jones and Little (1923) described a disease which they termed infectious ophthalmia of cattle. The symptoms of the disease were similar to those described for other infections of the bovine cornea, and the ocular discharge was yellowish white in color. The organism isolated from the eyes was similar morphologically to Haemophilus bovis. Symptoms were not produced in other animals except the bovine by experimental inoculation. Cultural examination revealed the presence of this bacillus in all infected eyes. Transmission studies made with pure cultures of the bacillus and secretions from field cases all resulted in active infection, except that experimental cases were not as severe as those encountered in the field. It was found by these workers that animals which had recovered were harboring the bacillus three to four months later.

Jones and Little (1924) in their second article on infectious ophthalmia noted that this condition occurs mainly during the summer months, which further led them to believe that an insect vector was involved. They found that the

diplobacillus was incapable of living in the gastrointestinal tract of flies for as short a period of time as five minutes. The maximum length of time the organism could survive on the surface of a fly was three hours. It was difficult to isolate the organism from nasal washings of infected cattle. Isolations from eyes were made only in the very early stages of the disease. It was thought that in the later stages of the disease, the tear duct is swollen and will not allow the downward passage of exudate from the eye. There is the possibility that in early stages of the disease, the spread may be by droplets from the nasal secretions.

Baldwin (1945) cultured the secretions from 90 eyes of clinically diagnosed cases of infectious keratitis. Moraxella bovis was found in 66 (73.3%) of the eyes by direct microscopic examination. The organism was found in 69 (76.6%) of the samples when the secretions were cultured. If the two methods were combined 74 (82.2%) of the secretions contained Moraxella bovis. Other bacteria frequently isolated in these studies were staphylococci, streptococci, diptheroids and Pasteurella multocida. The presence of these organisms was not considered to play a part in the pathology observed. No other species except the bovine were found to be susceptible to this infection. The disease was transmitted in 12 of 15 animals when inoculated into the conjunctival sac with Moraxella bovis. Systemic antibodies were not detected by bacteriolytic or precipitin tests. Spontaneous agglutination was reported on the agglutination trials. Baldwin (1945) advanced the theory that immunity to reinfection may be a tissue immunity.

Reid and Anigstein (1945) reported that keratoconjunctivitis was present during all months of the year in animals on the subtropical Gulf Coast of Texas. The majority of the cases occurred during the summer months in all breeds, sexes and ages of cattle. They indicated that many of the cases of "cancer eye" are

a direct sequel of keratoconjunctivitis. In their observations, the infection was bilateral in 10 per cent of the cases. The symptoms described were the same as those for infectious keratitis. On the basis of the experimental results, it was concluded the disease was transmitted from one animal to another by direct contact. The disease was easily reproduced by inoculations with eye and nasal secretions, and the nasal secretions were as virulent as the eye secretions. They also stated that the rate of natural infection in herds ranges from 30 to 70 per cent. They further stated that this disease was a systemic infection and conferred solid immunity. An agglutination test was described. A sheep and two goats were infected by instilling into the conjunctival sac a pure culture of a diplobacillus isolated from an active case.

Farley, Kliewer, et al. (1950) stated that cattle in all stages of condition and nutrition are equally as susceptible to infectious keratitis. It was stated that certain insects act as agents in transmission of the disease. They also found that all species, except the bovine, were resistant to infection. The disease was produced in three of nine animals, by using the eye secretions from active cases of infectious keratitis and placing the material under the eyelids and membrane nictitans. These animals were previously exposed to pure cultures of Hemophilus bovis by the same method of inoculation. They were normal after three weeks.

In another experiment (Farley, Kliewer, et al., 1950) fresh cultures of Hemophilus bovis were isolated from field cases of infectious keratitis. Five calves were kept under range conditions and inoculated with cultures of Hemophilus bovis. The calves remained normal after repeated exposures to 24 hour cultures. From the observations made during the course of these experiments, they concluded that Hemophilus bovis was probably not the cause of infectious keratitis.

They observed that infectious keratitis occurs most frequently in Oklahoma from May to October, and the incidence of the disease decreased rapidly after the first frost.

Farley, Foote, et al. (1950) stated that infectious keratitis is one of the most costly beef cattle diseases in Oklahoma. Serious loss in milk production is recorded among dairy cattle. Since both cattle and sheep show the same general symptoms, it would seem probable that the same causative agent is responsible for the disease in both species. However, the same symptoms that are observed in infectious keratitis may occasionally be due to mechanical injury. The disease is limited to the eye. The inflammation begins in the cornea and conjunctiva. The conjunctiva becomes pink primarily because of the concentration of white blood cells, red blood cells or degenerated epithelial cells. The small blood vessels of the conjunctiva become swollen and congested resulting in swelling and hyperemia of the conjunctiva. Although cattle of all ages and breeds are susceptible, cattle less than one year of age are the most susceptible. They stated that the three stages of infectious keratitis are mild, acute and chronic. When the disease is spreading through a herd, the condition may be seen in all three stages. In other herds only one stage of the disease is seen at any one time. They stated that a mild infection is manifested by a thin watery secretion from the eye, slight cloudiness of the cornea and slight congestion of the corneal vessels. This form of the disease responds best to treatment.

Acute infection occurs primarily in cattle (Farley, Foote, et al., 1950). The eye secretion is copious and often is observed in large quantities on the face of the animal. There is a cloudiness of the cornea and the conjunctiva is congested. The infection may appear in one or both eyes. A small raised area sometimes appears just below the center of the cornea and eventually

ruptures to form an ulcer.

According to these workers, chronic infection produces more extensive changes in the cornea. If the acutely infected eye does not clear in two weeks, the cornea ruptures. Pyogenic bacteria invade the chambers of the eye; the third eyelid becomes swollen and edematous; and the lens may escape through the break in the cornea and be completely lost to the eye. It is unlikely that this form of the disease will develop if cattle are placed in darkened stalls during the early stages of the disease.

These same authors stated that insects are the spreaders of this disease. Treatment is usually successful if animals are placed in a dark stall to avoid the intense sun, wind and insects. Sulfa powders applied to the eye are recommended.

Attempts made by Farley, Foote, et al. (1950) to transmit the disease to cattle with pure cultures of Hemophilus bovis were unsuccessful. Attempts to propagate the organism in chick egg embryos were unsuccessful. However, the disease could be transmitted to susceptible animals by eye secretions from field cases.

Hall-Patch (1951) reported an ocular disease of cattle in the area of the New Forest in England. The symptoms described for this disease are almost identical with those described for infectious keratitis in the United States. The disease spread rapidly when cattle were outside of buildings, but stabled animals did not contract the disease. The author was of the opinion that flies were the vector. The disease appeared and reappeared in a herd of cattle as the fly population raised and fell with the season of the year.

The treatment that gave the best results was chloromycetin both as a 0.25 per cent lotion and as a 0.5 per cent ointment. In the early stages, lachrymation ceased 48 hours following treatment. It was further recommended that

animals being treated be placed in darkened quarters.

Klussendorf (1952) reported that dairy cows affected with infectious keratitis may drop in milk production as much as 50 per cent. The incidence of infection is usually from 40 to 60 per cent in a herd, but may vary from 10 to 90 per cent. If infection occurs in an eye, this eye is resistant to reinfection. The unaffected eye, however, remains susceptible. Because of this fact, Klussendorf (1952) was of the opinion that the immunity is local tissue immunity and not systemic. The lacrimal fluids may remain infective for four months or longer following recovery. He stated that bacterins were used with some degree of success. Autogenous bacterins and foreign proteins were also used as symptomatic treatments in this condition.

It was stated by Jackson (1953) that infectious keratoconjunctivitis of cattle is spread by insect vectors. He was of the opinion that the incidence of infection was highest among the Hereford breed, followed by the Jersey and Aberdeen Angus. Aberdeen Angus-Hereford crosses seemed to be more resistant to this infection. He found that the Brahman breed was relatively resistant to natural infection but would contract the disease by inoculation. The forms observed were acute, subacute, chronic, fulminating and carrier. In the fulminating form the ulcer of the acute syndrome covers most of the cornea. The ulcer penetrates Descemet's membrane and the corneal endothelium in less than one week and a prolapse of the lens and iris occurs. This form usually attacks both eyes. The fever that accompanies the disease runs from 104 to 106°F. Of the 20 cases observed by the author, 11 died and the remainder became blind. This form occurs in calves one to six months of age. In the carrier form, Hemophilus bovis has been isolated for as long as eight months following infection.

Jackson (1953) used Bordet Gengou potato glycerin agar medium, with five

per cent blood, in all isolation procedures. Lyophilized cultures were found to remain viable up to three months. The Haemophilus bovis was characterized by having its growth enhanced by the addition of X and V factors; the optimum pH was 7.2 to 7.4; the optimum temperature was 37°C; the thermal death point was 60°C in four minutes; facultative aerobic; carbohydrates were not attacked; indol was not formed; litmus milk was alkaline after seven days, with the formation of a precipitate of casein after ten days; nitrates were not reduced to nitrites; the cultures of Haemophilus bovis were very pleomorphic after 48 hours at 37°C; a capsule was observed in recently isolated cultures; spontaneous agglutination appeared in smooth recently isolated cultures; the strain remained smooth up to the fourth or fifth serial transfer on artificial media; and the rough variant is a fixed type that is devoid of its capsule.

According to Jackson (1953) subconjunctival injections of the smooth type in rabbits gave rise to a severe conjunctivitis. The condition remained localized. Intrascular injection produced a severe ophthalmitis. Haemophilus bovis was isolated from the conjunctival and anterior chambers of the eye. The disease was reproduced in cattle with ease using the smooth strain of Haemophilus bovis. The rough strain was found to be avirulent.

Jackson (1954) reporting from Texas on field studies of infectious keratoconjunctivitis believes that the disease is transmitted readily by direct contact. The house fly may transmit the disease in dairy herds. The incubation period varies with the time of year and climatic conditions, being from 48 hours to one week during the spring, summer and early fall, whereas a three to four week incubation period is common in the winter months. It was the opinion of the author that the Hereford breed is the most susceptible to infection. Chloromycetin ophthalmic ointment one per cent was applied locally in the eyes of 110 Hereford calves showing symptoms. Such treatment resulted

in relief of symptoms of infectious keratoconjunctivitis when applied once a day for three to nine days. Animals were placed in dark quarters during the treatment. Other commercial pinkeye remedies were tried with poor results.

Anthony (1957) tested a commercial Moraxella bovis bacterin. He found that no immunity was conferred by the injection of this bacterin. The controls on the experiment contracted keratitis as readily as the vaccinated group when virulent cultures of Moraxella bovis were swabbed into the eyes. The incubation period varied from 3 to 78 days. He concluded that systemic immunity was not produced by the commercial bacterin used in these experiments. It was found that calves born to previously immunized mothers were susceptible to infection with virulent Moraxella bovis. Dust and sunlight were thought to aggravate the infection. The disease was found to be more severe in the summer months than in the winter months. Moraxella bovis organism could penetrate through epithelium to the cornea.

Bergey's Manual of Determinative Bacteriology (Breed, et al., 1957) stated that Moraxella bovis is a short plump rod that measures 0.5 by 1.5 to 2.0 microns. The organism has rounded ends and occurs in pairs with occasional short chains. The organism has a capsule, is non-motile and is Gram-negative. In gelatin media, when incubated at 22°C, there is slow growth and slow liquefaction. On blood agar after 24 hours the colonies are round, grayish white and translucent. There is a narrow zone of clear hemolysis around the colonies. This zone is usually 1.5 mm in diameter. After 48 hours colonies on the surface are somewhat flattened and are 3.5 to 4.0 mm in diameter. The subsurface colonies are ellipsoidal and biconvex with hemolytic areas 2.5 to 3.0 mm in diameter. On blood agar slants after 24 hours incubation at 38°C, the growth is heavy, viscid and grayish white in color. Moraxella bovis will liquefy coagulated serum. The organism grows slowly in broth with

slight turbidity and considerable sediment. The growth reaction in litmus milk is alkaline with partial coagulation. There is no growth on potatoes; indol is not produced; and no acid is formed from any carbohydrate. Nitrites are not produced from nitrates. The organism requires aerobic conditions for growth. The optimum growth temperature is 36°C. The thermal death time is 55 to 59°C in five minutes. The bacteria is not pathogenic for laboratory animals. The only known source of this organism is the eyes of cattle that have infectious keratitis or are infected carriers.

Barner (1952) was unable to infect rabbits, sheep or guinea pigs with cultures of Moraxella bovis instilled into the conjunctival sac. A heavy suspension was used from agar plates diluted to turbidity of tube No. 1 of McFarland's nephelometer scale. The culture used was transferred seven times before it was inoculated. The culture used was a smooth culture recently isolated from a field case. One milliliter of the inoculum was used. All animals were normal thirty days following inoculation. Four calves were inoculated with Moraxella bovis into both eyes using the same procedure as laboratory animals. All eyes became infected and symptoms characteristic of infectious keratitis were produced. The eyes were cultured and Moraxella bovis was recovered from all infected eyes. All eyes were cultured one year later and one calf was found to be a carrier.

According to Barner (1952) Moraxella bovis was easily isolated from the eyes of animals infected with infectious keratitis. Moraxella bovis may be isolated from nasal secretions often during the active stages of the disease. Blood cultures made during the acute stage of infectious keratitis were negative for Moraxella bovis. The epithelial cells of the conjunctiva from normal eyes and eyes infected with infectious keratitis were found to contain bodies giving the appearance of pleuropneumonia-like organisms or rickettsial-

like organisms. These were later identified as melanin granules.

He cultured both eyes of 35 normal cattle. All eyes were negative for the presence of Moraxella bovis.

The author found that proteose peptone agar enriched with five per cent bovine, ovine, or equine blood was satisfactory for initial isolations and cultivation of Moraxella bovis. Litmus milk was slowly peptonized and complete proteolysis took place in eight to ten days. A very small amount of casein precipitate was present in the bottom of the tube.

Cultures preserved by rapid desiccation under high vacuum in the frozen state were found to remain viable after eight months of storage.

The author demonstrated only smooth colonies from clinical cases and these cultures remained smooth when cultured for eight months. After this time intermediate, dwarf and rough colonies appeared. The dissociants had a different reaction on litmus milk producing only slight alkalinity with no evidence of peptonization.

Barnes (1952) stated that infectious keratitis (pinkeye) of cattle is caused by Moraxella bovis.

Kanawyer, et al. (1953) reported on a bacteriophage active against Moraxella bovis. Secretions were collected from the eyes of two calves that were previously found refractory to infection. The filtrate prepared from these secretions produced typical phage-like plaques on agar plates that were seeded with Moraxella bovis. It was found that the phage would multiply. It was hypothesized that the presence of a bacteriophage might explain some of the inconsistent and variable results in the transmission of infectious keratitis by cultures of Moraxella bovis.

Perlswieg and Keifer (1925) working with pneumococcus repeated an immunity produced by a bacterial fraction. They suggested that this immunity was pro-

duced by the polysaccharide or the polysaccharide containing fraction. They thought the polysaccharide portion of the bacterial cell may be important in all active immunity.

Reistrick and Topley (1934) showed that specific polysaccharides of Bact. aertrycke were antigenic. The authors stated the specific polysaccharides which are a major importance in the induction of active immunity are dependent for full antigenic activity on their linkage with some other component of the bacterial cell, possibly a protein. This linkage may be easily broken by the method utilized for isolation of the polysaccharide. The amount of immunity produced by the polysaccharide from bacterial species appears to be related to its toxicity.

Pittman (1935) stated that the bacterium, Haemophilus influenzae, does not form a well characterized bacterial species serologically and morphologically. It has long been recognized that the individual strains differ from one another in morphology and virulence. The smooth strains have capsules and are virulent. The rough strain is less virulent and has no capsule. The smooth strain when injected into rabbits will give rise to a positive serum that will precipitate the soluble substance in the supernatant fluid of a broth culture or in a fluid used to wash organisms grown on agar. This procedure could not be performed on the rough strain of Haemophilus influenzae. Therefore, it was concluded that the soluble substance which was antigenic was not present in the noncapsulated rough strain. Hence, the antigenic portion of this bacterial species was thought to be contained in the capsules.

Pennell and Huddleson (1937) produced a soluble antigen from Brucella abortus by tryptic digestion and trichloroacetic acid. The antigen yield was from 15 to 30 per cent of the dry weight of the bacteria. The antigen would precipitate when layered on a positive brucella serum. Using this antigen it

was possible to detect antibodies in low concentrations. These antigens were toxic for the guinea pig and the greatest toxicity existed shortly after the antigens were prepared. It was noted that antigenicity and toxicity correlate closely. When large doses of an antigen are not toxic to guinea pigs, this same antigen produces few, if any, antibodies. This material was found to be free from both protein and polysaccharide.

Miles and Pirie (1939a) working with Brucella melitensis that was grown on tryptose agar and killed with chloroform, phenol or acetic acid, found essentially four main compounds. The authors did not report on the serological activity of all of these compounds. The specific soluble substance, which is usually thought to be a polysaccharide, was best removed by using two per cent phenol. The bacteria were exposed to phenol for several days. Methods for the extraction of S.S.S. by acetic acid, urea, trypsin and trichloroacetic acid were discussed.

Miles and Pirie (1939b) reported that all four of the fractions from Brucella melitensis had some antigenic activity. The haptan portion was the most toxic. The specific soluble substance was exceptionally active serologically.

Miles and Pirie (1939c) isolated a specific soluble substance from Brucella melitensis. The authors were of the opinion that the soluble antigen with which they were working was a phospholipid mixture in its least modified form. Two per cent phenol is used to kill the bacteria for the isolation of the antigen in its crudest form. The suspension of bacteria with phenol is held at 0°C for several days or weeks. The bacteria were centrifuged out at 3500 rpm.

Morgan and Partridge (1940) extracted a Boivin type of antigen from Bact. dysenteriae using diethylene glycol. The yield of antigen from the bacteria was slightly more than one per cent of the dry weight of the organisms.

The procedure was carried out both on the rough and smooth strains of East. dysenteriae. The primary extracted product which came from the smooth strain of bacteria, contained a homogeneous molecular complex which was able to induce the formation of antibacterial immune-bodies similar to those produced by the injection of the entire organism. It was stated that the primary extracted product is, in reality, a form of the antigenic complex as it exists in the bacterial cell. When the primary product was injected into a rabbit, there was a production of agglutinins, precipitins and haemolysins. The antigen was also extracted by trichloroacetic acid and antigenically compared to diethylene glycol. The results were inconclusive, however they indicate that there are some differences in the chemical composition.

Kabat and Mayer (1948) stated that many Gram-negative bacteria contain complex antigens that are composed of carbohydrate, lipid and a protein or polypeptide-like material. Some of the bacteria that have antigens of this type are the *Salmonella*, meningococcus, gonococcus and *Brucella* groups. These antigens are toxic for mice and are antigenic for animals and man. The antigen obtained from the *Salmonella* group corresponds to the somatic O antigen. Three methods of extraction described were extraction with trichloroacetic acid, tryptic digestion and diethylene glycol.

Hunter and Colbert (1954) produced a soluble antigen from *Brucella abortus* which could be coated on cholesterol crystals. This antigen was used on a microscopic slide or a macroscopic tube flocculation test. The procedure gave reproducible consistent results. Results of these tests compared favorably to those recorded by the tube agglutination test. The authors believed the test would be as successful as the use of cholesterol coated with cardiolipin and lecithin as an antigen for the serological diagnosis of syphilis.

Hunter and Colbert (1956) reported on a flocculation test for brucellosis

in which the soluble antigen was prepared by phenol extraction. Brucella abortus was grown on three per cent tryptose agar and the growth was washed off with saline containing 0.25 per cent phenol. This phenolized solution was dialyzed in running tap water until free of phenol. The suspension was centrifuged to remove the bacteria and the supernatant was made isotonic by the addition of Na Cl. Merthiolate was added to make a dilution of 1:10,000. The extract was refrigerated or frozen until needed. In order to obtain a "working solution", the soluble antigen was mixed cholesterol, lecithin and Eagle's buffered saline. Inactivated serum was used in the test. The serum was inactivated at 56°C for 30 minutes prior to use in the test. The antibody content was titrated by using dilutions of serum. Either tube or microscopic slide flocculation procedures may be used for the test. The specificity of the test was checked by absorption experiments. The purpose of these absorption experiments was to determine whether the brucella antigen emulsion would remove the agglutinins from serum. Five milliliters of the antigen emulsion was added to five milliliters of a 1:10 dilution of positive serum and this mixture was allowed to stand for one hour at room temperature. At the end of this time the mixture was centrifuged. Although only part of the antibody content was removed by one absorption procedure a second absorption treatment resulted in removal of all agglutinins.

The authors selected a series of blood serum specimens from known human cases where the brucella organism had been isolated from the blood. The brucella titer was checked by both the flocculation and microscopic agglutination methods. The flocculation test results compared favorably with those obtained with the agglutination tests. No prezone reaction took place if the serum was inactivated at 56°C for 30 minutes. Fifty-five per cent of the sera showed prezone reactions prior to inactivation and 12 per cent after inactivation. The

soluble antigen of brucella when injected into rabbits stimulated the formation of agglutinins. A lipopolysaccharide has been chemically extracted from brucella organisms and used in the same manner as the soluble antigen emulsion. Preliminary results indicated that this antigen was as good as the soluble antigen.

Bornowski (1957) developed a flocculation test for pullorum disease that was suitable for either the macroscopic tube or the microscopic slide method. The soluble antigen from Salmonella pullorum was produced by extraction with two per cent phenol. The antigen emulsion, for use in the test proper, was prepared by the addition of cholesterol and lecithin to the dialyzed, cell-free, pullorum extractant. The results obtained by the two methods of performing the test compared well with results obtained with the tube agglutination test. The results were reproduced at a later date but the titers were usually lower when they were run a second time. This was explained by a loss of titer by the serum, rather than any fault of the antigen.

EXPERIMENTAL PROCEDURE

Experiment I: Extraction of a Boivin Antigen with Trichloroacetic Acid

Lipophilized cultures of Moraxella bovis were obtained from the Pathology Department of the School of Veterinary Medicine, Kansas State College, Manhattan, Kansas. The cultures were plated on proteose peptone agar, pH 7.3 to 7.4. The culture was incubated at 37°C for 72 hours. At the end of 72 hours incubation the growth was checked for purity by a Gram stain. Moraxella bovis appears as white flakes on the bottom of the flask, if there is good

growth in proteose peptone broth at 72 hours. The organisms were removed by placing the broth in 500 ml bottles and centrifuging at 1,500 rpm for one-half hour. All clear supernatant fluid was decanted, leaving only that portion which was turbid. The turbid portion of the broth was transferred to a centrifuge tube and centrifuged at 3,500 rpm for 20 minutes. This last procedure packed the bacterial cells on the bottom of the centrifuge tube.

Most of the procedures recommended the washing of the bacterial cells in alcohol and ether before extracting the Boivin antigen. This procedure was attempted with Moraxella bovis and was found that such treatment produced a mass of organisms too sticky to handle with normal procedures. Therefore, bacterial cells were washed from the tube with distilled water. This suspension of cells was shaken and recentrifuged. This procedure removed soluble material from the broth that might have remained with the bacterial mass. The packed bacterial cells were removed from the centrifuge tubes by washing with the smallest amount of distilled water that effectively removed all the cells.

The bacterial cells were dried by placing the aqueous suspension in an open Petri dish over a beaker of boiling water. When dry, the bacterial mass was scraped from the Petri dish and used for the extraction. Air drying was found to be unsatisfactory.

A solution of 0.5 N trichloroacetic acid was made and placed in a refrigerator at 17°C. A bottle of distilled water was also placed in the refrigerator. To one gram of the dried bacterial cells was added five ml of cold distilled water and five ml of cold 0.5 N trichloroacetic acid. This mixture was shaken vigorously for two minutes and placed in the freezing chamber of a refrigerator at 0°C. The bacteria cell reagent emulsion was left in the freezing chamber of the refrigerator for three hours. At the end of this period, the insoluble bacterial residue was removed by centrifugation for 20 minutes

at 3,500 rpm. The supernatant fluid was markedly opalescent. The opalescent fluid was filtered through a Seitz filter under vacuum pressure of 300 mm Hg to remove any media or bacteria that may have escaped removal by centrifugation. The material was then placed in cellophane dialyzing tubing. The tubing with the antigen emulsion was placed in running tap water for 48 hours. Little of the opacity was removed by the dialyzing process. The antigen emulsion was removed from the tubing and 68 per cent by weight of 95 per cent ethyl alcohol was added. When the alcohol was added, a cloudy precipitate formed. The antigen emulsion was allowed to stand at room temperature for 24 hours. The precipitate was the "so-called" Boivin antigen portion of the Moraxella bovis bacterial cell. The precipitate and supernatant fluid were then centrifuged at 3,500 rpm for fifteen minutes. The precipitate was removed from the centrifuge tube by washing with 95 per cent ethyl alcohol. This suspension was re-centrifuged and the recovered precipitate was washed in diethylether. Following the last centrifuging, the ether was poured off and 5 ml of distilled water added. The antigen emulsion was then placed in a water bath heated to 73°C for 15 minutes to remove any ethyl alcohol or diethylether that remained from the washings. The antigen went into solution with the addition of distilled water. The antigen solution was stored in the deep freeze at 0°C until it was used.

Testing the Antigen. Two calves were used to produce Moraxella bovis antiserum. Five ml of commercial Moraxella bovis bacteria was administered by subcutaneous injection every other day for two weeks; the other calf received a five ml injection which was repeated in seven days. At the end of two weeks following the first injection the calves were bled. The blood was allowed to clot at room temperature and the serum removed. This serum was used to test all of the antigens.

The flocculation test was performed according to Hunter and Colbert (1956).

Only the microscopic slide flocculation test was used.

Procedure of Making the Antigen Emulsion. 1. Double glass distilled water was added in 0.85 ml amounts to 30 ml glass stoppered Kline bottles.

2. The bottle was continually rotated while one ml of one per cent cholesterol (Pfanstiehl, ash free, precipitate from alcohol for Kline test) in absolute alcohol was added. Time taken for the addition of the cholesterol was 20 seconds.

3. Fifteen-hundredths of a one per cent lecithin solution was added to the cholesterol emulsion. (The lecithin was furnished by Dr. Sol Rosenberg^{*1} and diluted so the concentration of lecithin was one per cent in absolute alcohol.)

4. Soluble antigen was added in 0.15 ml and 0.2 ml amounts to the emulsion. The emulsion was shaken vigorously by striking the bottom of the antigen bottle against the palm of the hand. Two concentrations of the antigen emulsion were made from each solution of antigen.

5. The final reagent to be added to the antigen emulsion was 2.5 ml Eagle's buffered saline. The Eagle's buffered saline was made as follows:

Stock Solution	
Na Cl	170. g
KH_2PO_4	2.7 g
Anhydrous Na_2HPO_4	11.3 g
Distilled water	1,000 ml

To prepare the working solution, add five ml of the stock solution to 100 ml of distilled water and adjust the pH to 7.4 by the addition of 0.1 N HCl or 0.1 N NaOH.

Microscopic Slide Test Procedure. 1. Serum dilutions of 1:10, 1:20 and 1:40 were made by placing 1.8 ml saline in the first tube and one ml in the next two tubes. Two tenths ml of serum was added to the first tube and

^{*1} Sylvania Laboratories, Orange, New Jersey

serially transferred in one ml amounts from the first tube down to the last tube. Five tenths ml of the undiluted or diluted serum was placed in the ring of a paraffin ring slide.

2. One drop of the antigen emulsion was placed on the slide using a one ml syringe with an 18 gauge needle with the bevel ground off. This was found to deliver 81 drops of antigen.

3. The slides were rotated on a rotating apparatus, (Arthur H. Thomas Co., Edition 1950, Number 3623, Electric Rotating Apparatus, A.H.T.Co. Specification) for five minutes at a speed of 180 rpm.

4. The results were read immediately under the microscope at 100 X magnification.

5. A known negative serum and saline controls were run with all antigens tested.

All samples were negative with diluted sera. An occasional roughness was noted in the test when undiluted sera were tested.

A precipitin test was conducted by layering the antigen emulsion over serum. The same sera used with the flocculation test were used in the precipitin test. It was impossible to use the serum layer on top of the antigen in the precipitin tubes. If the serum was on top, the antigen dispersed up through the serum. If the antigen was put on top, a definite line between the antigen and serum could be seen. The precipitin test was incubated at 37°C for four hours and then overnight in the refrigerator. No positive results were observed with any of the sera.

**Experiment II:
Extraction of a Boivin Type Antigen with Diethylene Glycol**

The method of culture and preparation of the Moraxella bovis cells for this extraction was the same as that used in the trichloroacetic acid experiment. To 1.25 grams of the dried bacterial cells, 12.5 ml of diethylene glycol were added. The mixture of diethylene glycol and bacterial cells was placed in an incubator at 37°C. The flask containing the suspension was placed on an electric flask shaker and shaken vigorously for two hours. The suspension was removed from the incubator and maintained at room temperature for 24 hours. The suspension was slightly opaque after the digestive process. Clarification of the suspension was accomplished by centrifugation at 1,500 rpm for 30 minutes. This suspension was then placed in a cellophane dialyzing tube. Caution was taken to keep the tube wet at all times and to immerse the bag in running tap water as soon as it was filled. The suspension was dialyzed in running tap water for 72 hours. The antigen was precipitated by the addition of 68 per cent by weight of 95 per cent ethyl alcohol. This antigen-alcohol mixture was allowed to stand 24 hours at room temperature for the antigen to settle out. The antigen precipitate had a cloudy, greyish white color similar to the antigen extracted with trichloroacetic acid. The antigen was removed from the alcohol by centrifugation, washed once in 95 per cent ethyl alcohol, once in diethyl ether and handled in the same manner as the antigen prepared by the trichloroacetic acid extraction process.

The same procedure was used for testing the antigen by the flocculation and the precipitin test, as was used for the antigen that was extracted from Moraxella bovis with trichloroacetic acid. All results from these tests using the boivin antigen were considered negative.

One guinea pig and one rabbit were used to produce an antiserum against this antigen. The two Reivin antigen extraction solutions were mixed equal parts for this experiment. Each animal was injected with 1.5 ml of the combined antigen solutions. At the end of seven days, the injections were repeated. At the end of 14 days, both animals were bled by heart puncture. The serum was removed and heat inactivated at 56°C for 30 minutes. The flocculation test was performed on the diluted serum using dilutions starting 1:1 and the final dilution being 1:10. The results of the tests were all negative, with exception of the 1:1 dilution of the rabbit serum. The 1:2 serum dilution was a smooth negative.

A precipitin test on the serum was negative.

Experiment III: Attempted Extraction of a Surface Antigen

Moraxella bovis that was isolated from an experimental case at the Research Station, Kansas State College of Agriculture and Applied Science, Manhattan, Kansas, was used in this experiment. A Hiss stain revealed that approximately 50 per cent of the cells had a capsule. The organism was streaked on Bordet Gengou agar enriched with five per cent defibrinated blood. At the end of 34 hours, the plates were streaked opposite the direction of the first streaking to give maximum growth per plate. At the end of 48 hours, the plates were checked for purity by Gram stain of typical colonies. Phenolized saline was made in the concentrations of 0.25, 2.5, 5 and 10 per cent phenol in 0.85 per cent saline. Three ml of each phenol concentration was used to wash the growth from one plate. A flamed inoculating needle was used to scrape the growth from the agar surface. The suspension of bacterial cells in phenol-

ized saline was poured into a two ounce screw top bottle and placed in the refrigerator at 17°C for 14 days. At the end of this period, the bacterial suspension was centrifuged at 3,500 rpm for 20 minutes. The supernatant fluid was collected by decantation and placed in a cellophane dialyzing tube. The dialyzing tube was placed in running tap water for 72 hours. The dialyzed material was used as an antigen.

The sera used to test this antigen were prepared by injecting one rabbit and two guinea pigs subcutaneously with the one ml of the antigen suspension utilized to make the antigen. The injections were repeated at the end of seven days. Fourteen days following the first injection the animals were bled by cardiac puncture. The blood serum was collected and used for the microscopic slide test.

The antigen prepared from the culture of Moraxella bovis was tested by the microscopic slide flocculation test using the procedure previously described. The results obtained from these tests were all considered to be negative.

Experiment IV:
Attempted Extraction of a Polysaccharide by Heat
From a Culture of Moraxella Bovis

Proteose peptone broth was inoculated with both rough and smooth strains of Moraxella bovis. The broth was incubated for 48 hours at 37°C. At the end of the incubation period the culture was checked for purity by the Gram stain. The bacterial cells were removed from the broth by centrifugation at 3,500 rpm for 20 minutes. Supernatant fluid was collected by decantation. Enough of the broth supernatant fluid was left on the bacterial cells to resuspend them. The bacterial suspension thus obtained was placed in a boiling water bath for one hour. Distilled water was added during this period to keep the volume constant.

The fluid was allowed to cool at room temperature. The fluid was clarified by centrifugation at 3,500 rpm for 15 minutes. The supernatant fluid was removed by decantation and saved as an antigen.

The antigen was tested with the microscopic flocculation test, using the serum from the calves that had been injected with Moraxella bovis. The procedure was used as previously described. The results obtained were all considered to be negative.

**Experiment V:
Collection of the Enzymes Produced by Moraxella bovis
and Their Antigenic Properties**

A solution of dry milk was used as a medium for the extraction of the crude enzymes of Moraxella bovis. The dried milk was placed in solution by using 2 2/3 cups of a commercial dried milk with 7 1/2 cups of distilled water. This reconstituted milk was dispensed in 500 ml amounts into one liter flasks. The milk was autoclaved at 121°C temperature, 15 pounds pressure for 30 minutes. After autoclaving, the milk was incubated at 37°C for three days. At the end of three days incubation, the milk was checked for sterility by streaking out on a protease peptone agar plate enriched with five per cent defibrinated blood. Sterile milk media was inoculated with 20 ml of a three day old protease peptone broth culture of Moraxella bovis. The inoculated media was incubated at 37°C. Complete hydrolysis of the protein took place in 15 days. The long period of time required for complete hydrolysis of milk protein was probably due to the fact that an avirulent culture of Moraxella bovis was used as the inoculum. After complete hydrolysis of the protein had taken place, the milk serum was placed in 500 ml bottles and centrifuged at 2,000 rpm for 30 minutes. The supernatant fluid was decanted and placed in

the refrigerator at 17°C for 12 hours. At the time the milk serum was placed in the refrigerator, the volume was measured. Double this volume of 95 per cent ethyl alcohol was also placed in the refrigerator. At the end of 12 hours, the chilled 95 per cent ethyl alcohol and the milk serum were mixed. The ethyl alcohol was used to effect precipitation of the crude enzymes. The mixture was placed in the refrigerator for 12 hours to allow all of the precipitate to form. After the precipitate had formed, this sediment was removed by centrifugation at 3,500 rpm for 30 minutes. The supernatant fluid was poured off the precipitated material. The centrifuge tube was turned upside down on a towel for 30 minutes to allow the precipitate to dry. Distilled water was added drop by drop while stirring. The precipitate was dissolved with the smallest amount of distilled water possible. Dialyzation of the enzyme solution was accomplished by placing the solution in a cellophane bag. The filled bag was placed in a beaker of distilled water which was changed every 12 hours for 48 hours.

One guinea pig and one rabbit were injected in an attempt to develop an antienzymatic serum against the crude enzymes of Moraxella bovis. Each species received 0.5 ml of the crude enzymes subcutaneously. At the end of seven days, the injection of 0.5 ml of the enzymatic solution was repeated. Blood collection was accomplished by heart puncture 14 days following the initial injection and the serum was removed. Bovine serum collected from a calf two weeks following the acute stage of a bacteriologically confirmed experimental keratitis infection was also used.

The tests were conducted in the same manner as the previous flocculation and precipitin tests using the crude enzyme as an antigen. No positive results were obtained.

Four mature guinea pigs were used to determine if the enzymatic solution was capable of producing a local reaction in the eye. The enzymatic solution

was injected under the palprebra conjunctiva and poured over the surface of the eye.

Various concentrations of the enzymatic solution were prepared by diluting the stock solution with 0.85 per cent saline. The right eye was chosen for the enzymatic solution. The left eye was used as a control and 0.2 ml of 0.85 per cent saline was injected subconjunctival. The concentration of the enzymatic solution and the amounts are as follows:

Guinea pig No. 1 received 0.2 ml pure enzymatic solution
 Guinea pig No. 2 received 0.2 ml enzymatic solution diluted 1:4
 Guinea pig No. 3 received 0.2 ml enzymatic solution diluted 1:8
 Guinea pig No. 4 received 0.2 ml enzymatic solution diluted 1:16

One week following injection none of the guinea pigs showed any reaction to the enzymatic solution. The control eyes of all guinea pigs were normal.

Experiment VI:

An Attempt to Demonstrate a Local or Tissue Immunity Against Moraxella Bovis

In this trial, nine calves were used. Five of the calves were raised on the Veterinary Research Farm and weighed approximately 250 pounds. Calf No. 1 was a black and white Holstein heifer; calf No. 2 was an all black Holstein bull; calf No. 459 was a red Shorthorn heifer; calf No. 451 was a black, white face heifer; and calf No. 81 was a black, white face heifer. The four other calves used in this experiment were purchased through a local sale barn and ranged in weight from 300 to 475 pounds. Calf No. 3 was a black, white face heifer; calf No. 4 was a polled white face heifer calf with a white lined back; calf No. 5 was a horned white face steer; and calf No. 451 was a red, white face heifer.

Moraxella bovis was obtained in pure culture from an active experimental case of infectious keratitis. The initial isolation was made on Bordet-Gengou

potato agar enriched with five per cent defibrinated sheep blood. The subsequent cultivation of this strain was made on proteose peptone agar enriched with five per cent defibrinated ovine or caprine blood. After the second transfer on proteose peptone agar, the bacteria were inoculated into proteose peptone broth. The bacteria were transferred every 24 hours until three passages were completed. The last transfer was checked for purity by the Gram stain. Concentration of the bacterial cells was accomplished by centrifugation at 3,000 rpm for 15 minutes. The supernatant fluid was removed with a pipette, leaving enough of the broth on the cells to completely resuspend the bacterial cells. The concentration of bacterial cells was adjusted to the equivalent of McFarland's nephelometer tube No. 1. Approximately one-half of the suspension was placed in a water bath at 56°C for one hour. At the end of one hour the bacterial cell suspension was removed from the water bath and placed in a freezing chamber of a refrigerator for 24 hours. At the end of 24 hours, the suspension was thawed and used immediately for injection into the cornea. The other half of the suspension was used as live vaccine. The eyes of calves No. 1 and No. 2 were anesthetized with a two per cent butyn sulfate solution. The left eye of calf No. 1 was injected intracorneally with 0.1 ml of the live vaccine using a 27 gauge needle. The left eye of calf No. 2 was injected intracorneally with 0.1 ml of the heat and cold treated suspension of bacterial cells. The left eye of both calves were used for injection while the right eye served as a control eye. The left eye of both calves was swabbed the day after the injection was made. Swabs taken from the eyes were streaked out on Bordet-Gengou blood agar. Moraxella bovis colonies were observed on the plate swabbed with the material from the left eye of calf No. 1. A Gram negative bipolar staining bacillus was isolated from the left eye of calf No. 2. This organism was inoculated into maltose, mannitol, sucrose and dextrose. Acid

and gas were produced in sucrose and dextrose with no fermentation in the other carbohydrates. The organism was not Moraxella bovis.

Calves No. 1 and No. 2 were challenged with a culture of Moraxella bovis 14 days after inoculation. The bacterial growth from three proteose peptone blood agar plates were used to challenge both eyes of each calf. The bacterial growth was washed off of the plates by flooding the surface with from 1.5 to 2 ml of 0.85 per cent saline. The growth was scraped from the agar surface with an inoculating loop. The eyes were first swabbed with a dry swab until the mucous membranes were red. The second swab was soaked in the bacterial cell suspension and rubbed over the entire conjunctival sac and under the membrana nictitans. The remainder of the bacterial cell suspension was poured over the surface of the eye. After the challenge, the calves were placed in an outside pen giving them full access to sunlight.

After the calves were challenged the next 14 days were cloudy with little or no sunlight. No symptoms of infectious keratitis were noted in any of the calves during the first 15 days. On the 15th day after the previous challenge, the challenge procedure was repeated in the same manner as before. No symptoms of infectious keratitis appeared following the second challenge. An attempt was made to recover the culture of Moraxella bovis used seven days following the second challenge. At this time a challenge was repeated. No organisms were recovered on this trial. On the 25th day the challenge was repeated. Calf No. 1 died between challenges of causes unrelated to the experiment. The challenges were repeated on the 72nd and 100th days on calf No. 2. Calf No. 2 developed infectious keratitis in the vaccinated eye 14 days after the last challenge.

Two heifer calves that had been purchased at the local sale barn were used in the second vaccination trial. Calf No. 3 was a black, white face heifer that

weighed 325 pounds. Calf No. 4 was a polled white face, red calf that was approximately 400 pounds in weight. The vaccine used to vaccinate these calves was prepared by inoculating proteose peptone broth with the same strain of Moraxella bovis used in the first vaccine trial. Two 10 ml screw top bottles full of proteose peptone broth, along with two proteose peptone agar plates enriched with five per cent defibrinated blood, were inoculated with Moraxella bovis. These cultures were incubated at 37°C for 72 hours. At the end of 72 hours, the broth was centrifuged at 2,000 rpm for 10 minutes and the supernatant fluid was removed. The bacterial cell suspension was adjusted to the density of McFarland's nephelometer tube No. 1 by scraping growth off the plates with the inoculating needle and emulsifying it in broth. Immediately before using 0.1 ml of an aqueous solution, 15 turbidity units of hyaluronidase was added to 0.3 ml of the bacterial cell suspension in the proteose peptone broth. Hence, the inoculating dose used was 0.4 ml. When doing the actual injecting, no more than 0.1 ml was injected into any one position on the cornea. There was a noticeable hyperemic reaction in the inoculated eyes for the next four days. Moraxella bovis was isolated in pure culture from one of the inoculated eyes four days after they were injected.

A third portion of the vaccine was prepared in the same manner as it was for injection into the cornea of experimental calves. This vaccine was placed in an incubator at 37°C. At the end of 6 and 12 hours, the vaccine was streaked out on proteose peptone blood agar plates. It was found that the Moraxella bovis remained viable for six hours, but were not viable at 12 hours. This procedure was used to determine if Moraxella bovis would remain viable in the concentration of hyaluronidase used in the vaccine trials.

At the end of 16 days after inoculating, the eyes of the calves were challenged in the manner formerly described with a culture of Moraxella bovis

that had been recently isolated from an experimental case of infectious keratitis. Swabs of the eye secretions were taken at the end of 8 and 12 days. At the end of eight days, all of the eyes yielded cultures of Moraxella bovis. Of the cultures made at the end of 12 days, only the right (control) eyes yielded cultures of Moraxella bovis. No symptoms of infectious keratitis were noted after this challenge. This challenge was repeated at 44 and 72 days. Calf No. 3 developed infectious keratitis in the vaccinated eye 11 days after the last challenge. Calf No. 4 developed infectious keratitis in the control eye 16 days after the next to the last challenge and infectious keratitis in the vaccinated eye 14 days after the last challenge.

Two calves that had been purchased at the local sale barn were used for the third vaccine trial. Calf No. 5 was a horned, brockle face, red heifer weighing approximately 500 pounds. Calf No. 451 was a horned, white face, red steer weighing approximately 375 pounds. Proteose peptone broth was inoculated with the same strain of Moraxella bovis used in the previous vaccine trials. The broth was incubated at 37°C for 72 hours. At the time the broth was inoculated, two plates of blood enriched proteose peptone agar were also inoculated and incubated with the proteose peptone broth. At the end of 72 hours, the broth was centrifuged at 3,000 rpm for 15 minutes. The supernatant fluid was decanted down to the turbid portion of the broth. Growth from the agar plates was scraped off with the inoculating needle and emulsified in proteose peptone broth. The turbidity of the broth was brought up to the density of McFarland's nephelometer tube No. 1. The vaccine was used at once after the final preparation. The left eyes of the calves were anesthetized with a two per cent butyn sulfate solution. Four sites of injection were used in the left eye and the right eye was left as a control. The injection into the eye was made using 0.1 ml in two places into the sclera and in two places intracorneally. The calves

were maintained in a darkened stall for 13 days following injection. At no time after the injection did these calves show any reaction in the injected eyes. These calves were challenged with a virulent culture of Moraxella bovis in the formerly described manner on the 13th and 31st days. Calf No. 5 showed no evidence of infectious keratitis at any time during the course of the trial. Calf No. 451 developed infectious keratitis in the control eye 14 days after the first challenge.

Two calves that were raised at the Veterinary Research Farm were used for the fourth vaccine experiment. Calf No. 459 was an all red heifer calf that was approximately 250 pounds in weight. Calf No. 461 was a black and white spotted heifer calf. The vaccine used in this trial was prepared in the same manner as the vaccine used in the third vaccine trial. The vaccine was injected in two places on the eye. One injection of 0.2 ml was made intra-corneally and 0.2 ml was injected intrasclerally. The right eye was used on the red calf and the left eye was the control eye. The left eye of the black and white calf was used as the vaccinated eye and the right eye was the control eye. There was an area of corneal opacity on the right eye of calf No. 459 the day after vaccination. The eye was improved on the second day, so the reaction was considered to be due to trauma. Calves No. 459 and 461 were challenged with a virulent culture of Moraxella bovis 14 and 28 days after vaccination, in the formerly described manner. Calf No. 459 showed no evidence of infectious keratitis in the vaccinated eye three days after the first challenge.

The fifth vaccine trial was carried on with one 500 pound black, white face heifer designated as animal No. 81. The vaccine used to inoculate this heifer was prepared by inoculating a proteose peptone blood enriched agar plate with the same strain of Moraxella bovis used in the previous vaccine trials. This plate was incubated at 37°C for 48 hours. At the end of 48 hours

the growth was scraped off the plate and emulsified in 0.5 ml of 0.85 per cent saline until the concentration was approximately that of McFarland's nephelometer tube No. 1. The amount of vaccine used was 0.4 ml which was injected in two places intracorneally. This procedure was repeated in six days. This animal was never challenged with virulent Moraxella bovis as the vaccinated eye developed a clinical case of keratitis prior to challenge.

As calves developed infectious keratitis in only one eye, a challenge was made to ascertain if the other eye was susceptible to infectious keratitis. This procedure was often neglected when the vaccinated eye came down first. In this case it was felt that the necessary information about the immunity produced by the vaccine was gained, because in these instances the necessary information was gained about the product used.

Two calves remained refractory to the infectious keratitis in both eyes during the course of the experiment.

Five calves developed infectious keratitis in the vaccinated eyes. One calf developed the disease in both eyes and one calf developed the disease in only the control eye.

Moraxella bovis was recovered from all eyes that showed symptoms. Although all attempts to recover the organism were successful, there were various degrees of ease in recovery. On some eyes recovery was made on the first trial, while on other eyes several trials had to be made before the organism could be recovered.

DISCUSSION

These findings agree with previous workers (Baldwin, 1945) that spontaneous agglutination is a problem when working with Moraxella bovis. A problem

Table 1. Results of vaccine trials using vaccines and bacterins prepared from *Moraxella bovis* cultures.

Calf	: Dates		: Vaccine Preparations Used, : Amount and Route	: Dates		: Results
	: Vaccinated	: 4 Jan. 58		: Challenged	: Vaccinated Eye : Control Eye	
# 1	4 Jan. 58	0.1 ml vaccine intracorneally	18 Jan. 58	Med of causes unrelated to the experiment		
# 2	4 Jan. 58	0.1 ml bacterin intracorneally	18 Jan. 58	29 Apr. 58 infec- tious keratitis		
			23 Jan. 58			
			30 Jan. 58			
			27 Mar. 58			
# 3	11 Feb. 58	0.3 ml vaccine 0.1 ml hyaluronidase (15 tur- bidity units) intracorneally	14 Apr. 58	25 Apr. 58 infec- tious keratitis		
			27 Feb. 58			
			27 Mar. 58			
			14 Apr. 58			
# 4	11 Feb. 58	0.3 ml vaccine 0.1 ml hyaluronidase (15 tur- bidity units) intracorneally	27 Feb. 58	28 Apr. 58 infec- tious keratitis		12 Apr. 58 infec- tious keratitis
			27 Mar. 58			
			14 Apr. 58			
# 5	14 Mar. 58	0.4 ml vaccine in two areas (0.2 ml intracorneally and 0.2 ml intrasclerally)	27 Mar. 58			
			14 Apr. 58			
#451	14 Mar. 58	0.4 ml vaccine in two areas (0.2 ml intracorneally and 0.2 ml intrasclerally)	27 Mar. 58 14 Apr. 58			10 Apr. 58 infec- tious keratitis
#459	18 Apr. 58	0.4 ml vaccine in two areas (0.2 ml intracorneally and 0.2 ml intrasclerally)	2 May 58 13 May 58			
#461	18 Apr. 58	0.4 ml vaccine in two areas (0.2 ml intracorneally and 0.2 ml intrasclerally)	2 May 58 13 May 58	5 May 58 infec- tious keratitis		
# 61	29 Apr. 58	0.4 ml vaccine intracorneally 0.4 ml vaccine intracorneally	17 May 58 infec- tious keratitis			

Footnote: 1. The left eye was used as the vaccinated eye. The right eye was used as the control eye.
 2. *Moraxella bovis* was cultured from all experimental cases of infectious keratitis. The recovery was not always made on the first trial.

Table 2. Results of the microscopic slide flocculation test.

Antigen	Sera Tested	Number	
		Positive	Negative
Bovin antigen Trichloroacetic extracted	Sera from two bovines that were injected with <u>Moraxella bovis</u>	0	2
	Sera from two bovines that were injected with <u>Moraxella bovis</u>	0	2
Bovine Antigen Diethylene glycol extracted	Sera from one guinea pig and one rabbit that were injected with an equal mixture of the trichloroacetic and the diethylene glycol extracted antigens	0	2
	Sera from two guinea pigs and one rabbit that were injected with the phenol extracted antigen	0	3
Surface antigen Phenol extracted	Sera from two bovines that were injected with <u>Moraxella bovis</u>	0	2
	Sera from one guinea pig and one rabbit that were injected with crude enzymes	0	2
Polysaccharide Heat extracted	Sera from one bovine that had acute infectious keratitis two weeks previously	0	1
Crude enzymes Alcohol extracted			

of this type rendered the agglutination or agglutination lysis test as unreliable. Other serological tests were found (Baldwin, 1945) to be equally as unreliable for the detection of Moraxella bovis antibodies.

The use of various cellular and extracellular products that were extracted by chemical means coated on cholesterol with lecithin was tried in a microscopic flocculation test to overcome the problem of spontaneous agglutination. All attempts to demonstrate an immune response by this test ended in negative and inconclusive results.

Attempts to demonstrate a toxic principle by instilling various intracellular and extracellular products into the conjunctival sac of rabbits and guinea pigs gave negative results.

Experiments conducted to try to find if the crude enzymes produced by Moraxella bovis grown on milk were antigenic or toxic, also gave negative results.

It was concluded that with the present methods and knowledge, it is impossible to demonstrate systemic antibodies produced by Moraxella bovis. There is the possibility that Moraxella bovis does not produce systemic antibodies.

Previous workers (Farley, Foote, et al., 1950) have reported that once a bovine eye has passed through an attack of infectious keratitis caused by Moraxella bovis, that the eye is refractory to a second attack. It was also found that if one eye was affected, the other eye remained susceptible. This factor alone indicated that there are no systemic antibodies formed by an attack of infectious keratitis.

Other workers (Anthony, 1957) have shown that the injection of bacterins and vaccines gives no protection to an animal from an attack of infectious keratitis when challenged with a virulent culture of Moraxella bovis.

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All previous work on the subject have pointed to a conclusion that the immunity conferred by an attack of infectious keratitis was a tissue immunity. An attempt was made to demonstrate a local tissue immunity. No immunity could be demonstrated from the injection of alive or dead Moraxella bovis cells when challenged with a virulent culture of organisms. There are at least three possible explanations for this phenomenon. First, when a bovine eye undergoes an attack of infectious keratitis, there is a chemical alteration of the tissues that renders the eye refractory to a second attack of Moraxella bovis.

The second possibility was that Moraxella bovis acts with a virus to manifest its pathogenic process. The relative ease with which infectious keratitis can be produced with pure cultures of Moraxella bovis would tend to indicate that if a virus is involved it was probably carried with the cultures of Moraxella bovis. Tissue culture studies or other virus studies would be indicated.

The third possibility may be that the strain of Moraxella bovis used in the vaccine and serological tests was not antigenically active. There is the possibility that one of the cultures would be antigenically active.

It was found that the strain of Moraxella bovis used in the vaccine trials would reproduce in the eye. Viable organisms were cultured from vaccinated eyes five days after injection. One calf developed a case of infectious keratitis after vaccination intracorneally with the same strain of Moraxella bovis used to vaccinate the other calves.

Hence, it could be assumed the vaccine strain was virulent when injected intracorneally into a very susceptible animal and under conditions of environment that would favor the development of infectious keratitis.

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STUDIES OF THE MORAXELLA BOVIS ORGANISM

by

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Various extractions were made chemically from cells of Moraxella bovis and combined with cholesterol and lecithin in a microscopic flocculation test.

The first extraction was a Boivin antigen extracted with 0.5 N trichloroacetic acid. The resulting Boivin antigen was coated on cholesterol and lecithin to make a working antigen emulsion. This antigen emulsion was used to test bovine serum obtained from calves that had received injections of Moraxella bovis two weeks prior to bleeding. The results obtained with the microscopic flocculation test as well as the precipitin test were negative.

A second antigen extraction was made utilizing diethylene glycol. The same sera were tested with this antigen and all results were negative.

The two Boivin antigens, (extracted with trichloroacetic acid and diethylene glycol), were mixed in equal parts and injected into a rabbit and a guinea pig. Two weeks following injection these animals were bled and the serum tested against the mixture of antigens using the microscopic slide flocculation technique. The results obtained were considered negative.

An attempt was made to extract a surface antigen from Moraxella bovis cells obtained from growth on Bordet-Gengou blood agar. Phenolized saline of varying concentrations was utilized to wash the cells from this agar. This suspension of Moraxella bovis cells was placed in the refrigerator for 14 days. Phenol was removed by dialysis and the cells by centrifugation. The remaining fluid was used as an antigen for the microscopic slide flocculation test. The sera were obtained by injecting a rabbit and two guinea pigs with the surface antigen.

The results with this antigen and these sera were all considered negative.

Heat was used in an attempt to extract a polysaccharide from Moraxella bovis. A broth culture of these cells was heated; the bacteria were removed by centrifugation; and the supernatant was utilized as an antigen. The sera

utilized in testing this antigen were the same as those utilized in testing the Boivin antigen and all results were negative.

The crude enzymes produced by Moraxella bovis grown in sterile milk were precipitated with ethyl alcohol and centrifuged. The sediment thus obtained was suspended in distilled water and dialyzed in a cellophane bag in tap water. One guinea pig and one rabbit were injected with this enzymatic solution. The blood serum from these animals were collected two weeks following injection. The crude enzyme solution was used as an antigen in testing the sera from the two experimentally injected animals and the serum from a bacteriologically confirmed experimental case of infectious keratitis. No positive results were obtained with either the microscopic slide flocculation test or the precipitin test.

Various concentrations of the crude enzyme solution were tested for toxicity by injecting them into the conjunctiva of healthy guinea pigs. One week following injection none of these animals showed any gross lesions of the injected eye.

Nine calves were utilized in an attempt to demonstrate the existence of a local tissue immunity. Six of the animals were used to inject the living Moraxella bovis organisms and one animal was used in a bacterin trial. One animal died of causes not related to the experiment prior to the obtaining of any results.

A supposedly avirulent strain of Moraxella bovis was produced by repeated passage of artificial media. The killed bacterin was produced from the same strain of Moraxella bovis.

One calf received 0.1 ml bacterin intracorneally; two calves were injected with 0.3 ml of the vaccine and 15 turbidity units of hyaluronidase intracorneally; four calves received 0.4 ml of vaccine in two areas; and one calf

received 0.4 ml of vaccine intracorneally at two different times. Only one eye of each animal was vaccinated and the other was utilized as a control.

Challenge was made by placing a virulent culture of Moraxella bovis in both eyes of the calves. Five cases of infectious keratitis occurred in the vaccinated eyes while only two occurred in the control eyes.