IMMUNOLOGIC STUDIES WITH *MORAXELLA BOVIS* IN INFECTIOUS BOVINE KERATOCONJUNCTIVITIS

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

Infectious bovine keratoconjunctivitis has been recognized as an important disease of cattle in the United States since the late 1880's. The disease is characterized by inflammation of the cornea and conjunctiva and affects cattle of all ages and breeds. The incidence of the disease in Oklahoma appears to be greatest during the late spring and early fall, with a decrease during the mid-summer period. Active cases have been observed during December, January and February.

This disease has been reported to be initiated by the bacterium, Moraxella bovis. (Jackson, 1953). Various other organisms including viruses and rickettsiae have been suggested as the etiological agent in infectious keratitis. The combination of a virus and M. bovis has also been advanced as the cause of the disease.

There has been considerable disagreement among investigators concerning the classification of M. bovis and its exact etiological role in the disease. Sunlight, dust and flying insects have been suggested as playing an active part in the transmission of the disease. Transmission studies using M. bovis have fulfilled Koch's postulates in an environment free of dust or flying insects when ultraviolet radiation was used to make the eye susceptible. (Hughes et al., 1965).

The major economic losses from this disease are manifested as poor

weight gain and food utilization and a decrease in milk production. Even though various therapeutic measures have been helpful in controlling the disease, these losses usually occur before treatment is initiated.

A method of establishing immunity in all breeds of cattle would be beneficial to the industry. Attempts to produce a successful biological product which would impart an active immunity in susceptible cattle have been discouraging, but there is a need for further study of this method.

This investigation was designed to determine whether a simple macroscopic plate agglutination test could be used in studying outbreaks of clinical keratoconjunctivitis. Using this agglutination test, attempts were made to measure the serological responses in susceptible animals exposed to living and killed suspensions of M. bovis.

The relation of this serological response to the susceptibility of a herd to M. bovis infection was evaluated to see if the extent of possible outbreaks could be defined.

It was desired to ascertain if the use of bacterins and periodic serological testing might play a role in the control of the clinical disease.

CHAPTER II

REVIEW OF SELECTED LITERATURE

History and General Aspects of Infectious Bovine Keratoconjunctivitis

Billings (1889) first described a condition in Nebraska cattle he called keratitis contagiosa. A short, thin bacillus with rounded ends was isolated from the lacrimal fluid. The organism did not reproduce the disease when instilled into the conjunctival sac of healthy bovine eyes.

Allen (1919) reported an outbreak of infectious keratitis in cattle in Canada. Direct smears contained a short, thick, gram negative diplobacillus. The description of the organism conforms to several of the characterisitcs described by Morax and Axenfeld (1896, 1897). Lacrimal fluid when instilled into the conjunctival sac of healthy bovine eyes caused a disease similar to the original outbreak.

Jones and Little (1923, 1924) described a diplobacillus isolated from cattle in Ohio which when grown in pure culture and instilled into the conjunctival sac of healthy eyes caused an acute ophthalmia.

Creech (1942) pointed out that the terms, ophthalmia, conjunctivitis or keratitis, used by early investigators depend on the part of the eye involved or the extent of inflammation. The common name for all of these conditions is "pink eye", or keratoconjunctivitis.

Baldwin (1945) reported the isolation of a diplobacillus from cases of infectious keratitis in Ohio, Montana and California cattle herds. Isolation of a similar organism in Kansas, Oklahoma and Texas led investigators to believe that the disease was not limited to any specific geographical locality in the United States.

Farley et al. (1950) outlined three main types of clinical keratoconjunctivitis. The mild form was evidenced by lacrimation, conjunctivitis and slight cloudiness of the cornea. The acute form was characterized by lacrimation, photophobia, cloudiness of cornea and hyperemia of the scleral and conjunctival vessels. The chronic form was manifested by ulceration of the cornea, protrusion of Descemet's membrane and hypopyon.

Barner (1952) was able to reproduce the disease using M. bovis after a conjunctival incubation period of 15-21 days. He was unable to isolate the organism from any tissue or fluid except the conjunctival sac and lacrimal fluid. Moraxella bovis was not isolated from the conjunctival sac of cattle having no history of previous keratoconjunctivitis. Recovery of M. bovis from the conjunctival sac was successful in animals as long as one year after infection.

Jackson (1953) concluded that Hemophilus (Moraxella) bovis is the cause of infectious keratoconjunctivitis in Texas cattle. His classification of Hemophilus is based on the requirement of the organism for both the X and V factors. Freshly isolated organisms were reported to have a distinct capsule. Serum antibodies were present after spontaneous or experimental infection. These antibodies were detected by using a formalized antigen and serum in a simple

macroscopic agglutination procedure.

Rickettsia bovis has been isolated by Voight and Dietz (1956) in chicken embryos inoculated with lacrimal fluid from animals with infectious keratoconjunctivitis. The disease was reproduced in calves by injecting amnionic fluid into the anterior chamber of the eye.

Sykes et al. (1962) isolated a virus from lacrimal fluid of calves showing clinical "pink eye". This virus produced massive CPE in monolayers of fetal bovine lung cells. Intranasal inoculation of young calves using fluid from the tissue cultures produced classical infectious keratoconjunctivitis.

Hughes et al. (1964) reported the isolation of the IBR (Infectious Bovine Rhinotracheitis) virus in relation to a keratoconjunctivitis in which different clinical ocular lesions were manifested by lymphoid hyperplasia, hyperemia and edema of the palpebral conjunctiva and a fibrinonecrotic exudate.

Abinanti and Plumer (1961) described ocular lesions in feeder cattle exhibiting a syndrome similar to infectious keratoconjunctivitis. The eyes of these cattle were showing lacrimation and vascular hyperemia, but no corneal opacities or ulcers. IBR virus was isolated from the ocular fluid in these animals.

Kliewer (1958) repeatedly isolated M. bovis from naturally infected cases of keratoconjunctivitis. Using fresh isolates grown on blood agar, he was able to fulfill Koch's postulates.

The morphological and biochemical characteristics of Moraxella bovis have been described by many investigators. (Barner, 1952, Jackson, 1953, Henson, 1960). The organism is a gram negative,

non-motile diplobacillus, measuring one-half to one micron by one to two microns. Pleomorphism is evident even in fresh isolates. A capsule has been described in the early literature but has not been observed by recent authors. Good growth is obtained without the X and V factors provided the organism is grown on blood agar plates. Acid or gas is not produced in carbohydrate media, however, the organism apparently causes a pH change to a more alkaline state in most carbohydrates when phenol red is used as the indicator. Litmus milk also shows an alkaline digestion. Nitrate is not reduced. Gelatin is slowly liquified. Indole is not formed. A hemolytic toxin is produced which does not cause an increasing zone of hemolysis on blood agar upon prolonged incubation.

Henson and Grumbles (1960) described the pathogenicity of Moraxella bovis in selected laboratory animals. Mice were killed by intravenous, intraperitoneal and intracerebral inoculations. Dermonecrosis and ophthalmitis were demonstrated in rabbits. Chicken embryos were killed by cultures inoculated into the amnionic sac.

Several investigators (Ellis, 1954; Jackson, 1953) mentioned the presence of antibodies in the serums of naturally and experimentally infected cases of "pink eye". In unpublished data, Ellis has described the preparation of a plate antigen which appears useful in detecting serum agglutinins. Modifications of his method have been used throughout this investigation.

Chowdhury (1963) studied various antigenic and serological characteristics of different strains of Moraxella bovis. His results indicate a limited and somewhat transient antibody response in rabbits

inoculated with M. bovis. Using various chicken egg albumin fractions as antigens, Henson (1964) has shown that there is an antibody concentration differential between serum, aqueous humor and cornea of systemically immunized rabbits.

Zinsser and Parker (1923) established conclusively that it is the capsular material of Hemophilus influenza, a polysaccharide, which is serologically active. Since Moraxella bovis does not produce a capsule, the antigenic material is believed to be a polysaccharide contained within the cell wall complex.

Bövre (1964, 1965) discussed the transformation of resistance to streptomycin in Moraxella nonliquefaciens. This resistance has been demonstrated in transformation studies with M. bovis. A specific DNA component is responsible for this genetic ability of streptomycin resistance. It might be possible to demonstrate the transformation of a pathogenic factor between various strains of M. bovis that are otherwise similar.

General Aspects of the Immunochemical Properties of Bacterial Cell Wall Components

Various procedures for the preparation of agglutinins and hemoagglutinins are discussed by Kabat and Mager (1961). Numerous gram negative bacteria have been shown to possess the ability to agglutinate in the presence of homologous antibody.

Agglutination technics have become much more sensitive for the detection of small amounts of antibody, since it was shown that erythrocytes could adsorb various bacterial polysaccharides and that erythrocytes which had been treated with tannic acid could be coated

with protein antigens. (Boyden, 1951). In addition, polystyrene latex particles of uniform size have replaced the more erratic collodion particle technics used earlier. (Singer, 1956). This has made it possible to use agglutination tests with many antigen-antibody systems instead of being limited to systems in which the antigen was a constituent of the bacterium. Moreover, Stavitsky (1954) has shown that it is possible to titrate antigen in solution by its capacity to react with antibody and inhibit the antibody from agglutinating the coated cell.

Williams and Graber (1955) have studied the immunoelectrophoretic pattern of various human gamma globulins in rabbits and horses. Their results suggest an antigenic homogeneity against human gamma globulin. It is possible by immunoelectrophoretic methods to determine the mobility of specific precipitating antibodies and to relate these to normal components of the immune serum. These methods are also valuable in the study of normal antigen mixtures and of pathological and other modifications of these antigens. Bjorklund (1954) has shown that in immunological systems, antigens, when combined with antibodies in a gel, retain their affinity for certain dyes. This might make it possible to gain valuable information about the chemical nature of different antigens in a complex system.

While certain bacterial antigens like diphtheria toxin, streptolysin and the pneumococcal capsular polysaccharides pass into the culture media during growth or on autolysis and may be separated by chemical fractionation of the culture fluid, the usual procedure for obtaining antigens from bacteria is by extraction of washed, intact or

disintegrated cells with appropriate solvents. Disintegration of bacteria by the use of high frequency sound waves has been widely used. (Graber, 1953). These sound waves are produced by magnetostriction, the changes in length caused by application of an alternating magnetic field to a nickel rod clamped at its center or by piezo-electric oscillation, as a consequence of expansion and contraction of a quartz crystal subjected to an alternating potential difference. The former type creates frequencies of up to 50 kc/sec; these vibrations are transmitted to the suspension to be disintegrated. Heating effects must be avoided. The ultrasonic vibrations cause local reductions in pressure in the liquid with consequent boiling (cavitation). The rapid formation and collapse of these cavities causes enormous pressure changes which disrupt the bacteria.

Sonic vibrations of about 9 kc for one hour have been used with a number of gram negative organisms. (Rodenberg, 1958).

CHAPTER III

MATERIALS AND METHODS

Isolation and Preparation of the Plate Antigen

The original strains of Moraxella bovis were isolated from cattle with active disease from the Oklahoma State University Agronomy Farm at Perkins, Oklahoma in July, 1963. Cotton swabs were taken from the conjunctival sac and streaked on blood agar plates within one hour. The basic agar plates utilized tryptose blood agar base (Difco) with 5% defibrinated bovine blood being added prior to pouring of the plates. The plates were placed in an aerobic incubator at 37° C. Typical hemolytic colonies consisting of gram negative diplobacilli were inoculated into the following differential media: nitrate agar, indol, litmus milk and various carbohydrates enriched with 2% bovine serum. The carbohydrates used included dextrose, sucrose, maltose, lactose, salicin, raffinose, sorbitol and galactose. Young (9-12 hours) cultures were stained using Johne's method to demonstrate the presence of a capsule. Reference cultures were preserved by lyophilization using horse serum as the suspending medium.

Preparation of the plate antigen utilized 18-hours cultures of typical M. bovis. The typical M. bovis colonies were streaked on seed blood agar plates. After 18 hours incubation, the seed plates were

checked for contaminants. Individual agar plates were streaked utilizing sterile cotton swabs saturated in brain-heart infusion broth (Difco). Thirty to forty plates were prepared at one time and incubated for 18 hours. Plates containing atypical colony characteristics or contaminants were discarded.

The organisms were washed off the agar plates using 2 ml. of formalized saline (0.5% formalin). Rubber tipped applicators aided in removing the organisms. Ten ml. of suspension were placed in screw capped culture tubes with 10-15 glass beads, 4 mm. in diameter. About 25 to 30 ml. of the suspension was recovered from 40 agar plates. Tubes containing harvested bacterial cells were placed in flowing steam for one hour to prevent autoagglutination of the organisms. The individual tubes were cultured to assure death of Moraxella bovis and to assess the possibility of contamination. One ml. of a 0.3% aqueous brilliant green dye and 10 ml. of glycerine were added to each 89 ml. of antigen.

Standardization of the Plate Antigen

Serums from animals with chronic keratoconjunctivitis were used to determine the most feasible concentration of the bacterial cells in preparing the plate antigen. One-half ml. of serum was added to each of ten Kahn tubes. Tenfold dilutions of the cell suspension were made with normal saline. One-half ml. of the various dilutions were added to the Kahn tubes. The dilution of the cell suspension giving the most easily recognizable agglutination in one hour was

selected as the concentration to be used in the plate antigen. This corresponds to a suspension having a turbidity equal to that of a MacFarland nephelometer tube #6 and representing 1.6 x 10^8 organisms/ml.

Cell counts were made using a Coulter counter equipped with a 100 micron aperture tube. Using a cell suspension with a density of a MacFarland tube #6, the cell counts ranged from 1.2 to 1.9 x 10^8 cells/ml.

Subsequent standardization of the cell suspension was made with a Bausch and Lomb Spectronic 20 Colorimeter. Various MacFarland tubes were scanned at a wave length of 530 millimicrons. A base-line graph was established for the MacFarland tubes. The cell suspension antigens were diluted with normal saline to obtain a 30% transmission at 530 millimicrons wave length of light. This served as the standard concentration of all cell suspensions used as an antigen and contains 1.6 x 10^8 organisms/ml.

Plate Agglutination Testing Procedure

The plate agglutination procedure similar to the standard Brucella plate agglutination test was employed in this study. Normal and titered serums were pipetted onto a glass plate containing 72, 1.5 inch squares with a serologic pipette delivering 0.08, 0.04, 0.02, 0.01 and 0.005 ml. of serum. The 0.05 ml. of antigen was added and mixed with a glass rod or wooden applicator stick. The plate was rotated frequently during a ten minute incubation period. The degree of agglutination was recorded at the various dilutions as: 4+, 3+, 2+, 1+ and negative; with a 4+ reaction being the maximum agglutination visible

grossly and 1+ agglutination the minimal agglutination visible. The mixture of serum and antigen resulted in the final dilution of 1:25, 1:50, 1:100, 1:200 and 1:400.

Tube Agglutination Procedure

Negative and titered serums were diluted 1 to 10 (.9 ml. saline plus .1 ml. of serum) and inactivated by incubation in a water bath at 56° C. for 1 hour. Each was then diluted two fold up to a dilution of 1:640, and 0.05 ml. of antigen was added. The mixture was allowed to incubate at 37° C. in a water bath overnight. The highest dilution showing evidence of irregular clumping that failed to break up upon gentle shaking was recorded as a positive reaction. No attempt was made to designate the reaction from 4 to 1+, since this is an arbitrary classification.

Sheep Erythrocyte Agglutination Procedure

Fresh sheep erythrocytes were obtained by bleeding from the jugular vein and washed by the following technic. Forty ml. of freshly drawn sheep blood was added to 150 ml. Erylenmeyer flasks containing 6 ml. of a 3% solution of sodium citrate and 100 ml. of normal saline. One ml. of the above dilution (20% erythrocytes) was rewashed with enough normal saline to obtain a 3% erythrocyte suspension as determined by a microhematocrit procedure.

One ml. of the 3% erythrocyte suspension was added to an equal amount of M. bovis cell wall suspension (procedure described later) and allowed to incubate in a water bath at 37° C. for 3 hours. The

complex was washed in normal saline and centrifuged at 1800 rpm to separate the erythrocytes and the cell wall material.

An alternate procedure employed the treatment of sheep erythrocytes with tannic acid in a buffered physiological saline solution. The sheep erythrocytes were suspended in buffered normal saline containing equal volumes of normal saline and a buffer solution of KH_2PO_4 (0.15 M) and Na_2HPO_4 (0.15 M) mixed to obtain a pH of 7.2. Two ml. of a 2.5 percent suspension of buffered sheep erythrocytes were added to 2 ml. of a 1:20,000 dilution of tannic acid. The mixture was allowed to incubate 10 minutes at 37° C. The tanned erythrocytes were washed with buffered saline and centrifuged at 1800 rpm to separate the erythrocytes from the tanning solution. The erythrocytes were resuspended in 2 ml. of buffered saline and 0.5 ml. of the suspension added to 0.5 ml. of M. bovis cell wall components. After 10 minutes at room temperature the cells were centrifuged and washed in normal saline, then resuspended in 0.5 ml. of the same medium. The tanned and untanned sheep erythrocytes were used as a tube antigen. The bovine serum was diluted twofold from 1:10 to 1:640. To one ml. of each dilution, two drops (0.10 ml.) of the antigen was added. The tubes were incubated overnight at room temperature. A positive reaction was one that showed evidence of clumping that did not break up upon gentle shaking. The negative tubes showed the typical "button" in the bottom of the tube.

Preparation of M. bovis Cell Wall Component

Moraxella bovis cell wall component was prepared in the following manner. Eighteen hour cultures were washed from blood agar plates with

normal saline allowing 3 ml. of saline for each plate. Harvested cells were pooled and sonicated with the Branson ultrasonic sonifier (Model LS-75), delivering 2 amps for 60 minutes. The temperature of the cell complex was maintained at 2° C. during the ultrasonic treatment. Smears of the sonicated complex were stained with crystal violet to assess the completeness of disruption of the cells. The cell wall complex and protoplasmic matter were separated by centrifugation at 3500 rpm for 35 minutes at 0° C.

Preparation of Experimental Bacterins

Moraxella bovis isolates from clinical cases of infectious bovine keratoconjunctivitis from the Rossander herd of Stillwater, Oklahoma were used as seed cultures in the production of bacterins. These isolates were designated as strain #729, 8 and 2.

Organisms were grown in mass and harvested by the same procedure described for the production of the antigen. The harvested cells were killed by treatment in flowing steam for one hour. Various adjuvants were added to the bacterins. The bacterin was then transferred to 125 ml. vaccine bottles, 0.4 ml. of 40% formaldehyde added and the bottles capped with rubber stoppers. The bacterin was stored at room temperature for 7 days after which time the sterility of the product was evaluated by aerobic and anaerobic culture methods.

Several products were used as adjuvants. M. bovis was harvested and diluted to a density of a MacFarland Nephotometer tube #10. To 50 ml. of this suspension, 2.33 ml. of a 10% aqueous aluminum chloride was added. Sufficient 20% sodium hydroxide was added to adjust the pH

to seven. The cell suspension and the adjuvant mixture was diluted to 100 ml. with normal saline.

Freund's complete adjuvant was used with strain #729. Twenty-five ml. of the adjuvant was added to 25 ml. of the M. bovis cell suspension, density of a MacFarland #10.

Amphogel[1] was used as the aluminum hydroxide source with strain #8. Twenty-five ml. of Amphogel was added to 25 ml. of M. bovis cell suspension, density of a MacFarland #10

Some of the bacterins were used without adjuvants. Strain #2 was prepared as previously described and contained approximately 1.6×10^8 organisms per milliliter.

Strain #8, M. bovis was used as a bacterin after lysis of the cells by sonication. A cell suspension containing 1.6×10^8 organisms per ml. was sonicated for five minutes with 4 amp of direct current in a Branson ultrasonic sonifier, model LS-75. The cell suspension was examined by gram staining to determine the degree of lysis. Five minutes of sonification caused 95-99% lysis of the cells if the cells had previously been subjected to flowing steam for one hour.

Methods of Immunization

The experiment to determine the antigenicity of the bacterin was divided into two major parts using rabbits and cattle. Young adult rabbits were divided into treatment groups, each group consisting of 4 animals. Each group was injected with one of the prepared bacterins according to the following immunization schedule: Day 1, 0.5 ml. of

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[1]Amphogel, Wyeth Laboratories, 20% suspension of aluminum hydroxide gel.

bacterin; day 7, 0.5 ml; day 14, 0.75 ml; day 28, 1.0 ml. The route of inoculation was varied for each group between subcutaneous, intramuscular and intravenous inoculations. Serum samples were obtained via cardiac puncture at the time of each injection. The serum samples were examined for agglutinins utilizing the plate agglutination testing procedure described previously.

Twenty-three, 4 to 5 month old Hereford calves were obtained from the Pawhuska, Oklahoma area in May, 1964 (Group IV). These animals had no history of previous clinical infectious keratoconjunctivitis; however, these animals were mixed with mature cattle prior to purchase. The animals were well developed but in poor nutritional condition. Preinjection serum samples were obtained and analyzed as previously described. At the same time, conjunctival swabs were cultured. Cultural results indicated a preexisting M. bovis infection in several animals. Each animal was given a subcutaneous injection of 5 cc. of aluminum hydroxide precipitated bacterin on day 1 and day 14. Serum samples and conjunctival cultures were obtained from each animal at 14-day intervals until day 60 when the interval was changed to 30 days between collections. This was continued until October, 1964. The final examination was conducted in January, 1965, by collecting serum samples and conjunctival cultures on the animals remaining in the test group.

During December and January of 1964-65, young calves from the Pawhuska station were divided into two groups. The first group consisted of 10 purebred calves, 2 to 3 months of age, nursing dams that had been purchased and brought to the station the previous spring.

This group was untreated and served as a control group. The second group (Group V) of 12 grade calves, 2 to 3 months of age, also nursing dams that had been purchased the previous spring and kept at the Station were given injections of bacterin. Two, 5 cc. injections of bacterin were given at 14-day intervals. Serum samples were collected at the beginning of the test and at 14-day intervals until postinoculation day forty-five.

During May of 1965, the grade calves and their dams used during the winter of 1964-65 were again used in a field trial (Group VI). These calves and dams along with five, 2 year old heifers were given two doses of 5 cc. of bacterin at 14-day intervals. The bacterin was the same as previously described except Amphogel was added as the adjuvant. Nine yearling animals used during the field study in the summer of 1964 were given the same bacterin and at the same dosage as the above animals. Ten purebred Hereford cows and their 10 calves were not vaccinated and were used as controls (Group VII). Bimonthly serum samples and eye cultures were obtained on the vaccinated group. The purebred animals were not available for this sampling procedure. The control group was sampled approximately every 4 weeks. The field studies continued until mid August of 1965.

Infectivity Studies

The eyes of four young adult rabbits were inoculated with fresh cultures of M. bovis #2 and subsequently cultured daily for 21 days (Group I). Serum samples were drawn weekly and evaluated as previously described to detect the presence of serum agglutinins.

Two Hereford calves were obtained from the Pawhuska Station and kept in individual screened stalls (Group I). Direct sunlight and wind were kept to a minimun in these stalls. The conjunctival sacs were cultured and serums obtained prior to the inoculation of strain #2, M. bovis, into the conjunctival sacs of each animal. The conjunctiva was cultured at 2-day intervals. Serum samples were drawn via jugular vein puncture at 2-day intervals. These procedures were continued for 26 days.

Six, 4 to 5 month old Hereford cattle used in the infectivity trials were bred and raised at the Pawhuska Station (Group III). Seven days prior to and throughout the studies these calves were housed in individual fly proof stalls. Preinjection serum samples and conjunctival cultures were obtained and analyzed as previously described. Each animal was given a subcutaneous injection of 5 cc. of aluminum hydroxide precipitated bacterin on two occasions, 14 days apart. Serum samples and conjunctival swabs were obtained every 7 days, from day one through day forty-five. The animals were then challenged with fresh 18-hour cultures of M. bovis isolated from a clinical case of infectious keratoconjunctivitis (#746) by rubbing a cotton swab over the cornea and conjunctiva. Conjunctival swabs were cultured daily after initial challenge. A second challenge was conducted in a similar manner 7 days after the first challenge. The animals were released to summer pasture 14 days after the first challenge.

A second group of 4 yearling Herefords raised at the Pawhuska Station were placed in the isolation stalls (Group II). These animals had been naturally exposed to M. bovis the previous summer but showed

no evidence of clinical keratoconjunctivitis. After acclimatization to their new environment, the eyes of these animals were challenged with strain #729, M. bovis. Conjunctival swabs were cultured at two-day intervals to determine the growth phase of the organism. Serum agglutinations were run periodically during the period of confinement. These animals were confined for twenty-five days, then returned to summer pasture.

Isolation Facilities

The isolation facilities consisted of six screened pens, 10 by 12 feet in size, a small calf stock and a storage area with a solid wall on the north and west sides. The pens were open on the east and south. The pens were covered with screens having an average 0.5 mm. square aperture. Heavy guage wire screen was used around the inside of the pens to prevent puncturing of the smaller screen. Sunlight and dust were not significantly reduced. Periodic spraying aided in eliminating the fly problem caused by opening and closing the individual pens.

CHAPTER IV

RESULTS

Growth Requirements and Biochemical Properties of M. bovis

M. bovis was readily isolated and maintained on laboratory media consisting of tryptose blood agar base (Difco) enriched with 5% defibrinated bovine blood. Typical colonies were 3 to 4 mm. in diameter with narrow zone of β-hemolysis, approximately 1 mm. in diameter surrounding the colony. This zone of hemolysis did not increase upon prolonged incubation. Poor growth of M. bovis was obtained with brain-heart infusion broth (Difco) enriched with 5% bovine serum.

The organism did not produce acid or gas in any of the carbohydrate media, in fact, a change to a more alkaline state was occasionally observed. Litmus milk underwent a comparable change. Nitrate is not reduced, nor is indole formed.

Repeated attempts to demonstrate the presence of a capsule utilizing Johne's methods in both old and young (8 hour) cultures were unsuccessful.

Rabbit Immunization

Group I - Conjunctival inoculation with M. bovis #2.

Moraxella bovis could not be isolated from the eyes after inoculation of the organism. Serum agglutinins were not demonstrated.

Group II - Subcutaneous injection with M. bovis #2.

The serum of this group contained no detectable agglutinins.

Group III - Intravenous injection with M. bovis #2.

There was no evidence of serum agglutinins in this group.

Group IV - Intramuscular injection with M. bovis #2.

There was no evidence of serum agglutinins.

Group V - Subcutaneous injection with M. bovis #2 plus aluminum hydroxide.

Transient titers of 1:50 were obtained about 21 days after the first injection, but disappeared by the 30th day post-inoculation.

Group VI - Subcutaneous injection with M. bovis #729 with Freund's Adjuvant.

There was no evidence of serum agglutinins. Large, well-encapsulated, fibrotic masses developed at the injection sites.

Group VII - Subcutaneous injection with M. bovis #8 with Amphogel.

Rabbit #2 had a measurable antibody response after the second injection, positive 1:25. This rose to 1:50 after the 3rd injection, but was negative at the 28th day. Fibrous masses were present at the injection sites.

Group VIII - Subcutaneous injection with M. bovis #8 - sonicated.

All serums were negative to the agglutination test.

Group IX - Intravenous injection with M. bovis #8 - sonicated.

All serums were negative to the agglutination test.

Group X - Intravenous inoculation of fluid culture media after removal of M. bovis.

All serums were negative to the agglutination test.

Bovine Infectivity Studies

Group I - Stillwater calves inoculated with M. bovis #2 in conjunctiva.

The organism was recovered from the eyes of both calves on the 6th-day post-inoculation and was still present on 26th-day post-inoculation. Lacrimation began on day six. Hyperemia of the conjunctiva began on 12th-day with severe conjunctitivis beginning on 16th-day. The serum agglutination test was positive at 1:50 on 16th-day and remained at this level until the 26th-day at which time the animals were turned to pasture. There was no evidence of clinical pinkeye at this time, but M. bovis could still be isolated from the eye.

Group II - Pawhuska calves inoculated with M. bovis #729 in conjunctiva.

The organism was recovered from the eyes of all calves on the 4th-day post-inoculation and remained in the eye throughout the experimental period. Lacrimation and conjunctivitis appeared on the 4th-day post-inoculation in all calves. Calf #755 developed a positive titer at 1:25 on day ten. The other calves remained negative to the agglutination test.

Bovine Immunization Studies

Group III - Pawhuska calves inoculated subcutaneously with M. bovis #8 plus aluminum hydroxide.

These animals were injected with the bacterin and kept in individual isolation stalls. Two weeks after the second injection, animals #591 and 594 had a positive titer at 1:50. The animals were challenged initially two weeks after the second injection with strain #746, M. bovis, and again 7 days later. The organism was recovered from #593

and BC 3 days after the 2nd challenge. Seven days after the second challenge, animals #591, 594 and 754 had a positive titer at 1:50. These animals did not develop clinical keratoconjunctivitis.

Group IV - Pawhuska calves incoulated subcutaneously with M. bovis #8 plus aluminum hydroxide adjuvant. (Table I).

These animals were injected with two, 5 cc. doses of bacterin 14 days apart and kept under normal range conditions. Two weeks after the second injection, 8 of 23 animals had an increase in titer of at least one dilution. One of the eight was carrying M. bovis and had clinical pinkeye. Four weeks after the second injection, two additional animals had an increase in titer of at least one dilution. M. bovis was not isolated from the latter two animals. During the entire experimental period, 14 of 23 animals demonstrated M. bovis. Thirty days after the beginning of the injection period #729 and 746 went through a moderate clinical syndrome of pinkeye evidenced by lacrimation and slight corneal opacity. Even though all animals were grouped together, there was no acute outbreak of pinkeye during the summer and early fall periods. Members of this same group were again injected and studied during the 1965 summer period. (Table II).

Group V - Pawhuska calves inoculated subcutaneously with M. bovis #8 plus Amphogel as the adjuvant (nursing calves).

These calves were inoculated with two, 5 cc. doses of the bacterin while nursing their dams. There was no evidence of increased titer due to the injection of the bacterin.

Group VI - Pawhuska cattle inoculated subcutaneously with M. bovis #729 with Amphogel as the adjuvant. (Tables III and IV).

TABLE I

1964

COMPARISON OF SERUM TITERS AND RESULTS OF CONJUNCTIVAL CULTURES IN WEANED CALVES INOCULATED SUBCUTANEOUSLY WITH M. BOVIS #8 PLUS ALUMINUM HYDROXIDE ADJUVANT

Animal No.	5-15*		5-29*		6-15		6-30		7-14		8-15		9-24		1-6-65	
	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer
726		200		100		100		50		0		50	+	100		100
727		0		25		50		50		50		0		0		0
728		25		200		50		0		0		0		0	A	
729		25	+	50	+	25		0		0	+	0		0		0
730		0		0		25		0		0		50		0		0
731		0		25		50	+	50		100		0	+	0	A	
732	+	200		100		0		25		0		50	+	100	+	200
733		100		400		50		0		50		25		50	A	
734		50		25		0		25		50		0		0		0
735		50		200	+	50	+	50		25		0		0		0
736		50		0		50		50		50		0		0		0
737		50		100		100		0		0		0		0	A	
738		0		100	+	50		25		25		0		25		0
739	+	50	+	0		50		25		0		0		0		200
740		50		200		50		50		0		0		0		0
741		25		0		50		0		0		0		0		0
742		400		25		50		25		50		25	+	100	A	
743		50		0		50		50		50	+	50		50	A	
744		100		50	+	0		25		0		0		25	A	
745	+	0		0		50		50		25		0		0		0
746	+	0	+	50	+	100	+	0		0		25	+	0		50
747		25	+	0		25		25		0		0		0		0
748		25	+	0	+	0		25		50		50		0	A	

* Bacterin administered.
\+ Isolation of M. bovis.
A Indicates animals sold from herd.
The numbers 25, 50, 100, 200, 400 indicate serum dilutions having a 1+ positive reaction.

TABLE II

1965

COMPARISON OF SERUM TITERS AND RESULTS OF CONJUNCTIVAL CULTURES IN YEARLING ANIMALS INOCULATED SUBCUTANEOUSLY WITH M. BOVIS #8 PLUS AMPHOGEL

Animal No.	4-28*		5-5		5-12*		5-19		5-26		6-2		6-16		6-30		7-14		7-28		8-11		8-25		9-11		9-22	
	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer
726	+	0		25		50		25		50		50		0		50	+	50	+	0		0		0	+	25		0
729	+	0	+	0	+	0	+	0		25	+	25		25	+	0		0		25	+	0		25	+	0		0
730		0	+	0		0		25		0		25		0		0		0		0		0		0		25		0
739		50	+	50		0		0		50		25		0		0		0		0		25		50		25		0
740		0	+	25		25	+	25	+	50		25		0		0		0		0		50	+	25		25		0
741		0		0		25		25		50		25		0		0		0	+	0		0		25		25		0
745		0		25		25		0		25		25		0		0		50		50		25		50		0		0
746	+	0		25		0		0		25		50		25		0		0		0		0		25	+	50		0
747		0		25		25		50		50		50		25		0		0		25		0		25		0		0

* Bacterin administered.
\+ Indicates the isolation of M. bovis from the eye.
25 Indicates a titer at a 1:25 dilution.
50 Indicates a titer at a 1:50 dilution.
x These same animals were used in 1964 and results appear on Table I.

TABLE III

1965

COMPARISON OF SERUM TITERS AND RESULTS OF CONJUNCTIVAL CULTURES IN ADULT CATTLE INOCULATED SUBCUTANEOUSLY WITH M. BOVIS #8 PLUS AMPHOGEL

Animal No.	4-28*		5-5		5-12*		5-19		5-26		6-2		6-16		6-30		7-14		7-28		8-11		8-25		9-11		9-22	
	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer
1		0		0		0		25		50		50		0		25		0		0		0		0	+	0		0
2		0		0		0		0		0		0		0		25		0		25		0		25		---		50
3		0		25		0		25		25		25		0		25		0		50		0		25	+	25		50
4		0		0		25		0		0		25		0		25		0		25		0		0		25	+	0
5		0		0		0		0		50		25		25		25		0		25		0		25		0	+	0
6		0		0		0		0		0		25		0		0		0		25		0				0		0
7		0		0		25		25		25		25		0		25		0		0		0		25		0		0
8		0		0		25		25		25		25		0		25		0		25		25				50		0
9		0		25		25		0		50		50		25		25		0		25		0		50		50		0
10		0		0		25		25		50		50		25		25		25		25		0		25		25		0
417	+	0		0		25		0	+	50		25	+	0		25	+	0		25		0		25		0		0
707		25		0		25		25		25		50		0		50		0		50		0		25		50		0
710		0		25		25		25		50		25		0		0		0		50		0		0		50		0
723		0		0		25		25		25		50		0		50		25		25		0		50		50		0
822		0		25		0		25		0		25		0		25		0		25		0		0		25		0
976		0		25		25		0		50		25		0		0		0		0		0		0		25	+	0

* Bacterin administered.

\+ Isolation of M. bovis.

The numbers 25, 50, 100 indicate serum dilutions having a 1+ positive reaction.

TABLE IV

1965

COMPARISON OF SERUM TITERS AND RESULTS OF CONJUNCTIVAL CULTURES IN NURSING CALVES INOCULATED SUBCUTANEOUSLY WITH M. BOVIS #8 PLUS AMPHOGEL

Animal No.	4-28*		5-5		5-12		5-19*		5-26		6-2		6-16		6-30		7-14		7-28		8-11		8-25		9-11		9-22	
	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer	Culture	Titer
704		0		0		0		0		50		25		0		25		25		25		0		50		50		0
707		0		0		0		25		0		0		0		25		0	+	25	+	25		0		25		0
711		0		0		25		25		25		25		25		0		0		25	+	0	+	0	+	0		0
714		0		25		0		0		0		0		0		0		0		0		0		0	+	50		0
716		0		0		0		0		0		0		0		0	+	0		25	+	0		0		0		0
717		0		0		0		0		25		25		0		0		0		0		25		0		0		0
719		0		0		0		0		0		0		0		0		0		0	+	0		25	+	0		0
722		0		25		0		0		0		50		0		0		0	+	0	+	0		50		50		0
773		0		0		50		0		0		0		0		0		0	+	0		25		25	+	0	+	0
774	+	0		0		25		0	+	0		0		0		0		25		0		0		25		0		50
775		0		0		25		0		0		0		0		0	+	0	+	0	+	0		25		0		0
951	+	0		50		0		0		0		0		0		0		0		25	+	25		0	+	0		0

* Bacterin administered.
\+ Indicates the isolation of M. bovis.
25 Indicates a 1+ reaction at a dilution of 1:25.
50 Indicates a 1+ reaction at a dilution of 1:50.

Twelve calves, eleven mature cows and 5 first calf heifers were given two, 5 cc. doses of the bacterin 14 days apart beginning April 28, 1965. These animals were bled and conjunctival swabs cultured weekly until June 2, 1965, when the sampling period was extended to a two-week interval. Several animals (#711, 729, 745) had small areas of corneal opacity in one eye. These areas were never observed to be over 5 mm. in diameter. An acute phase of the disease was not observed in any of these animals even though several animals were shown to harbor M. bovis for relatively long periods of time; up to 10 weeks.

Group VII - controls.

Ten purebred cows and their calves served as controls for this field trial. These animals were not available for the routine sampling procedure. They were therefore bled and cultured approximately every four weeks. Several of the calves (2R, 9R, 3R) showed lacrimation and varying degrees of corneal opacity. These focal areas of opacity ranged from 2 to 15 mm. in diameter. The degree of lacrimation appeared to be related to the severity of the corneal opacity. The mature cows also showed varying degrees of lacrimation, but little evidence of corneal opacity. There was no acute epidemic of infectious kerato-conjunctivitis in the control animals. The isolation of M. bovis was sporadic throughout the summer but the prevalence of the organism appeared to increase during the August and September period.

CHAPTER V

DISCUSSION

Evaluation of Testing Procedures

The failure of rabbits to consistently respond to injection of M. bovis with the production of agglutinins conforms to the observations of other workers. (Chowdhury, 1963; Henson and Grumbles, 1960).

One group of rabbits given a bacterin employing Amphogel as the adjuvant showed evidence of transient agglutinin formation (Group VII). The demonstration of these antibodies failed shortly before the 28th-day after the first inoculation. The absorption of antigen in these animals occurred at least to a limited extent, even though fibrous masses developed at the sites of inoculation. A latent challenge of these animals failed to demonstrate an anamnestic reaction.

Using Freund's complete adjuvant, small fibrous masses also developed at the sites of inoculation. The fibrous masses may have been responsible for the failure of absorption. The absorption could have been delayed to the extent that the antigen was released in quantities too small to effect stimulation.

There may be several reasons for the failure to consistently detect agglutinin production in rabbits. Failure of absorption of antigen due to the formation of the fibrotic swelling has already been

discussed. The interval between injections may have been too short. If this happened so that the antigen was reintroduced into the body while antibody resulting from the previous injection was still present, there might result a negative phase in which the level of humoral antibodies was depressed. A more acceptable theory suggests that if the injection of M. bovis does indeed stimulate specific agglutinins, it is possible that the use of the plate and tube agglutination test was not sufficiently specific to detect antibodies.

The antigenic properities of M. bovis have thus far been assumed to be due to components of the cell wall, probably a complex mucopolysaccharide. There is no evidence that an exotoxin is produced by M. bovis. Henson and Grumbles (1961) indicated the presence of a dermonecrotic toxin in the walls of viable and non viable M. bovis cells. Although chemical extraction procedures on the cell walls were not carried out in this investigation, attempts to use sonicated cell wall components as antigens failed to produce any evidence of specific agglutinin formation.

It is well to consider the reasons for developing and using an agglutination test. Attempts were made to develop a simple test by which immune and susceptible animals could be identified. It seemed feasible to assume that most veterinary practitioners have facilities to run a simple agglutination test using serum and an antigen. The development of a precipitin test was considered. The precipitation reaction depends on the agglutination of soluble antigens by the action of antibody. Since the antigen complex of M. bovis is considered to be in or on the cell wall, these antigens would be considered insoluble

antigens. Secondly, it has been shown that titration could not be carried out by dilution of serum with addition of a fixed amount of antigen to all tubes in the series because the tubes containing the higher dilutions of serum failed to show precipitation despite the presence of antibody. This failure is due to an excessive amount of antigen. For these reasons the precipitin test was not employed in this study.

The reaction of agglutination is in principle like that of precipitation; both are aggregative, but in this case particulate rather than soluble antigens are brought together by antibody. Titrations of the agglutinating ability of antisera are ordinarily carried out by serial dilution of serum and the addition of a constant quantity of antigen suspension to all dilutions. The possibility of prozone reaction is minimized because the agglutination reaction is between particulate antigens attached to or on the bacterial cell wall. Thus, an excess of reaction sites available for antibody attachment are reduced. It is then possible for lattice formation and visible agglutination to occur.

The development of the plate agglutination test was patterned after unpublished data from Ellis (1954). From the beginning of the project it was noted that serums from adult animals with no previous history of clinical keratoconjunctivitis may show agglutinins at a dilution of 1:25. The possibility of isoantibodies was considered. However, it is interesting to note that young animals, calved during the winter from dams showing no evidence of agglutinins also remained negative until exposure to the natural infection. Kliewer (unpublished data) has observed titers corresponding to a 1:200 or higher dilution

in young and mature animals recovering from the clinical disease.

The plate agglutination test as outlined previously appears to have some reliability in the detection of agglutinins. However, it was necessary to correlate the macroscopic test with a more reliable but more time consuming procedure. The antibodies in serum can be more accurately titrated by the tube agglutination test. The serial titers from 1:25 to 1:200 were used in the plate agglutination procedure. This is only an approximation of the actual serum dilution achieved since the serum was diluted not with saline but by the antigen itself. Correlation between the plate and tube procedures indicates that serums with a titer of 1:25 (plate method) have a titer of 1:40 with the tube agglutination procedure. Serums that contain no agglutinins as evidenced by the plate test were also negative to the tube test. However, in the arbitrary designation of reactions from 1 to 4 plus with the plate test, it is difficult to determine a constant correlation with the tube test. The tube test may go up to a 1:80 dilution with serums that react to the plate dilution of 1:25 but are negative at a dilution of 1:50. It, therefore, seemed necessary to designate all positive dilutions in the plate test as positive at the existing dilution and not try to distinguish between a 1, 2, 3 or 4 plus reaction.

Attempts were made to further define the specific antigens by their adsorption onto sheep erythrocytes. Even though sonicated cell wall components contained a complex protein and carbohydrate mixture, untreated, intact sheep erythrocytes adsorb the carbohydrate component of the cell wall complex.

The untreated sheep erythrocytes and antigen showed agglutination

similar to the tube agglutination test, but the reactions were generally one dilution lower. The test requires fresh sheep cells and it is necessary to prepare the antigen-cell material fresh each day. Even though these technics require considerable time to overcome, evidence indicates that the sheep erythrocytes did indeed adsorb some of the specific carbohydrate from the cell wall complex.

Sheep erythrocytes treated with tannic acid have been shown to adsorb specific protein antigens. (Boyden, 1951). This procedure was attempted in order to learn some specific information about the antigenic complex of M. bovis. Technical difficulties in using sheep cells and the damage caused by tannic acid itself made the use of this procedure impractical in this study.

The separation of the cell wall complex after sonic treatment is at best crude. Chemical separation of the complex is necessary to clarify the extent of protein-carbohydrate totals within the complex. The adsorption of some component by the sheep erythrocytes only suggests that the antigenic component is carbohydrate in nature. The failure of the treated sheep cells to perform in agglutination in no way eliminates the possibility of an additional protein type antigen.

Evaluation of Infectivitity Studies in Cattle

Attempts were made to obtain young calves with no previous history of exposure to or clinical evidence of infectious keratoconjunctivitis. Although animals were selected on the basis of these criteria, the serum of many animals contained agglutinins against the disease. It was therefore difficult to obtain animals under the conditions of

purchase that were free of exposure to or clinical signs of infectious keratoconjunctivitis.

Typical isolates of M. bovis were placed on the cornea and beneath the bulbar conjunctiva of negative calves to study the pathogenesis and immune reactions stimulated in these calves. M. bovis could usually be isolated from these animals for periods up to 365 days. Although these animals were not kept in isolation for this long a time, isolation of the organism during the winter months indicates that such animals remain as carriers rather than reinfection occurring.

Clinical evidence of infection usually developed about the 5th or 6th day after exposure as evidenced by lacrimation. Hyperemia of the scleral vessels followed beginning about the 12th to 13th day. Small, 1-2 mm. in diameter, opaque areas developed on the cornea with no ulcer formation. This is somewhat different than the results obtained by Kliewer (1958), who observed the development of ulcers on the center of the cornea. The development of agglutinins began about the time of the severe hyperemia and conjunctivitis. This observation may be important in the development of immune titers since animals going through the acute phase of the disease will usually show agglutinins in their serums. The animals that develop agglutinins rarely remain positive to the agglutination test for over thirty days. Even though some animals developed a titer, these same animals continued to harbor M. bovis in the eye.

The development of pinkeye lesions is distinctly different from those described for infectious bovine rhinotracheitis, even though the IBR virus may be isolated by the conjunctiva of calves harboring

M. bovis. The lesion of infectious bovine keratoconjunctivitis starts with a small ulcer in the center of the cornea and then progresses to a diffuse corneal opacity and conjunctivitis. The keratitis caused by IBR virus developes secondary to inflammation of the conjunctiva. The marked swelling of the conjunctiva and lids characterized by lymphoid hyperplasia suggests that this reaction is a primary effect of the virus. (Hughes, 1964).

The series of animals kept in the isolation facilities for the infectivity or pathogenicity studies were unique in that these animals harbored no M. bovis organisms and were negative to the agglutination test prior to the start of the experiment. The isolation facilities provided excellent control of the fly population as well as protection from the prevailing north and west winds. Sunlight was not significantly reduced by the metal screen.

These animals were kept in isolation 14 days after inoculation of the organism. The organism was recovered from the eye during the stages of lacrimation and hyperemia. The organism was not recovered at the time the animals were released from the isolation facilities. Forty-five days after the animals were turned out, one calf had a typical syndrome of infectious keratoconjunctivitis. M. bovis was isolated at this time. This evidence may indicate a cyclic growth pattern of the organism in the conjunctiva. The incubation phase of the organism is probably quite long in the conjunctiva, possibly as long as 30 days.

Not all animals that develop clinical signs of the infection develop detectable agglutinins. This would seem to imply that even

in natural outbreaks of the disease little or no immunity ensues. Further evidence for this conclusion was obtained when a group of animals at Pawhuska showed signs of acute infection during January, 1964. Many of these animals had experienced acute infectious keratoconjunctivitis in the fall of 1963 during pathogenicity studies of various strains of M. bovis.

Evaluation of Experimental Bacterin Under Field Conditions

The presence of agglutinins in the serums of young nursing calves with no previous history of infectious keratoconjunctivitis suggested the possibility of immune globulin absorption from the colostrum or milk of the dam. Previous experience (Dahlgren, 1960) indicates that the absorption of immune globulin occurs within the first 48 hours of postnatal life. Thereafter, intestinal absorption of such large particles is not possible. The passive immunity due to absorption of colostral antibody begins to wane within six weeks after absorption. The agglutinins being present in the calves after this 6-week period implies that the agglutinins may be in response to a specific antigen.

A young orphan calf in another experiment was unintentionally given 10 cc. of the M. bovis bacterin. This animal developed agglutinins to a titer of 1:100. During this same period, nursing calves given 10 cc. of the same bacterin showed no evidence of agglutinin development. No attempts were made to artificially challenge these calves. The failure to develop agglutinins in these calves was assumed to be due to the age of the animals or to the fact that these animals were still nursing heavily. This trial was conducted during January

when there was no known source of M. bovis available.

The same calves with their dams were inoculated again in the spring along with five heifers using the procedure previously outlined. The animals were herded together under typical Oklahoma range conditions. Purebred cows and calves served as controls, although they were not kept in the same pasture. Contact was possible at the ranch buildings and water tanks.

By beginning the immunization program in late April, it was planned to have as much immunity developed as possible before the critical disease period. This period in Oklahoma has usually been late May and early June with a second season occurring in late summer and early fall. The early season usually coincides with periods with moderate moisture and winds, while the later season is quite dry with light wind and dust conditions.

Except for one of the replacement heifers, none of the remaining twenty-seven animals had any evidence of agglutinins in their serum at the beginning of the field trial. (Table III). Three of the animals were carrying M. bovis in their eyes. There was no correlation between the isolation of the organism and the presence of the agglutinins. During the five month trial period, the mature animals remained relatively free of M. bovis on conjunctival culture. Within 14 days after the first injection of the bacterin and adjuvant, 10 of the 16 mature animals began showing titers at a 1:25 dilution. (Table III).

A somewhat similar pattern developed with the 12 offspring calves. (Table IV). The isolation of M. bovis was sporadic until the late August and early September period. The agglutinins in these calves did

not develop in any logical pattern as observed with the mature cows.

There was no acute outbreak of keratoconjunctivitis in the test animals; while in similar herds in the Osage area, the disease was prevalent in the later period especially in yearlings and older weaned calves with severe ulceration and blindness.

The group of 9 yearlings used in the 1964 trial were again inoculated with the bacterin in the same manner as previously described. (Table II). These animals were in poor nutritional condition. These animals developed agglutinins for M. bovis. However, M. bovis was cultured from at least several of these animals during all periods throughout the trial as well as at the beginning of the trial. It is difficult to establish the relationlship between the experimental bacterin and the development of the titers in this group. Two of these animals did progress through the subacute stage of the disease, but there were no extensive ulcers observed. There was no evidence of an acute outbreak of keratoconjunctivitis in this group.

The purebred cows and their offspring which served as controls in this trial could not be cultured and bled at frequent intervals. However, they were observed almost daily in small lots. There was some evidence of lacrimation in both the cows and calves. M. bovis was isolated from many of the controls during the late summer period. The presence of specific agglutinins could not be correlated with natural exposure to the organism. There was no acute syndrome of the disease within this control group until late October when cows 945H and 929H showed lacrimation and ulceration. M. bovis was isolated from these eyes at this time.

Critical evaluation of results obtained from the group of calves inoculated in 1964 indicated that these animals were carrying agglutinins against the M. bovis organism before the trial period began. (Table I). The herd history prior to purchase of this group suggested numerous severe acute and chronic cases of infectious keratoconjunctivitis. It is difficult to evaluate the ability of the experimental bacterin to produce serum agglutinins or the ability to provide immunity against the disease in the presence of the pathogenic organism even though this group did not experience an acute clinical outbreak of the disease.

The group of mature cows and heifers represents the most homogeneous group used in the field trials. (Table III). These animals were not carrying agglutinins against the organism, nor was the organism isolated from the eyes except for rare instances. It appears then, that in this group the development of the agglutinin was due to the experimental bacterin. It is well to keep in mind that the response measured by the agglutination test does not necessarily indicate an accurate measure of the agglutinins developed, nor does it define the degree of immunity a given animal might have attained.

The development of agglutinins in this group began one week following the first injection. (Table III). The number of animals showing agglutinins increased after the second week postinoculation. There is a stable period of two more weeks when the number of animals showing agglutinins did not increase. Following this period or by the fifth week postinoculation, another increase in the number of animals showing agglutinins was observed.

Figure 1 indicates the overall percentage of animals showing agglutinins on a given date for periods up to ten weeks after the first injection. This does not measure a titered response to the experimental bacterin. The cyclic rise and fall in the number of animals showing agglutinins cannot be explained. The serums of animals taken during the peak and minimal period shown by this graph have been tested by the plate and tube agglutination procedures to eliminate the possibility of erroneous results. Both procedures have shown the absence of agglutinins during the minimal periods.

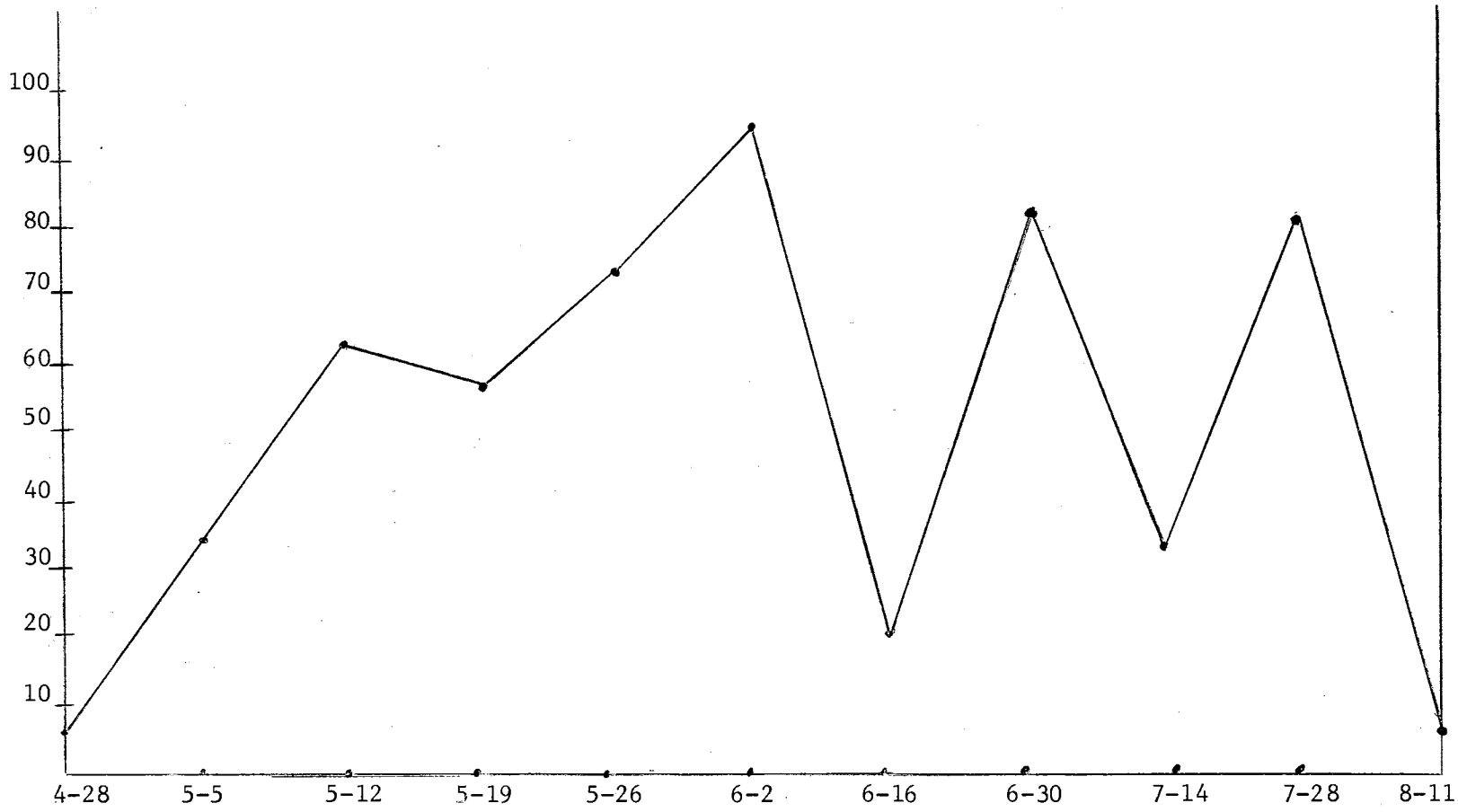

Fig. 1 Percentage of animals from Table II showing a titer on a specific date.

CHAPTER VI

SUMMARY AND CONCLUSIONS

This investigation was divided into two phases. Phase I concerned the development of a simple, accurate laboratory procedure to detect the presence of specific seroagglutinins in cattle manifesting clinical signs of infectious keratoconjunctivitis or in cattle which had recovered from the disease. Phase II involved the attempted *in vivo* evaluation in cattle of an experimental bacterin composed of killed *M. bovis* organisms and an adjuvant.

The first part of this study describes the preparation and application of a plate agglutination test. This test is accurate in detecting seroagglutinins stimulated in response to a subacute or chronic infection of keratoconjunctivitis, with the agglutinins developing within 10 to 14 days after initial infection. The exact level of agglutinins is more accurately determined using the tube agglutination and the sheep erythrocyte hemagglutination procedures. However, the level does not rise above the initial level regardless of the persistence of the infection or even a delayed appearance of clinical infection.

In the second part of this study, several experimental bacterin preparations containing various adjuvants were used in an attempt to stimulate the development of specific agglutinins. The most

significant rise in titers were obtained in a group of mature cattle injected with a bacterin containing aluminum hydroxide as the adjuvant. These animals, which were from the permanent herd, were carrying minimal detectable M. bovis infections in their eyes and had few pre-inoculation titers. The development of specific agglutinins in response to the injection of the same bacterin-adjuvant complex in their calves and other cattle was not consistently apparent in the results and was found not to be statistically valid. The response was apparently masked by infections represented by the presence of preinoculation titers, the isolation of M. bovis from the conjunctiva of many animals and a lack of immunologic stimulation in many of the animals.

It is not possible to equate immunity in relation to the presence of specific seroagglutinins for M. bovis in infectious bovine kerato-conjunctivitis. Natural and artificial infections of M. bovis can be established in cattle even in the presence of specific seroagglutinins. Repeated field trials might prove helpful in determining the value of a bacterin. Even though none of the vaccinated cattle at the Pawhuska Station have experienced any acute clinical outbreaks of infectious keratoconjunctivitis, the inferred conclusions are statistically invalid because of the multinomial type problems which could not be controlled in the circumstances under which this study was conducted.

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