A study on the immune response to *Mycoplasma bovoculi* in calves

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EXPLANATION OF THESIS FORMAT

This thesis consists of an introduction, literature review, two separate manuscripts, references and acknowledgements. The masters candidate, Barik Abdul-Kadir Salih, is the senior author and principal investigator for each of the manuscripts.

INTRODUCTION

A variety of microorganisms have been incriminated in early studies on the etiology of infectious bovine keratoconjunctivitis (IBK). These included bacteria, viruses, rickettsia like organisms (RLO) and chlamydia. However, no mycoplasmas were isolated from such cases (Wilcox, 1968).

Gourlay and Thomas (1969) first reported on the isolation of several strains of mycoplasmas from the eyes of cattle with IBK in Great Britain. Those isolates were T-mycoplasma, Mycoplasma bovirhinis, Acholeplasma laidlawii and an unidentified strain from four out of 20 cases. Langford and Dorward (1969) also isolated a mycoplasma from the eyes of calves in two naturally occurring outbreaks of IBK and from a third group of calves exposed to an animal which had ocular exudates instilled into his eyes. The preliminary typing studies indicated that this mycoplasma was not serologically related to any known bovine mycoplasmas.

Some years later Langford and Leach (1973) characterized the new strain of mycoplasma that had been isolated in 1969, on the basis of its cultural, morphological, biological and serological properties and they proposed the name Mycoplasma bovoculi for the new species.

Following its characterization, several reports on the isolation of Mycoplasma bovoculi from the eyes of cattle with IBK have been published (Nicolet, 1974; Friis and Pedersen, 1979; Rosenbusch and Knudtson, 1980 and Kelly, 1983).

In an attempt to understand the role of Mycoplasma bovoculi in IBK, Friis and Pedersen (1979) instilled M. bovoculi broth culture into the eyes of colostrum-deprived calves followed by Moraxella bovis and 5 days later produced conjunctivitis and then keratitis. This indicated that M. bovoculi might have an enhancing effect on the pathogenicity of Moraxella bovis.

In a separate trial Rosenbusch and Knudtson (1980) instilled M. bovoculi and ureaplasma broth cultures into the conjunctival sac of six calves. Mild localized conjunctivitis and serous lacrimation appeared 3-4 days following exposure to M. bovoculi. Diffuse conjunctivitis and watery lacrimation was noticed after the same period following exposure to ureaplasma. Recently, Rosenbusch (1983) studied the role of M. bovoculi and ureaplasma species in IBK. Calves infected with M. bovoculi followed by Moraxella bovis developed bilateral keratitis. No keratitis was seen in calves exposed to ureaplasma and Moraxella bovis. Also, M. bovoculi appeared to extend Moraxella bovis colonization in all calves while ureaplasma showed this

property in two calves only. These results confirm the work done by Friis and Pedersen (1979).

The objectives of this work were to study the antibody response to M. bovoculi in calves, to obtain information on its protective role in vitro, and to attempt to produce conjunctivitis in sheep as a model for future studies.

LITERATURE REVIEW

Bovine immunoglobulins

Four antigenically distinct bovine immunoglobulin classes have been described, these include IgG, IgM, IgA and IgE. The biological activity of each class have been reviewed by Butler (1983). The IgG class, which had been most extensively studied contains two subclasses IgG_1 and IgG_2 Both subclasses fix bovine complement, but IgG_1 fix guinea pig and rabbit complements more efficiently (Butler, 1983). Cytophilic activity for bovine alveolar macrophages was found with IgG_1 while IgG_2 had similar activity for polymorphonuclear leucocytes. The IgM class was found more effective than IgG in fixing complement and also had agglutinating activity. Secretory IgA had blocking and neutralizing activities. It acts by preventing bacterial or parasitic adherence or colonization and by inhibiting virus penetration (Butler, 1983). It also had agglutinating activity like IgM (Butler, 1983). In humans, serum IgA blocks direct binding of complement components or indirect complement binding mediated by IgM or IgG (Griffiss, 1983). It was found that IgA function as a regulatory immunoglobulin by shunting a small antigenic mass away from polymorphonuclear leukocytes (PMN's) into monocyte-macrophage immune mediator cells

(Griffiss, 1983). Monocyte opsonization by IgA for complement independent bactericidal activities was demonstrated previously (Lowell et al., 1980). Bovine IgE activates rat mast cells in a manner similar to human IgE.

The concentration of these different classes and subclasses of immunoglobulins in different body fluids depends on many factors. Spiegelberg (1974) reported that the concentration of a particular class in serum will depend on the number of plasma cells, the rate of synthesis, the rate of catabolism and the rate of exchange between intra- and extravascular spaces. The percentage of plasma cells forming a particular class of immunoglobulin was found to be different. Hanson et al. (1983) reported that more than half of all immnoglobulin producing cells may be synthesizing IgA. Osserman et al. (1964) indicated that the rate of synthesis of immunoglobulin per plasma cell was similar for all classes. However, according to Hanson et al. (1983) IgA appeared to be produced at a higher rate than any other immunoglobulin. The rate of catabolism of an immunoglobulin is dependent in part on the structure of the constant region of the heavy chain. Nakamura et al. (1968) reported that the distribution between plasma and lymph spaces was dependent on the primary structure of the immunoglobulin.

In external secretions, IgA was found to predominate

(Mach and Pahud, 1971; Butler, 1983). Two factors appeared to be responsible for the increased concentration of IgA in secretions. First, IgA combines with a secretory component formed by the epithelial cells of the glands. Second, it is mainly produced locally in lymphoid tissues around the glands (Butler, 1983).

Immunoglobulins of newborn calves

It is well-documented that there is no prepartum transfer of immunoglobulins between maternal and fetal tissues in cattle, and that the newborn calves obtained their antibodies mainly from colostrum (Spiegelberg, 1974; Porter, 1979). It has been demonstrated that bovine fetal and newborn calf sera collected prior to colostrum ingestion contains both IgM and IgG immunoglobulins (Porter, 1979). Cells producing these classes were present by 59 and 145 days of gestation (Schultz et al., 1973). This fetal stimulation might be derived from its own antigens or from maternal or microbial antigens (Schultz et al., 1973).

Smith and Ingram (1965) studied the presence of immunoglobulins in colostrum-deprived and colostrum fed calves. An average of 7.4% of gamma-globulins were found in sera of one day old colostrum-deprived calves, while an average of 31.3% were found in sera of calves 12-24 hours

after feeding colostrum. Colostrum-deprived calves had a steady increase in gamma-globulin levels from one day to two months of age. The colostrum fed calves showed a drop in gamma-globulin level throughout the first month and a slight rise during the second month.

Immunoglobulin A producing cells were not demonstrated in the bovine fetus and its production is normally not observed until after birth, a time when massive stimulation with environmental antigens occurs (Schultz et al., 1973). Arora et al. (1980) studied the appearance of immunoglobulins in lacrimal secretions of newborn calves and found that immediately after birth no known immunoglobulins were detected. IgG appeared first shortly after birth while secretory IgA appeared after 4 weeks.

The production of antibodies to different types of antigens in colostrum-deprived and colostrum fed calves was investigated by Husband and Lascelles (1975). Both groups responded similarly when injected intramuscularly with egg albumin, an antigen to which there was no circulating maternal antibodies. Greater responses were observed to other antigens injected at birth and at three weeks of age in colostrum-deprived calves than colostrum fed calves. Specific antibodies to these antigens were present in maternal circulation. These observations were in agreement

with the work done by Smith and Ingram (1965). The immunologic unresponsiveness of neonates appeared to be due to the presence of maternal antibodies in circulation rather than due to the immaturity of the lymphoid system. The age at which these calves were challenged also seems to affect the immune response, younger calves (less than 1 month of age) responded less than older calves (Smith and Ingram, 1965; Husband and Lascelles, 1975).

Antibody responses to mycoplasmas

The major mycoplasmal surface antigens (glycolipids and proteins) are located on the plasma membrane since mycoplasmas lack cell wall (Fernald, 1979). Glycolipids are generally poor immunogens unless attached to protein. It was found that glycoproteins isolated from the surface membrane of Mycoplasma pneumoniae were more immunogenic than glycolipids (Kahane and Brunner, 1977). These antigens together with other internal antigens which might become exposed after degradation of the mycoplasma are important in eliciting the immune response.

Local and systemic immune responses to different mycoplasmas have been studied. Brunner et al. (1973) reported that nasal secretions and sputa of humans showed a predominant IgA activity detected as early as one week

following inoculation with M. pneumoniae. Similar results were demonstrated in calves naturally infected with M. bovis (Boothby et al., 1983). In mice, the intranasal inoculation of M. pulmonis revealed IgG_1 and IgG_2 in tracheobronchial washings (Casell et al., 1974). Sera of pigs inoculated with M. hyorhinis had increased IgG activity (Ross et al., 1973) while sera of calves naturally exposed to M. bovis showed a relatively high IgM and low IgG and IgA activities (Boothby et al., 1983). The route of inoculation appeared to play a role in stimulating a specific immunoglobulin class response. One calf given M. bovis antigen in one dose subcutaneously and 5 weekly doses intravenously developed IgM after one week followed by IgG after 4 weeks. Another calf given 6 weekly intravenous doses developed only IgM antibody after 4 weeks with no IgG response (Carroll et al., 1977).

The possibility that local and systemic immune systems exist as separate compartments in responding to mycoplasmal organisms is still not clear. Gourlay and Howard (1982) reported that several studies conducted with respiratory mycoplasmas revealed that the inoculation of mycoplasmal antigens locally followed by parenteral route gave better protection against respiratory infection than with a single route.

Several serological tests have been used for the detection of antibodies to mycoplasmal infections. These include growth inhibition, metabolic-inhibition, mycoplasmacidal activity, agglutination, indirect hemagglutination, latex agglutination, inhibition of film production, radial growth precipitation, complement fixation, immunodiffusion, radial hemolysis, immunofluorescence, radioimmunoprecipitation and the enzyme-linked immunosorbent assay. The sensitivity of these tests vary and only some of them detect specific immunoglobulin classes.

The correlation between the presence of antibodies and protection against mycoplasmas is still contraversial. Whittlestone (1976) reported on the conflicting studies by others in regards to the protective activity of antibodies detected by different serological tests against several respiratory mycoplasmas. A majority of these tests detected antibodies that had no correlation with protection. The metabolic-inhibiting and hemagglutinating antibodies were among those whose protective activities was questioned. Taylor and Howard (1981) showed that neither convalescent serum nor immunoglobulin fractions inhibited the growth of M. pulmonis in the metabolic-inhibition test. Antibodies detected by the mycoplasmacidal (complement dependent) test were shown to possess protective activities. Brunner et al.

(1973) found that these mycoplasmacidal antibodies were correlated with resistance to M. pneumoniae infection in humans. Taylor and Howard (1981) demonstrated similar results with M. pulmonis.

Activation of the complement system seems to be of importance in the defense mechanism. According to Bredt et al. (1977), M. pneumoniae was demonstrated to have the capability of activating the complement system of guinea pigs via the classical and the alternative pathways, however the killing effect was shown to be a property of the classical pathway.

In a study done by infecting guinea pigs with M. pneumoniae intranasally, a significant increase in the complement components C1, C2, C3 and C4 in bronchial secretions were observed, which then decreased after two weeks, a time when the antibody titer in serum started to increase (Loos and Brunner, 1979). It was shown that the first complement component had higher binding capacity for M. pneumoniae and M. hominis than M. orale and M. fermentans. Rounding and killing of these organisms by the activated complement system suggested that an antibody-independent mechanism may contribute to the immune defense against some mycoplasmas (Bredt et al., 1977).

The protective activity of different immunoglobulin

classes in vivo was studied by Taylor and Howard (1981). Incubation of M. pulmonis with either convalescent serum, IgG_1 or IgG_2a for 30 min in vitro, followed by intranasal inoculation into mice revealed no mycoplasma isolation from the lungs after 24 hours. Mice inoculated with mycoplasmas treated with IgG_2b or IgA showed significantly lower numbers of isolated mycoplasmas than controls. They concluded that the presence of these immunoglobulin classes in the lungs of mice were capable of mediating resistance to M. pulmonis. Immunoglobulin A does not fix complement but it might mediate resistance by inducing a complement independent bactericidal activity of monocytes (Lowell et al., 1980).

Studies on the protective activity of whole serum antibodies have also been conducted. According to Taylor (1979), the passive transfer of convalescent serum to recipient animals protected cattle from M. mycoides var mycoides, pigs against M. hyorhinis and M. hyopneumoniae and mice against M. pulmonis. It was suggested that sensitization of lymphocytes by the mycoplasmas was inhibited by the serum which in turn suppresses the accumulation of lymphoid cells in the peribronchial and perivascular areas of the lungs. Cole et al. (1969) showed that passive transfer of convalescent serum from animals infected with M. arthritidis protected the recipient animal from infection.

Pigs inoculated intranasally with M. hyosynoviae developed strong serum antibody responses and this was found important in protection against the infection (Zimmerman and Ross, 1982). However, Gourlay et al. (1975) detected high serum antibody titers in cows inoculated with M. dispar into the mammary gland. These antibodies did not appear to protect against the infection. Also, it was found that the bovine mammary gland reinfected with M. dispar possesses immunity only in the quarter previously infected rather than in the whole gland. Similar observations were reported by Bennett and Jasper (1978). In contrast, Gourlay et al. (1975) demonstrated that ureaplasma infection of the mammary gland was found to provide generalized immunity. The role of serum antibodies developed in cattle challenged with M. bovis into the mammary gland was not clearly shown to be associated with providing resistance (Bennett et al., 1980). In humans, the presence of serum antibodies to M. pneumoniae in healthy individuals did not appear to protect against infection (Smith et al., 1967; Fernald and Clyde, 1970; Brunner et al., 1977).

The prevalence of mycoplasmas in host tissues might also play a role in the immunological mechanisms. Mycoplasma bovis was isolated from the bronchioalveolar region of 25 out of 30 healthy calves and from the nasal region of 15 of those

calves (Boothby et al., 1983). It was found that M. pneumoniae could be isolated from healthy young children as frequently as from diseased children (Brunner et al., 1977). This indicated that mycoplasmas exhibit tissue tropism within the respiratory tract. The fact that calves naturally infected with M. bovis showed no increase in IgA response indicates that M. bovis is a common symbiont of the respiratory tract (Boothby et al., 1983). Such immunologic unresponsiveness in experimental animals to indigenous microflora has been reported (Berg and Savage, 1975).

Biological mimicry between mycoplasmas and their hosts is another important subject in their immunologic responsiveness. Cole et al. (1970) showed a positive correlation between the ability of mycoplasmas to induce disease and lack of metabolic-inhibition (MI) antibody formation by its natural host. Mice, rats and guinea pigs immunized with M. pulmonis, M. neurolyticum, M. arthritidis and M. felis had no or very low MI-titer in their natural hosts, however high titers were obtained in unnatural hosts. Cahill et al. (1971) indicated that the occurrence of a common antigen between M. arthritidis and rat tissues may be rendering the organism less immunogenic which in turn might enable it to resist the host immune defenses.

Enzyme-linked immunosorbent assay

Several techniques were used for the preparation of mycoplasmal antigens for the test. Howard et al. (1982) obtained good results using a cell lysate antigen. Nicolet et al. (1980) compared the specificity of Tween 20 solubilized antigen and SDS-solubilized antigen for the detection of antibody activity to M. hyopneumoniae in pigs. They reported that both antigens showed good activity but Tween 20 antigen had higher specificity.

Metabolic-inhibition (MI) test

Antibodies to a specific mycoplasma slow the growth of the organism or stop it completely in the MI test. The tests described by Purcell et al. (1966) and Taylor-Robinson et al. (1966) differ basically in substrate utilization. Mycoplasma multiplying in liquid medium metabolize the specific substrate and produce a pH change that is detectable by an appropriate pH indicator. Specific antibodies inhibit multiplication and indirectly prevent color change. Unheated guinea pig serum may be added as a source of complement in the test. It may either enhance the MI serum antibody titer or retard the decrease in antibody titer that may otherwise occur on prolonged incubation time.

Ovine infectious keratoconjunctivitis

Infectious keratoconjunctivitis in sheep is not as complex as that in cattle, where a number of microorganisms and several predisposing factors complicate the etiology of the disease (Wilcox, 1968). Among the mycoplasmas most frequently isolated from cases of keratoconjunctivitis in sheep was M. conjunctivae which can be considered also as the sole cause of the disease. Other mycoplasmas such as M. arginini, M. ovipneumoniae, A. oculi and ureaplasma were also recovered from such cases and in some instances together with M. conjunctivae (Cottew, 1979).

Two forms of the disease have been described, the so-called follicular conjunctivitis is characterized by the development of numerous enlarged lymphoid follicles on the lower and third eyelids and the development of chemosis. The non-follicular form comprises a transient conjunctivitis followed by keratitis which becomes very severe in some animals (Jones et al., 1976).

In goats, a number of the mycoplasmas mentioned above were also recovered from animals suffering from kerato-conjunctivitis (Trotter et al., 1977).

Experimental reproduction of the disease with M. conjunctivae in sheep and goats has been conducted by several workers (Jones et al., 1976; Trotter et al., 1977).

PART I. ANTIBODY RESPONSE IN CALVES EXPERIMENTALLY OR NATURALLY EXPOSED TO MYCOPLASMA BOVOCULI

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ANTIBODY RESPONSE IN CALVES EXPERIMENTALLY OR NATURALLY EXPOSED TO MYCOPLSMA BOVOCULI

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ABSTRACT

An antiglobulin-ELISA has been developed to detect antibody activity to Mycoplasma bovoculi in sera, nasal fluids and lacrimal fluids of field and experimental exposed calves. Low IgG activity with no IgM or IgA was detected in sera of experimental calves. In nasal and lacrimal fluids, IgA appeared as early as the first week following exposure to M. bovoculi. This isotype predominated in both of these fluids throughout the 9 week observation period. Sera from field exposed animals showed high IgG and IgM activities.

The metabolic-inhibition test was applied to detect growth inhibition of M. bovoculi in those fluids. This property was found only in sera of exposed animals and thus could be used to test for M. bovoculi infection.

The ELISA test was found to be more specific than the metabolic-inhibition test in detecting antibodies to M. bovoculi and was considered a reliable test for its diagnosis. The implication of the finding of negative growth-inhibiting activity in nasal and lacrimal fluids concurrent with a high IgA activity are discussed.

INTRODUCTION

Mycoplasma bovoculi has been isolated frequently from the eyes of cattle with infectious bovine keratoconjunctivitis (IBK) together with Moraxella bovis (Langford and Leach, 1973; Friis and Pedersen, 1979). It has been reported that M. bovoculi produces conjunctivitis in calves (Rosenbusch and Knudtson, 1980) and that it plays an important role in the pathogenesis of Moraxella bovis (Rosenbusch, 1983).

The purpose of this study was to develop assays for the detection of specific antibody responses to M. bovoculi and to attempt to identify serum, nasal and lacrimal fluid immunoglobulin classes which inhibit the growth of M. bovoculi in vitro.

MATERIALS AND METHODS

Mycoplasma

The FS8-7 strain of M. bovoculi (Rosenbusch, 1983) grown in dialysate medium as previously described (Kenny and Cartwright, 1977) was harvested by centrifugation (18,000 X g for 20 min at 4°C) washed three times in Hepes-saline (0.005 M pH 7.4) and resuspended either in distilled water or in 0.0125 M phosphate buffer at 4 mg/ml (Lowry et al., 1951).

Preparation of antigens for ELISA

Two methods were used: In a Tween 20 solubilization method (Nicolet et al., 1980), washed M. bovoculi cells in 0.0125 M phosphate buffer pH 7.1 were solubilized with 1% Tween 20 and incubated for 90 min at 37°C. The supernatant collected following centrifugation (48,000 X g for 60 min at 4°C) was designated as the Tween 20 antigen. An osmotic lysis technique was the second method (Razin and Rottem, 1976). Breifly, 1 ml of crude M. bovoculi antigen contains 4 mg protein was brought up to 3 ml with 0.25 M sodium chloride, centrifuged at 48,000 X g for 30 min. The pellet was resuspended in 2 ml of 2 M prewarmed glycerol and incubated for 10 min at 37°C, then loaded into 5 ml syringe and injected very rapidly into 250 ml of prewarmed distilled water and incubated for 15 min at 37°C. The

pellet was collected after centrifugation (as above), washed three times first with 25 ml of cold (4°C) distilled water, 25 ml of cold sodium chloride (0.05 M) in 0.01 M phosphate buffer pH 7.5 and finally with another 25 ml of cold distilled water, and then resuspended into 1 ml of 1:20 dilution of beta-buffer (0.15 M NaCl, 0.05 M Tris-HCl and 0.01 M 2-Mercaptoethanol pH 7.4). The 1 ml suspension was layered on 4.5 ml of a 45% sucrose cushion, centrifuged at 27,000 X g for 30 min using a swinging bucket rotor (SW-39). The floating bands in the interphase were collected and used for the ELISA.

Antisera, nasal and lacrimal fluids

Antisera were collected from cattle of various ages from two different herds naturally exposed to M. bovoculi (group A and B). Sera were collected from 14 colostrum-deprived isolation raised calves and from 6 colostrum fed calves (group C and D). The sera were heat-inactivated at 56°C for 30 min and stored at -20°C. Nasal and lacrimal fluids were obtained from another group of cattle naturally exposed to M. bovoculi (6 animals) and from 6 colostrum-deprived isolation raised calves that were negative for M. bovoculi by culture (group E and F). Lacrimal fluids were collected by placing a piece of sterile gauze in the lower conjunctival sac. Nasal fluids were collected by introducing a small gauze pledget into the nasal

cavity. The moistened gauze pieces were removed after several minutes. Fluids were expressed from gauze by centrifugation (1,500 X g for 10 min) and a final concentration of 1 mM EDTA was added to the samples before being stored at -20°C.

Experimental animals

Three colostrum-deprived dairy-type calves were used. Each calf was raised from birth in an individual isolation room and fed twice daily with antibiotic-free milk replacer (Instant nursing formula, Land O Lakes, Fort Dodge, IA). Solid food (mixed grains and alfalfa pellets) was given in increasing amounts from the first week on. All 3 calves were sampled twice before infection and for 6 to 9 weeks following infection with M. bovoculi (These samples included sera, nasal fluids and lacrimal fluids). Sampling was done every 7 days and eye swab samples were taken and processed to detect the presence of mycoplasmas. Samples taken following infection were used to quantitate the conjunctival colonization by M. bovoculi as described previously (Rosenbusch and Knudtson, 1980). One ml of modified Bovarnick's solution (0.005 M mono-sodium glutamate and 0.28 M lactose in 0.02 M phosphate buffer pH 7.3 with 10% heat inactivated horse serum), was added to the eye swabs that were kept in tightly-capped tubes and stored for 1 h at 4°C. The swabs were discarded after expressing the absorbed fluid, then

centrifuged at 150 X g for 5 min. From the top layer of the supernatant, 0.2 ml was transfered to 1.8 ml of Friis's broth and 10-fold serial dilutions were made. The tubes were incubated for 14 days at 37°C and examined daily for growth indicated by increased turbidity and change in pH.

Infection of calves

Calves were infected by the bilateral instillation into the lower conjunctival sac of 1.5 ml of a broth culture of M. bovoculi. The broth culture was filtered through a sterile 450 nm Millipore membrane (Millipore Corp., Bedford, MA) and cloned by plating onto Friis's agar for single colonies. Further characterization of these cloned cultures were done by growth-inhibition and immunofluorescence tests (Erno and Jurmanova, 1973; Bradbury, et al., 1976). The organism was cloned twice and used at the 8th passage with a titer of 10^8 (CCU/ml) color changing units/ml. Calves number 91 and 108 were infected at the age of 18 days while calf number 107 was infected at the age of 21 days.

Enzyme-linked immunosorbent assay

Indirect-ELISA The method described by Engvall and Perlmann (1972) was used to determine the best antigen preparation reacting specifically with antibodies to M.

bovoculi. The assay was performed using polystyrene microtiter plates (Dynatech, Alexandria, VA). The plates were incubated with 100 ul of Tween 20 antigen or osmotic-lysis antigen diluted to 1 ug/ml (Wang and Smith, 1975; Lowry et al., 1951) with 0.1 M sodium carbonate for 3 h at 37°C, then stored at 4°C until used. Just before testing, the plates were washed four times with 0.15 M sodium chloride containing 0.5% Tween 20 (NaCl/T) to remove unattached antigen. The coated plates were then incubated for 1 h at 37°C with 100 ul of test antisera diluted to 1:100 with 0.05 M Tris-HCl pH 7.4 containing 0.15 M sodium chloride, 0.1% bovine serum albumin and 0.05% Tween 20 (TSBT). After washing three times with NaCl/T, 100 ul of horseradish peroxidase conjugated rabbit anti-bovine IgG (Cappel Laboratories, Cochranville, PA) diluted to 1:100 with TSBT was added and incubated for 2 h at 37°C. The plates were then washed three times with NaCl/T. Just before testing, substrate solution was prepared as follows: 16 mg of 5-aminosalicylic acid (Sigma Chemical Co., St. Louis, MO) was dissolved in 20 ml hot (60°C) 0.02 M sodium phosphate pH 6.0, filtered and 0.1 ml of 1% hydrogen peroxide solution was added. A 100 ul volume of this substrate solution was added to each well and incubated for 15 min at room temperature. The reaction was stopped by the addition of 100 ul of 1 N NaoH and the color change was measured spectrophotometrically at 488 nm with an ELISA-plate reader.

Antiglobulin-ELISA The method described previously (Howard et al., 1982) was employed for the detection of antibody class responses to M. bovoculi in serum, nasal and lacrimal fluids. The ELISA plates were coated with 100 ul of Tween 20 antigen diluted to 1 ug/ml in 0.1 M sodium carbonate and incubated for 3 h at 37°C, then stored at 4°C until used. The plates were washed four times with NaCl/T just before testing. The coated plates were then incubated for 1 h at 37°C with 100 ul of sera, nasal fluids or lacrimal fluids diluted to 1:100 with TSBT for IgG and IgM and to 1:10 for IgA class detection. After washing three times with NaCl/T, 100 ul of rabbit anti-bovine IgG, IgM or IgA (Nordic Laboratories, Tilburg, CA) diluted to 1:100 with TSBT was added and incubated for 2 h at 37° C. The plates were then washed three times and 100 ul of horseradish peroxidase conjugated goat anti-rabbit IgG (Cappel) diluted to 1:1000 with TSBT was added and incubated for 30 min at 37°C. After washing three times with NaCl/T, the substrate solution was added as described for the indirect-ELISA.

Metabolic-inhibiton (MI) test

A microtitration plate test previously described (Taylor-Robinson et al., 1966) was used. A test culture of M. bovoculi. was grown for 36 h in a modified base medium (Friis, 1975). The medium contained 50 ml of 1.23% w/v Brain Heart Infusion (Difco

Laboratories, Detroit, MI), 1.3% w/v PPLO Broth (Difco), 6.25% v/v Hank's balanced salt solution (Difco), 0.004% w/v DNA (Sigma), 0.01% w/v L-arginine (Matheson-Coleman and Bell, Rutherford, N.J.), 0.016% w/v L-glutamine (Sigma), 0.003% w/v phenol red (Baker Co., Phillipsberg, N.J.), 0.018% w/v NAD (Sigma) and 0.003% w/v cysteine (Matheson-Coleman and Bell); 2 ml PPLO serum fraction (Difco); 1 ml Fleischmann's dry yeast extract (Standard Brands Incorporated, New York, NY); 1 ml Thallium acetate (Fisher Scientific Co., Fair Lawn, N.J.); 1 ml Penicillin (Sigma); 45 ml double deionized water and 1 ml of 10% glucose. The medium was adjusted to pH 7.8 and used both as a diluent and growth medium. Aliquots of test culture of M. bovoculi were frozen at -70°C. This antigen was titrated in 10-fold serial dilutions by adding 0.05 ml of test culture dilutions to 0.15 ml of the medium and incubated for 6 days at 37°C. The highest dilution giving a change in color indicated the number of color changing units (CCU/ml) in the frozen antigen. For the MI test, two-fold serial dilutions were made by adding 0.025 ml of test sample to 0.025 ml of medium. Then 0.05 ml of antigen (300 CCU/ml) was added followed by 0.125 ml of medium. The plates were then incubated at 37°C for 44-48 h and read when control wells containing antigen and medium only (no test sample) had changed approximately 0.5 pH unit.

RESULTS

Enzyme-linked immunosorbent assay

Both antigen preparations used in the indirect-ELISA detected serum antibodies in cattle naturally exposed to *M. bovoculi* (group A). However, Tween 20 antigen gave lower values when tested with negative sera from unexposed calves (group C) than osmotic-lysis antigen (Table 1). On that basis, Tween 20 antigen was chosen to be used in the antiglobulin-ELISA.

Serum antibody responses of experimental calves were low (Table 2). Two calves reponded with very low IgG activity and no detectable IgM or IgA (data not shown) in the last two weeks of the experiment. Calf 108 died after the 6th week (severe enteritis) and did not give serum antibody response. Nasal fluids from these calves (Table 3), showed IgA activity detected at the 1st week following exposure in calf 107 and at the 2nd and 3rd weeks in calves 108 and 91. No IgG or IgM were detected (data not shown). In lacrimal fluids, IgA was detected as early as the first week following exposure in all three calves (Table 4). Low IgG activity was also detected in these fluids at the 8th and 9th weeks, but no IgM was found. Table 5 show the antibody responses of cattle naturally exposed to *M. bovoculi* and of unexposed calves. Antisera of group A and B showed a predominant IgG and low IgM activities. Sera of group C and D

Table 1. Results of an indirect-ELISA using a Tween 20 antigen and an osmotic-lysis antigen of Mycoplasma bovoculi for detecting IgG in sera of cattle naturally exposed to M. bovoculi and in sera of controls[a]

Animal group	Sample (# of animals)	Antigen	O.D. at 488 nm Mean (min - max)[b]
A	Serum (13)	Tween 20	3.50 (0.57-5.41)
		Osmotic lysis	5.02 (2.64-6.45)
C	Serum (14)	Tween 20	0.10 (0.02-0.23)
		Osmotic lysis	0.55 (0.12-1.52)

[a]The controls were colostrum-deprived calves (group C).

[b]Mean optical density at 488 nm, minimum and maximum values.

Table 2. Results of antiglobulin-ELISA for detecting IgG in sera of three calves before and following exposure to *Mycoplasma bovoculi*[a]

	Weeks before			Weeks after							
Calf #	2	1	0	1	2	3	4	5	6	7	8	9
91	0.02	0.04	0.04	0.07	0.09	0.14	0.14	0.12	0.22	0.21	0.31	0.23
107	0.03	0.02	0.03	0.04	0.02	0.04	0.12	0.10	0.08	0.16	0.25	0.31
108	0.02	0.02	0.03	0.05	0.03	0.04	0.05	0.14	NT[b]	NT	NT	NT

[a]The figures given are optical density at 488 nm. Values of $\leq$ 0.23 were considered as negative.

[b]NT, Not tested.

Table 3. Results of antiglobulin-ELISA for detecting IgA in nasal fluids of three calves before and following exposure to Mycoplasma bovoculi[a]

Calf #	Weeks before 2	1	0	Weeks after 1	2	3	4	5	6	7	8	9
91	NT[b]	0.16	0.14	0.15	0.09	0.34	0.43	0.95	0.60	0.69	0.41	0.31
107	0.08	0.29	0.08	0.32	0.36	0.39	0.79	0.56	1.49	0.93	0.60	0.38
108	0.11	0.13	0.11	0.19	0.34	0.89	0.94	1.12	NT	NT	NT	NT

[a]The figures given are optical density at 488 nm. Values of $\leq$ 0.23 were considered as negative.

[b]NT, Not tested.

Table 4. Results of antiglobulin-ELISA for detecting IgA in lacrimal fluids of three calves before and following exposure to Mycoplasma bovoculi[a]

	Weeks before			Weeks after							
Calf #	2	1	0	1	2	3	4	5	6	7	8	9
91	NT[b]	0.12	0.16	0.38	0.40	0.98	1.16	1.16	2.46	3.97	2.71	0.94
107	NT	0.19	0.15	0.31	0.34	0.29	0.44	0.62	2.82	4.23	2.14	0.84
108	NT	0.02	0.04	0.39	0.47	0>89	0.76	0.72	NT	NT	NT	NT

[a]The figures given are optical density at 488 nm. Values of $\leq$ 0.23 were considered as negative.

[b]NT, Not tested.

had no detectable IgG or IgM except two animals from group D showed higher optical density values for IgG and one animal from the same group for IgM. Optical density values of ≤0.23 as measured for group C were considered as negative. Nasal fluids showed low IgA activity with no detectable IgG antibody (group E). In lacrimal fluids, IgA predominated over low IgG activity (group E). Nasal and lacrimal fluids from negative animals (group F) had no detectable antibodies.

Metabolic-inhibition antibodies

Table 6 show the metabolic-inhibition antibody titers of samples from three experimental calves, those with a titer of 16 or less were considered negative. Sera from all 3 animals had inhibiting activities noticed as early as the 1st, 2nd or 3rd week following infection. However, nasal and lacrimal fluids did not show such activity. A serum sample obtained from calf 107 two weeks before inoculation had a positive titer of 32 which became negative by the next week.

Samples obtained from field exposed animals and from unexposed animals were also tested by the MI test (table 7). Antisera of almost all animals of group A and B showed inhibitory effect while sera of group C and D did not. Nasal and lacrimal fluids of group E and F had a titer of 16 or less, which indicates their non-inhibitory activity.

Table 5. Antibodies in sera, nasal and lacrimal fluids of cattle naturally exposed to *Mycoplasma bovoculi* and of unexposed calves detected by the antiglobulin-ELISA[a]

Animal group	Samples	*M. bovoculi* isolated	IgG Mean(min - Max)[b]	IgM Mean(min - max)	IgA Mean(min - max)
A	Serum	+	2.73(0.35-6.56)	0.36(0.10-0.47)	NT[c]
B	Serum	+	1.88(0.17-4.58)	1.04(0.09-4.04)	NT
C	Serum	-	0.13(0.04-0.23)	0.06(0.02-0.14)	NT
D	Serum	-	0.19(0.04-0.73)	0.14(0.02-0.64)	NT
E	Nasal fl.	+	0.08(0.02-0.14)	NT	0.23(0.18-0.65)
E	Lacrimal fl.	+	0.34(0.12-0.45)	NT	1.88(0.64-2.54)
F	Nasal fl.	-	0.05(0.02-0.08)	NT	0.03(0.02-0.08)
F	Lacrimal fl.	-	0.08(0.04-0.12)	NT	0.10(0.05-0.17)

[a]The figures given are optical density at 488 nm.

[b]Mean optical density, minimum and maximum values.

[c]NT, Not tested.

Table 6. Metabolic-inhibition antibody titers in sera, nasal and lacrimal fluids of three calves exposed to Mycoplasma bovoculi[a]

Calf	Samples	Weeks before 2	1	0	Weeks after 1	2	3	4	5	6	7	8	9
	Serum	4[b]	8	16	16	16	32	32	32	32	32	32	32
91	Nasal fl.	NT[c]	8	8	2	8	8	4	16	4	8	8	8
	Lacrimal fl.	8	4	4	2	4	16	8	16	16	4	2	16
	Serum	32	8	8	16	32	32	32	32	32	32	64	64
107	Nasal fl.	2	8	16	8	8	16	4	4	8	16	16	16
	Lacrimal fl.	NT	16	8	4	4	4	4	4	4	8	16	16
	Serum	8	16	8	32	32	32	32	32	32	NT	NT	NT
108	Nasal fl.	8	16	16	16	4	8	16	16	16	NT	NT	NT
	Lacrimal fl.	NT	4	16	4	4	8	8	8	8	NT	NT	NT

[a]End points were read when the control wells only containing M. bovoculi and medium changed approximately 0.5 pH units.

[b]The figures given are the reciprocals of the titers (mean of triplicate wells). Titers of 16 or less were considered as negative.

[c]NT, Not tested.

Table 7. Metabolic-inhibition antibody titers in sera, nasal and lacrimal fluids of cattle naturally exposed to *Mycoplasma bovoculi* and of unexposed calves[a]

Animal group	Samples	*M. bovoculi* isolated	Metabolic-inhibition antibody titers
A	Serum	+	4[b], 128, 64, 64, 64, 32, 128, 64, 64, 128, 64, 64, 32
B	Serum	+	128, 64, 64, 64, 32, 32, 32, 64, 4
C	Serum	-	0, 0, 2, 2, 2, 8, 2, 2, 4, 2, 2, 0, 16, 0
D	Serum	-	4, 2, 8, 8, 4, 2
E	Nasal fl.	+	4, 8, 8, 8, 8, 8
E	Lacrimal fl.	+	0, 16, 8, 2, 4, 8
F	Nasal fl.	-	16, 8, 8, 4, 8, 8
F	Lacrimal fl.	-	16, 4, 8, 4, 8, 8

[a]End points were read when the control wells only containing *M. bovoculi* and medium changed approximately 0.5 pH units.

[b]The figures given are the reciprocals of the titers (mean of triplicate wells). Titers of 16 or less were considered as negative.

Colonization of M. bovoculi

As shown in table 8, extended colonization of the conjunctivae with M. bovoculi was seen in all 3 calves. In calf 91, a titer of 10^6 CCU/swab (color changing units/swab) was maintained for one or two weeks and then declined gradually. The other two calves had a titer of 10^6 for the first 5 weeks after challenge.

Table 8. *Mycoplasma bovoculi* colonization of the conjunctivae of three colostrum-deprived calves[a]

Weeks after exposure	Calf 91		Calf 107		Calf 108	
	L[b]	R[c]	L	R	L	R
0	0	0	0	0	0	0
1	6	6	6	6	6	6
2	6	5	6	6	6	6
3	5	5	6	6	6	6
4	3	5	6	6	6	6
5	3	3	6	6	6	6
6	2	2	NT[d]	NT	4	5
7	1	2	NT	NT	NT	NT
8	1	2	NT	NT	NT	NT
9	0	0	NT	NT	NT	NT

[a]Colonization is expressed as $\log_{10}$ CCU/swab (color changing units/swab).

[b]L, Left eye.

[c]R, Right eye.

[d]NT, Not tested.

DISCUSSION

Non-ionic detergents like Tween 20 are known to have specific properties in solubilizing cell membranes. They selectively solubilize proteins that are exposed on the surface of the membrane (Johansson and Hjerten, 1974; Helenius and Simon, 1975). A glycoprotein isolated from the surface membrane of Mycoplasma pneumoniae was shown to be immunogenic (Kahane and Brunner, 1977). Internal proteins might also trigger an immune response if they become exposed to the host defense mechanisms. The osmotic-lysis technique, which separates the whole plasma membrane and thus contains both internal and external membrane protein was applied for this purpose. Our data show that the osmotic-lysis antigen had higher antigenic activity than the Tween 20 antigen but was less specific. Solubilization of proteins with another neutral detergent (Triton X-100) was also attempted and the results were not superior to those obtained with Tween 20. Similar observations were reported by others (Nicolet et al., 1980). We also observed that storage of the antigen solubilized with Tween 20 at 4°C for 4 months did not affect its activity.

The low level of IgG antibody detected by the antiglobulin-ELISA in sera of two experimental calves indicates a minimal contact between the mycoplasma and the internal immune system.

In such a case, the host local defense system is expected to play a major role in responding to these organisms. In field and presumably multiply exposed animals, higher levels of serum IgG were found. Mycoplasmal infections are known to be persistent in nature and possibly these organisms might be phagocytosed and then presented to the systemic antibody producing cells.

Immunoglobulin A constitute the major immunoglobulin class in most bovine external secretions (Mach and Pahud, 1971; Bienenstock and Befus, 1980; Butler, 1983). We also found IgA to predominate in nasal and lacrimal fluids of both naturally and experimentally exposed animals. Brunner et al. (1973) also detected IgA antibody in nasal secretions of humans infected with M. pneumoniae.

In the metabolic-inhibition test, we found that only serum samples from cattle naturally exposed to M. bovoculi inhibited the growth of M. bovoculi. These results were in contrast with those of Taylor and Howard (1980) who examined how different classes of antibodies might exert their protective activities against Mycoplasma pulmonis in mice and found that neither convalescent serum nor immunoglobulin fractions inhibited the growth of M. pulmonis in the MI test. Further separation and purification of immunoglobulin classes in our serum samples needs to be done. The non-specific inhibition of M. bovoculi

growth by sera from experimental animals that lack any detectable antibodies by the antiglobulin-ELISA might be attributed to the developement of other inhibitory factors (Goodwin et al., 1969).

Nasal and lacrimal fluids showed no inhibitory effects despite the presence of high levels of IgA antibody. Secretory IgA is known to possess blocking and neutralizing activities (Butler, 1983). Brunner et al. (1973) suggested that the presence of IgA antibody in nasal secretions of humans might be related to their resistance to M. pneumoniae infection. In mice, resistance to M. pulmonis infection was found to be related to the presence of either IgG_1, IgG_2 or IgA class in the respiratory system (Taylor and Howard, 1980). However, IgA did not show any MI, mycoplasmicidal or opsonic activities when tested against M. pulmonis infection in mice (Taylor and Howard, 1981).

Our data show that M. bovoculi colonized the eyes of one calf for a period of 8 weeks. This property of extended colonization which was demonstrated by Rosenbusch and Knudtson (1980) appeared to be important for stimulating the immune response in our experiment. Even though conflicting results have been reported on the protective role of IgA, one calf in our experiment had no M. bovoculi at the time a sharp decline of IgA level in lacrimal fluids was noticed which might suggest the

mediation of other cellular responses by this antibody. It has been shown that IgA has monocyte-mediated bactericidal activity in vitro (Lowell et al., 1980). Further investigation using a larger number of animals is needed.

This report indicates that the antiglobulin-ELISA had greater specificity than the MI test for the detection of antibodies to M. bovoculi infection. Antibodies in nasal and lacrimal fluids were found to be predominantly of the IgA class while systemic antibodies were of the IgG class. The MI test detected antibodies to M. bovoculi in sera of field exposed animals but not in nasal or lacrimal fluids.

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PART II. EXPERIMENTAL EXPOSURE OF SHEEP TO MYCOPLASMA BOVOCULI: AN ATTEMPT TO PRODUCE CONJUNCTIVITIS

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EXPERIMENTAL EXPOSURE OF SHEEP TO MYCOPLASMA BOVOCULI: AN ATTEMPT TO PRODUCE CONJUNCTIVITIS

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ABSTRACT

Mycoplasma bovoculi, a bovine mycoplasma was instilled into the eyes of 6 sheep in an attempt to produce conjunctivitis. Two sheep reponded with mild hyperemia and watery lacrimation from days 3 to 5 following exposure, other sheep showed no symptoms. The organism was isolated from the eyes of 3 sheep at day 2 and from the left eye of one sheep at day 5 following exposure.

M. bovoculi did not appear to produce conjunctivitis and colonized sheep eyes only for a short period of time.

INTRODUCTION

According to Cottew (1979) several mycoplasmal species have been isolated from infectious keratoconjunctivitis in sheep and goats. These include Mycoplasma conjunctivae which was the most frequently isolated, Mycoplasma arginini, Mycoplasma ovipneumoniae, Acholeplasma oculi and ureaplasmas. Many of these mycoplasmas like M. arginini, A. oculi and ureaplasmas were also isolated from cases of infectious bovine keratoconjunctivitis (IBK) (Leach, 1970; Kelly, 1983; Goto et al., 1979).

Mycoplasma bovoculi, isolated frequently from the eyes of cattle with IBK (Langford and Leach, 1973; Friis and Pedersen, 1979) is known to produce conjunctivitis in calves (Rosenbusch and Knudtson, 1980). Stalheim et al. (1982) isolated a mycoplasma from the eyes of goats with keratitis which reacted with a specific antibody to M. bovoculi.

In this report, we attempted to produce conjunctivitis in sheep and to determine if M. bovoculi does colonize the sheep conjunctival epithelium.

MATERIALS AND METHODS

Six 5 month-old sheep (3 Suffolk lambs, 2 Columbia lambs and 1 Columbia ewe) were divided into two groups of three, and kept in separate isolation rooms. The eyes were examined grossly for the presence of any lesions. Eye swab samples were taken and processed for the presence of mycoplasmas as described previously (Rosenbusch and Knudtson, 1980). One ml of modified Bovarnick's solution (0.005 M mono-sodium glutamate and 0.28 M lactose in 0.02 M phosphate buffer pH 7.3 with 10% heat inactivated horse serum), was added to the eye swabs that were kept in tightly-capped tubes and stored for 1 h at 4°C. The swabs were discarded afer expressing the absorbed fluid, then centrifuged at 150 X g for 5 min. From the top layer of the supernatent, 0.2 ml was transfered to 1.8 ml of Friis's broth and a 10-fold serial dilutions were made. The tubes were incubated for 14 days at 37°C an examined daily for growth indicated by increased turbidity and change in pH. Two species of mycoplasmas were isolated from the eyes of sheep number 1 and 2. The colonies of these mycoplasmas were identified by indirect immunofluorescence (IIF) (Bradbury et al., 1976) using 8 different sheep and goat antisera. Mycoplasma conjunctivae was identified from the left eye of sheep number 2 and M. ovipneumoniae from both eyes of sheep number 1. No mycoplasmas

were isolated from the eyes of other sheep. Conjunctival scrapings were examined by the IIF technique for the presence of M. bovoculi.

The animals were infected two weeks after they were obtained. All sheep were challenged by the bilateral instillation into the cojunctival sac of 0.5 ml of broth culture of M. bovoculi strain FS8-7. The broth culture was filtered through a sterile 450 nm Millipore membrane (Millipore Corp., Bedford, MA) and cloned by plating onto Friis's agar for single colonies. Further characterization of these cloned cultures were done by growth inhibition and immunofluorescence tests (Erno and Jurmanova, 1973; Bradbury, et al., 1976). The organism was cloned twice and used at the 8th passage with a titer of 10^8 CCU/ml (color changing units/ml). The eyes were then examined daily for the presence of any lesions. Eye swab samples were taken at days 2, 5, 7, and 14 following exposure. Colonies grown on culture media were identified by the IIF technique, those that were positive were passed into broth culture and then further identified by the growth-inhibition test (Erno and Jurmanova, 1973).

RESULTS AND DISCUSSION

Two sheep (number 17 and 19) had mild hyperemia and watery lacrimation in both eyes. These symptoms appeared within two days and persisted until 5 days following exposure. No symptoms were noticed in other sheep. Mycoplasma bovoculi was isolated from both eyes of sheep 17 and 19 and from the right eye of sheep 3 at 2 days and from the left eye of sheep 17 at day 5. Other sheep were negative as shown in table 1.

Reproduction of conjunctivitis and keratoconjunctivitis with some of the ocular mycoplasmas has been reported. Al-Aubaidi et al. (1973) produced mild conjunctivitis in goats by the instillation of few drops of broth culture of A. oculi into the conjunctival sac 3 times in one day. Trotter et al. (1977) inoculated 6 goats with M. conjunctivae subconjunctivally and produced mild conjunctivitis in two animals and severe keratoconjunctivitis in three others. In sheep, the instillation of a broth culture of M. conjunctivae into the conjunctival sac also produced keratoconjunctivitis (Jones et al., 1976).

In our trial, it appeared that M. bovoculi does not colonize sheep conjunctival epithelium for extended periods as it colonized calf conjunctival epithelium (Rosenbusch and Knudtson 1980). Although many mycoplasmas are known to be host

Table 1. Isolation of Mycoplasma bovoculi from the eyes of 6 sheep following exposure

Sheep #	Eyes	Days after exposure 2	5	7	14
1	L[a]	-	-	-	-
	R	-	-	-	-
2	L	-	-	-	-
	R	-	-	-	-
3	L	-	-	-	-
	R	+	-	-	-
6	L	-	-	-	-
	R	-	-	-	-
17	L	+	+	-	-
	R	+	-	-	-
19	L	+	-	-	-
	R	+	-	-	-

[a]L, Left; R, Right.

specific, Freundt (1974) indicated that several species of mycoplasmas have been isolated from unrelated hosts. Such an example was reported by Rosendal (1974) who isolated M. bovigenitalium from the conjunctiva and the lungs of a dog with pneumonia.

We conclude that M. bovoculi strain FS8-7 does not produce conjunctivitis in sheep as it does in calves although 2 sheep responded with mild hyperemia and watery lacrimation.

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SUMMARY AND CONCLUSIONS

The purpose of this work was to develop an ELISA test for the detection of antibodies to M. bovoculi in calves. An indirect-ELISA was optimized (see appendix) and applied for the differentiation between the two antigen preparations of Mycoplasma bovoculi (Tween 20 antigen and osmotic lysis antigens). The antiglobulin-ELISA was used to detect different immunoglobulin classes. The samples tested with the latter included sera, nasal and lacrimal fluids from animals naturally and experimentally exposed to M. bovoculi. Immunoglobulin A was found predominantly in secretions of both groups of animals while IgG predominated in the sera. These samples were also tested by the metabolic-inhibition (MI) test to detect their growth inhibitory effect on M. bovoculi in vitro. Nasal and lacrimal fluids showed no inhibitory effect while sera did.

We also investigated the possibility of producing conjunctivitis in sheep by the direct instillation of a broth culture of M. bovoculi into the conjunctival sac.
The conclusions that could be drawn from these experiments are:

1. Tween 20 antigen had higher specificity than osmotic-lysis antigen in ELISA tests.

2. The antiglobulin-ELISA is more specific than the MI test for the detection of antibodies to M. bovoculi.
3. The metabolic-inhibition test detects antibodies to M. bovoculi in sera but not in nasal or lacrimal fluids.
4. M. bovoculi colonizes sheep eyes for a very short period of time and thus did not produce conjunctivitis.

Further investigation is needed in these areas:

1. Determine the inhibitory effect in the MI test of purified bovine immunoglobulin classes.
2. Several strains of M. bovoculi may be tried on larger numbers of sheep to determine conjunctival colonization and reproduction of the disease.

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APPENDIX

Indirect-ELISA

Determination of optimal antigen concentration: Different concentrations of Mycoplasma bovoculi Tween 20 solubilized antigen were coated on a microtiter plate. Four antiserum dilutions (1:250, 1:500, 1:1000, 1:5000) were added to each antigen concentration. One serum from a naturally exposed calf to M. bovoculi was compared with a serum from an unexposed calf. The conjugate (rabbit anti bovine IgG horseradish peroxidase) was used at 1:100 dilution. In Fig. 1A, it can be seen that 1 ug/ml of antigen resulted in best differential binding with the antiserum dilutions. The control serum had optical density value below 0.20.

Determination of optimal antiserum dilution: Antisera from four calves naturally exposed to M. bovoculi and from an unexposed calf were diluted (10^{-1}–10^{-6}) and added to plates coated with 1 ug/ml of antigen. The conjugate dilution was kept at 1:100. As shown in Fig. 2A, a titer of 10^{-2} was found to detect specific antibodies for M. bovoculi in all four sera. The control serum showed optical density value below 0.20.

Determination of optimal conjugate dilution: Six

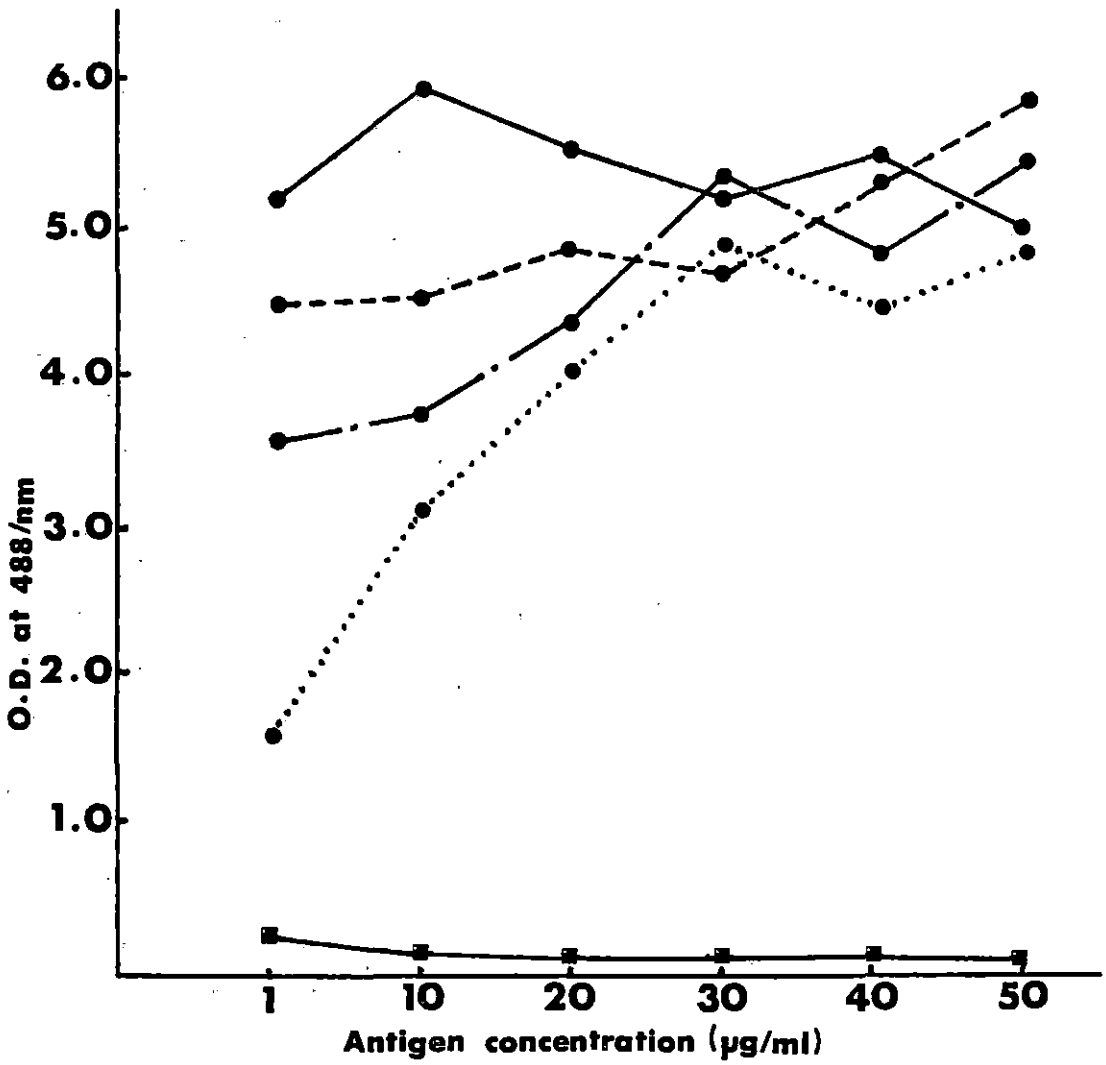

Fig. 1A. Determination of optimal antigen concentration measured by the indirect-ELISA. Coating was done for 3 h and evaluated with a serum from a calf naturally exposed to M. bovoculi diluted 1:250 (●—●), 1:500 (●- -●), 1:1000 (●—·—●) and 1:5000 (●·····●) and with serum from an unexposed calf diluted 1:250 (■—■). Conjugate dilution at 1:100.

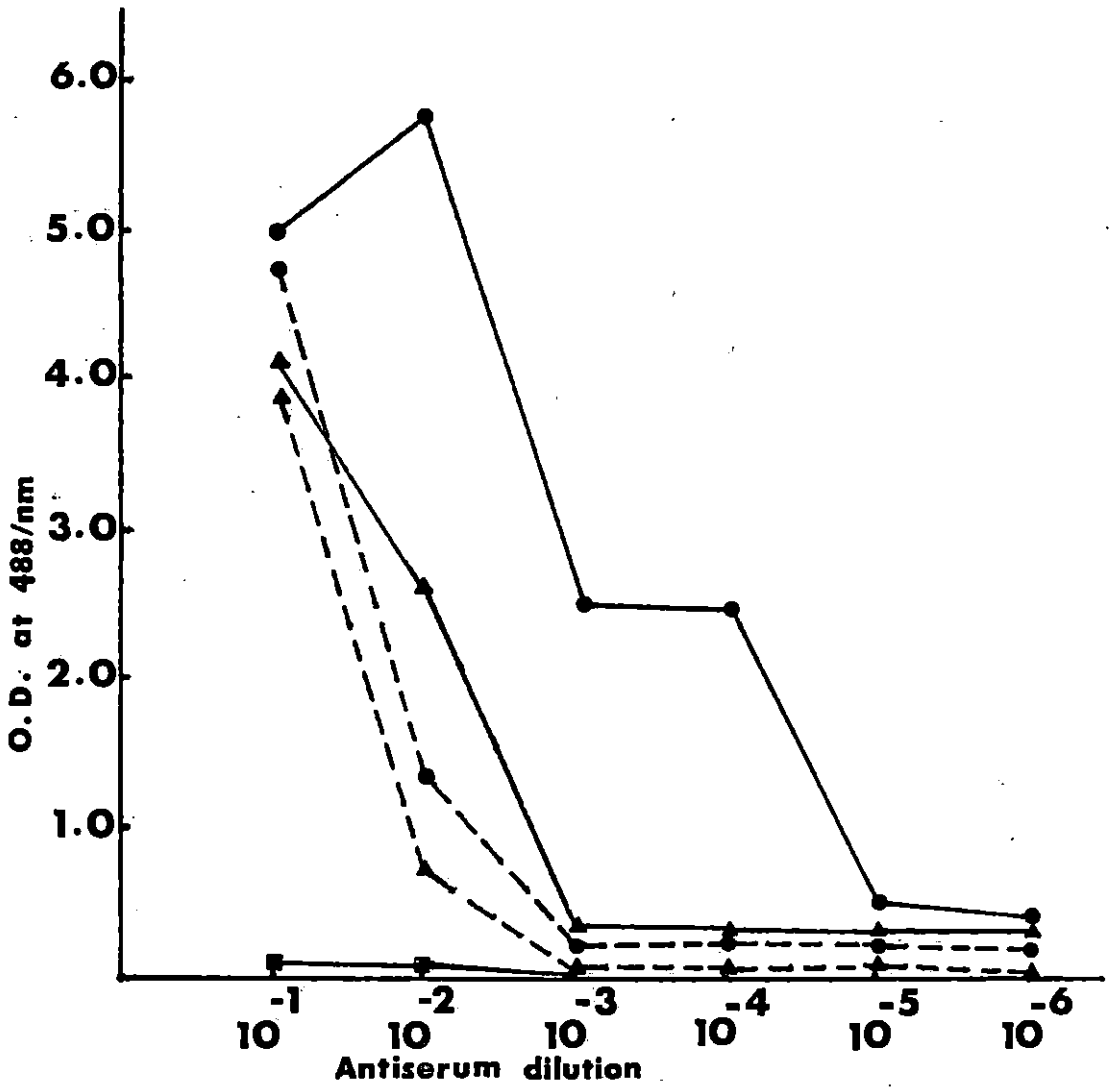

Fig. 2A. Determination of optimal antiserum dilution measured by the indirect-ELISA. Antisera from four calves naturally exposed to M. bovoculi <calf #: 182 (●——●), 103 (●- -●), 161 (▲——▲), 18 (▲- -▲)> and from an unexposed calf # 72 (■——■). Conjugate dilution at 1:100.

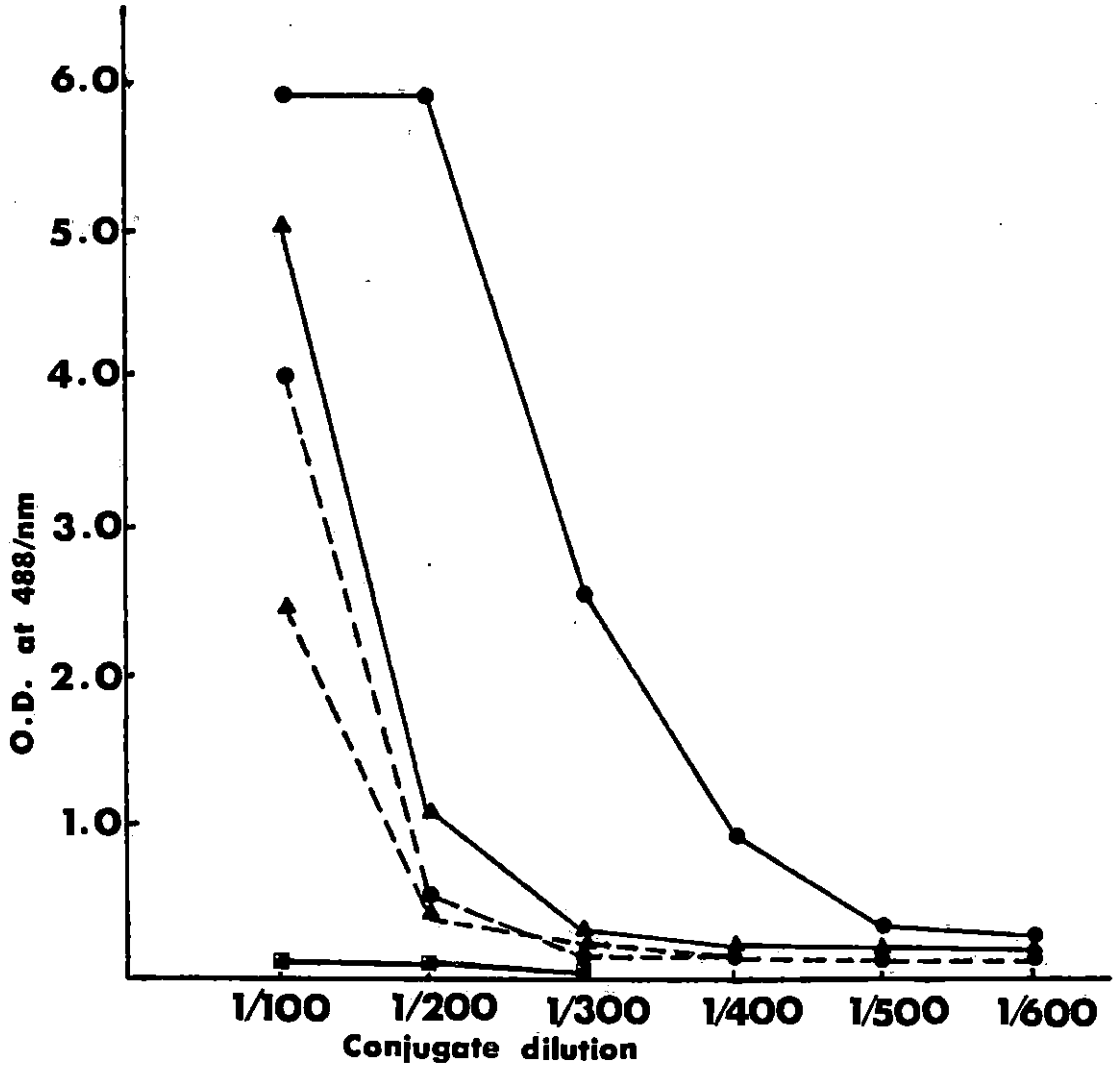

Fig. 3A. Determination of optimal conjugate dilution measured by the indirect-ELISA. Six conjugate dilutions were tested with anti-sera from four calves naturally exposed to M. bovoculi <(calf #: 182 (●—●), 103 (●--●), 161 (▲—▲), 18 (▲--▲)> and with serum from an unexposed calf # 72 (■—■).

conjugate dilutions were used as shown in Fig. 3A. With high conjugate concentrations, high optical density values were detected. The control serum had low values even when tested with high conjugate concentrations. The antigen concentration was kept at 1 ug/ml, and the antibody dilution at 1:100.

Antiglobulin-ELISA

The optimal antigen concentration and optimal antiserum dilution determined for the indirect-ELISA was applied for this test also.

Determination of anti-class antibody dilution: Three dilutions of anti-class specific antibody (rabbit anti-bovine IgG antibody) were used 1:100, 1:200 and 1:500. Higher optical density values were obtained with high concentrations of anti-class antibody (Fig. 4A).

Determination of optimal conjugate dilution: Three conjugate dilutions (1:1000, 1:1500, 1:2000) were used (Fig. 5A). Higher optical density values were obtained with high concentration of conjugate.

Reproducibility of the antiglobulin-ELISA: Following the establishment of the optimal conditions for the test, the test was repeated at 8 different occasions to determine its consistency using four different antisera from calves

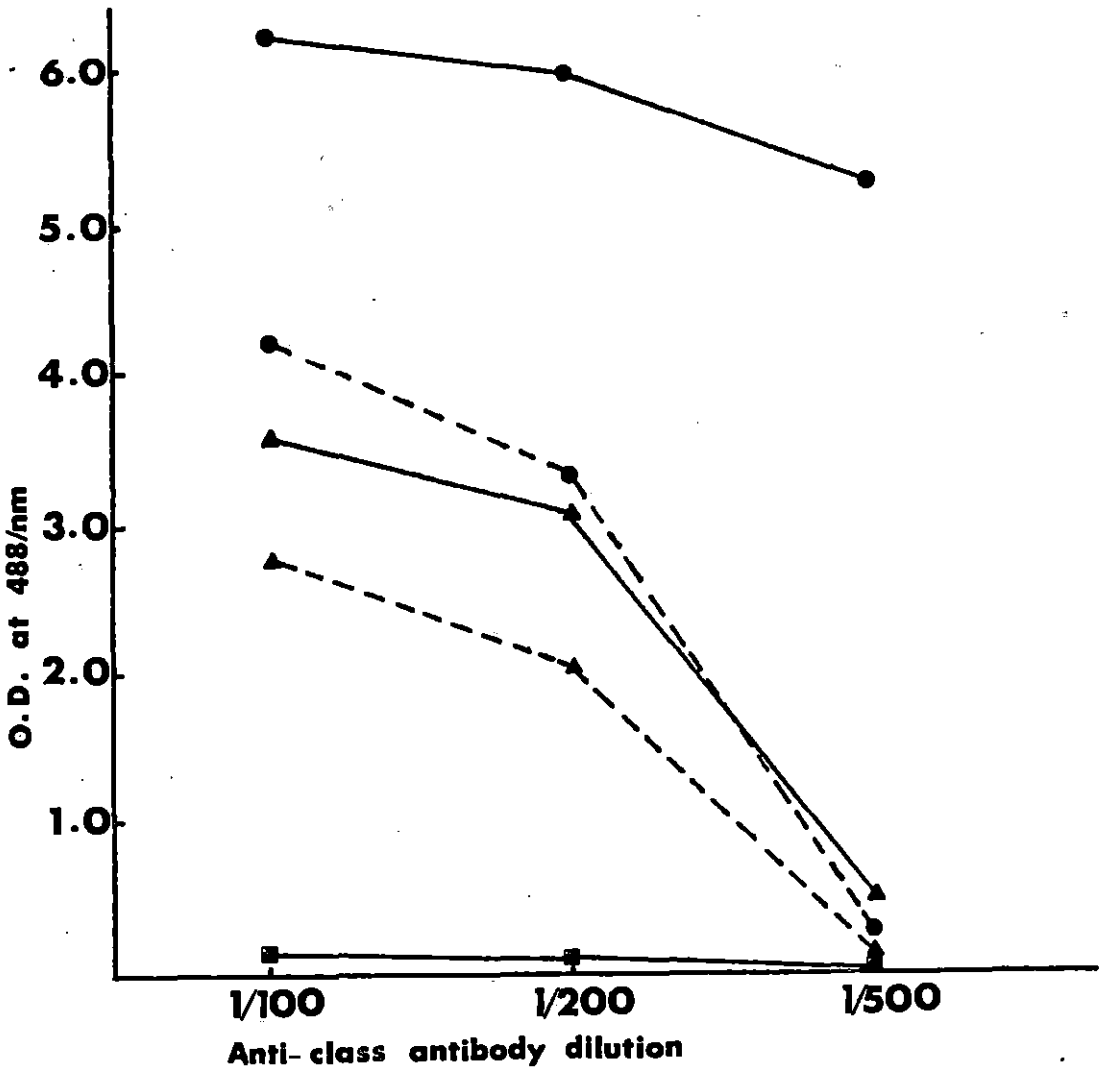

Fig. 4A. Determination of optimal anti-class antibody dilution measured by the antiglobulin-ELISA. Three anti-class antibody dilutions were tested with antisera from four calves naturally exposed to M. bovoculi <calf #: 182 (●——●), 19 (●- -●), 159 (▲——▲), 181 (▲- -▲)> and with serum from an unexposed calf # 72 (■——■). Conjugate dilution at 1:1000.

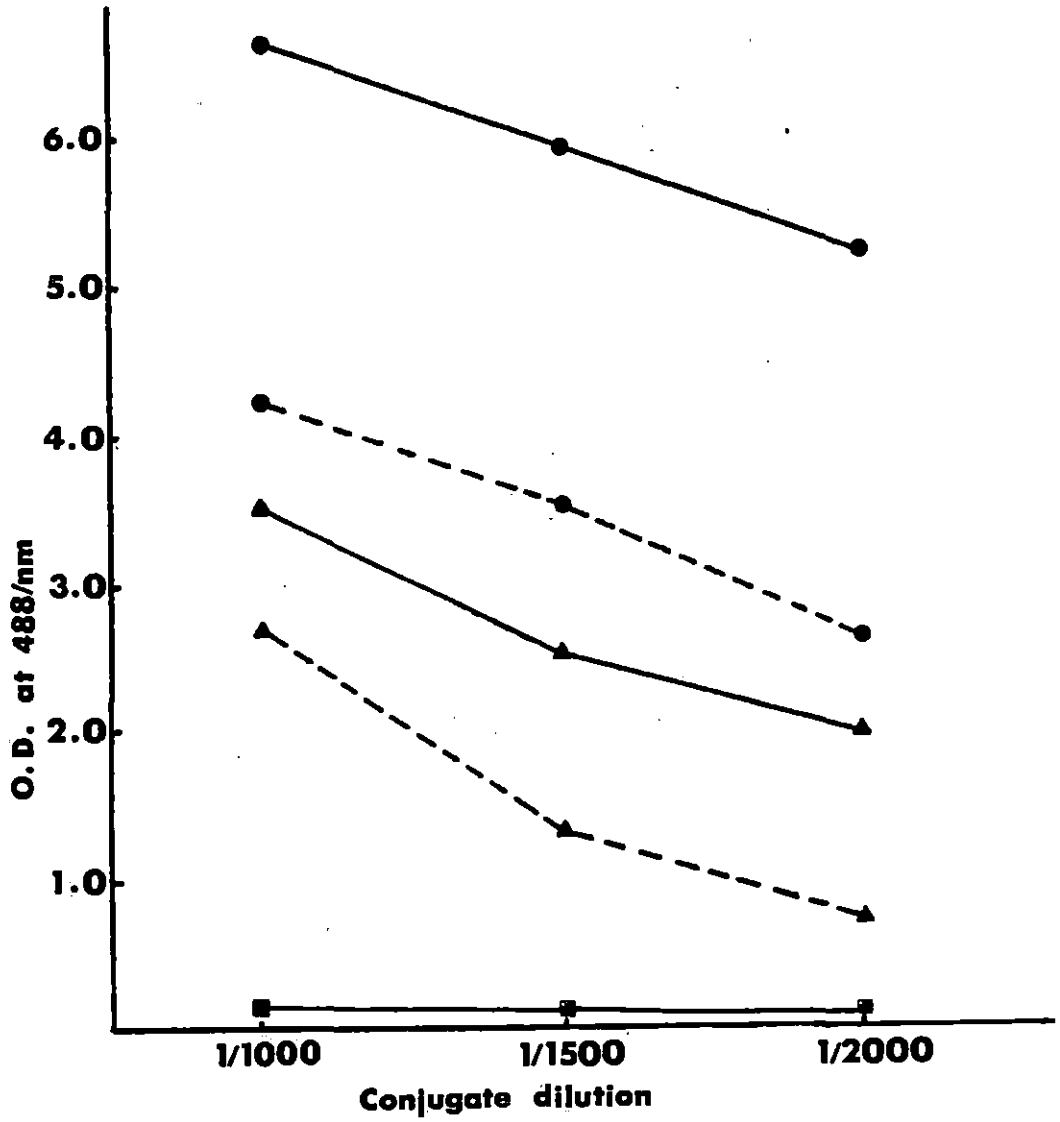

Fig. 5A. Determination of optimal conjugate dilution measured by the anti-globulin-ELISA. Three conjugate dilutions were tested with antisera from four calves naturally exposed to M. bovoculi <calf #: 182 (●——●), 19 (●- -●), 159 (▲——▲), 181 (▲- -▲)> and with serum from an unexposed calf # 72 (■——■).

naturally exposed to M. bovoculi together with serum from an unexposed calf. High degree of reproducibility was demonstrated in Fig. 6A.

Controls: The following controls were used.

1. Substrate control: antigen coated wells with substrate (no antiserum and no conjugate).
2. Conjugate control: antigen coated wells with anti-class antibody, conjugate and substrate (no antiserum).
3. Antiserum control: uncoated wells (no antigen) with antiserum, anti-class antibody conjugate and substrate.
4. The substrate control served as a blank for adjusting the machine to zero.

Values of the reaction product were obtained by subtracting the optical density value of the conjugate control from the optical density value of the tested antiserum. Four positive and four negative sera were included in each run.

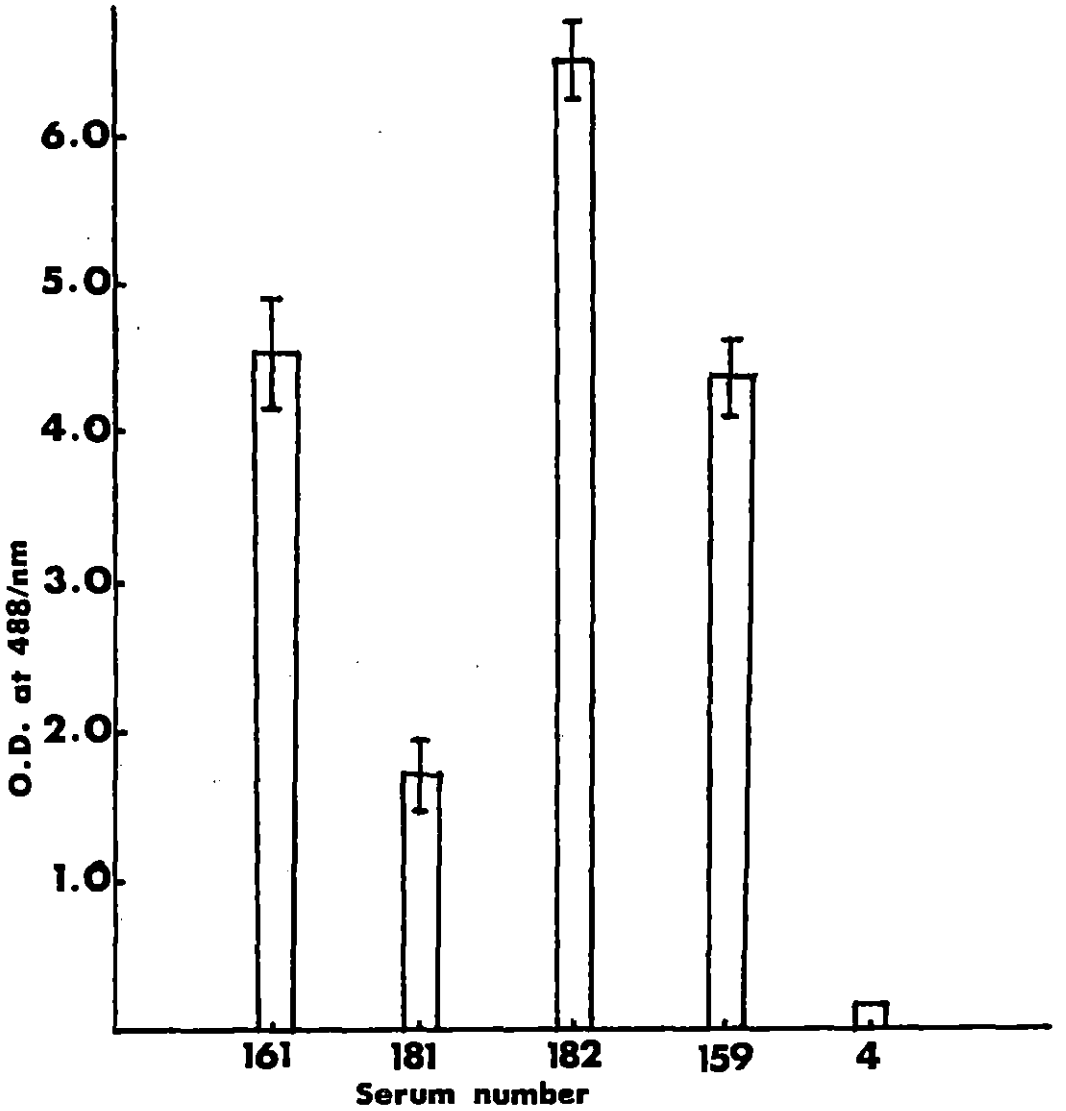

Fig. 6A. Reproducibility of the antiglobulin-ELISA. Antisera from four calves naturally exposed to M. bovoculi (161, 181, 182, 159) were tested at 8 different occasions together with serum from an unexposed calf # 4.