Evaluation of an enzyme immunoassay for use in diagnosis of *Leptospira interrogans* serovar *hardjo* infection in cattle

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by

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A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

Interdepartmental Program: Immunobiology
Major: Immunobiology

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1989

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GENERAL INTRODUCTION

Leptospira interrogans is a ubiquitous bacterial pathogen among domestic and wild animals. Leptospirosis has an economic impact on the animal industry, and is important for public health because leptospirosis is transmissible to humans. Leptospirosis is considered a major occupational zoonotic disease of people in rural areas working in contact with animals. Being a common disease in pets and rodents, leptospirosis is also a health hazard in certain urban areas. Leptospirosis causes economic losses in livestock because of reproductive failure, decreased milk production, and death. Losses due to health care may result from human infections.

New diagnostic techniques and improvement of isolation procedures have provided information and led to better understanding of the epidemiology of diseases. Some organisms such as human retroviruses were regarded as uncommon pathogens or even innocuous ones in the past because of technical limitations in their diagnosis. Today the same agents are considered important pathogens because of the development of more sensitive diagnostic tests. In other cases newer simplified techniques have replaced expensive, inaccurate, and time consuming techniques of the past contributing to a broader surveillance of certain infec-

tious diseases. Leptospirosis is such a disease.

Diagnosis of leptospirosis is usually accomplished by detection of the organism in body tissues and fluids and by demonstration of serum antibodies against leptospires. Despite the availability of improved culture media and use of selective substances to avoid contamination, bacteriologic culture of leptospires remains an impractical approach for routine use. The high cost of materials, time required, and the fastidious nature of some leptospires are disadvantages of this procedure. Other techniques used to detect leptospires in tissues including fluorescent antibody procedures and histopathology are not routinely available and are not sensitive. Therefore diagnosis of leptospiral infection is usually based on serologic tests.

The microscopic agglutination (MA) test is the standard method for diagnosis of leptospiral infections in animals. The test is carried out using a battery of different serovars of L. interrogans as antigens. Skilled personnel are required to conduct the MA test because experience is necessary to interpret the results. The MA test also involves extensive manipulation of live cultures of L. interrogans which is a health hazard to laboratory workers. Moreover the MA test is a laborious, multi-step technique that requires maintenance of live cultures of 15 different serovars. Despite the experience of the person-

nel, the concentration of organisms used to conduct the test varies from day to day and may influence the results.

These features of the MA test often exceed the capabilities of most diagnostic laboratories. Consequently, the application of the MA test is limited to only a few laboratories which have the staff and resources to conduct this test on a daily basis. These factors contribute to the lack of epidemiologic information about bovine leptospirosis.

Bovine leptospirosis is a major problem in many countries. Several serovars of _L. interrogans_ have been isolated from cattle. Among those serovars, _hardjo_, _pomona_, and _grippotyphosa_ are the most commonly isolated from cattle in the United States.[93]

Increasing interest has developed during the last 7 years in the infections produced by _L. interrogans_ serovar _hardjo_.[93] Unlike leptospirosis caused by other serovars, this organism causes inapparent to mild symptoms in cattle.[16,17,36,95]

The MA test is insensitive in detecting cattle which have been either naturally or experimentally infected with serovar _hardjo_.[16,17] Isolation of serovar _hardjo_ from MA-negative cattle has also been reported in naturally exposed animals.[93] Considering these aspects of infection produced by serovar _hardjo_, the MA test is not an adequate

test for diagnosis. It is necessary to improve the diagnosis of leptospirosis using more sensitive methods and with less risk for workers. It is also important to determine that any new diagnostic technique is not more complex than the MA test.

Enzyme immunoassay (EIA) is a better alternative serologic test because of safety, sensitivity, and relative simplicity. The versatility of the EIA allows the technique to be used for assessing the type of immunoglobulin generated during infection and for measuring the magnitude of the immune response. This test has been used to detect antibodies in serum as well as body fluids and secretions.[98] Enzyme immunoassay is a readily automated technique that can be used for diagnosis of numerous diseases using a similar protocol. These features are desirable for use in a diagnostic laboratory.

Several protocols using EIA for detection of antibodies against Leptospira have been described and show promising results. However it is difficult to compare results obtained by different laboratories because of the variety of antigens and procedures used.

The objectives of this research were to prepare and evaluate different extracts of L. interrogans serovar hardjo as potential antigens for an EIA and to obtain information on development and persistence of serum anti-

bodies in cattle experimentally exposed to *L. interrogans* serovar *hardjo*. Detection of vaccination titers and the possibility of discriminating between vaccination titers and those produced by infection were investigated.

LITERATURE REVIEW

Leptospirosis in Cattle

Leptospirosis is a common disease in cattle[93,95]; the three serovars most commonly isolated from cattle in the United States are: hardjo, pomona, and grippotyphosa.[93,95] Animals infected with most serovars of L. interrogans commonly develop agalactia accompanied by an atypical mastitis (soft udder) and abortions. The most severe symptoms are seen in young animals in which leptospirosis can be an acute disease and characterized by renal and hepatic failure which may cause death.[95]

Leptospira interrogans serovar hardjo is the serovar most commonly isolated from cattle in the United States and other countries.[26,65,84,93] Two types of L. interrogans serovar hardjo have been described.[36,66] These two types of serovar hardjo have different DNA restriction enzyme digestion profiles but are serologically indistinguishable using routine techniques.[65] Type hardjoprajitno is the reference strain for serovar hardjo and has been isolated from cattle in Europe. The type hardjo-bovis is isolated from cattle throughout the world and is the only type of serovar hardjo isolated in the United States.[66]

Comparative studies suggest that the pathogenicity of type hardjo-bovis and hardjoprajitno may be different.[36]

It is apparent that both types of serovar _hardjo_ produce subclinical disease in most cases.[16,17,36] Infection produced by type hardjo-bovis however, is thought to produce less severe clinical signs than type hardjoprajitno.[36]

The main route of infection of cattle with _L. interrogans_ serovar _hardjo_ is by penetration of the conjunctiva and nasal mucosa. Urine is the most important means of infection. Since serovar _hardjo_ can also persist in the genital tract, it is likely that genital transmission also occurs.[95]

The organisms successfully enter the body and after 4 to 10 days leptospiremia occurs. Leptospires disappear from the blood with the development of circulating antibody and localize in different organs, including kidneys and upper genital tract.[34,95] Placental and fetal infection occurs during leptospiremia. Common consequences of infection of cattle with serovar _hardjo_ are abortion, stillbirths, and birth of weak calves.[16,35,33,95] Sudden agalactia is not often associated with infections produced by _L. interrogans_ serovar _hardjo_ type hardjo-bovis.[36]

Poor fertility has been described in cattle infected with serovar _hardjo_. The cause of infertility is not known but serovar _hardjo_ can persist in the female reproductive tract for up to 142 days.[34,94] Leptospires have been also isolated from semen.[95]

Serovar hardjo may establish a persistent infection without stimulating humoral immune responses detectable by the MA test. Paradoxically, a lack of agglutinating antibodies is more evident after infection of previously vaccinated animals.[16,17] The reasons for the relatively poor humoral immune response of cattle infected with serovar hardjo are unknown. Lipopolysaccharide (LPS) may be involved in this process. L. interrogans cells bind C3 molecules but interfere with the formation of the killing complex.[67] Similar mechanisms of interference with the classical pathway of complement activation are described for other gram negative bacteria.[56] Additionally decreased phagocytosis results from lack of chemotactic and opsonic factors.[56] Antiphagocytic properties have been described for outer membrane components of Leptospira.[68] Low amounts of antibody produced by vaccination did not protect cattle against infection with serovar hardjo type hardjo-bovis. These animals did not have a secondary humoral response following challenge.[17] Perhaps antibodies coat the organism neither affecting its motility nor harming it; but rather masking some of the epitopes required to bring about secondary immune response. Immunocomplexes in general are found to be to be immunosuppressive to lymphocytes in other systems.[22,62,79]

It has been reported that a variation of the antigenic

make up of some serovars of L. interrogans serogroup Hebdomadis occurred when cultured with homologous antibodies.[53] Therefore it is possible that antigenic variation in vivo may be involved in this lack of antibody induction.

Lesions in calves infected with serovar hardjo are mild. Renal and hepatic hyperemia are the major finding.[16] In adult cattle, the lesions are limited to the kidneys. Multifocal lymphoplasmacytic interstitial nephritis and tubular degeneration are the major features.[16] The mechanism by which serovar hardjo induces tissue damage remain unknown. Some researchers suggest that circulating immune complexes produced during leptospiral infection could play an important role in the pathogenesis of the disease produced by L. interrogans serovar icterohaemorrhagiae.[42] Cattle experimentally infected with L. interrogans serovar hardjo which had been vaccinated twice showed more severe kidney lesions than those vaccinated once.[16,17] These findings may suggest circulating immunocomplexes may be involved in the pathogenesis of serovar hardjo infection, however the lack of apparent glomerular damage detracts from this possibility.

Persistent infection with this serovar, which may last for several months, is responsible for most of the attention that the disease is receiving. During persistent infection, the animal can spread the infection to other

animal herds or to humans.[15,21] Lack of a measurable humoral response may accompany this shedding state.[16] Because serologic methods are commonly used for diagnosis of leptospirosis it is logical to conclude that the incidence of infection of cattle with serovar hardjo is underestimated.

Serologic Procedures

Microscopic agglutination test

Microscopic agglutination (MA) is the standard method for diagnosis of leptospirosis in animals and it has been considered to be a reliable test.

Dilutions of serum to be analyzed are mixed individually with an antigen battery of different serovars. These antigens are live cultures of serovars of *L. interrogans*. Presence of antibodies is measured by agglutination of the bacteria which is observed by means of a microscope with dark-field illumination.

The MA test is relatively serogroup specific so that a serovar used as an antigen detects antibody against most of the serovars belonging to a specific serogroup. Traditionally a positive serum is one that agglutinates at least 50% of the organisms at a dilution of 1:100. Comparative studies between the MA test and other serologic techniques have shown that both immunoglobulin G (IgG) and immunoglob-

ulin M (IgM) are involved in the microscopic agglutination reaction.[28,96] It is generally accepted that the MA test requires a relatively high titer of antibodies to occur. IgM is more efficient in this test and lower concentrations than those of other immunoglobulins can cause agglutination.[72] The disadvantages of the MA test are numerous and can be summarized by the following points:

1. It is a hazardous procedure because handling live pathogens is necessary.

2. It requires recent subcultures of several serogroups of *L. interrogans*.

3. It fails to detect acute disease and persistent infection produced by serovar *hardjo* type hardjobovis.[16,17,93]

4. It is difficult to standardize.

5. The results are observed and a subjective interpretation is made.

6. It is a time consuming procedure to conduct.

7. The technique is not easy to conduct, requires trained personnel, and a well equipped laboratory.

8. It is a costly procedure since it requires large amounts of expensive culture media.

Enzyme immunoassay

Enzyme immunoassays have proven to be sensitive tests for many diseases. EIA gives additional information about the immune response generated during infection because it allows measurement of specific IgG and IgM.

EIA has been used in other laboratories for diagnosis of leptospirosis in animals as well as humans.[10,14,27,29,44,92,96,102,107] Comparative studies have shown that EIA is more sensitive than the MA test for detection of antibodies against *L. interrogans*.[9,10,44,93]

Different antigen extracts have been tested, some of which are genus specific others are serogroup specific. Genus specific antigens are prepared by means of detergents.[44] Serogroup specific systems have been obtained by extracting LPS[92] or by sucrose gradient.[14] Other preparations described were extracted by sonication,[10,102] and heating of whole cells.[92]

These systems have been adapted to detect IgM, IgG, or both. Differences in the concentration of each immunoglob-

ulin are found to be related to time after infection.[96] Differences in classes of immunoglobulin produced are also found between different infected species and serovar of Leptospira. For example, antibodies can be detected more easily in acutely infected dogs using anti-IgM conjugate.[45,46] Humans do not seem to develop high titers of IgG when infected whereas high titers IgM are detected.[5,10,92,107] In other species, such as swine or cattle, IgG is detected.[27,94,102] Cattle infected with serovar pomona seem to produce higher antibody titers than cattle infected with serovar hardjo.[96]

High sensitivity is a major advantage of EIA. Reports indicate that the MA test did not detect cattle infected with L. interrogans serovar hardjo.[16,93] Consequently EIA may be useful to detect cattle infected with this serovar. However, due to its high sensitivity, the EIA test appears to detect titers resulting from both vaccination and infection.[27,44,94]

Hemagglutination test

Different approaches have been used to coat red blood cells with leptospiral antigens. The test is mainly genus-specific.[37,71,90] It has been found to be more sensitive than the MA test in cattle[72] but is very difficult to perform and standardize. The hemagglutination test

is not used for diagnosis of leptospirosis in animals.

Taxonomy of *Leptospira*

Leptospires belong to the order Spirochaetales which is composed of 2 families: Spirochetaceae and Leptospiraceae. The family Leptospiraceae includes two genera, *Leptospira* and *Leptonema*. The genus *Leptospira* contains two species; *Leptospira interrogans* which is composed of pathogenic serovars and *Leptospira biflexa* which is composed of saprophytes. Based on serology, *L. interrogans* is composed of 172 serovars that are grouped for convenience in 19 serogroups; *L. biflexa* is composed of 38 serogroups.[55]

Structure of *Leptospira*

The structure of leptospires is quite complex and not fully known. The three major components of the leptospiral cell are: outer envelope, cytoplasm, and axial filament.[106]

The structure and chemical composition of the outer envelope of *Leptospira* resembles the outer membrane of other gram-negative bacteria.[30,49] *Leptospira*, however, appears to have a multi-layered outer membrane.[49,76] The outer membrane is composed of 46% protein, 27% carbohy-

drate, and 22% lipid.[49] Like gram-negative bacteria, these substances form lipoproteins, phospholipids, and LPS in leptospiral outer membranes.[23,49] It is common in Leptospira to find the presence phosphatidylethanolamine composed mainly by hexa and octadecenoic acid.[49] Leptospiral LPS differs from the classical LPS because it lacks 2-keto-3-deoxy-octulosonic acid (KDO) and L-glycero-D-manno-heptose.[49,86,101] Sugars such as arabinose, rhamnose, xylose, galactose, mannose, erythrose, fucose, and glucose have been found in Leptospira.[86,100]

Lipid A also seems to be absent in this organism.[86] These differences may account for the low cytotoxicity, pyrogenicity, mouse lethality, and mitogenicity of leptospiral LPS.[51,86,87,101]

In the periplasmic space, beneath the outer membrane, there are two axial filaments which are independent structures; each one is anchored by means of a knob to one end of the cytoplasmic cylinder.[19,49] Axial filaments overlap only in a small central portion of the cell.[49] These filaments, like bacterial flagella are responsible for motility of the organism. The axial filaments have a similar amino acid composition and morphology as flagella of gram-negative bacteria.

The leptospiral axial filament is composed of a sheath and inner core.[70] Six proteins have been identified as

part of the axial filament.[70] More recent studies report four axial-filament proteins. A doublet composed of a 33-34 kilodaltons (kDa) band and a 37 kDa band were the most prominent in polyacrylamide gels.[60] Leptospiral axial filaments have protein electrophoretic profiles similar to those of treponemes.[60]

The cytoplasmic cylinder has an internal cell wall structure that is composed of a peptidoglycan layer under which a cytoplasmic (inner) membrane is present. These structures maintain the cylindrical shape of the cell.[49]

Antigens of Leptospira

The outer membrane which is the outer most component of leptospires, is an important element in interactions between these bacteria and the host.[11,61,68] As mentioned before, a variety of molecules are present; some of them are accessible to interaction with the host, others which are submerged in the membrane may be accessible only when the cell is lysed. Differences in exposed antigens are the basis for serotyping of L. interrogans. Serovars are identified by serum cross-agglutination and absorption and those serovars that share exposed antigens are known as a serogroup.

The term "main antigens" was used by Kmety in 1966.[63] These antigens were described as **"antigenic factors elicit-**

ing the highest antibody fractions, which are responsible for the homologous titer and coagglutinations of the closely related serotypes are designated as main antigens" This concept was based on MA test results. However, different serologic techniques have shown that other cellular components induce production of antibodies; but, these antibodies do not agglutinate leptospires.[19] Therefore, it is premature to postulate that the antigens responsible for induction of agglutinating antibodies also elicit the highest antibody responses. However, antigens that are recognized by agglutinating antibodies are located in the outer membrane[57] and are likely to be one of the earliest targets of the humoral and cellular immune response.[11] The location of the epitopes may render these antigens immediately available to interact with antibodies, lymphocytes, and phagocytic cells. Since there is crossagglutination among members of a given serogroup it is also possible that presence of antibodies cross-protect the host against infection produced by other members of the same serogroup.

Early work demonstrated that carbohydrate-rich fractions of L. interrogans showed a high degree of serogroup specificity.[83] Several approaches to extract the serogroup specific antigens have been studied. A modification of the phenol/water method developed by Westphal and Jann[104] to extract LPS was used to extract a serovar specific antigen

called the "type specific main antigen" (TM). However, in contrast to the classic approach, the TM antigen was extracted from the phenol phase and purified by means of precipitation and resuspension using different organic compounds.[88] TM antigen was subject of extensive study. It was called type specific because this preparation was demonstrated to contain serovar specific determinants.[88]

This TM antigen generated a great deal of attention when further investigations were performed to study the nature of the epitopes. A total loss of antigenic activity of the TM antigen occurred after treatment with proteolytic enzymes. This was evidence that protein was an important component of the TM antigen.[1,2,77] However, the susceptibility of TM antigen to proteolytic enzymes was tested later using serovar canicola and it was not possible to reproduce the decrease in antigenic activity.[100]

Periodic acid oxidation reportedly decreased the antigenic activity of the TM antigen and this was considered evidence of the importance of carbohydrates as a component of the type specific epitopes.[58,100] Chemical analysis indicated that this antigen was not a homogeneous molecule but a complex of lipids and carbohydrate similar to a gram-negative bacterial LPS.[49] More evidence of the lipopolysaccharide nature of the type specific antigens has been provided using serovar specific monoclonal

antibodies.[7,24,40,75,85]

Studies were conducted to determine the immunogenicity of different preparations of TM antigen. For this purpose a substance similar to the TM antigen was produced by mild alkali extraction and was called F4 antigen.[4,6,38,77,100] This F4 antigen extracted from Leptospira showed inconsistent results as an immunogen. For instance, immunization with F4 failed to induce agglutinating antibody and to protect against chronic kidney infection.[4,6,39] However, leptospiral LPS may not behave as a complete antigen but as a hapten.[62] Also, LPS from enterobacteria acts as a T cell-independent antigen (mitogen) that does not stimulate the production of memory cells.[62] None of these properties have been fully investigated in Leptospira. Purified leptospiral LPS (F4) does not stimulate a measurable humoral immune response, however this does not imply that LPS is not the protective antigen when it is complexed in the leptospiral cell. The way in which the LPS is presented to lymphocytes is also an important factor in determining the efficiency of the immunogen. For instance, LPS molecules in an entire gram-negative bacterial membrane stimulate a greater protective immunity than small particles or aggregates of LPS.[54]

Recent studies provide evidence that LPS epitopes are important in protection. Monoclonal antibodies against LPS

epitopes of L. interrogans serovars hardjo, and copenhageni were able to mediate opsonization.[7,40,52,91] Moreover, monoclonal antibodies against LPS determinants of L. interrogans serovar copenhageni protected hamsters, dogs and monkeys from challenge with the homologous serovar.[85]

Leptospiral LPS antigens are likely to be the outer most epitopes on the surface of the bacterial cell and they are required to initiate antibody mediated phagocytosis.[64] This component is responsible for serotype and serogroup specificity.[24] Using monoclonal antibodies, it appears that leptospiral LPS is composed of a mixture of different epitopes.[40,75] Moreover, the lack of discrete bands observed in immunoblot analysis indicates that these epitopes are compounded in the same LPS molecule.[40]

Leptospiral LPS appears to be a virulence factor because it may play a role in leptospiral evasion of the host immune response.[50] One of the mechanisms seems to be interference with phagocytosis.[50] Additionally, L. interrogans, unlike L. biflexa, seems to bind the C-3 molecule and prevents C-3 from activating other components of complement without sialic acid intervention.[67] This property of neutralizing components of the complement cascade has been associated with LPS molecules in other gram-negative bacteria.[56]

There are many antigenic leptospiral proteins de-

scribed. Most of them were characterized by electrophoretic analysis and immunoblotting of lysates of leptospiral cells. Some of these proteins have serogroup cross reactive properties as demonstrated by the same techniques.[8,20,74] The number of cross-reactive electrophoretic bands observed depends on the genetic relatedness of the organisms used to produce the hyperimmune sera.[20] The cross-reactive proteins seem to have different localization in the intact cell. Some of the major cross-reactive antigens are part of the axial filaments.[60] Two proteins of the axial filament with molecular weights from 30 kDa to 40 kDa are the most evident in SDS-polyacrylamide gel electrophoresis.[60] The axial filament has immunogenic properties.[19,43] These filaments have been found to have conserved epitopes even within the family Leptospiraceae.[60]

Other cross-reactive proteins may be associated with the bacterial outer membrane and are described as genus specific antigens.[81,41] A genus specific antigen that seems to be located beneath the leptospiral surface of the cell has also been described.[81] Evidence of other antigenic outer membrane proteins was provided by Nunes-Edwards et al.[74] These workers using radioimmunoprecipitation suggested the presence of some exposed type specific proteins in the outer membrane. However the possibility of LPS being bound to these proteins can not be discarded.

T lymphocytes recognize leptospiral outer membrane antigens and axial filaments after immunization as measured by leukocyte migration inhibition test and lymphocyte blastogenesis assay.[13,89] Unlike leptospiral LPS, antibodies directed against outer membrane proteins fail to agglutinate whole cells and fail to induce passive protection in guinea pigs.[57] This information suggests that although proteins are immunogenic, they are not readily accessible to antibodies on the intact leptospiral cell.

PART I: PREPARATION OF ANTIGENS AND DEVELOPMENT OF AN ENZYME IMMUNOASSAY

ANTIGEN PREPARATION

Phenol Extract

This extract was based on a modification of the procedure followed by Baum and Joens to obtain lipopolysaccharide from Treponema hyodysenteriae.[12]

One liter of a 15 day culture of L. interrogans serovar hardjo type hardjo-bovis (strain 93u) in bovine serum albumin polysorbate-80 medium (BAP-80 medium)[32] was harvested by centrifugation (32,000 x g for 20 minutes). The pellet was resuspended in phosphate buffer saline (PBS) and centrifuged. The washing step was repeated twice to eliminate media components. Finally, the pellet was resuspended in 10 ml of hot distilled water (68°C) and transferred to glass tubes; 10 ml of 88% hot phenol (68°) was added, and vortexed. This mixture was incubated at 68°C for 15 minutes and shaken every 3 minutes. Water and phenol phases were separated by cooling the tube in an ice bath for 30 minutes. Once cooled, the mixture was centrifuged at 32,000 x g for 20 minutes in a swinging-bucket rotor and the water phase was collected. Ten milliliters of hot water (68°C) was added to the phenol phase and the extraction was repeated. Water phases were combined and dialyzed against 50 volumes of distilled water for 24 hours with six changes of water. The extract was concentrated by

leaving the dialysis tube on the bench at room temperature overnight. The phenol phase was dialyzed and concentrated using the same procedure described for the water phase.

Butanol-Water Extract

The method used was a modification of the procedure described by Morrison and Leive to extract smooth and rough LPS from various gram-negative bacteria.[69]

One and half liters of a 15 day culture of L. interrogans serovar hardjo type hardjo-bovis (strain 93u) in BAP-80 medium was harvested by centrifugation (32,000 x g for 20 minutes). The pellet was resuspended in PBS and centrifuged. The pellet was washed twice and resuspended in 0.85% saline at 4°C. The mixture was transferred to a polypropylene centrifuge tube. Twenty ml of water-saturated butanol-1 was added and vortexed for 15 minutes. The mixture was then centrifuged at 35,000 x g for 20 minutes and the aqueous phase was collected. The extraction was repeated twice with 10 ml of saline each time and the aqueous phases were combined. Pronase[a] solution was prepared in 0.2 M Na_3PO_4 buffer (pH 7).

Pronase solution was added to achieve a final concentration of 20 μg of enzyme per milliliter of water phase.

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[a]Boehringer Mannheim Biochemical, Indianapolis, IN.

This preparation was placed in an incubator at 37°C overnight. A white flocculent layer was removed from the top of the tube; the remaining fluid was centrifuged at 10,000 x g for 40 minutes to eliminate precipitate. The supernatant was removed and dialyzed against 50 volumes of distilled water at 4°C for 6 hours with 4 changes of water.

Proteinase K Extract

The method was based on that used by Hitchcock and Brown technique to obtain LPS from *Salmonella*.[48]

One liter of a 15 day culture of *L. interrogans* serovar *hardjo* type hardjo-bovis (strain 93u) in BAP-80 medium was centrifuged (32,000 x g) for 20 minutes. The pellet of cells was washed twice with PBS as for the other extracts. The final pellet was suspended in 10 ml of PBS. Aliquots of this suspension (1.5 ml) were transferred to Eppendorf microfuge tubes and centrifuged in a microfuge for 1.5 minutes. Pellets were resuspended using 50 μl of lysing buffer containing 2% of sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol and 1 *M* Tris (pH 6.8). Lysates then were heated at 100°C for 10 minutes. Ten microliters of lysate solution containing 25 μg of Proteinase K[a] was added to each tube. The tubes were incubated for 60

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[a]Sigma Chemicals, St.Louis, MO.

minutes at 60°C. The extract was dialyzed against 4 changes of water. Proteinase K activity was stopped by adding 25 μg/ml of phenylmethylsulfonyl fluoride.

Desoxycholate Extract

This method was a modification of Schneider's procedure used to extract leptospiral antigens.[82]

One liter of a 15 day culture of Leptospira interrogans serovar hardjo type hardjo-bovis (strain 93u) in BAP-80 was pelleted and washed with PBS as described previously. Finally the cells were suspended in 2% of the original volume of PBS (pH 7.2) The organisms were lysed by adding an equal volume of a solution containing sodium chloride (0.1 M), sodium citrate (0.2 M), and sodium desoxycholate (0.4%). The mixture was vortexed and kept at room temperature for 2 hours, and the suspension was held at 4 °C for 24 hours. The extract was dialyzed against 50 volumes of water with 4 changes.

Salt Altered Cell Antigen

This method was described by Auran et al. and was used to extract the outer membrane antigens from Leptospira.[11]

One liter of a 15 day culture of serovar hardjo type hardjo-bovis (strain 93u) was washed as in the previous extractions. The final pellet was resuspended in 2 ml of

distilled water and 30 ml of a 1 M solution of NaCl was added. The mixture was allowed to stand for 30 minutes at room temperature. Presence of spherical forms of leptospires produced by osmotic shock were confirmed by dark field microscopy. The salt altered cells were centrifuged at 31,000 x g for 20 minutes at 4°C and the supernatant discarded. The pellet was suspended in 2 ml of distilled water and 30 ml of 0.04% solution of SDS was added. This procedure lyses and releases the outer membrane. This was confirmed by the presence of thin filaments when the preparation was observed by dark field microscopy. Lysates were centrifuged at 31,000 x g for 20 minutes, the supernatant collected, and dialyzed against 2 liters of distilled water to eliminate the SDS.

Mechanically Disrupted Cell Antigen

Leptospires were cultured, harvested, and washed as described previously. Leptospires were suspended in 2 ml of PBS (pH 7), split into aliquots, and placed in three 1.8 ml-Eppendorf tubes containing 400 μl of zirconium beads (0.1 mm). Tubes were shaken in a Biospec mini bead-beater for 2 minutes and then were cooled in an ice bath. The procedure was repeated twice. Tubes then were centrifuged in a microfuge for 5 minutes to remove debris. The supernatant was used as mechanically disrupted cell antigen.

Soluble Proteins and Membrane Antigens

A portion of the mechanically disrupted leptospires was centrifuged at 100,000 x g for 90 minutes to pellet the membrane fraction.[74] The supernatant was used as the soluble protein antigen. The pellet was suspended in 2 ml of PBS and used as the mechanically disrupted membrane antigen.

ENZYME IMMUNOASSAY DEVELOPMENT

Enzyme Immunoassay

The EIA protocol was based on the procedure described by Thiermann and Garret.[96] Immulon-I plates[a] were coated for 18 hours at 4°C with 250 μl of the optimum concentration of each antigen diluted in a solution containing 0.05 M carbonate-bicarbonate buffer, pH 9.6. Contents of the plate were aspirated and unoccupied binding sites were blocked for 1 hour with 300 μl of a solution of 2% ovine serum albumin[b] dissolved in 0.01 M PBS, pH 7.2 (0.22 μm-filter-sterilized). The blocking reagent was aspirated and 200 μl of serum diluted 1:100 in 0.01 M PBS (pH 7.2) was placed in each well. Serum was allowed to react for 2 hours at 37°C. Each serum sample was tested in two wells on the same plate. After aspiration of serum, plates were washed 4 times with a solution of 1% Tween 20 in PBS (pH 7.2). The last three washes were done at 3 minute intervals. Goat anti-bovine IgG or goat anti-bovine IgM antibodies conjugated to horseradish peroxidase[c] were diluted

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[a]Dynatech Laboratories Inc., Alexandria, VA.

[b]Sigma Chemicals, St. Louis, MO.

[c]Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD.

1:1000 in PBS; 250 µl of this solution was placed in each well and incubated for 90 minutes at 37°C. When rabbit serum was used, horseradish peroxidase goat anti-rabbit IgG conjugate was diluted 1:2000 in PBS and incubated for the same time. The contents of the plate were aspirated and the wells washed as before. During all incubations steps, plates were kept in a plastic bag to avoid evaporation. The substrate buffer was 20 ml of 0.96% citric acid buffer (pH 4), mixed with 100 µl of a 1.87% peroxide solution (substrate), and 100 µl of ABTS[a] (2,2`-azino-di-3-ethyl- benzythiazoline sulfonic acid) as indicator. Each well received 250 µl of substrate solution and the reaction was allowed to take place for 15 minutes at 37°C. To stop the reaction, 50 µl of a 37 mM solution of sodium cyanide were added to each well. The reactions were monitored by a Dynatech 3900 EIA reader at 490 nm of wavelength.

Every time that the test was conducted controls were used. One blank well which received everything but serum, two positive controls wells which received known positive serum (MA test), and two negative controls which received known negative serum were included in each plate.

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[a]National Veterinary Service Laboratory, Ames, IA.

Hyperimmune rabbit serum

New Zealand White rabbits which lacked detectable anti-leptospiral antibodies as determined by MA test were used. Rabbits were inoculated intravenously with 2 x 10^8 leptospires in 1 ml of BAP-80 semisolid medium.[32] Seven days later, 4 x 10^8 leptospires were inoculated intravenously and on the 14th day, 6 x 10^8 leptospiras in 3 ml of medium were inoculated. Ten days later, blood was collected from each rabbit and those rabbits that had more than 1:5000 MA test titer were exsanguinated by cardiac puncture. Serum was extracted and lyophilized or frozen until needed.

Cattle serum

Sera were collected from nineteen 12-month-old-steers experimentally infected with 1 x 10^7 cells of serovar hardjo type hardjo-bovis (isolate 203) by conjunctival instillation. Serum samples were collected before challenge and 3 weeks after challenge.[17]

Optimum concentration

Optimal concentration of antigens and conjugate were determined in a checker board titration scheme. Two fold dilutions of antigen and two fold dilutions of conjugate were tested. Antigens were diluted from 1:250 to 1:12,800

and conjugates were diluted 1:500 to 1:4000 (Appendix).

Serum from a cow three weeks after experimental infection with serovar hardjo was used as a positive serum and serum from a non-exposed, MA-negative cow was used as negative serum. Both positive and negative sera were analyzed on the same plate. The optimum concentration of antigen and conjugate were determined using two criteria: first, the optimal dilution must produce a high optical density with a 1:100 dilution of a positive serum; at the same time, the optimal concentration of the antigen must not produce an optical density greater than 0.200 with a 1:100 dilution of negative serum.

This titration procedure allowed for the selection of four antigen preparations which were superior based on their reactivity. These antigens were: desoxycholate extract, salt altered cells, butanol extract, and mechanically disrupted membranes.

The optimum dilution was 1:1000 (0.11 μg of carbohydrate/ml) for desoxycholate antigen, 1:500 (0.28 μg of carbohydrate/ml) for butanol/water extracted antigen, 1:4000 (0.03 μg of carbohydrate/ml) for salt altered cells, and 1:16000 (0.06 μg of carbohydrate/ml) for mechanically disrupted membranes. Optimal concentration of the peroxidase-labeled goat anti-bovine IgG conjugate was determined to be 1:1000 for all the antigens (Appendix).

Further evaluations of the butanol/water extract were performed to determine the optimal conditions for detecting bovine IgM. The optimal concentration of peroxidase-labeled goat anti-bovine IgM conjugate was 1:1000. The optimal concentration of goat anti-rabbit IgG conjugate (1:2000) was determined by checker board titration using rabbit anti-hardjo-bovis hyperimmune serum.

Optimum time

The optimal enzyme-substrate-indicator reaction time was established by using positive serum from a infected cow and the same negative serum used before. Fourteen replications of each serum were tested in the same plate. Standard deviation and the background were obtained and analyzed. The optical density was recorded at 10, 12, and 15 minutes after addition of the substrate (Table 3). The purpose of this experiment was to determine a time interval within which the minimum variation between EIA readings and low background are observed.

Positive samples

For determination of the positive-negative limit, the criteria described by Adler et al.[10] were used. Two negative sera were analyzed in each plate and the mean of the optical density obtained with these negative sera was added

to three times a preestablished standard deviation for negative samples. This value was considered to be the upper limit of a negative value. The preestablished standard deviation was the standard deviation of the optical density readings obtained on 5 consecutive days using 6 negative sera. This value for the preestablished standard deviation was 0.033.

Table 1. Comparison of means and standard deviations of enzyme immunoassay values recorded after 10, 12, 15, and 20 minutes of the peroxidase-peroxide-ABTS reaction (at 490nm wavelength). Negative serum was obtained from a negative cow at the time of infection and positive serum came from the same animal 3 weeks after experimental infection with serovar hardjo. Fourteen replications of a 1:100 dilution of each sample were analyzed. Butanol-water extracted antigen was used

	TIME							
	10 MINUTES		12 MINUTES		15 MINUTES		20 MINUTES	
	Neg.[a]	Pos.[b]	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
	0.069	1.061	0.089	1.238	0.105	1.371	0.196	≥2.000
	0.060	1.064	0.089	1.256	0.103	1.389	0.194	≥2.000
	0.067	0.978	0.088	1.245	0.105	1.362	0.194	≥2.000
	0.061	0.990	0.084	1.220	0.107	1.355	0.199	≥2.000
	0.050	1.031	0.087	1.266	0.110	1.385	0.205	≥2.000
	0.077	1.020	0.098	1.277	0.100	1.393	0.189	≥2.000
	0.096	1.049	0.110	1.260	0.134	1.388	0.215	≥2.000
	0.073	0.995	0.099	1.210	0.115	1.341	0.220	≥2.000
	0.060	0.999	0.140	1.198	0.116	1.333	0.207	≥2.000
	0.070	0.870	0.109	1.146	0.115	1.298	0.225	≥2.000
	0.069	0.924	0.092	1.163	0.123	1.269	0.227	1.880
	0.066	0.967	0.090	1.157	0.110	1.290	0.207	≥2.000
	0.082	0.920	0.099	1.200	0.112	1.334	0.212	≥2.000
	0.120	1.088	0.130	1.270	0.113	1.394	0.202	≥2.000
MEAN	0.073	0.997	0.100	1.222	0.112	1.350	0.207	1.991
STD.DEV.	0.016	0.059	0.016	0.072	0.008	0.039	0.012	0.031

[a]Negative serum.

[b]Positive serum.

CHARACTERIZATION OF THE ANTIGENS

Selection of Antigens

Four antigens were selected for further study and evaluation based on their performance in the titration assays. The other antigens were discarded because their lack of reactivity such as was observed in proteinase K extract and phenol-water extract or because of high background observed in the mechanically disrupted cells, and soluble protein antigens (Appendix).

Composition of Antigen

Protein concentration

The technique used was described by Bio-Rad Laboratories.[a] Two-fold dilutions of protein standard[a] were made; the lowest concentration was 0.2 mg/ml and the highest was 1.4 mg/ml. One hundred microliters of standard, undiluted leptospiral antigens and PBS (blank) were transferred to 5 ml-tubes. Five milliliters of dye reagent was added to each tube and the mixture was vortexed. After a period of 30 minutes optical density was determined using a Ultrospec II-LKB spectrophotometer (595 nm wavelength). A standard curve was plotted and concentrations were calculated.

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[a]Bio-Rad Chemical Division, Richmond, CA.

Carbohydrate concentration

The method was by Dubois et al.[31] Standards were prepared by placing 0 μg, 17.5 μg, 35 μg, and 70 μg of dextrose in 2 ml of distilled water. Samples were diluted in distilled water 1:10 and 0.5 ml of each dilution was added to a 1.5 ml of distilled water. One milliliter of a solution of 5% phenol was added. Using a 5 ml pipette, 5 ml of concentrated sulfuric acid were added vigorously against the water surface. The mixture was allowed to stand for 10 minutes and then was shaken and placed at 28°C for 20 minutes. Optical density was determined at a wavelength of 490 nm in a Ultrospec II spectrophotometer and concentrations of carbohydrate in the antigens were determined (Table 1).

Polyacrylamide gel electrophoresis (PAGE)

Discontinuous SDS-PAGE was conducted using each antigen under reducing conditions. The gel thickness was 1.5 mm, the concentration of polyacrylamide was 12.5% in the resolving gel and 3.75% in the stacking gel. Gels were stained for protein and for LPS.

Resolving gel The resolving gel was prepared by mixing 25 ml of a monomer solution (30% acrylamide, 0.8% Bis), 7.5 ml of resolving gel buffer (3.0 M Tris-HCl; pH 8.8), 0.6 ml of 10% SDS, 23.9 ml of distilled water, final-

ly 3 ml of 1.5% ammonium persulfate, and 30 μl of N,N,N',N'-tetramethylethylenediamide (TEMED).

Stacking gel   This buffer contains 2.5 ml of the monomer solution, 5 ml of stacking gel buffer (0.5 M Tris-HCl, pH 6.8), 0.2 ml of 10% SDS, 11.3 ml of distilled water, 1 ml of 1.5% ammonium persulfate and 15 μl of TEMED.

Sample treatment buffer   The treatment buffer was a solution of 0.125 M Tris, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.011% of bromophenyl blue. Samples and protein molecular weight standards[a] were treated for 10 minutes at 100°C.

Tank buffer   This buffer was composed of 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% SDS. The pH was 8.3.

Silver stain for proteins   For this purpose a Bio-Rad[b] silver stain kit was used. The gels were first fixed with 10% ethanol and 5% acetic acid in a total solution of 400 ml for 30 minutes. Then the proteins were oxidized using 200 ml of a 10% solution of the oxidizer for 10 minutes. Three washes with 400 ml deionized water with a 10 minutes interval between them were done. Two hundred milliliters of a 10% solution of silver reagent were added and allowed to react for 30 minutes. The gels was washed

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[a]Bethesda Research Laboratories, Gaithersburg, MD.

[b]Bio Rad Chemical Division, Richmond, CA.

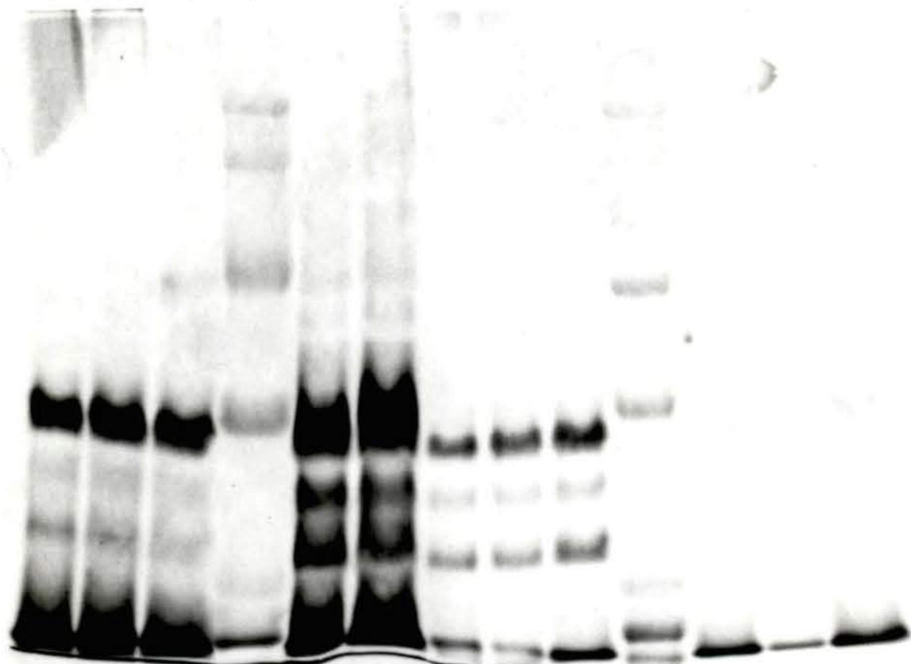
as before. Developer was prepared by dissolving 32 gm of concentrate in one liter of deionized water at 23°C under constant stirring. Two hundred milliliters of this solution was added for approximately 30 seconds and was replaced when the solution changed to yellow color. The reaction was stopped with a 5% acetic acid solution for 5 minutes.

Silver stain for lipopolysaccharide The procedure was based on Tsai and Frasch technique[99] modified by Hitchcock and Brown[48] The gels were fixed overnight in a solution of 25% isopropanol and 7% acetic acid. An oxidizing solution containing 1.05 gm of periodic acid in 150 ml of distilled water plus 4 ml of a 25% solution of isopropanol in a 7% solution of acetic acid was prepared. This solution was poured over the gel and allowed to react for 5 minutes. Eight washes with 200 ml of distilled water at 30 minutes interval were done. The stain solution consisted of 28 ml of 0.1 N NaOH, 1 ml of 29.4% NH_4OH, 5 ml of 20% $AgNO_3$, and 115 ml of distilled water. This solution was allowed to react with the gel for 10 minutes. Gels were washed with distilled water 8 times at 30 minutes interval.

To develop the gel, 200 ml of a solution containing 50 mg of citric acid, 0.5ml of a 37% solution of formaldehyde solution was used. The reaction was maintained at 25 °C and the reaction was observed for color development. When

LPS bands were visible, but before protein molecular weights standards were colored, the reaction was stopped by washing the gel in 200 ml of distilled water plus 10 ml of a 7% solution of acetic acid. The gels then were covered to protect them from light and photographed.

Figure 1. SDS-Polyacrylamide gel electrophoresis of L. interrogans serovar hardjo. Duplicated gels were stained; A silver stain for lipopolysaccharide and B silver stain for proteins. Molecular mass markers were Coomassie Blue prestained myosin H-chain (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (68 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa), and β-lactoglobulin (18.4 kDa). Lanes 1-3 contain decreasing concentrations of salt altered cells antigen; lanes 4 and 10, molecular weight standards; lanes 5 and 6, increasing concentrations of mechanically disrupted membrane; lanes 7-9, butanol extract; lanes 11-13, desoxycholate extract


A.
1
2
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8
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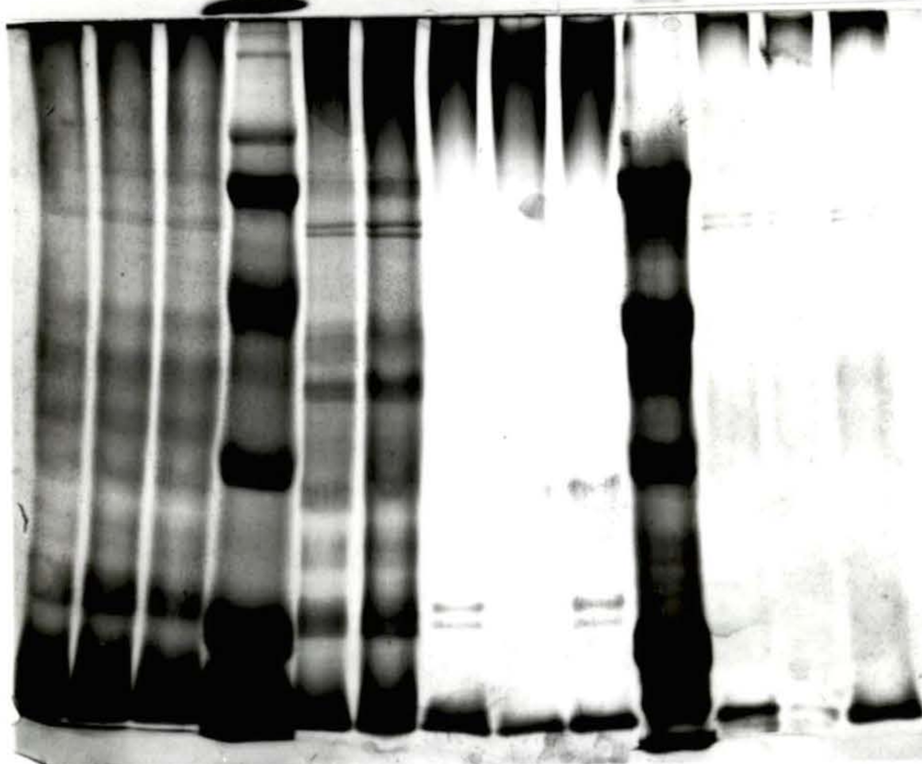
12
13


B.

ANTIGEN EVALUATION

Determination of Background

Twenty sera which lacked detectable anti-hardjo antibodies by MA test, were analyzed on 3 consecutive days, and the mean of the values obtained for each antigen were compared with those of the other antigens. All the antigens showed a low nonspecific binding of antibody. Wells without serum were analyzed also (Table 2).

Sensitivity

To evaluate the relative sensitivity of the antigens, 15 sera from cattle experimentally infected with serovar _hardjo_ were utilized at a 1:400 dilution. There were no major differences in sensitivity among antigens (Figure 2).

Specificity

Specificity of the antigens for antibodies against serovar _hardjo_ was assessed using rabbit hyperimmune serum against _L. interrogans_ serovars _hardjo_ type hardjo-bovis, _pomona_, _canicola_, _tarassovi_, _grippotyphosa_, _wolffi_, _copenhageni_, _bratislava_, and _istrica_ (Figures 3-6). Mechanically disrupted membranes and the butanol-water extract showed a high degree of serogroup specificity. There is serological cross reactivity with antibodies against _tarassovi_.

Crossreactivity between these two serovars has been found in other serogroup specific EIA and MAT.[27] These antigens displayed no crossreactivity with serovars pomona and grippotyphosa which together with serovar hardjo are the most prevalent serovars of Leptospira infecting cattle in the United States.[93]

Salt altered cells and desoxycholate extract had broad crossreactivity (Figures 3-4). Based on these results the butanol-water extracted antigen was selected as the most adequate antigen to be applied to detect cattle infections with serovar hardjo.

Table 2. Concentration of protein and carbohydrate as measured by Bio-Rad technique (595nm wave length) and Dubois technique (490nm wavelength) respectively. Values presented were calculated based on the dilution at which the optimal EIA reactions were observed. Butanol/water extract (BUT), mechanically disrupted membrane antigen (MEM), desoxycholate extract (DOC), and salt altered cell extract (SAC)

	ANTIGENS			
COMPONENT	SAC	MEM	DOC	BUT
PROTEIN (μg/ml)	170	2000	130	70
CARBOHYDRATE (μg/ml)	120	990	110	140
PROTEIN (μg/well)	0.011	0.031	0.033	0.035
CARBOHYDRATE (μg/well)	0.008	0.015	0.027	0.070

Table 3. Comparison of enzyme immunoassay results using different antigens to determine nonspecific binding of antibody. Twenty sera from non-infected cattle (MA negative) was diluted 1/100; another 20 wells were left without serum. Values are the means of the absorbance obtained at 490 nm wavelength using butanol-water extracted antigen (BUT), desoxycholate extracted antigen (DOC), salt altered cell antigen (SAC), and mechanically disrupted membranes (MEM). Peroxidase labeled goat anti-bovine IgG was used with ABTS as indicator

ANTIGEN	NEGATIVE SERA	NO SERA
BUT	0.061	0.011
DOC	0.087	0.017
SAC	0.107	0.027
MEM	0.068	0.026

Figure 2. Comparison of enzyme immunoassay results using four different antigens and sera from experimentally infected cattle. Mean values (± standard error of the mean) of the optical density using 14 positive sera diluted 1:400 from cattle exposed to L. interrogans serovar hardjo. Antigens used were butanol-water extract (BUT), mechanically disrupted membranes (MEM), desoxycholate extract (DOC), and salt altered cell (SAC). Peroxidase labeled goat anti-bovine IgG was used with ABTS as indicator

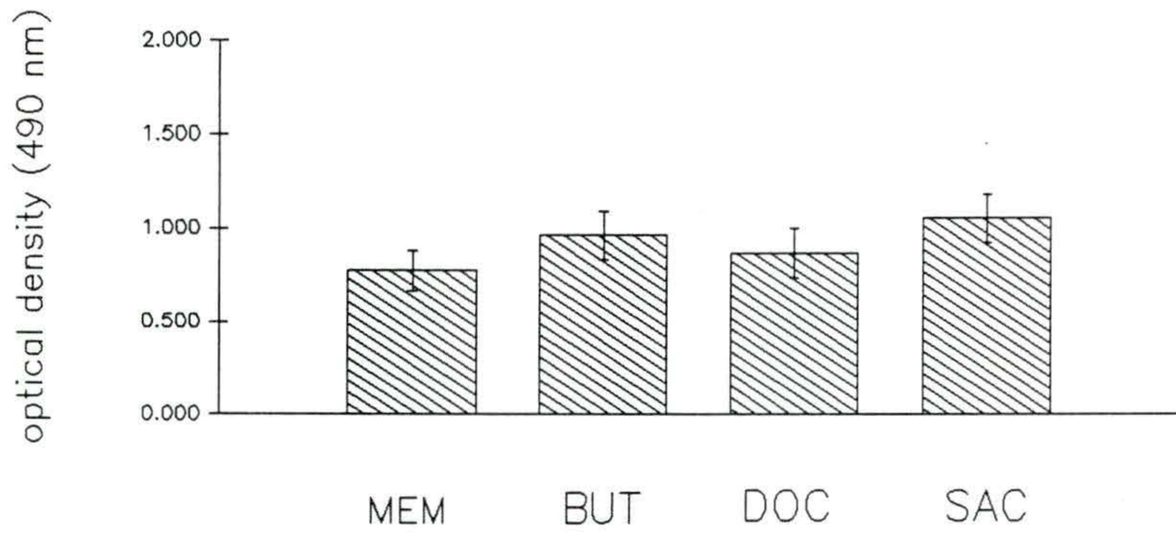

optical density (490 nm)
2.000
1.500
1.000
0.500
0.000
MEM
BUT
DOC
SAC

Figure 3. Comparison of enzyme immunoassay results obtained using salt altered cell antigen and rabbit hyperimmune serum against different serovars of *L. interrogans*. Optical density values recorded of rabbit hyperimmune sera (diluted 1:100) against *tarassovi*, *copenhageni*, *pomona*, *grippotyphosa*, *wolffi*, *hardjo*, *istrica*, *canicola*, and *bratislava*. Conjugate used was peroxidase labeled goat anti-rabbit IgG with ABTS as indicator. (*) Antiserum raised against serovars belonging to the same serogroup as *hardjo*

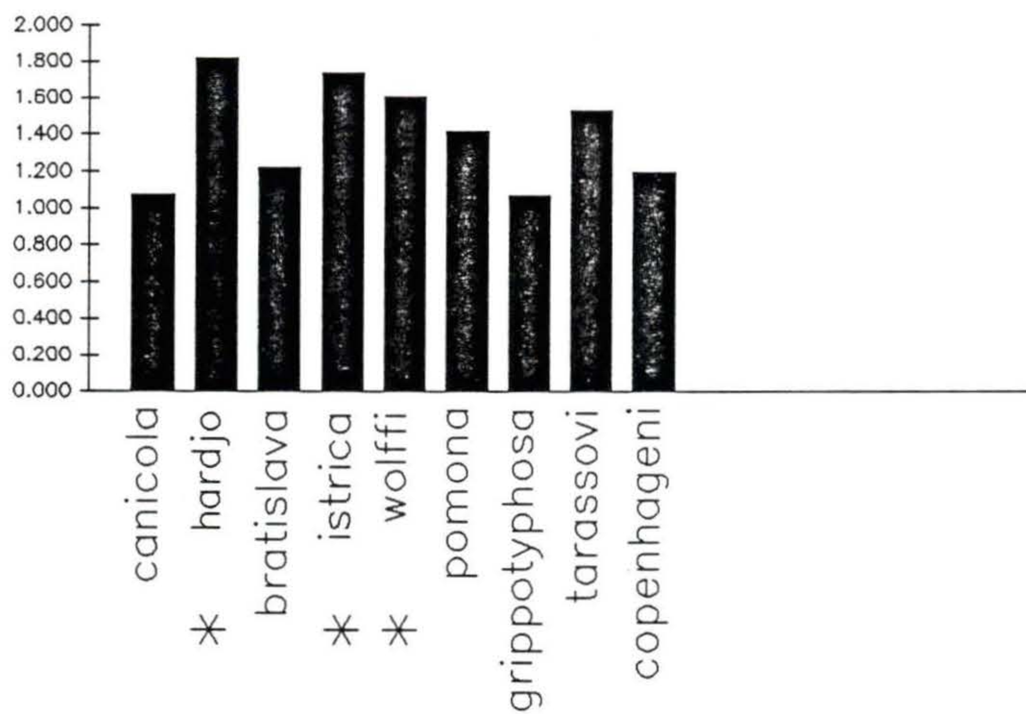

OPTICAL DENSITY (490 nm)
2.000
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
canicola
* hardjo
bratislava
* istrica
* wolffi
pomona
grippotyphosa
tarassovi
copenhageni

Figure 4. Comparison of enzyme immunoassay results obtained using desoxycholate extracted antigen and rabbit hyperimmune serum against different serovars of _L. interrogans_. Optical density values recorded of rabbit hyperimmune sera (diluted 1:100) against _tarassovi_, _copenhageni_, _pomona_, _grippotyphosa_, _wolffi_, _hardjo_, _istrica_, _canicola_, and _bratislava_. Conjugate used was peroxidase labeled goat anti-rabbit IgG with ABTS as indicator. (*) Antiserum raised against serovars belonging to the same serogroup as _hardjo_

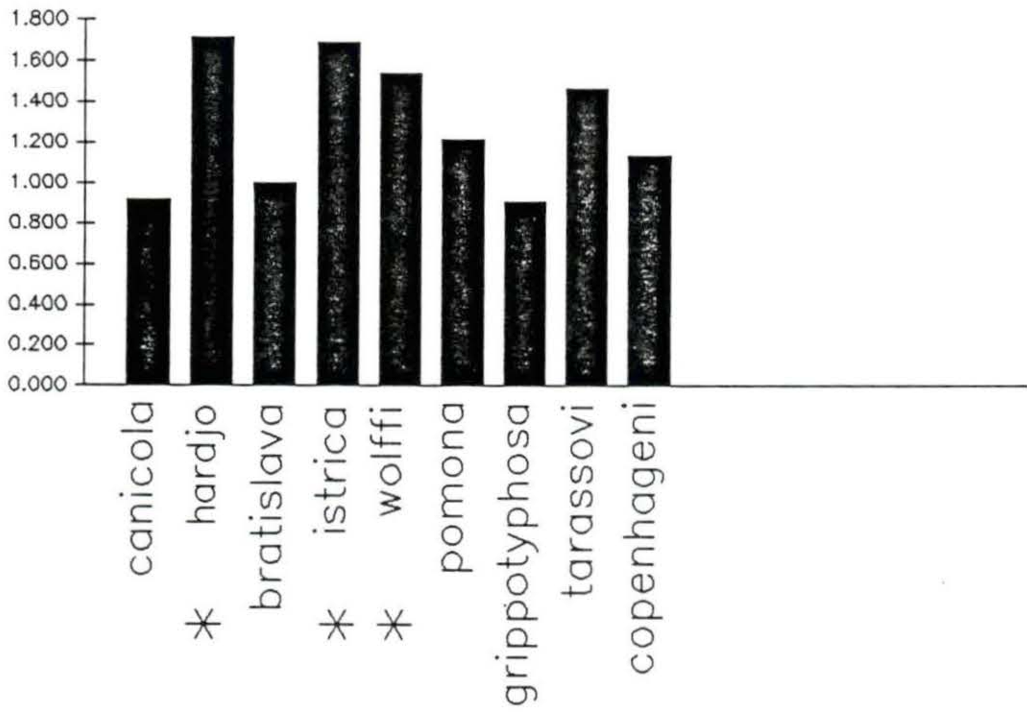

OPTICAL DENSITY (490 nm)
1.800
1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
canicola
* hardjo
bratislava
* istrica
* wolffi
pomona
grippotyphosa
tarassovi
copenhageni

Figure 5. Comparison of enzyme immunoassay results obtained using mechanically disrupted membranes antigen and rabbit hyperimmune serum against different serovars of _L. interrogans_. Optical density values recorded of rabbit hyperimmune sera (diluted 1:100) against _tarassovi_, _copenhageni_, _pomona_, _grippotyphosa_, _wolffi_, _hardjo_, _istrica_, _canicola_, and _bratislava_. Conjugate used was peroxidase labeled goat anti-rabbit IgG with ABTS as indicator. (*) Antiserum raised against serovars belonging to the same serogroup as _hardjo_

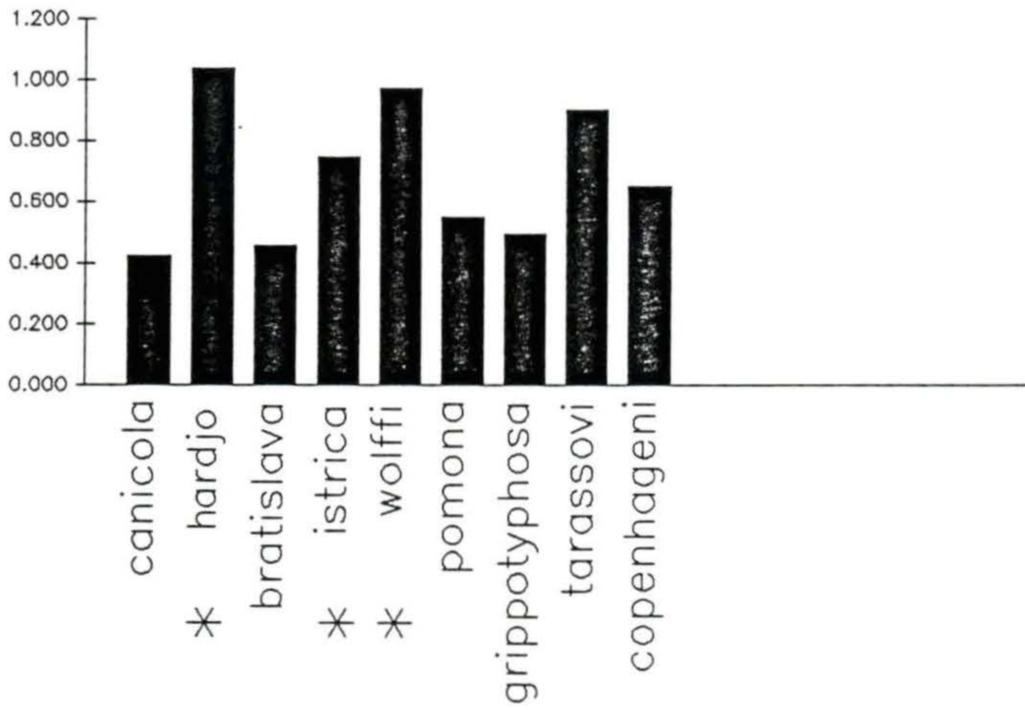
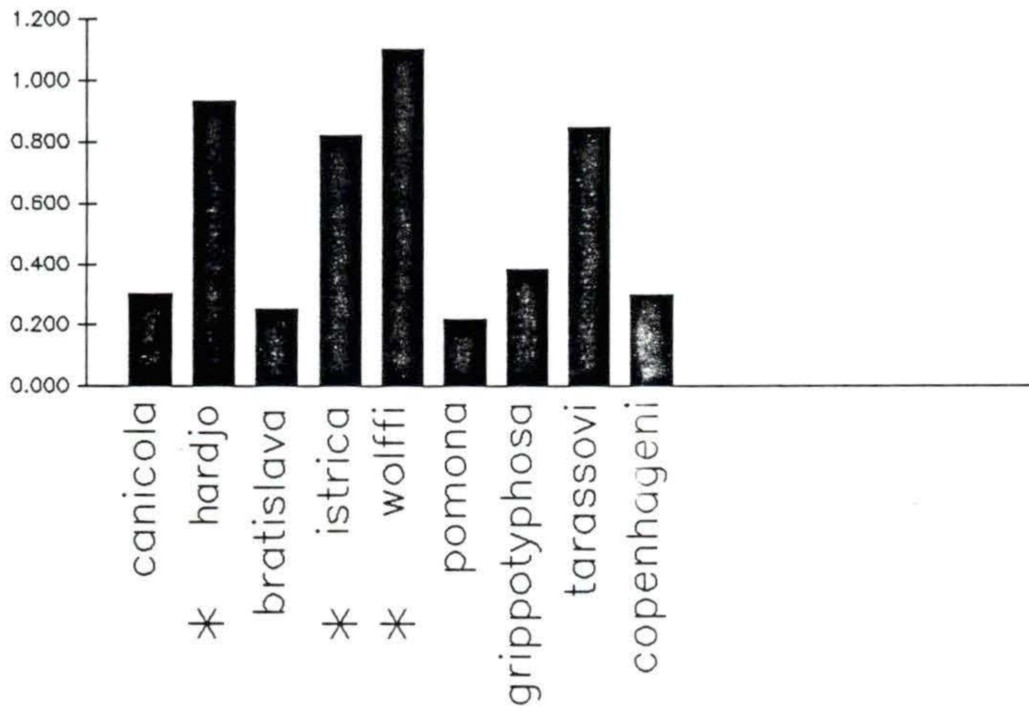

OPTICAL DENSITY (490 nm)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
canicola
* hardjo
bratislava
* istrica
* wolffi
pomona
grippotyphosa
tarassovi
copenhageni

Figure 6. Comparison of enzyme immunoassay results obtained using butanol-water extracted antigen and rabbit hyperimmune serum against different serovars of _L. interrogans_.
Optical density values recorded of rabbit hyperimmune sera against _tarassovi_, _copenhageni_ _pomona_, _grippotyphosa_, _wolffi_; _hardjo_, _istrica_, _canicola_, and _bratislava_. Conjugate used was peroxidase labeled goat anti-rabbit IgG with ABTS as indicator. (*) Antiserum raised against serovars belonging to the same serogroup as _hardjo_



OPTICAL DENSITY (490 nm)
1.200
1.000
0.800
0.600
0.400
0.200
0.000
canicola
* hardjo
bratislava
* istrica
* wolffi
pomona
grippotyphosa
tarassovi
copenhageni

DISCUSSION

The results of these experiments demonstrate that outer membrane antigens produce the most suitable reactions in enzyme immunoassay, because they produced a high optical density and a low background. The extraction of the outer membrane using detergents seems to free cross reactive antigens that are not readily available to antibodies in the outer membrane surface of an intact organism. Lipopolysaccharide-like components extracted using the butanol-water procedure had a high degree of serogroup specificity. The results presented here provide evidence that the butanol/water extract of Leptospira contains the sejroe serogroup and hardjo serovar specific epitopes.

The outer membrane disrupted by mechanical means showed some serogroup specific reactivity. Unlike detergent extracts, the mechanical disruption does not dissolve the outer membrane. Therefore some of the determinants may remain unaltered.

Butanol-water extracted antigen was selected to be applied to the study of bovine leptospirosis produced by serovar hardjo type hardjo-bovis. This antigen also has valuable features for investigating the immune response against leptospiral LPS. The relative purity of this antigen may allow for study of the immune responses of cattle

against LPS. There is also a potential for use of butanol/water extracted antigen for diagnosis of cattle naturally infected with serovar hardjo type hardjo-bovis.

Cross-reactive properties of the outer membrane proteins have been demonstrated by other workers.[57,80] These proteins may be responsible for cross-reactivity observed with antigens such as desoxycholate and salt altered cell antigens. These crossreactive determinants also could have a valuable application on research of the cattle immune response against infection produced by any serovar of L. interrogans. Comparative studies of humoral responses directed against LPS and those against protein components of the outer membrane may provide information about protective immune responses against the disease. Cross reactive antigens also may be useful in diagnosis of human leptospirosis.

Lack of antigenic reactivity of some extracts could be due to alteration or absence of some epitopes, lack of attachment to the polystyrene EIA plate, or low yield of antigens. Future work will be oriented to extract antigens from other serogroups of Leptospira and improve a serogroup specific enzyme immunoassay.

PART II: EVALUATION OF AN ENZYME IMMUNOASSAY FOR DETECTION OF ANTIBODIES AGAINST *Leptospira interrogans* SEROVAR *hardjo* TYPE HARDJO-BOVIS IN CATTLE SERUM

INTRODUCTION

Leptospira interrogans serovar *hardjo* type hardjo-bovis is the only serovar *hardjo* isolated from cattle in the United States.[35,66] This organism is also the most common *Leptospira* isolated from cattle worldwide.[26,65,93] Type hardjo-bovis causes chronic leptospirosis in cattle characterized by relatively low MA titers.[16,17,93]

The MA test is the standard diagnostic test for leptospirosis in animals. However the MA test is insensitive for detection of cattle infected with type hardjo-bovis. In addition, the MA test is a dangerous, laborious, and time consuming procedure.

Comparative studies have demonstrated that the sensitivity of EIA is greater than MA for detection of antibodies in cattle experimentally infected with hardjo-bovis.[16,93,97] Variations in the magnitude of serologic reactions of experimentally infected animals seems associated with the route of challenge.[97] Intramuscular or intravenous routes of challenge apparently induce a measurable humoral response earlier than that observed following conjunctival route as measured by EIA and MA.[3,27,97] Additionally, a lack of correlation between EIA and MA results in experimental infections has been found.[3,27]

Immunoglobulins M and G against serovar *hardjo* have been detected during different stages of the disease.

Immunoglobulin M is the first class of antibody detected.[3,27] Immunoglobulin G is produced later and the titer tends to persist for long periods of time.[3,27] Detection of anti-leptospiral IgM has been suggested to be useful for detection of early infection. Likewise detection of anti-hardjo IgG has been regarded as a means to detect chronic infections.[27] Antibodies produced by vaccination are also detected by EIA.[7,94]

The purpose of this research was to study the development of the cattle humoral immune response against hardjo-bovis lipopolysaccharide (butanol-water extract) after vaccination and after infection. The research was oriented to determine the advantages and limitations that EIA might have as compared with the MA test as a diagnostic test. The possibility of discrimination between vaccination titers and infection titers in terms of amount or class of immunoglobulins was also assessed.

MATERIALS AND METHODS

Antigen Extraction

This extraction was carried out as described by Morrison and Leive[69] and modified by Wannemuehler et al.[103] Serovar hardjo type hardjo-bovis organisms were harvested by centrifugation (32,000 x g) and resuspended in PBS (pH 7.2); the washing procedure was repeated twice and finally, the pellet suspended in 0.85% saline at 4°C. The mixture was transferred to a polypropylene centrifuge tube, 20 ml of water-saturated butanol-1 was added, and vortexed for 15 minutes. The extract was then centrifuged at 35,000 x g for 20 minutes. The aqueous phase was collected. The extraction was repeated twice, adding 10 ml of saline each time. The aqueous phases were combined. Pronase[a] was prepared in 0.2 M of Na_3PO_4 buffer, pH 7. This solution was added to the combined aqueous phases to obtain a final concentration of 20 μg of enzyme per ml of aqueous phase. This preparation was incubated at 37°C for 18 hours. After removing the white flocculent layer from the top of the tube, the contents were centrifuged at 20,000 x g for 40 minutes. The supernatant was dialyzed against 50 volumes of distilled water (4 changes).

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[a]Boehringer Mannheim Biochemicals, Indianapolis, IN.

Cattle Serum

Eighteen 4 to 8 month-old mixed breed steers that lacked detectable serum antibodies against serovar *hardjo* (hardjoprajitno) by the microscopic agglutination test were used. Steers 101 and 9863 were given one dose of pentavalent vaccine containing hardjoprajitno; steers 1020, 1030, 1060, and 9900 were given two doses of the same vaccine, with 3 weeks between vaccinations. Steers 105, 211, 212, and, 214 were given one dose of pentavalent vaccine containing hardjo-bovis; steers 111, 973, 1028, and 9875 were given two doses of the same vaccine, with 3 weeks between vaccinations. Steers 1003, 1023, 1052, and 1025 were maintained as nonvaccinated controls. All vaccine was administered intramuscularly with a 2 ml dose.

Serum obtained from all steers during post vaccination (initial) week 0, 9, and 26 was tested for antibodies against serovar *hardjo*. Twenty-six weeks after vaccination, steers were challenged with a field isolate of serovar *hardjo* type hardjo-bovis.

Challenge was by conjunctival instillation of 1×10^7 cells of serovar *hardjo* type hardjo-bovis on 3 occasions. Nonvaccinated animals were challenged in a similar manner.[17]

Sera collected at 1, 2, and 4 weeks postchallenge were analyzed. Each of the vaccinated and non vaccinated con-

trol steers became infected and shed type hardjo-bovis in the urine. Results and experimental data were previously published.[17]

An additional 216 cattle sera and MA test data from a diagnostic laboratory survey were kindly provided by Dr. David Miller.[a] Ninety eight of these sera contained MA detectable antibodies at a 1:100 dilution against serovar hardjo.

Enzyme Immunoassay

The EIA protocol was based on that described by Thiermann and Garret.[96] Immulon I plates[b] were coated with 250 µl of 1:500 dilution of butanol/water extracted antigen diluted in a solution containing 0.05 M carbonate-bicarbonate buffer, pH 9.6. Antigen was incubated for 18 hours at 4°C. Contents of the plate were then aspirated and unoccupied binding sites were blocked for 1 hour with 300 µl/well of a filter sterilized solution of 2% ovine serum albumin[c] dissolved in 0.01 M PBS (pH 7.2) at room temperature. After aspiration of the blocking agent, 200 µl of

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[a]National Veterinary Services Laboratory, USDA, Ames, IA.

[b]Dynatech Laboratories Inc., Alexandria, VA.

[c]Sigma Chemical Co., St. Louis, MO.

serum diluted 1:100 in 0.01 M PBS (pH 7.2) were placed in each well and allowed to react for 2 hours at 37°C. The first well in the plate was not reacted with serum for use as a blank. After aspiration of serum, plates were washed 4 times with a solution of 1% Tween 20 in PBS (pH 7.2). The last three washes were done at 3 minutes intervals.

Goat anti-bovine IgG and goat anti-bovine IgM conjugated to horseradish peroxidase[a] were diluted 1:1000 in PBS and 250 μl of this solution was placed in each well. Plates were incubated for 90 minutes at 37°C. After this period of time, the conjugate was aspirated and the plate washed as before. During all incubation periods, plates were placed in a plastic bag to avoid evaporation. The substrate was prepared with 20 ml of 0.96% of citric acid buffer (pH 4), mixed with 100 μl of a 1.83% peroxide solution and 100 μl of ABTS[b] (2,2`-azino-di-[3-ethyl-benzythiazoline sulfonic acid]). Two hundred μl of the substrate was added to each well and the reaction was allowed for 15 minutes at 37°C. To stop the reaction, 50 μl/well of a 37 mM solution of sodium cyanide was added. Reactions

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[a]Kirkegaard and Perry Laboratories Inc., Gaithers burg, MD.

[b]National Veterinary Service Laboratories, USDA, Ames, IA.

were read using a Dynatech 3900 EIA reader at 490 nm wavelength.

Sera collected from individual cattle on the day of challenge and during weeks 1, 2, and 4 after challenge were analyzed within the same plate to minimize variations. Samples collected on the day of vaccination and 9 weeks after were analyzed in another plate. Individual plates were used for IgG and for IgM determinations (Tables 6-9). To establish the cut-off point between positive and negative values, the criterion described by Adler et al.[10] was used. Two known negative sera were analyzed in each plate and the mean of the optical densities were added to three times a preestablished standard deviation (0.033). All optical density readings exceeding this value were considered positive.

Two positive control sera were also analyzed in each plate. Serum from cattle experimentally infected with serovar hardjo was used for this purpose.

Microscopic Agglutination Test

The technique used was that described by Cole et al.[25] A L. interrogans serovar hardjo type hardjoprajitno culture with a concentration of 27 nephelometer units was used as antigen.

Flat-bottom disposable plastic microtiter plates were

used. For dilutions, 50 µl of PBS was added to each well of a row except the first well that received 84 µl. Sixteen µl of serum was added to the first row of wells and two-fold dilutions were made with a microdiluter, beginning with 1:12.5 to a final dilution of 1:12,800. After serum dilution, 25 µl of <u>hardjo</u> antigen was added to each well. Plates were covered and incubated at room temperature for 2 hours. Agglutination was evaluated using a dark-field microscope equipped with a long-working-distance 10X objective. The MA titer of a serum was recorded as the reciprocal of the highest dilution at which 50% or more of the leptospires were agglutinated. Geometric mean titers were calculated using the reciprocal of the lowest dilution at which < 50% of leptospires agglutinate, i.e., the first dilution at which a negative reaction was observed.

RESULTS

Vaccinated and nonvaccinated cattle were challenged with a field isolate of serovar _hardjo_ type hardjo-bovis. Regardless of the vaccination status, all animals became chronically infected and shed leptospires in their urine.[17]

A relative high level of nonspecific IgM binding were observed in some cattle before vaccination. Values of anti-hardjo IgM were increased 9 weeks after vaccination in 11 of the 14 cattle. Levels of anti-_hardjo_ IgM diminished to negative values by 26 weeks after vaccination (Table 4).

Titers of anti-hardjo IgG were clearly increased 9 weeks after vaccination in all 14 cattle. The IgG levels remained positive 26 weeks after vaccination (Table 5).

Microscopic agglutination titers were also increased 9 weeks after vaccination. Seven of 14 of these sera had titers ≥ 100 (Table 6), which by standard procedures would be considered positive.[25] Microscopic agglutination titers were ≤ 25, 26 weeks after vaccination.

Vaccinated and nonvaccinated cattle showed a completely different humoral immune response after challenge. Nonvaccinated control cattle showed no anti-_hardjo_ IgM or IgG antibodies before challenge. One week after challenge, 1 of 4 sera from nonvaccinated animals had detectable anti-_hardjo_ IgM (Table 8). An overall increase in IgM

titers occurred during the first week (Figure 7). Likewise, 1 week after challenge the IgG antibody titers of nonimmunized animals were also increased but positive readings were not recorded until the second week after challenge (Table 9). The appearance of IgG antibodies was characterized by a simultaneous decrease of anti-*hardjo* IgM levels (Figure 7).

Microscopic agglutination titers of nonvaccinated cattle were also increased 1 week after challenge but titers greater than 100 were not observed. The peak post-challenge MA titers were observed during the second week after challenge; all the animals had titers between 400 and 800 (Table 7).

As mentioned before, all vaccinated cattle had high IgG levels and very low IgM levels at the time of challenge. These levels remained practically unaltered during the 4 weeks following challenge (Figure 8). No secondary IgG or IgM humoral response was found after challenge as assessed by EIA.

Similarly, post challenge MA titers of vaccinated animals were different from those obtained from nonvaccinated animals. Low titers (≤ 25) remained basically the same during the four weeks after challenge (Table 6).

Microscopic agglutination titers of sera obtained from the diagnostic laboratory survey were 99.5% coincident with

EIA results in positive samples. However only 58.3% of the MA negative results were coincident with EIA negative results; 41.6% of the samples with titers lower than 100 in MA were EIA positive. Only one serum was found to have a MA titer greater than 100 and negative to EIA (Appendix, Table A14). A regression analysis between means of EIA optical density values and log of the MA titers showed a R^2 of 0.95 (Figure A1).

Table 4. Results of enzyme immunoassay using serum from 14 cattle collected at: 0 and 9 weeks after vaccination and 0 (26 weeks after vaccination), 1, 2 and 4 weeks following conjunctival exposure to *Leptospira interrogans* serovar *hardjo* type hardjo-bovis. Optical density measured at 490 nm wavelength using butanol-water extract (antigen) and peroxidase labeled goat antibovine IgM with ABTS as indicator

Steer	Time (weeks)					
	Vaccination[a]		Challenge[b]			
	0	9	0[c]	1	2	4
101	0.137	0.258	0.114	0.057	0.073	0.052
105	0.110	0.256	0.058	0.037	0.033	0.052
111	0.133	0.218	0.029	0.02	0.023	0.022
211	0.172	0.215	0.086	0.042	0.036	0.062
212	0.256	0.233	0.074	0.167	0.062	0.042
214	0.233	0.227	0.072	0.073	0.038	0.083
973	0.257	0.538	0.080	0.068	0.047	0.072
1020	0.150	0.263	0.191	0.06	0.116	0.090
1028	0.136	0.162	0.018	0.019	0.025	0.037
1030	0.107	0.190	0.107	0.226	0.171	0.294
1060	0.369	0.400	0.107	0.172	0.131	0.211
9863	0.201	0.239	0.036	0.034	0.042	0.042
9875	0.113	0.384	0.072	0.021	0.041	0.029
9900	0.200	0.193	0.063	0.029	0.046	0.055

[a]Limit between positive and negative value is 0.213.

[b]Limit between positive and negative value is 0.156.

[c]26 weeks after vaccination.

Table 5. Results of enzyme immunoassay using serum from 14 cattle collected at: 0 and 9 weeks after vaccination and 0 (26 weeks after vaccination), 1, 2 and 4 weeks following conjunctival exposure to *Leptospira interrogans* serovar *hardjo* type hardjobovis. Optical density measured at 490nm of wavelength using butanol-water extract (antigen) and peroxidase labeled goat antibovine IgG with ABTS as indicator

Steer	Time (weeks)					
	Vaccination[a]		Challenge[b]			
	0	9	0[c]	1	2	4
101	0.108	0.880	0.805	0.640	0.614	0.567
105	0.093	0.645	0.533	0.421	0.426	0.405
111	0.170	1.092	0.713	0.556	0.535	0.487
211	0.110	1.058	0.560	0.528	0.735	0.447
212	0.140	0.868	0.680	0.517	0.501	0.587
214	0.149	0.907	1.118	1.080	1.032	1.020
973	0.123	1.026	1.100	0.939	1.008	0.879
1020	0.117	0.598	0.356	0.236	0.206	0.163
1028	0.051	0.775	0.263	0.268	0.226	0.203
1030	0.078	0.855	0.257	0.263	0.424	0.322
1060	0.094	0.846	0.762	0.741	0.659	0.528
9863	0.188	0.691	0.425	0.474	0.775	0.524
9875	0.165	1.191	0.815	0.768	0.739	0.595
9900	0.072	0.880	0.535	0.417	0.418	0.338

[a] Limit between positive and negative value is 0.213.

[b] Limit between positive and negative value is 0.156.

[c] 26 weeks after vaccination.

Table 6. Results of microscopic agglutination test using 14 cattle sera collected 0 and 9 weeks after vaccination and 0 (26 weeks after vaccination), 1, 2 and 4 weeks following conjunctival exposure to *Leptospira interrogans* serovar *hardjo* type hardjo-bovis. Titers are the reciprocal of the last dilution of serum at which a 50% of leptospires were agglutinated

Steer	Time (weeks)					
	Vaccination		Challenge			
	0	9	0[a]	1	2	4
101	< 12.5	50	12.5	25	12.5	25
105	< 12.5	50	< 12.5	25	12.5	12.5
111	< 12.5	200	12.5	25	12.5	25
211	< 12.5	25	< 12.5	12.5	12.5	25
212	< 12.5	100	12.5	25	12.5	25
214	< 12.5	100	12.5	25	50	50
973	< 12.5	400	25	25	25	25
1020	< 12.5	50	12.5	12.5	12.5	12.5
1028	< 12.5	100	12.5	25	12.5	12.5
1030	< 12.5	25	< 12.5	12.5	25	12.5
1060	< 12.5	200	25	25	25	25
9863	< 12.5	25	12.5	25	25	25
9875	< 12.5	400	25	25	25	25
9900	< 12.5	50	12.5	25	12.5	12.5

[a] 26 weeks after initial vaccination.

Table 7. Results of microscopic agglutination test using sera from nonvaccinated animals collected at 0, 1, 2 and 4 weeks following conjunctival exposure to *Leptospira interrogans* serovar *hardjo* type hardjo-bovis. Data are the reciprocal of the last dilution of serum at which a 50% of leptospires were agglutinated

Steer	Time after infection (weeks)			
	0	1	2	4
1003	< 12.5	12.5	400	200
1023	< 12.5	12.5	800	400
1025	< 12.5	12.5	800	400
1052	< 12.5	12.5	400	50

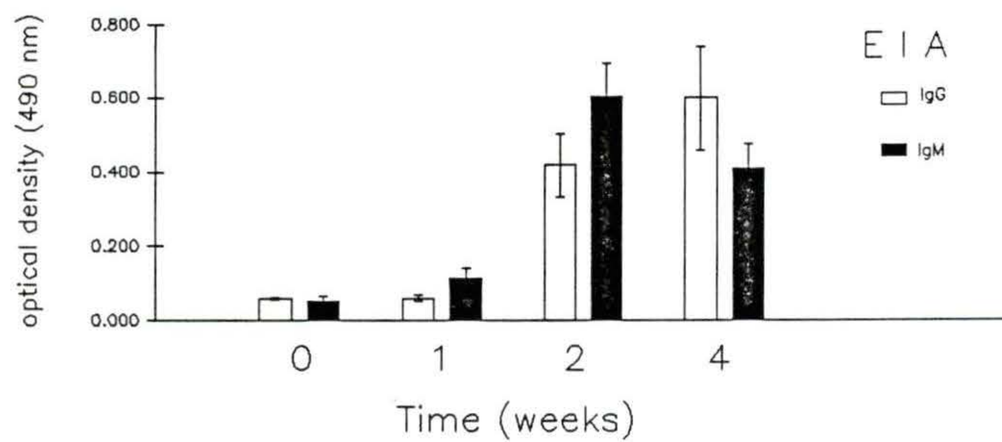
Table 8. Results of enzyme immunoassay using sera from nonvaccinated animals collected at: 0, 1, 2 and 4 weeks following conjunctival exposure to Leptospira interrogans serovar hardjo type hardjo-bovis. Optical density measured at 490 nm wavelength using butanol-water extract (antigen) and peroxidase labeled goat antibovine IgM with ABTS as indicator. Limit between positive and negative values is 0.156

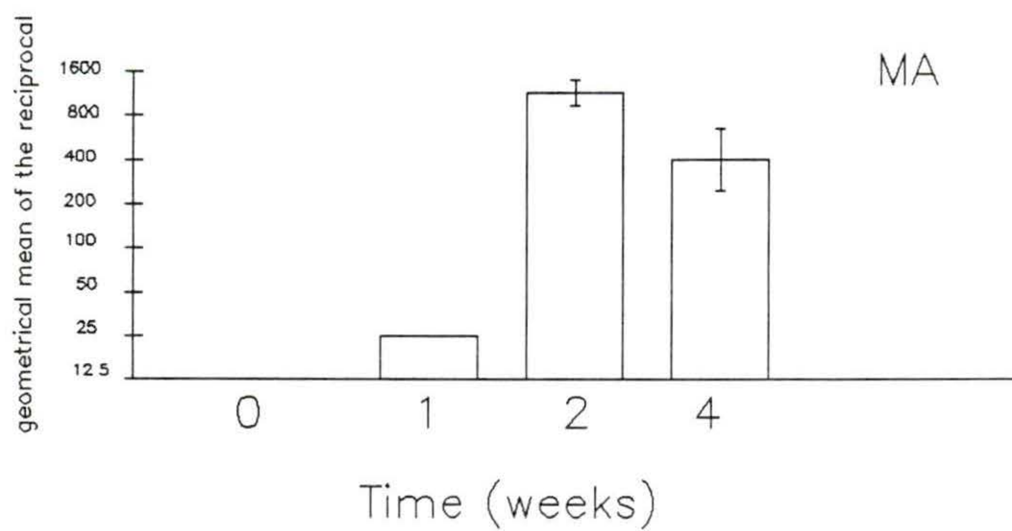
Steer	Time (weeks)			
	0	1	2	4
1003	0.078	0.128	0.532	0.686
1023	0.054	0.193	0.614	0.349
1025	0.083	0.068	0.825	0.558
1052	0.013	0.081	0.380	0.269

Table 9. Results of enzyme immunoassay using sera from nonvaccinated animals collected at 0, 1, 2 and 4 weeks following conjunctival exposure to Leptospira interrogans serovar hardjo type hardjo-bovis. Optical density measured at 490 nm wavelength using butanol-water extract (antigen) and peroxidase labeled goat antibovine IgG with ABTS as indicator. Limit between positive and negative values is 0.156

Steer	Time (weeks)			
	0	1	2	4
1003	0.057	0.061	0.509	0.608
1023	0.067	0.077	0.608	0.939
1025	0.052	0.066	0.236	0.249
1052	0.067	0.042	0.322	0.597

Figure 7. Comparison of results of enzyme immunoassay (EIA) and microscopic agglutination (MA) test using serum collected from non vaccinated cattle at 0, 1, 2, and 4 weeks following conjunctival exposure to serovar *hardjo* type hardjo-bovis.
Optical density mean values (± standard error of the mean) measured at 490 nm wavelength using a butanol-water extracted anti gen, peroxidase labeled goat antibovine IgM and IgG with ABTS as indicator.
Geometrical means (± standard error of the mean) of micoscopic agglutination test (MA).
Note: For calculation of geometrical means, microscopic agglutination titers were calculated as described in materials and methods


optical density (490 nm)
0.800
0.600
0.400
0.200
0.000
E I A
IgG
IgM
0
1
2
4
Time (weeks)


geometrical mean of the reciprocal
1600
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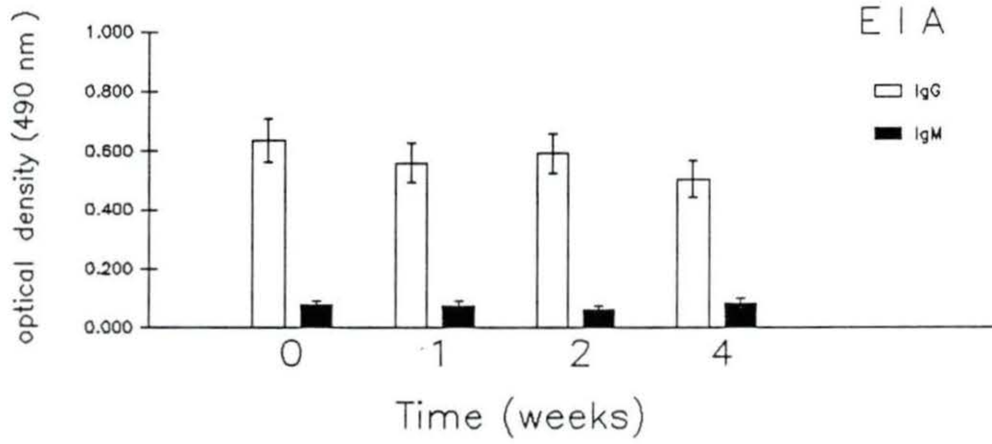
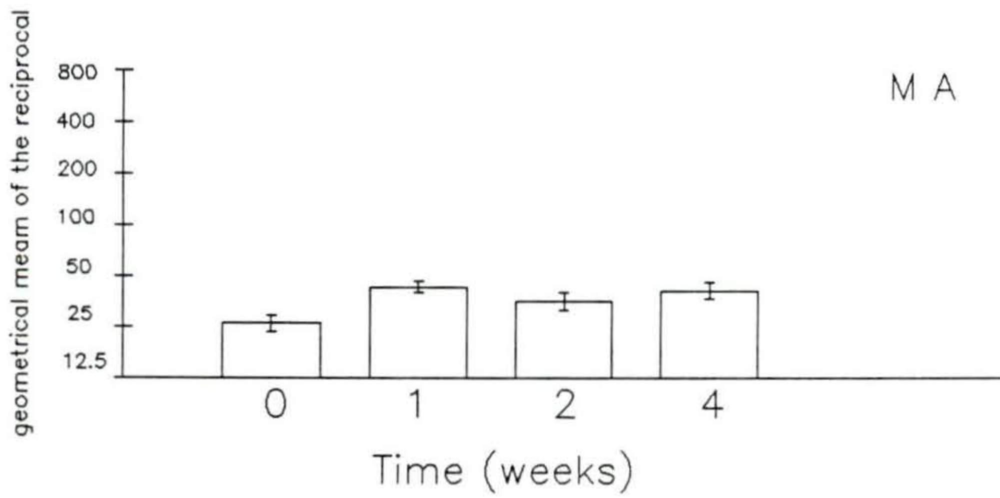
400
200
100
50
25
12 5
MA
0
1
2
4
Time (weeks)

Figure 8. Comparison of results of enzyme immunoassay (EIA) and microscopic agglutination test (MA) using sera collected from vaccinated animals (26 weeks) at 0, 1, 2 and 4 weeks following conjunctival exposure to serovar *hardjo* type hardjo-bovis.
Optical density mean values (± standard error of the mean) as measured by EIA (at 490nm of wavelength) using a butanol-water extracted antigen, peroxidase labeled goat antibovine IgM and antibovine IgG with ABTS as indicator.
Geometrical means (± standard error of the mean) of microscopic agglutination test.
Note: For calculation of geometrical means, microscopic agglutination titers were calculated as described in materials and methods



E I A
IgG
IgM
optical density (490 nm)
1.000
0.800
0.600
0.400
0.200
0.000
0
1
2
4
Time (weeks)



M A
geometrical meam of the reciprocal
800
400
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100
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25
12.5
0
1
2
4
Time (weeks)

DISCUSSION

The results of this research demonstrate that enzyme immunoassay was more sensitive than a microscopic agglutination test for detection of serovar hardjo infection in cattle. Comparison of EIA results with those obtained by MA test clearly demonstrated a greater capability of EIA to detect chronically infected cattle (Table 5). The MA reactions during chronic infection may be comparable to those obtained using sera from nonexposed animals (Table 6.

Enzyme immunoassay was also more sensitive than the MA test during the first week after infection in nonvaccinated cattle. Detection of antibodies resulting from vaccination was also greater on EIA than MA test. Despite the sensitivity of the test, it was not possible to appreciate any difference in titers or immunoglobulin isotype in vaccinated animals following infection (Figures 7 and 8).

Lack of clinical history of the sera obtained from the field make it difficult to conclude that all EIA positive samples correspond to infection titers. However it is unlikely that all antibodies detected by EIA which were not detected by MA were produced by vaccination.

The analysis of sera from experimentally infected cattle by EIA showed that IgG is the predominant immunoglobulin produced in cattle infected with hardjo-bovis.

The apparent rapid decline in IgM titers in nonvaccinated cattle 4 weeks after infection may be an artifact of the test resulting from competition between IgG and IgM. In addition, it has been suggested that rising levels IgG_1 may negatively influence IgM-producing B-lymphocytes in cattle.[73] This mechanism could also account for the absence of anti-hardjo IgM levels in vaccinated animals.

Although reports indicate that EIA results do not correlate with MA results,[3,27] some degree of coincidence between peaks on MA reactions and IgM EIA results was observed here. Additionally some degree of correlation was found between anti-hardjo IgG EIA results and MA titers of field samples. Immunoglobulin M is generally considered to have a greater capacity of agglutination.[62,72] These observations indicate that anti-hardjo IgM and IgG are involved in the MA reactions as observed in the immune response of nonvaccinated animals (Figure 7) and the correlation observed between EIA (IgG) results and MA test titers (Figure A1).

Results of this research show that determination of IgG titers was more efficient and could have a more practical applications than determination of IgM titers. Two major advantages were appreciated using the IgG system; first, IgG responses predominated during the infection, and second, nonspecific binding was observed in some samples

using the IgM system. However, IgM titers appeared earlier than IgG titers and this could be valuable in detection of recent infections (Table 4).

A secondary humoral response to serovar hardjo was not observed in vaccinated cattle after challenge. The reasons for this are not clear. However, the presence of relatively high levels of IgG may block the epitopes on the challenge leptospire and keep them from eliciting a secondary immune response. Similarly these IgG molecules or immunocomplexes may produce a suppressive effect on B-lymphocytes as observed in other systems.[22]

Use of leptospiral LPS in an EIA was demonstrated to be efficient in diagnosis of serovar hardjo infections in cattle. Additionally, leptospiral LPS is receiving significant attention because of findings suggesting that this molecule contains epitopes that are protective.[40,85,91] However the results of this research do not concord with previous findings since cattle with relatively high humoral response to hardjo-bovis LPS became chronically infected with the homologous leptospire.

EIA can be readily applied for diagnosis of bovine leptospirosis produced by *L. interrogans* serovar hardjo type hardjo-bovis. The extraction of antigens from *Leptospira* is simple and productive in terms of yield. In addition, the EIA described here is more easily standardized

and less hazardous than the MA test. Despite the greater detection of vaccination titers the enzyme immunoassay is a good alternative to microscopic agglutination test. The EIA described here would be particularly useful in areas where cattle are not vaccinated against serovar hardjo. More testing is required in order to establish the advantages and the limitations of the test.

GENERAL SUMMARY AND DISCUSSION

This thesis is composed of two sections. The first section is devoted to the development of techniques to be applied in the second section. It includes a brief discussion of the rationale to select antigens and procedures. The second is the application of the EIA using LPS as antigen for detection of antibodies against L. interrogans serovar hardjo in cattle sera.

Outer membrane antigens were analyzed as potential antigens for an enzyme immunoassay. Extraction of the outer membrane components using detergents produced serogroup crossreactive antigens. Lipopolysaccharide components extracted by a butanol/water procedure were shown to be sensitive and serogroup specific antigens.

An EIA was used to detect antibodies against L. interrogans serovar hardjo type hardjo-bovis in cattle serum from vaccinated, experimentally infected and naturally exposed cattle to serovar hardjo. Butanol-water extracted lipopolysaccharide was used as antigen.

Sera from experimentally vaccinated animals were positive by EIA after 9 and 26 weeks. Microscopic agglutination titers ≥ 100 were detected in 50% of the samples 9 weeks after vaccination but none were ≥ 100 26 weeks after vaccination.

It was not possible to detect any secondary humoral response by either test in vaccinated animals following challenge by either test. Immunoglobulin G was the predominant immunoglobulin detected in sera of vaccinated and infected cattle. Immunoglobulin M was detected only in nonvaccinated cattle for a short period of time and the presence of IgM coincided with the peak of MA test titers.

Results of the research indicate that a serogroup EIA could provide a more sensitive and more practical test for diagnosis of leptospirosis and without hazard of human exposure. One of the major disadvantages observed is the inability to discriminate vaccination titers from infection titers.

The availability of a lipopolysaccharide EIA may allow an assessment of the reason that a secondary immune response does not occur in vaccinated cattle that became infected with hardjo-bovis. Immune humoral responses are classified according to the T-lymphocytes contribution into thymus-dependent and thymus-independent immune responses.[62] Likewise, mechanisms involved in down regulation of humoral immune responses are either dependent or independent of T-lymphocyte function.[22] Leptospiral LPS does not appear to share the same biological properties observed in LPS from other gram-negative organisms.[87] However leptospiral LPS appears to induce mitogenic effects on B lymphocytes, and

adjuvant effects inducing production of non-specific antibodies and antitumor activity.[51,87]

T-cell-independent responses are characterized by production of high amounts of IgM, low amounts of IgG and absence of immunological memory.[62] The features of T-cell-independent responses contrast with findings of this research because IgG is the predominant immunoglobulin. However, feed back inhibition effects of IgG have been shown to play important roles in modulation of IgM responses directed against Salmonella O antigen.[105] Similar mechanisms have been suggested to operate with IgM response against Brucella abortus LPS in cattle.[73] T-lymphocyte involvement has been found in the immune response against various antigens of Leptospira,[13,89] however, no characterization of the antigens has been done. The possibility of T cell-dependent immune suppression of the antibody response against leptospiral LPS can not be eliminated.

The inefficacy of vaccines to prevent kidney infections has been described.[16,17] Some authors attribute this lack of protection of the vaccines to alterations of immunogenic proteins produced by treatments with formalin, heat etc.[18,78] It is necessary to investigate the immunogenicity of leptospiral LPS and the importance of the anti-LPS antibodies in protection against renal infection.

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ACKNOWLEDGMENTS

I would like to thank my major professor Dr. Charles Thoen for guidance and direction in preparation of this thesis. I also would like to express my sincere appreciation to Dr. Carole Bolin for supervision of this work and encouragement, guidance and patience throughout the course of this research. Also I wish to acknowledge Dr. Michael Wannemueller, Dr. Jerome Sacks, Dr. David Miller, and Dr. Lyle Miller for their assistance in this research. I would like to extend my recognition to the staff at National Animal Disease Center and USDA for providing me the opportunity to carry out this project especially to Dr. Alejandro Thiermann.

I must mention that the research would not have been possible without the valuable contributions Annette Handsaker and John Foley to whom I express my sincere gratitude.

Additionally I would like to thank my parents and my parents-in-law for their encouragement and sacrifices. Finally, I dedicate this manuscript to my wife Elena and my two daughters Ana Francisca and Gabriela for their continuous support and love.

APPENDIX

Table A1. Optical density at 490 nm wavelength of enzyme immunoassay values recorded of rabbit hyperimmune sera against L. interrogans serovars tarassovi, copenhageni, pomona, grippotyphosa, hardjo, canicola, wolffi, istrica, and bratislava. Conjugate used was goat anti-rabbit IgG in a peroxidase-ABTS system

HYPERIMMUNE SERA SEROVARS	ANTIGENS			
	BUT	MEM	DOC	SAC
canicola	0.313	0.422	0.918	1.069
hardjo[a]	0.933	1.036	1.717	1.823
bratislava	0.253	0.460	0.999	1.220
istrica[a]	0.822	0.745	1.689	1.740
wolffi[a]	1.100	0.971	1.536	1.603
pomona	0.219	0.550	1.212	1.415
grippotyphosa	0.382	0.495	0.908	1.068
tarassovi	0.844	0.900	1.463	1.528
copenhageni	0.298	0.648	1.126	1.196

[a]Serovars belonging to the same serogroup as hardjo.

Table A2. Enzyme immunoassay values and standard deviation of the values of randomly selected negative sera from 30 nonexposed cattle. Negative sera were tested during four consecutive days using horse-radish peroxidase labeled goat anti-bovine IgG and ABTS as indicator

	DAY 1	DAY 2	DAY 3	DAY 4
	0.066	0.107	0.064	0.122
	0.039	0.135	0.083	0.184
	0.064	0.047	0.095	0.102
	0.065	0.097	0.092	0.105
	0.120	0.092	0.097	0.102
	0.086	0.013	0.038	0.139
	0.040	0.033	0.053	0.067
	0.065	0.045	0.074	0.068
	0.188	0.106	0.048	0.099
	0.048	0.036	0.066	0.100
	0.066	0.043	0.039	0.063
σ	0.041	0.038	0.020	0.033

σ = Standard deviation.

Table A3. Comparison of enzyme immunoassay results using four different antigens and sera from experimentally infected cattle. Means and standard error of means of the optical density at 490nm wave length obtained using 8 sera diluted 1/400 from cattle 3 weeks after conjunctival exposure to *L. interrogans* serovar *hardjo*. Plate was divided in four parts each of which was coated with one antigen: butanol-water extracted antigen (BUT), mechanically disrupted membranes (MEM), desoxycholate extracted (DOC), and salt altered cells (SAC). Goat antibovine IgG was used in a peroxidase ABTS system

	ANTIGENS			
SERUM	MEM	BUT	DOC	SAC
1	1.058	1.171	1.206	1.362
2	0.959	1.224	1.175	1.308
3	0.832	1.112	0.971	1.151
4	0.167	0.206	0.170	0.280
5	0.452	0.607	0.416	0.725
6	1.003	1.107	1.075	1.223
7	0.875	1.106	0.919	1.090
8	0.837	1.155	1.011	1.280
MEAN	0.773	0.961	0.868	1.052

Table A4. Titration of the butanol-water extracted antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horse-radish peroxidase labeled goat antibovine IgG at 490 nm of wave-length with ABTS as an indicator. Serum from a cow 3 weeks after conjunctival exposure to L. interrogans serovar hardjo type hardjo bovis and serum from a nonex posed animal (MA negative) were used

Conjugate — Antigen Dilutions (positive serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	1.198	1.173	1.237	1.200	1.030	0.746	0.474	0.188
1:1000	1.152	1.186[a]	1.179	1.041	0.921	0.602	0.368	0.120
1:2000	0.809	1.026	1.031	0.691	0.573	0.374	0.110	0.002
1:4000	0.583	0.536	0.431	0.310	0.199	0.053	0.056	0.000

Conjugate — Antigen Dilutions (negative serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.284	0.248	0.146	0.078	0.065	0.012	0.000	0.000
1:1000	0.188	0.106[a]	0.076	0.045	0.034	0.023	0.007	0.000
1:2000	0.089	0.045	0.045	0.008	0.000	0.000	0.000	0.000
1:4000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

[a]Optimal concentration.

Table A5. Titration of the desoxycholate extracted antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horse-radish peroxidase labeled goat antibovine IgG at 490 nm of wave-length with ABTS as a indicator. Serum from a cow 3 weeks after conjunctival exposure to L. interrogans serovar hardjo type hardjo bovis and serum from a nonexposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	2.000	2.000	1.347	0.967	0.799	0.327	0.000	0.000
1:1000	1.595	1.418	1.163[a]	0.932	0.674	0.254	0.100	0.000
1:2000	1.257	1.091	0.884	0.610	0.301	0.200	0.066	0.000
1:4000	0.937	0.780	0.394	0.450	0.410	0.270	0.051	0.012

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.293	0.211	0.205	0.173	0.136	0.105	0.098	0.044
1:1000	0.278	0.198	0.154[a]	0.101	0.089	0.055	0.058	0.055
1:2000	0.146	0.098	0.076	0.043	0.065	0.005	0.021	0.011
1:4000	0.106	0.007	0.012	0.003	0.000	0.003	0.000	0.000

[a] Optimal concentration.

Table A6. Titration of the mechanically disrupted membrane. Results of enzyme immunoassay using two fold dilutions of antigen and horseradish per oxidase labeled goat antibovine IgG at 490 nm of wavelength with ABTS as a indicator. Serum from a cow 3 weeks after conjunctival exposure to _L. interrogans_ serovar _hardjo_ type hardjo bovis and serum from a nonexposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1:500	1.216	1.305	1.445	1.515	1.487	1.565	1.45	1.144
1:1000	1.189	1.207	1.283	1.465	1.521	1.487[a]	1.323	0.949
1:2000	0.93	1.124	1.231	1.263	1.312	1.29	0.919	0.753
1:4000	1.17	0.847	1.107	1.157	1.112	1.071	0.838	0.515

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1:500	0.841	0.862	0.612	0.539	0.387	0.402	0.303	0.223
1:1000	0.555	0.527	0.464	0.343	0.220	0.183[a]	0.171	0.152
1:2000	0.463	0.418	0.310	0.259	0.195	0.174	0.145	0.129
1:4000	0.444	0.368	0.266	0.234	0.162	0.173	0.135	0.141

[a]Optimal Concentrations.

Table A7. Titration of the salt altered cell antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horseradish peroxidase labeled goat antibovine IgG at 490 nm of wavelength with ABTS as a indicator. Serum from a cow 3 weeks after conjunctival exposure to *L. interrogans* serovar *hardjo* type hardjo bovis and serum from a non-exposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1:500	2.000	2.000	2.000	2.000	1.731	1.464	1.102	0.736
1:1000	1.878	1.794	1.810	1.666[a]	1.463	1.255	0.929	0.591
1:2000	1.674	1.595	1.593	1.527	1.295	1.018	0.660	0.408
1:4000	1.668	1.533	1.429	1.390	1.130	0.762	0.475	0.279

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1:500	0.726	0.412	0.252	0.172	0.132	0.109	0.110	0.092
1:1000	0.563	0.331	0.193	0.133[a]	0.105	0.092	0.091	0.078
1:2000	0.452	0.237	0.135	0.097	0.082	0.068	0.064	0.062
1:4000	0.344	0.183	0.115	0.081	0.087	0.064	0.020	0.020

[a] Optimal concentration.

Table A8. Titration of the butanol-water extracted antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horseradish peroxidase labeled goat antibovine IgM at 490 nm of wavelength with ABTS as a indicator. Serum from a cow 3 weeks after conjunctival exposure to <u>L.</u> <u>interrogans</u> serovar <u>hardjo</u> type hardjo bovis and serum from a non-exposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgM	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	1.195	1.100	1.237	0.988	0.838	0.710	0.488	0.400
1:1000	0.882	0.733[a]	0.780	0.660	0.524	0.360	0.234	0.162
1:2000	0.490	0.544	0.448	0.480	0.330	0.193	0.121	0.091
1:4000	0.324	0.290	0.233	0.175	0.169	0.105	0.093	0.065

Conjugate — Antigen dilutions (negative serum)

ANTI IgM	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.337	0.320	0.320	0.285	0.300	0.256	0.250	0.221
1:1000	0.165	0.185[a]	0.185	0.180	0.176	0.170	0.154	0.151
1:2000	0.112	0.073	0.084	0.098	0.087	0.077	0.070	0.080
1:4000	0.067	0.070	0.069	0.055	0.063	0.060	0.056	0.053

[a] Optimal dilution.

Table A9. Titration of the butanol extracted antigen. Results of enzyme immu noassay using two fold dilutions of antigen and horseradish peroxidase labeled goat anti rabbit IgG at 490 nm of wavelength with ABTS as a indicator. Serum was obtained from hyperimmunized rabbit against L. interrogans serovar hardjo type hardjo bovis and serum from a non-exposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive rabbit serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	1.820	1.835	1.532	1.249	1.100	0.674	0.677	0.658
1:1000	1.627	1.708	1.761	1.503	1.284	0.806	0.764	0.533
1:2000	1.623	1.499[a]	1.708	1.315	0.974	0.849	0.617	0.591
1:4000	1.561	1.613	1.597	1.412	1.112	0.922	0.668	0.462

Conjugate — Antigen dilutions (negative rabbit serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.317	0.285	0.182	0.163	0.085	0.041	0.054	0.058
1:1000	0.221	0.167	0.185	0.095	0.040	0.045	0.034	0.035
1:2000	0.323	0.108[a]	0.054	0.056	0.023	0.021	0.021	0.020
1:4000	0.047	0.061	0.036	0.028	0.028	0.028	0.020	0.022

[a] Optimal dilution.

Table A10. Titration of the phenol-water extracted antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horse-radish peroxidase labeled goat antibovine IgG at 490 nm of wave-length with ABTS as a indicator. Serum from a cow 3 weeks after conjunctival exposure to L. interrogans serovar hardjo type hardjo bovis and serum from a non-exposed animal (MA negative)

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.491	0.307	0.302	0.229	0.235	0.212	0.176	0.197
1:1000	0.245	0.162	0.155	0.117	0.099	0.080	0.080	0.089
1:2000	0.212	0.131	0.086	0.086	0.070	0.064	0.039	0.079
1:4000	0.087	0.068	0.071	0.037	0.037	0.043	0.018	0.033

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1:500	0.150	0.179	0.113	0.111	0.103	0.153	0.181	0.165
1:1000	0.108	0.080	0.083	0.077	0.070	0.099	0.077	0.101
1:2000	0.062	0.040	0.050	0.043	0.044	0.063	0.045	0.066
1:4000	0.028	0.010	0.023	0.015	0.020	0.024	0.041	0.0380

Table A11. Titration of the proteinase K extracted antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horseradish per oxidase labeled goat antibovine IgG at 490 nm of wavelength with ABTS as an indicator. Serum from a cow 3 weeks after conjunctival exposure to L. interrogans serovar hardjo type hardjo bovis and serum from a non-exposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.418	0.704	0.681	0.676	0.702	0.594	0.527	0.422
1:1000	0.288	0.437	0.512	0.526	0.479	0.406	0.282	0.229
1:2000	0.272	0.362	0.405	0.463	0.425	0.335	0.236	0.152
1:4000	0.247	0.314	0.314	0.325	0.303	0.223	0.18	0.134

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.184	0.292	0.343	0.410	0.289	0.241	0.214	0.181
1:1000	0.145	0.223	0.235	0.288	0.242	0.216	0.147	0.136
1:2000	0.128	0.197	0.219	0.242	0.196	0.175	0.145	0.106
1:4000	0.089	0.146	0.220	0.180	0.169	0.134	0.087	0.108

Table A12. Titration of the soluble protein antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horseradish peroxidase la beled goat antibovine IgG at 490 nm of wavelength with ABTS as an indicator. Serum from a cow 3 weeks after conjunctival exposure to L. interrogans serovar hardjo type hardjo bovis and serum from a non-exposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	1.220	1.119	0.887	0.640	0.510	0.396	0.295	0.301
1:1000	1.017	0.801	0.626	0.371	0.306	0.159	0.168	0.143
1:2000	0.895	0.545	0.421	0.212	0.153	0.119	0.079	0.081
1:4000	0.541	0.351	0.243	0.122	0.097	0.047	0.053	0.026

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
1:500	0.535	0.274	0.262	0.229	0.170	0.152	0.172	0.146
1:1000	0.378	0.174	0.134	0.130	0.134	0.072	0.083	0.106
1:2000	0.246	0.136	0.078	0.080	0.059	0.041	0.037	0.051
1:4000	0.203	0.093	0.081	0.065	0.051	0.056	0.062	0.0420

Table A13. Titration of the mechanically disrupted cell antigen. Results of enzyme immunoassay using two fold dilutions of antigen and horseradish peroxidase labeled goat antibovine IgG at 490 nm of wavelength with ABTS as an indicator. Serum from a cow 3 weeks after conjunctival exposure to L. interrogans serovar hardjo type hardjobovis and serum from a nonexposed animal (MA negative) were used

Conjugate — Antigen Dilutions (Positive serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.890	0.950	1.177	1.220	0.876	0.699	0.511	0.398
1:1000	0.756	0.857	1.101	1.095	0.700	0.589	0.467	0.253
1:2000	0.697	0.755	0.892	0.821	0.598	0.433	0.287	0.177
1:4000	0.539	0.622	0.715	0.689	0.407	0.355	0.155	0.009

Conjugate — Antigen dilutions (negative serum)

ANTI IgG	1:250	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000
1:500	0.644	0.709	0.711	0.558	0.415	0.302	0.200	0.098
1:1000	0.521	0.660	0.675	0.403	0.254	0.207	0.142	0.099
1:2000	0.453	0.503	0.527	0.386	0.153	0.099	0.067	0.054
1:4000	0.388	0.455	0.492	0.255	0.071	0.089	0.052	0.092

Table A14. Comparison between microscopic agglutination (MA) titers and IgG enzyme immunoassay (EIA) optical densities on a peroxidase-ABTS system (wavelength = 490 nm) recorded using cattle field serum samples

EIA		MA
0.531	0.605	
0.128	0.306	
0.069	0.320	
0.384	0.491	
1.338	0.366	
0.142	0.145	
0.854	0.130	
0.134	0.765	
0.155	0.901	
0.067	0.048	
0.088	0.116	
0.675	0.093	
0.714	0.067	
0.404	0.088	
0.181	0.698	
0.328	0.085	
0.411	0.270	
0.139	0.145	
0.627	0.158	
0.208	0.039	
0.171	0.070	
0.090	0.216	
0.354	0.523	
0.133	0.229	
0.243	0.092	
0.127	0.241	
0.183	0.169	
0.101	0.316	MA < 1:100
0.178	0.944	EIA mean = 0.313
0.833	0.057	range(0.039-1.400)
0.088	0.299	
0.043	0.601	
0.070	0.798	
0.367	0.259	
0.338	0.317	
0.382	0.175	
0.084	0.390	
0.047	0.341	
0.071	0.513	
0.250	0.749	
0.086	0.152	
0.091	0.263	
0.125	0.335	
0.047	0.290	
0.107	0.247	
1.400	0.509	
0.163	0.243	
0.303	0.355	
0.356	0.120	
0.410	0.379	
0.211	0.609	
0.159	0.535	
0.153	0.380	
0.266	0.489	
0.643	0.181	
0.900	0.145	
0.223	0.102	
0.252	0.579	
0.131	0.333	
0.480		
0.861		
0.979		
0.906		
0.877		
0.735		
0.731		
0.459		
0.689		
0.469		
1.326		
0.397		MA = 1:100
0.339		EIA mean = 0.720
0.788		range(0.339-1.326)
0.748		
0.711		
0.561		
0.913		
1.038		
0.731		
0.404		
0.692		

EIA	MA
1.290	
0.883	
1.198	
0.483	
1.014	
1.301	
0.530	
0.722	
0.432	
1.254	
0.803	
0.575	MA = 1:200
0.549	EIA mean = 0.787
0.266	range (0.266-1.301)
0.548	
1.120	
1.117	
0.348	
0.634	
0.976	
0.620	
0.588	
0.569	
0.756	
1.105	
1.117	
1.201	
1.060	
1.201	
0.993	
1.440	
1.336	
1.218	
1.147	
0.824	
0.797	MA = 1:400
0.361	EIA mean = 1.016
0.865	range(0.772-1.440)
0.942	
0.825	
1.320	
1.065	
0.772	
0.823	
1.456	
1.288	
1.208	
1.473	
1.203	
1.209	
1.218	
0.981	
1.361	MA = 1:800
1.248	EIA mean = 1.088
0.674	range(0.640-1.473)
1.057	
1.344	
0.640	
0.703	
0.773	
0.660	
1.453	
1.362	
1.317	
1.339	
0.682	
0.903	MA = 1:1600
1.183	EIA mean = 1.175
1.206	range (0.682-1.453)
1.147	
1.306	
1.347	
0.860	
1.043	MA = 1:6400

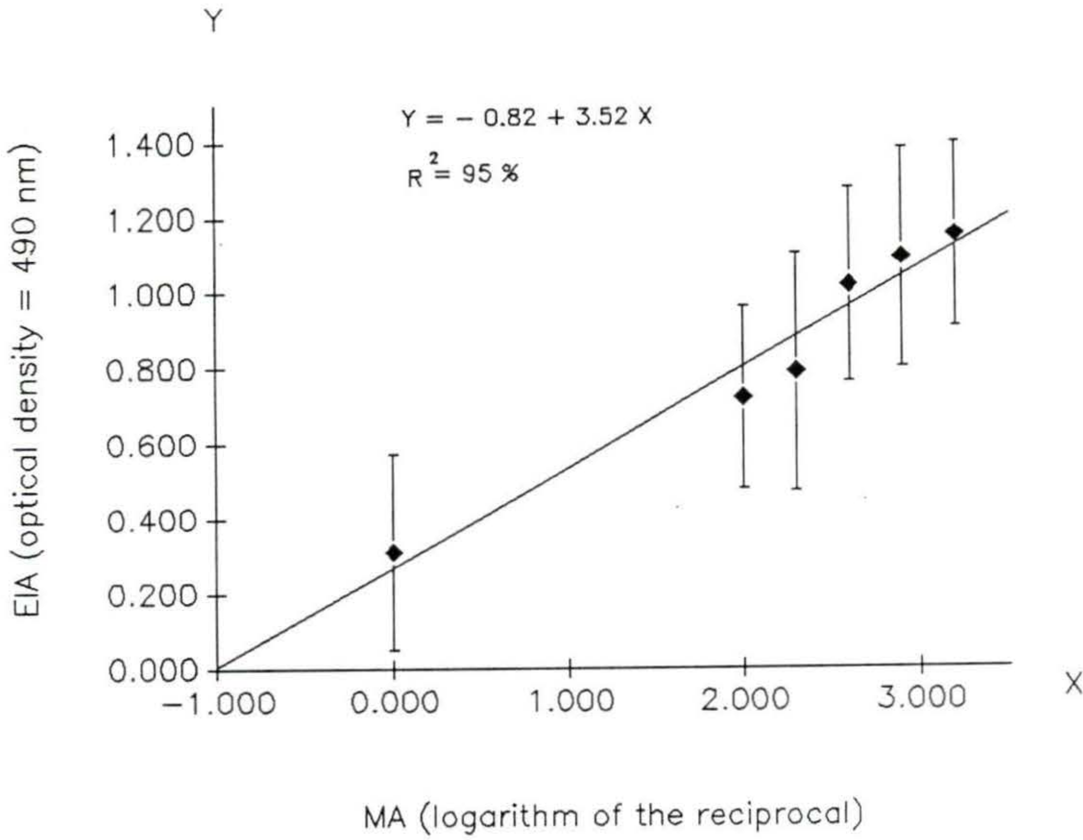

Figure A1. Regression analysis of IgG enzyme immunoassay optical density (peroxidase-ABTS, wavelength = 490 nm) and logarithms of reciprocal of the microscopic agglutination titer of cattle sera from field survey.