EVALUATION OF THE ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TEST FOR THE DETECTION OF LEPTOSPIRAL ANTIBODIES IN BOVINE SERA

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ABSTRACT

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Evaluation of the Enzyme-Linked Immunosorbent Assay (ELISA) Test for the Detection of Leptospiral Antibodies in Bovine Sera

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The Micro-Enzyme-Linked Immunosorbent Assay (ELISA) test was evaluated as a presumptive test for the diagnosis of bovine leptospirosis. Disposable flat bottom Micro-ELISA plates were utilized as antigen carriers and test vehicles. The antigen was prepared from a soluble alcohol extract of serovars, hardjo, Hardjoprajitno and illini, 3055 and stored at -70 C until ready for use in the test. Serology on each bovine serum sample was performed by using the Microscopic Agglutination Test (MA), the Indirect Hemagglutination Test (IHA), and the Enzyme-Linked Immunosorbent Assay (ELISA) Test. The comparison of the ELISA test with the MA and IHA was done with coded sera, randomized as to order of testing and stored at -20 C. A total of 142 different bovine serum samples was tested for the presence of leptospiral antibodies, using antigens serovar hardjo (a pathogen) and serovar illini (a saprophyte). Reproducibility was checked by duplicating 83 serum samples and triplicating 58 serum

samples, resulting in a final total of 482 bovine serum samples being tested. All sera were tested by MA, IHA, and ELISA tests before being decoded for comparison of results. The total agreement of hardjo sera for both positive and negative sera was 48% among all 3 test procedures, whereas the total agreement of illini sera for both positive and negative sera was 92% among all 3 test procedures. The test was safer to perform since there was no need to use live antigens in the test; the test did not require pretreatment of sera; the test was read visually, and the test was a simple and rapid procedure. The sensitivity and specificity appear to have been low; however, in order to obtain further definitive results regarding the specificity, sensitivity, and the role of the ELISA test in the detection of leptospiral antibodies in bovine sera, other investigations will be needed in the future.

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CHAPTER I

INTRODUCTION

The disease, leptospirosis, is caused by any one of the more than 184 pathogenic organisms that belong to the genus Leptospira. The World Health Organization (WHO), Reference Laboratory, Center for Disease Control, lists some 184 serovars. Although some of these are in their provisional stage, they are considered to be pathogenic. The WHO Taxonomic Subcommittee on Leptospira recommends that the genus Leptospira be divided into two main species: Leptospira interrogans, which includes leptospires parasitic for animals and man (pathogens) and Leptospira biflexa, which includes the free-living aquatic leptospires that are harmless to animals and man (saprophytes). The serovar is the basic taxon of the various serologically heterologous strains of the genus Leptospira. Epidemiologically, leptospirosis is a zoonotic disease. It is an infection transmitted from vertebrate animals to man. In 1964, Van der Hoeden stated that leptospirosis is probably the worlds most widespread contemporary zoonotic illness. The chief reservoirs are wild rodents, and wild (skunks, raccoons and opossums) and domesticated (dogs, cats, pigs, horses and cattle) animals. Man becomes infected through indirect contact with infected animals that may harbor leptospires in the liver, kidney, bloodstream, tissue or urine.

Leptospirosis can vary considerably in severity from subclinical to fatal with a wide variety of symptoms. Seroreactors are found

among animals (livestock, dogs) which have never suffered any obvious illness. Some laboratory animals such as rats or mice may maintain enzootic leptospirosis in their colonies without any signs of ill health. In livestock the disease is often mild: fever, no appetite and depression may be the only signs. Jaundice and hemoglobinuria may be present. Infections of leptospires during pregnancy in bovine leptospirosis may result in abortion, stillbirths, or feeble progeny. Hanson (1976) states that leptospirosis is one of the major cattle diseases in the United States, and that the disease has an economic impact on the livestock industry which is of great public health significance. Because of such a great variety of symptoms, leptospirosis may mimic other acute infections of many other diseases and is liable to be overlooked in differential diagnosis. Because the disease mimics so many other diseases like brucella, tularemia, malaria, Q fever, etc., the diagnosis of the disease has been a big problem. Therefore, leptospirosis should always be considered in the differential diagnosis of any pyrexia of unknown origin (PUO), especially when the host, through the nature or place of his occupation or recreational activities, could have been exposed to infection.

Leptospira are often difficult to isolate from infected cattle and diagnosis usually depends on the detection of specific antibodies. Acute leptospirosis is diagnosed most accurately by isolating and identifying the organism. Cultures of pathogenic leptospires are sometimes extremely difficult, dangerous, impractical and unsatisfactory for routine use, making it highly desirable that a simple and safe test for the rapid

diagnosis of leptospirosis be developed, or at least a test that would be of ease to perform.

The purpose of this research was: 1) to determine the sensitivity, reproducibility, and specificity of the enzyme-linked immunosorbent assay test (ELISA) for detecting leptospiral antibodies in bovine serum, and (2) to evaluate the application of the ELISA test as a presumptive test by comparing it to the Microscopic Agglutination Test (MA) and Indirect Hemagglutination Test (IHA), which are serological tests currently being used as diagnostic tools in the detection of leptospiral antibodies.

CHAPTER II

REVIEW OF LITERATURE

Morphology of Leptospires

The morphologic appearance of leptospires is basically the same for all members of the genus Leptospira. Leptospires are spiral organisms, about 0.1μ in diameter and 6μ to 20μ long, although they may be as long as 30μ to 40μ. The organisms are tightly coiled and are so closely set together that they are difficult to distinguish, except in the living state, by dark-field microscopy or by electron microscopy. They usually have hooked ends but on rare occasions the ends may be straight. The active motility is mainly rotary, with the organism spinning rapidly on its long axis. Leptospires are highly motile and capable of passing through Seitz F-K filters and other similar bacteriological filters, such as Millipore (0.45μ and 0.22μ pore size) filters. Leptospires cannot be seen in wet mounts by light-field microscopy, but they can be seen with dark-field illumination. Darkfield microscopy will demonstrate lashing movements of individual organisms (Sulzer and Jones, 1978). Electron microscopy shows a protoplasmic cylinder with two flagella-like axial filaments inserted subterminally, with their free, non-overlapping, ends directed toward the center of the organism. An external membrane envelops the whole organism (Sulzer and Jones, 1978).

The organisms stain poorly with the usual bacterial stains-Gram, Wright or Giemsa methods; however, they can be readily stained by

several silver impregnation techniques, by fluorescent antibody techniques and negative staining with Congo red (Sulzer and Jones, 1978).

Leptospires are readily cultured in fluid media. The saprophytic strain, Leptospira biflexa, can be grown on simple broths. Parasitic strain, Leptospira interrogans, requires a medium containing 5% to 10% enrichment, addition of animal serum, or a substitute provided by a fraction of bovine serum albumin. Leptospires grow best at temperatures between 28 C and 30 C. The optimal pH for growth is about 7.2 (Sulzer and Jones, 1978).

Leptospires in Animals and Man

Leptospirosis is primarily a disease of wild and domestic non-human mammals that is caused by Leptospira interrogans. Man becomes infected through indirect contact with an environment contaminated by virulent leptospires originating from a convalescent or reservoir host. Leptospires usually enter the body through the mucous membranes of the conjunctivae, the nose or mouth, and through skin abrasions. Leptospires excreted in the urine of infected animals may be present in the urine during the second week of illness and continue to be excreted intermittently for 4 to 6 weeks. Infected animals may excrete leptospires in the urine for some months after recovery (Sulzer, personal communication). Vaccination of cattle with leptospiral bacterins has become the most effective method of leptospirosis control in a susceptible herd, using specific bacterins against the serotype prevalent in the area (Tripathy et al., 1975, 1976).

In man there are generally two overlapping phases following the incubation period. The first phase lasts about 7 days and is

characterized by leptospiremia, with symptoms and signs of a severe systemic infection. During this phase, patients are acutely ill. The second phase, leptospiruria, which is characterized by an increasing concentration of antibodies, the disappearance of leptospires from the blood, and the appearance of localized signs and symptoms. Leptospiruria may persist in the convalescent for months. In fatal cases, the cause of death can often be acute renal failure.

Domestic animals, cattle, and pigs may become infected through grazing in fields or from fodder contaminated with rodent urine. Leptospires in bovine usually enter through abrasions of the skin of feet and legs when cattle are wading in contaminated streams, field ponds and marshy areas, or in surface water around cattle feeding areas. In the acute phase of bovine leptospirosis there may be an increase in body temperature and anemia. Jaundice, hemoglobinuria, and death may occur in some severe infections. Abortion and stillbirths are the signs most frequently recognized in bovine leptospirosis (Hussain et al., 1978). Harrington (1975) performed an investigation to determine the frequency of leptospiral antibodies in serum from cattle and swine herds with histories of abortion. His findings detected leptospiral antibodies in 50 (57.5%) of 87 cattle herds and in 7 (41.2%) of 17 swine herds.

Serologic Procedures in the Diagnosis of Leptospirosis

A variety of tests have been developed for the serodiagnosis of leptospirosis. The Microscopic Agglutination (MA) is most widely employed as the standard reference test, Sulzer and Jones (1978). A

modified semimicro method (Sulzer and Jones, 1973) and a microtechnique (Lewis, 1978) for the MA test which save much time and material are being used in many laboratories. A modification of the microtechnique (Cole et al., 1973) is beneficial in that the 96 flat-bottom wells plates are used and the plates can be read easily on the stage of the dark-field microscope by using an AU.22UM-long-working-distance objective. A hemagglutination test for human leptospirosis (Sulzer and Jones, 1973) and wild mammals (Cirone, 1978), and an indirect hemagglutination test (Sulzer and Jones, 1975) are also used in the diagnosis of leptospirosis. Hodges and Weddell (1977) adapted a complement fixation test for large scale serological diagnosis of bovine leptospirosis.

The Microscopic Agglutination (MA) test, a confirmatory test, is performed with 23-26 live antigens (Appendix A) and is the serological test of choice in the Reference Laboratories. The MA test requires the maintenance of leptospiral serovars in the active growth phase on a routine basis (weekly). The Leptospiral strains used for antigen production are maintained in Ellinghausen and McCullough, as modified by Johnson and Harris (EMJH) with C.D.C. Enrichment medium and transferred every seven days (Sulzer and Jones, 1978). A ½ MacFarland standard can be used to check the density of antigens for serology. Very dense antigens will be undersensitive and if very light, the antigens tend to be too sensitive and the titers will be higher. The MA test detects leptospiral antibodies from the 6th to the 12th day of disease.

The Indirect Hemagglutination (IHA) test is a presumptive test in the diagnosis of leptospiral antibodies. In comparison with the MA test, only a single antigen is required, the test is genus-specific. The test uses a soluble antigen which is sensitized to sheep erythrocytes which have been fixed with glutaraldehyde. The test is simple and convenient, and sensitized fixed cells may be stored at a 10% suspension for at least a year. The IHA test detects leptospiral antibodies as early as the 4th day after the onset of illness. The test is positive only with sera from persons with current leptospiral illness.

Recently, an Enzyme-Linked Immunosorbent Assay (ELISA) procedure has been developed for the serological diagnosis of various infectious disease (Engvall and Perlmann, 1971, 1972). This assay is proving to be a simple, rapid, and reliable means for detecting humoral antibodies to disease agents. The basic ELISA depends on two assumptions: (1) that antigen or antibody can be attached to a solid-phase support yet retain immunological activity, and (2) that either antigen or antibody can be linked to an enzyme and the complex retain both immunological and enzymatic activity.

To date for immunodiagnosis of infections, antibodies to viruses, parasites, fungi, and bacteria have been measured in a microplate system. Bacteriologists have adapted the ELISA test for the detection of Streptococcal M protein antibodies (Russell et al., 1976), Fasciola hepatica antibody (Burden and Hammet, 1978), Salmonella O antibodies (Carlsson et al., 1972; 1975), Brucella abortus antibodies (Carlsson

et al., 1976), Legionnaires disease antibodies (Farshy et al., 1978; Tilton, 1979), diagnosis of gonorrhea (Buchanan, 1978; Glynn and Ison, 1978), Cholera serology (Holmgren and Svennerholm, 1973), quantitation by subclass for bovine antibodies (Sloan and Butler, 1978), diphtheria toxin antibodies (Svenson and Larsen, 1977), syphilis antibodies (Veldkamp and Visser, 1975), antibodies to Mycobacterium tuberculosis (Nassau et al., 1976), and detection of Clostridium botulinum toxin type A (Notermans et al., 1978).

The method has also been developed by virologists, as a serological procedure for detection of antibodies (Voller et al., 1976; 1978). Carthew (1978) employed the technique for the rapid differentiation of murine enteric viruses in tissue culture. Castellano et al. (1977) adapted the test for detection of antibody to Cytomegalovirus. Virologists have also adapted the test for quantitation of rotavirus antibodies (Ghose et al., 1978), serotyping of herpes simplex virus (Mills et al., 1978), identification of rubella virus isolates (Schmidt et al., 1978) and detection of rubella antibodies (Voller and Bidwell, 1975).

In the field of Parasitology the ELISA is also being employed as a serological procedure. Denmark and Chessum (1978) and Walls et al. (1977) used the procedure for the detection of Toxoplasma antibody. Luckins and Mehlitz (1978) evaluated an indirect fluorescent antibody test and ELISA in the diagnosis of bovine trypanosomiasis. Serodiagnosis of Trichinella spiralis infection (Ruitenberg et al., 1975) and the diagnosis of amebiasis (Sorice et al., 1977) were also used in the field of Parasitology.

Employing the indirect method of the ELISA, antigen is coated on the inside of microELISA plate wells (sensitization). Serum is added and any specific antibody attaches to the antigen. Enzyme-labelled antiglobulin is added, which attaches to antibody/antigen complex. Enzyme substrate is added, which is hydrolysed by captured enzyme and gives a color, the optical density of which is directly proportional to the amount of unknown antibody in the test serum. This method uses a single, host-specific, enzyme-labelled anti-globulin for any animal species (Voller et al., 1978).

The ability of the ELISA to be made immunoglobulin type specific makes it an attractive serological method for the detection of antibodies in all areas of microbiology. Jarvis (1978) has made an excellent conclusion, that ELISA ---- is the new girl around town.

CHAPTER III

MATERIALS AND METHODS

Growth of Organisms

Serovars, hardjo and illini were prepared for use in the ELISA test using the method of Sulzer and Jones (1978). A 10% inoculum of each of the serovars, hardjo and illini, was seeded into separate flat-bottom flasks containing 1 liter amounts of polysorbate 80 medium EMJH modified medium (Appendix B). The cultures were placed in a 30 C incubator for 10-14 days, then checked for contamination. The contents of each flask were centrifuged at 5000 X G for 30 min to pack the organisms. After centrifugation, the supernatant was discarded and the packed organisms were reconstituted with 37 ml of triethanolamine-buffered salt solution (Kent; Appendix C) and 37 ml of absolute ethanol to bring the antigens to a 50% alcohol solution. The 50% alcohol solution was placed at 56 C for 2½ hr. After incubation, the alcohol solution was refrigerated overnight at 4 C. The next day the solution was centrifuged and the supernatant was saved. The supernatant should be clear. The packed particulate was discarded. The 50% alcohol solution (supernatant of 37 ml of Kent and 37 ml of alcohol) was adjusted with cold absolute ethanol to bring the alcohol content up to 90%. The 90% solution was incubated at 4 C for 6-7 days. A fine white-grayish precipitate was formed. At the end of the incubation period, the 90% solution was centrifuged and the precipitate was saved. The precipitate was the ELISA antigen. Excess alcohol was drained off

the precipitate. The precipitate from each flask was resuspended with 37 ml of sterile distilled water and dispensed in 1.0 ml amounts for storage at -70 C until ready for use in the ELISA test.

Preparation of Antigen

There were two antigens used in the ELISA test: serovar hardjo, strain Hardjoprajitno, and serovar illini, strain 3055 obtained from the Leptospirosis Reference Laboratory at CDC. The optimal antigen dilution used in the ELISA test was determined by using twofold dilutions of antisera (1:25-1:25,600) along with 0.1 ml of a constant dilution of conjugate (predetermined) and 0.1 ml of varying concentration of antigen in all wells. Eight dilutions were tested 1:4-1:512, using twofold dilutions. The optimum dilution was the highest dilution giving maximum reactivity with the positive control serum, and no reaction with the negative control serum. All antigen control tests were negative.

Sensitization of Microtitration Plates

The ELISA test performed in this study is a modification of a procedure described previously (Walls et al., 1977). Microtitration plates (Cooke #1-223-29, Substrate plates) were sensitized with the optimal antigen dilution (described under the heading: Preparation of Antigen). The required amount of optimal antigen dilution was prepared in 0.06 M pH 9.5 sodium carbonate buffer (Appendix D). The optimal antigen dilution was dispensed in 0.1 ml into each well of the plates. The prepared plates were sealed with plate sealers (Cooke #1-220-30) and incubated in a 37 C water bath for 3 hr. The water

level was maintained such that the plates were immersed in about 6-7 mm of water resting on their support in the water bath. A temporary plate cover may be used if the plates are to be used on the same day. After incubation, the sensitized plates were stored at 4 C until ready for use in the ELISA test.

Serum Specimen

The bovine serum samples were collected from various cattle herds in Texas, by Dr. Paul Tallamy and fellow workers, Texas A & M University, Department of Veterinary Medicine, College Station, Texas, as a survey sera and sent to the Center for Disease Control, Bacterial Immunology Branch: Leptospirosis Laboratory for testing for leptospiral antibodies. Experimental serum samples were also included in this research project. The forty-one experimental serum samples were obtained from two sources, eleven serum samples plus the positive and negative controls were obtained from Dr. H. C. Ellinghausen, Jr., USDA Agricultural Research Service, Ames, Iowa, and 30 serum samples from Dr. Lyle Hanson, University of Illinois, Department of Veterinary Medicine, Urbana, Illinois. All serum samples were coded and stored at -20 C until ready for testing. An initial dilution of 1:25, made in sodium phosphate buffered saline with 0.05% Tween 20 (PBS/T; Appendix D) was prepared for each serum sample. Twofold serum dilutions were made in microtitration plates through 1:25,600 (Micro ELISA plates) using a 0.05 ml microdiluter. Inactivation of the serum was not needed for this procedure.

Conjugate

The conjugate used in the ELISA test was peroxidase antibovine

IgG (Rabbit) globulin (Miles-Yeda Ltd., Research Products, Elkhart, Ind.). A working solution of conjugate (optimal conjugate dilution) was determined for use in the ELISA test by using a constant amount of antigen (0.1 ml per well) and varying the concentration of conjugate in the test. Five dilutions were used (1:100-1:1600) in twofold dilutions. The optimum dilution was defined as that dilution giving maximum reactivity with the positive control serum, and little or no reactivity with the negative control serum.

Substrate

A stock solution of substrate was prepared weekly by adding 100 mg of O-phenylenediamine (Eastman Kodak Company, Rochester, New York) per 10 ml of methanol. A working solution of substrate for use in the ELISA test was prepared daily by adding 1 ml of stock substrate solution per 99 ml of distilled water and mixing thoroughly, after which 0.1 ml of 3% hydrogen peroxide solution was added to each 100 ml of working substrate solution and mixed thoroughly.

Performance of the Enzyme-Linked Immunosorbent Assay (ELISA) Test

The sensitized microtitration plates were removed from storage and the plate sealers removed from each plate. The optimal antigen solution was aspirated from each well using a Pasteur pipette attached to a vacumn source. A microtiter washer/aspirator, Bullock (1978), is available commercially. With a pointed tip fluid delivery device, the plates were flooded with sodium phosphate buffered saline with 0.05% Tween 20 (PBS/T). The PBS/T remained in the wells for 3 min. Afterward, it was removed from the wells and the plates then flooded

again with fresh PBS/T. This washing procedure was repeated three times. Plates were labeled for the bovine sera and the positive and negative control sera. The serum dilutions tested ranged from 1:25-1:25,600 in twofold increments. A 0.05 ml microtitration dropper (Cooke #1-220-36) was used to drop 0.05 ml of PBS/T into each of the 11 wells of all rows to be used, the first well of each row receiving the initial dilution of sera (1:25). The last well (12th well) was used for the antigen control. Twofold serum dilutions were prepared in the plates across the 11-well rows. The serum dilutions were made with the aid of 0.05 ml microtitration diluters (Cooke #1-220-34). The contents of the first wells were mixed by slowly twirling the 0.05 ml microdiluters. The microdiluters are transferred to the second wells of each row and continued transferring and mixing was carried through the eleventh well. The remaining 0.05 ml in the microdiluters was discarded. This procedure was repeated with all bovine serum specimens and with the positive and negative controls. The contents of each plate were mixed on a mechanical vibrator. The plates were sealed with plate sealers and incubated in a 37 C water bath for 30 min. The plates were removed from the water bath and the serum dilutions aspirated from each well of the plates. Each plate was washed three times with PBS/T with three minute intervals between each wash. A 0.1 ml of optimal conjugate dilution (pre-determined), diluted in PBS/T, was added to each serum dilution and antigen control. The plates were sealed and again incubated in a 37 C water bath for 30 min. After incubation the plates were

removed from the water bath and the conjugate was aspirated from each well. Each plate was washed with PBS/T three times with three minute intervals between each wash. Substrate working solution in 0.1 ml amounts was added to each well of each plate. Plates were covered and placed in the dark at room temperature for 30 min. After the 30 min incubation a 0.025 ml drop of 8 N sulfuric acid was added to each well of each plate to stop the enzyme-substrate reaction, with a 0.25 ml microtitration dropper (Cooke #1-220-35). The results were read by visually against a white, well-lighted background. The titer was the highest dilution of serum which showed a distinct color difference from the initial well of the negative control serum dilution series. The negative control shows little or no color development in any wells of the dilution series.

The Microscopic Agglutination Test

The reference test for the Enzyme-Linked Immunosorbent Assay (ELISA) test was the Microscopic Agglutination test (MA), a confirmatory test for the detection of leptospiral antibodies. The (MA) was used previously by Morris and Hussaini (1974) for the detection of leptospiral antibodies in bovine leptospirosis. The Microscopic Agglutination test (MA), was performed with 7 live antigens; RGA, pomona, wolffi, georgia, borincana, hardjo and illini. Flat bottom tissue culture plates with 96 wells (Linbro Scientific, Inc., Cat. #76-001-05) were used for the performance of the test. Serial twofold dilutions of serum were prepared in Sorensen's phosphate buffered saline solution (Appendix E); 0.1 ml of serum was added to wells on the first row, and 0.05 ml of saline solution

to the remaining wells. With the 0.05 ml microdiluters serial dilutions were made through 1:12,800 (11th well) leaving the 12th well for a saline control for each antigen used in the test. An equal volume of antigen, 0.05 ml, was added to each serum dilution. The plates were shaken gently to mix the serum and antigen and covered with sheets of plastic, then incubated on the table top at room temperature for 2 hr. The tests were read with the dark-field microscope using an AU.22UM-long-working distance, 10X objective (No. 599-003, E. Leitz, Inc., Rockleigh, N.J.) and 10X ocular.

The degree of agglutination was read as 4+, 3+, 2+, 1+ and negative. A 4+ reaction is recorded when 75% to 100% of the leptospires appear clumped, 3+ reaction when 75% of the leptospires are agglutinated, 2+ when about 50% of the leptospires are agglutinated, and 1+ when at least 25% of the leptospires were agglutinated. The end point titer in a positive test was the highest diulution in which at least 50% of the leptospires were agglutinated, a 2+ reaction.

Controls for this test were bovine normal and rabbit normal serum diluted in the same manner as above.

The Indirect Hemagglutination Test

The Indirect Hemagglutination test (IHA), is a presumptive test for the detection of leptospiral antibodies. The serum was diluted and inactivated at 56 C for 1 hr, absorbed with a 1% suspension of red blood cells (RBC) for every 0.1 ml of serum used, and placed in a 37 C water bath for 20 min. The serum dilution was centrifuged and drawn off the RBC for use in the test. Serial dilutions were made in the

microtitration plates, in 0.1 ml amounts, leaving the 11th well for the heterophile control and the 12th well for the saline control. The IHA test was performed with a soluble antigen which was sensitized with 1% sheep erythrocytes. An equal volume of sensitized cells (0.1 ml) were added to each well in the plate except the 11th well (heterophile control), which received non-sensitized cells. The plates were shaken and incubated at room temperature overnight. The test was read visually. The test was considered positive (+) when agglutination patterns were formed on the bottom of the wells and negative (-) when a smooth mat (button) was formed on the bottom of the well (Sulzer and Jones, 1978).

CHAPTER IV

EXPERIMENTAL RESULTS

Table 1 shows the distribution by number of the comparisons of serovar hardjo and serovar illini antigens and the agreement among the three tests, MA, IHA, and ELISA. The total agreement of hardjo sera for both positive and negative sera was 48% among all 3 test procedures, whereas the total agreement of illini sera for both positive and negative sera was 92% among all 3 test procedures.

Tables 2 and 3 show the correlation of titers obtained with the ELISA test versus those obtained with the MA test using serovar hardjo and serovar illini and, Tables 4 and 5 show the correlation of titers obtained with the ELISA test versus those obtained with the IHA test using serovar hardjo and serovar illini. The IHA and the MA tests were more reactive than the ELISA test. This was true for both the hardjo and illini antigens. Lesser reactivity is not objectionable if sensitivity is not also reduced. It was apparent that the ELISA test was less sensitive than the MA and IHA tests. This indicated that it may require more antibody to produce a reaction in the ELISA test as compared to the MA and IHA tests. This lower sensitivity is not objectionable unless there is a reduction in detection rate of individual cases. Lowered sensitivity is usually accompanied by greater specificity, but whether this is the case in these results is questionable.

The IHA and MA tests are considered to be quite sensitive and specific, especially at the greater titer levels, therefore it is

Table 1. Comparison of serovar hardjo and serovar illini showing agreement among three tests.

Serovar		MA-Positive IHA-Positive	MA-Negative IHA-Negative	MA-Positive IHA-Negative	MA-Negative IHA-Positive
hardjo	ELISA Positive	59[a] (42%)	9 (6%)	3 (2%)	47 (33%)
	ELISA Negative	1 (1%)	9 (6%)	1 (1%)	13 (9%)
illini	ELISA Positive	125 (88%)	0 (0%)	4 (3%)	2 (1%)
	ELISA Negative	4 (3%)	6 (4%)	1 (1%)	0 (0%)

[a]The number of sera with the indicated result.

Table 2. Correlation of ELISA titers with MA titers using serovar hardjo.

	MA TITERS serovar hardjo									
ELISA titers	<25	25	50	100	200	400	800	1600	3200	6400
<25	40[a]	0	3	4	3	2	0	0	0	0
25	45	0	8	14	10	12	8	4	2	0
50	4	0	1	3	0	5	6	2	1	0
100	0	0	0	0	2	4	1	1	0	1
200	0	0	0	1	0	1	4	2	1	0
400	0	0	0	0	0	0	1	0	0	0
800	0	0	0	0	0	0	0	0	0	0

[a]Number of sera with the indicated titer. Each titer was determined by averaging three experiments.

Table 3. Correlation of ELISA titers with MA titers using serovar *illini*.

	MA TITERS serovar *illini*									
ELISA titers	<25	25	50	100	200	400	800	1600	3200	6400
<25	1[a]	0	3	4	1	0	0	0	0	0
25	0	0	8	22	34	30	15	6	1	0
50	0	0	2	4	10	17	19	5	4	1
100	0	0	0	1	3	4	4	4	2	0
200	0	0	0	0	1	1	0	0	0	0
400	0	0	0	0	0	0	0	0	0	0
800	0	0	0	0	0	0	0	0	0	0

[a]Number of sera with the indicated titer. Each titer was determined by averaging three experiments.

Table 4. Correlation of ELISA titers with IHA titers using serovar hardjo.

ELISA titers	IHA TITERS serovar hardjo										
	<25	25	50	100	200	400	800	1600	3200	6400	12800
<25	20[a]	7	5	1	1	4	5	8	1	2	1
25	11	24	16	7	6	5	10	13	8	1	1
50	2	5	2	1	1	2	4	3	1	0	0
100	1	2	2	1	0	1	1	0	0	1	0
200	1	1	0	1	0	4	1	2	0	1	0
400	0	0	0	0	0	2	0	0	0	0	0
800	0	0	0	0	0	1	0	0	1	0	0
1600	0	0	0	0	0	1	2	2	0	0	0

[a]Number of sera with the indicated titer. Each titer was determined by averaging the experiments.

Table 5. Correlation of ELISA titers with IHA titers using serovar *illini*.

ELISA titers	IHA TITERS serovar *illini*										
	<25	25	50	100	200	400	800	1600	3200	6400	12800
<25	4	0	0	0	0	1	2	2	1	0	0
25	14	30	11	9	9	18	15	7	1	1	0
50	1	8	11	3	11	12	11	2	1	1	1
100	0	0	1	2	1	3	9	0	0	0	0
200	0	0	0	0	0	0	0	1	1	0	0
400	0	0	0	0	0	0	0	1	0	0	0
800	0	0	0	0	0	0	0	0	0	0	0
1600	0	0	0	0	0	0	0	0	0	0	0

possible that certain infections were missed by the ELISA test. This may be due to the time in the course of the disease when the serum samples were taken, chronic disease or acute disease, but can only be determined by history of the individual serum sample.

Table 6 shows the distribution by number and percentage of the titers among the various dilutions tested by ELISA. If titers of 1:25 and 1:50 are considered significant then 62% of the serum samples tested by hardjo and 82% of the serum samples tested by illini were significant.

Table 7 shows the comparison of the MA, IHA, and ELISA test results with MA results on the same sera from other investigations. The results among the four investigations were reproducible.

Table 6. The distribution of ELISA titers for sera tested.

ELISA Titers	hardjo		illini	
	Number	Percent	Number	Percent
25	23	16	10	7
25	64	45	71	50
50	24	17	45	32
100	12	9	14	10
200	10	7	2	1
400	2	1	0	0
800	2	1	0	0
1600	5	4	0	0
Totals	142	100	142	100

Table 7. Comparison of results of this investigation with collaborative investigations.

Results	Previous Investigations[a]	Present Investigations		
	MA	MA	IHA	ELISA
Positive	99[b]	87	130	121
Negative	43	55	22	21
Totals	142	142	142	142

[a]Dr. Catherine Sulzer, C.D.C., Atlanta, Georgia
Dr. H. C. Ellinghausen, Ames, Iowa, and
Dr. Lyle Hanson, Urbana, Illinois

[b]The number of sera with indicated results

CHAPTER V

DISCUSSION

The reliability or any immunological assay depends on the strict standardization of all reagents and procedures used. Without such standardization, comparison of results from assay to assay would be difficult, if not impossible. The present investigation illustrates that after controlling the different factors affecting the test, the ELISA technique can become a useful addition to the currently available methods (MA and IHA) for detection of leptospiral antibodies in bovine sera.

A major factor which plays a role in the success of any solid-phase immunoassay for detection of antibody is the preparation of a uniform solid-phase coupled antigen. In this investigation the methods described previously by Engvall and Perlmann (1971), Ruitenberg et al. (1976), and Walls et al. (1977) were employed, in which the solid-phase coupled antigen was prepared by physical adsorption of the antigen to the plastic plate. The ELISA test can be used for the assay of antibodies to any infectious agent if the specific antigen can be adsorbed satisfactorily to the solid-phase surface. The uneven adsorption of antigen to the solid-phase surface may create serious problems in using the method for laboratory diagnosis, especially when acute and convalescent-phase specimens are tested. Difference in titers may be due to unevenly coated surfaces rather than to an actual difference in the amount of the specific antibodies in the sera.

Greater specificity of the ELISA test may have been possible if purification of the antigen material had been done. Preparation of concentrated, purified antigen was a cumbersome and time-consuming procedure. The growth of leptospiral organisms and the production of a purified antigen was studied for two months with very small amounts of antigen materials produced. This inadequate supply of purified antigen was a limiting factor in this research project. Further work is needed for the concentration and purfication of an antigen to investigate coating the solid surface of the plates to check for better specificity.

Nonspecific reactivity is another important factor in all enzyme-labeled assays (Saunders, 1975; Saunders and Clinard, 1976). This poses difficulties both in identifying low positive reactions and in interpreting high negative values. The addition of Tween-20 to PBS for washings reduces partially the background reactivity but does not eliminate it completely. Therefore, the appropriate numbers of washings after each addition of reagents are necessary. The reduction in the background color permits reading visually rather than colorimetrically for direct measurement of antibodies in the microplates (Clem and Yolken, 1978; Ruitenberg et al. 1976).

The sensitivity of the ELISA test in this investigation was less than that of the MA and IHA tests being used routinely by the Reference Laboratory C.D.C. Engvall and Perlmann (1971) have suggested that it may be possible to make the ELISA test more sensitive by extending the

duration of the enzyme reaction and/or adding more conjugate; however, this would need to be investigated further.

The ELISA offers several advantages over the IHA and MA tests. No pretreatment of sera is required for the ELISA, whereas sera tested by the IHA procedures must be treated by receptor-destroying enzymes to remove nonspecific inhibitors of hemagglutination. The test is safer to perform since there is no need to use live leptospiral antigens in the test, as compared with the MA test. The ELISA test may also be used as a third test along with the MA and IHA for testing for detection of leptospiral antibodies in bovine sera.

Even though the MA test was the control test for the ELISA procedure employed in this investigation, each assay must stand on its own merits. The sensitivity and specificity appear to have been low in this research project but the ELISA might have been detecting the antibodies present because the role of the ELISA and its detection of antibodies with bovine sera is not known. The comparison shown in Table 5 with the ELISA and IHA are very significant, but this is also the first study using the IHA procedure with bovine sera. Additional reagents and procedure modification applicable to detection of antibodies which were not detected in this system must be developed in conjunction with further studies to better define the role of the ELISA with detection of leptospiral antibodies in bovine sera.

The use of ELISA represents a new and important development in the serology of bacterial, viral, parasitic and fungal diseases. ELISA employs stable reagents, simple to perform, suitable for automation

and is a good alternative to many diagnostic tests presently used.

Most existing methods, such as IHA, do not distinguish immunoglobulin type and can only detect IgM antibodies after separation from other immunoglubins. The ability of the ELISA to be made immunoglobulin type specific makes it an attractive method for detection of leptospiral antibodies in survey and experimental bacterin assays in bovine sera.

CHAPTER VI

SUMMARY

The Enzyme-Linked Immunosorbent Assay (ELISA) test was evaluated for the detection of leptospiral antibodies in bovine sera. The test was performed in disposable polystyrene plates sensitized with serovar hardjo and serovar illini. The total agreement of hardjo sera for both positive and negative sera was 48% among all 3 test procedures, and total agreement of illini sera for both positive and negative sera was 92% among all three test procedures. The test has the advantages of more stable reagents, fewer requirements of specialized equipment, and the ability to be made immunoglobulin class specific. The ELISA was suitable for detection of leptospiral antibodies in bovine sera for sero-epidemiological survey samples and experimental bacterin assay samples.

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APPENDICES

APPENDIX A

Representative antigens used for serological testing

Serovars, Reference Strains	Serovars, Reference Strains
ballum, Mus 127	cynopteri, 3522 C
canicola, Hond Utrecht	mankarso, Mankarso
copenhageni, M-20	celledoni, Celledoni
icterohaemorrhagiae, RGA	djasiman, Djasiman
bataviae, Van Tienen	borincana, HS 622
grippotyphosa, Andaman	panama, CZ 214 K
pyrogenes, Salinem	javanica, Veldrat Batavia 46
autumnalis, Akiyami A	butembo, Butembo
pomona, Pomona	andamana, CH 11
wolffi, 3705	fort-bragg, Fortbragg
australis, Ballico	hardjo, Hardjoprajitno
Tarassovi, Perepelicin	illini, 3055
georgia, LT 117	
alexi, HS 616	

APPENDIX B

Formula for *Leptospira* complete medium including C.D.C. Enrichment

Sorenson's phosphate buffered saline

a. Na_2HPO_4 16.6 g

b. KH_2PO_4 2.172 g

c. Add to one liter of H_2O to make stock solution (25X)

Diluent for bovine albumin fraction V

a. Stock solution (25X) of phosphate buffered saline 40 ml

b. Distilled H_2O 960 ml

CDC Enrichment

a. Diluent for bovine albumin fraction V 240 ml

b. Pentex Bovine albumin fraction V (Miles Laboratories)* 12g

c. Dissolve and filter through stacked Millipore filters in this order 1.20μ, 0.45μ, and 0.22μ pore size filters, respectively.

d. Add 20 ml of Vitamin B_{12}.

e. Mix 1.2 ml Tween 80 to 120 ml H_2O and add to total volume

f. Bring up to 400 ml and filter through stacked Millipore filters in this order: 1.20μ, 0.45μ, and 0.22μ pore filters.

Basal Medium - Ellinghausen, McCullough, Johnson and Harris (EMJH-Dehydrated)

a. Add 2.3 g of EMJH basal salts (Difco Laboratories)* to 600 ml H_2O

b. Bring total volume to one liter

*The use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service or the Department of Health, Education and Welfare.

APPENDIX C

Preparation of 1 Liter of Stock (10X Conc.)

Triethanolamine Buffered Salt Solution (TBS)-Kent Buffer

COMPONENTS

NaCl	75.0 g
HCl, (1N)	180.0 ml
TEA (a)	28.0 ml (b)
$MgCl_2 \cdot 6H_2O$ (4.16M)	1.2 ml (c)
$CaCl_2 \cdot 2H_2O$ (1.25M)	1.2 ml (c)

In a 1-liter volumetric flask, dissolve the NaCl in 700 ml of distilled water and then in the order given, add the indicated volumes of the other components. Adjust the volume to 1 liter with distilled water.

(a) Triethanolamine (2, 2^1, 2^{11} nitrilotriethanol) - the various lots of TEA procured from Matheson-Coleman 3rd Belt consistently have been satisfactory.

(b) The TEA was measured in a 50-ml graduated cylinder, taking precautions to prevent the chemical from coming in contact with the wall of the graduate above the 28-ml mark; this may be accomplished by pouring the TEA down a glass rod or pipette that touches the graduate at point below the mark. The final 2-3 ml was added with a 10 ml pipette. The measured TEA was poured into the solution of other components. The cylinder was allowed to drain for 2-3 minutes, then thoroughly rinsed three times with 10 ml portions of distilled water and the rinsings were added to the stock solution. Note: Special

care should be taken to assure the complete transfer of the TEA from the graduate.

(c) The divalent cation concentrations in the stock (10X conc.) TBS should be: 5X 10^{3} M $MgCl_2$ and 1.5 x 10^{-3} M $CaCl_2$. The working solution of Kent Buffer (1X Conc.) was prepared by mixing 100 ml of stock Kent buffer (10X conc.), 900 ml of sterile distilled water, and trace amounts of dry bovine fraction V powder (which acts as a stabilizer). The working solution of Kent buffer was mixed well and kept cold at 4 C. The pH was adjusted to 7.3-7.4 at 20 C.

APPENDIX D

Formula for ELISA buffers

Phosphate buffered saline (PBS), pH 7.2 (0.01 M)

a. 0.5 M Na_2HPO_4 (10.96 gm in 154.4 ml distilled H_2O)

b. 0.5 M $NaH_2PO_4 \cdot H_2O$ (3.1 gm in 45.6 ml distilled H_2O)

c. Combine 154.4 ml of 0.5 M Na_2HPO_4 and 45.6 ml of 0.5M $NaH_2PO_4.H_2O$ to make stock solution (10X)

d. Combine 40 ml stock to 100 ml 8.5% aqueous NaCl (Note: 10X physiologic) and dilute to 1 liter with distilled H_2O.

Carbonate buffer, pH 9.6 (0.06M) for dilution of antigen

a. 1.0 M $NaHCO_3$ (8.40 gm in 100 ml distilled H_2O solution)

b. 1.0 M Na_2CO_3 (10.60 gm in 100 ml distilled H_2O solution)

c. Combine 45.3 ml of 1.0 M $NaHCO_3$, 18.2 ml 1.0 M Na_2CO_3 and dilute to 1 liter with distilled water to make the Carbonate buffer, pH 9.6 (0.06 M).

Phosphate buffered saline with 0.05% Tween 20 (PBS/T).

Combine 0.05 ml of Tween 20 and 99.95 ml PBS.

APPENDIX E

Formula for Sorensen's Buffered Saline Solution

Sorensen's buffer: (pH 7.6)

a. Sodium phosphate (anhydrous)
   (Na_2HPO_4) ..... 8.33 g

b. Potassium phosphate (Monobasic) KH_2PO_4 ....1.09 g

c. Distilled water ..... 1 liter

Sterile buffered saline solution:

a. Physiological saline solution (0.85%)..... 1,840 ml

b. Sorensen's buffer ..... 160 ml

c. Autoclave at 6.8 kg pressure for 15 min. Determine the final pH after autoclaving. It should be pH 7.5.