AN EVALUATION OF THE ENZYME LABELED ANTIBODY TEST FOR THE DIAGNOSIS OF BOVINE BRUCELLOSIS

A Thesis

By

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ABSTRACT

An Evaluation of the Enzyme Labeled Antibody
Test for the Diagnosis of Bovine Brucellosis

(December 1977)

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Two conjugates, horseradish peroxidase labeled rabbit anti-bovine IgG and rabbit anti-IgM, were utilized to test 100 reactor and 100 non-reactor cattle with the enzyme labeled antibody (ELA) test. Specificity of each conjugate was evaluated by immunoelectrophoresis and the direct ELA test. Both antiserums showed specificity to their respective antigen when evaluated by immunoelectrophoresis, however, when antiserums were labeled with peroxidase and tested with the direct ELA procedure, cross reactivity was evident. It was suggested that this cross reactivity resulted from homologous kappa and lambda light chains common to both bovine IgG and IgM. Both conjugates were tested against serums of reactor and non-reactor cattle. Sensitivity of the anti-IgG and anti-IgM conjugates was 97.09% and 99.01%, and the specificity was 99.01% and 87.72% respectively.

Approximately 5,120 bovine serum samples were tested to compare the enzyme labeled antibody test with buffered Brucella antigen (card), standard agglutination tube (SAT), rivanol precipitation-plate agglutination (rivanol), and complement fixation (CF) tests. Percent agreement between the ELA test and other procedures was determined. The mean and standard deviation of ELA readings within test reactions was calculated and graphically illustrated.

Each serologic test was evaluated for sensitivity in detecting different concentrations of specific antibody from a pooled reactor serum. The ELA test proved to be the most sensitive assay, producing a positive reading at a 1:32 dilution, followed by SAT, CF, rivanol, and card at 1:10, 1:10, 1:8, and 1:6 respectively.

Card and ELA tests were run on serums from four consecutive bleedings over a five months period. These serums were collected from 15 non-vaccinated cattle and 15 vaccinated cattle which had Brucella abortus type 4 isolated from their tissues. In four cases, ELA detected specific antibody before the card test and in no instance did the card test detect antibody before ELA.

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INTRODUCTION

Brucellosis is a disease produced in man and animals by bacteria belonging to the genus Brucella. Brucellosis generally manifests itself in vivo after a short bacteremia. It is characterized by inflammation, and occasionally necrosis of various organs and tissues. Characteristic lesions of the placenta and uterus are produced in cattle, sheep, goats, and pigs. Death and abortion of the fetus is also common during pregnancy. Abortion is most common in cattle. In swine, males are more severely affected than in other animal species. In humans the most common symptoms are undulating or intermittent fever, night sweats, chills, asthenia, anoroxia, myalgia, and arthralgia (22).

Brucellosis in cattle occurs in nearly all parts of the world. This disease has apparently been eradicated in a few countries, and other countries are attempting to achieve that goal. However, there exist countries or areas on every continent where brucellosis prevails in 10 to 30 percent of the breeding livestock.

The citations on the following pages follow the style of the Journal of Clinical Microbiology.

The economic losses from brucellosis in cattle within these countries arise from four main causes: 1) abortions, stillbirths or birth of weak calves; 2) decreased milk yields due to reduction or absence of the stimulatory effect of a full-time pregnancy or from damage to the milk-secreting tissues; 3) arthritis or bursitis; 4) subsequent effects on fertility, mainly from secondary infections of the genitalia and interference with the desired sequence of calving (27).

In 1887, Bruce described a bacterium that was isolated from patients on the island of Malta who had died of the disease known as Malta fever, Mediterranean fever, or undulant fever. The bacterium that caused this disease in humans was termed Micrococcus melitensis (10). Ten years later, in Denmark, Bang isolated a microorganism that induced contagious abortion in cattle which he designated Bacillus abortus (10). The illness which accompanies this syndrome has since been termed Bang's disease. In 1910, McNeal and Kerr first isolated the bacterium of Bang's disease in the United States (10). In 1914, Traum (10) isolated a microorganism from aborted swine fetuses which was morphologically similar to the bacterium of undulant fever and Bang's disease. Not until the work of Evans (10) in 1918 was the etiologic bacterium of

undulant fever and Bang's disease considered to be related. Evans demonstrated a morphological, cultural, and serologic relationship between these organisms.

Bacteria of the genus Brucella are small, Gram negative bacilli or coccobacilli which are non-sporeforming and non-motile. They are aerobic but may need added CO_2 for primary isolation. Brucellae grow best on enriched media and ferment no carbohydrates. All are considered obligate parasites (27).

The three classical species are Brucella abortus, Brucella melitensis, and Brucella suis, their natural hosts being cattle, goats, and swine, respectively. Other species are Brucella ovis, Brucella canis, and Brucella neotomae, whose natural hosts are sheep, dogs, and rats, respectively. Bacteria from all species of Brucella can infect man and most domestic animals (4).

Wilson and Miles (27) alleviated much confusion over the antigenic relationship of the species of Brucella. They found that B. melitensis could be distinguished from B. abortus and B. suis by agglutination/adsorption tests and concluded that the three species contained homologous A and M antigens but in different porportions. Later, Miles (27) demonstrated that B. abortus had an A : M ratio of 20:1 and B. melitensis, 1:20. This wide difference allowed

production of monospecific antisera to either the A or M antigen by cross adsorption with Brucella cells. Renoux and Mahaffey (10) demonstrated two other antigens, z and r. The z antigen was found in B. ovis and in the rough phase of B. abortus and B. melitensis, while the r antigen exists in the rough phase of B. melitensis. They concluded that the serologic behavior of the Brucellae is the result of concentric distribution of antigens within the bacterial cell.

Virulence of Brucella is associated with the smooth colony type, but these bacteria may dissociate easily to produce rough avirulent forms which are altered antigentically (10). Dissociation can result from unfavorable factors such as temperature, availability of nutrients, and oxidation-reduction potential (27).

Brucellosis, caused by B. abortus, is present in almost all countries except where strict eradication and quarantine programs have been instituted. B. melitensis infections in goats is found in the Mediterranean countries, Latin America, southern areas of the U.S.A., U.S.S.R., and certain countries in the Middle East. Brucella melitensis infections occur in sheep in parts of the U.S.S.R. and Central Europe, and in cattle in the Mediterranean countries. In the U.S.A., infection with B. melitensis has been reported

in cattle and swine. Brucella suis infects swine in the United States and Latin America, and this species has been reported to infect both swine and hares in Europe (27).

The immune system of an individual with brucellosis may produce three types of antibodies detectable by serologic tests. These are agglutinins, complement-fixing and incomplete antibodies. Agglutinins are bivalent antibody molecules which cross-link Brucella cells or stroma. The resultant lattice formation provides the basis of seroagglutination tests. Apparently these antibodies are incapable of fixing complement. Complement-fixing antibodies form antigen-antibody complexes capable of binding complement at the Fc portion of the antibody. This capability is utilized in serologic testing as another method of detecting antibody. Incomplete antibodies are incapable of agglutination or complement fixation and may be detected by serologic tests (e.g. Coomb's test) involving anti-globulin antibodies. It has been suggested that the different reactions of these serologic tests are not different expressions of a single population of immunoglobulin molecules (8).

Results from studies completed in the 1950's and 1960's involving the examination of immunoglobulins from cattle with brucellosis greatly increased the knowledge about this disease and its serologic diagnosis. Two

important classes of immunoglobulins were found to predominate, immunoglobulin M (IgM) and immunoglobulin G (IgG). The IgM class was found to be of large molecular weight (19S), heat labile, and sensitive to mercapto-ethanol reduction. The IgG class was of lower molecular weight (7S), heat stable, and insensitive to mercapto-ethanol reduction. Within the IgG class there existed two subclasses, IgG_1 and IgG_2. IgG_1 was most common in serum and lacteal secretions. IgM was found to be a very efficient agglutinin while the IgG subclasses differed in agglutination activity. IgG_1 agglutinated Brucella cells poorly in the presence of acid pH or 5% sodium chloride. IgG_2 agglutinated antigen in saline, but agglutination was inhibited in acid solutions or high salt concentrations. Cattle with brucellosis produce IgM in the early stages of infection, IgG appears shortly thereafter and becomes the predominant class which persists for the duration of the infection (13). Generally, IgG_1 persists in higher concentrations than IgG_2 due to the anamnestic response (1). Animals vaccinated with Strain 19 vaccine normally produce IgM first, with IgG appearing as early as 10 days after inoculation. The concentration of IgG normally decreases with time (13). Six months post-inoculation, IgG_2 was not detectable in the serum but IgG_1 and IgM may persist (1). Persistent agglutinins were believed to be

IgM, however, this has not been adequately proven (13).

Definitive diagnosis of bovine brucellosis requires the recovery of Brucella from the infected animal; however, because isolation is not always practical, serologic tests are utilized more often for diagnosis (8). Serodiagnostic tests were developed to detect IgG, which was at first believed to be the most reliable indication of infection and because IgM was associated with residual vaccination titers and non-specific reactions. The results of these IgG specific tests indicated that Brucella antigens react with antibodies other than IgG. Also, that Brucella specific IgM, which was not detectable by these tests, is important in diagnosis in some cases (13).

Many factors have a marked influence on the significance and reliability of serologic testing. Increase in age of animal can affect immune responsiveness which may influence the reaction of the serologic tests. The incubation period, defined as the period between exposure to the infectious organism and the onset of clinical signs, varies from about six weeks to eight months of longer. During the incubation period the results of the serologic test are usually negative or equivocal in titer. The most dramatic clinical sign of the disease is abortion, which may be caused by factors other than brucellosis. Animals can abort and then

become chronic carriers, often excreting the organism after subsequent normal calving. These excretors are very effective transmitters of brucellae, therefore the disease can be easily reintroduced into the herd. The immunologic response of an infected animal is influenced by the size of inoculum, virulence of strain, and stage of pregnancy. The immunologic response of a vaccinated animal is influenced by the size of inoculum, vaccine used, and age at vaccination. Also, the individual response to any of the Brucella vaccines can vary from complete resistance to no protection from infection. After vaccination, serologic test results may be negative, yet the animal may completely resist infection upon subsequent challenge, or the animal may exhibit a serologic response and still contract the disease. The test or tests used, the manner in which these tests are performed and interpreted, and uniformity of results within a country and between countries are other factors which influence the significance and reliability of serologic testing (15).

Diagnostic tests for bovine brucellosis should: 1) detect infection early during the long and variable incubation period, 2) not be influenced by presence of "non-specific" antibodies, 3) detect chronic carriers, and 4) differentiate an immune response to vaccination from that of field infection (15).

Serologic tests which are most often employed for the diagnosis of bovine brucellosis include: buffered Brucella antigen or Rose Bengal (card) (29), serum tube agglutination (SAT) (28), rivanol precipitation-plate agglutination (rivanol) (29), mercaptoethanol agglutination (ME) (29), and complement-fixation (CF) (11) tests. Recently a new test, the indirect enzyme labeled antibody (ELA) test (23) has been developed and utilized for the sero-diagnosis of several diseases. An ELA test protocol, which utilizes specific antibody-enzyme conjugates to detect serum antibodies, has been developed for diagnosis of brucellosis but has yet to be applied on a large scale.

Conjugation of enzymes was first developed for use as an immunohistochemical marker (18). Unlike other markers, such as fluorescein (7) and ferritin (26), enzyme-antibody complexes may be utilized in both light and electron microscopy systems (14,16). Early procedures (3,6,18) for conjugation of horseradish peroxidase (HRPO) and IgG resulted in more IgG polymers than HRPO-IgG complexes. These low yields necessitated modification of the labeling protocols. The procedure by Nakane and Kawaoi (17) permits approximately 70% coupling of the HRPO with IgG and approximately 99% of the IgG to be labeled with HRPO. Biologic activity of the IgG and HRPO is not significantly altered by this

method.

The enzyme labeled antibody method was developed to detect intracellular antigens. This principle was later applied for development of a quantitative serologic test (9,30). This procedure was not applicable to large scale screening of serums. Factors detrimental to this procedure were incubation time, impractical reaction vehicles, and large volumes of reagents. To overcome these problems, a new test system (12,20,24) evolved using cellulose acetate filters as the antigen carriers. This system proved sensitive and rapid but not the ideal screening procedure. Problems arose from time consuming and incomplete washings, unstable reaction products, cumbersome handling of filters, and the inability of some antigens to bind to filters. These problems led to the development of the microtiter plate method. By utilizing this method (23), which employs disposable 96-well microtiter trays as the antigen carrier, these problems were eliminated without loss of sensitivity.

The ELA test has proven to be a highly sensitive and specific assay, but there is a need to evaluate its potential as a mass screening test. This test has been successfully applied for the diagnosis of hog cholera (21), trichinosis (22), and several viral infections (31). It has the potential to be a sensitive diagnostic test for microbial toxins, carcinogens,

pesticides, drug residues as well as other bacterial, viral or parasitic antigen. In addition, the procedure is adaptable to automation.

The objectives of this study are twofold: 1) to compare the ELA test conducted with enzyme labeled conjugates prepared against bovine IgG and IgM antibodies for sensitivity and specificity, 2) to compare the ELA test with the card, SAT, rivanol, and CF tests for the diagnosis of brucellosis in naturally infected herds including some of which have adult vaccinated animals.

MATERIALS AND METHODS

Antiserum Preparation

Lyophilized rabbit antiserums, anti-bovine IgM and anti-bovine IgG (Miles Laboratories, Elkhart, Indiana), were reconstituted with distilled water, and the globulins were precipitated with ammonium sulfate (5). The globulin fractions were utilized for conjugate preparation. Another antiserum, rabbit anti-bovine gamma globulin, was prepared in the Brucellosis Research Laboratory at Texas A&M University. The globulin fraction of a bovine serum pool was precipitated (5) and adjusted to a 1% protein concentration with distilled water. New Zealand white rabbits were immunized with bovine gamma globulin for a minimum 26 days before being bled for antiserum. The animals were given subcutaneous injections in the nuchal region at four different sites. The injection on the first and fifth day consisted of 0.4 ml of equal parts of 1% bovine gamma globulin emulsified in Freunds complete adjuvant. Subsequent injections were given intradermally at one week intervals with 0.4 ml of a 1% bovine gamma globulin in saline. Animals were bled by cardiac puncture and 20 ml collected per bleeding. Serum was collected, lyophilized in five milliliter aliquots and stored at $4^{o}C$.

Conjugate Preparation

Conjugates of peroxidase and antibody proteins were prepared according to a modified version of the procedure of Nakane and Kawaoi (17). Five milligrams of horseradish peroxidase (type VI, Sigma Chemical Company, St. Louis, Missouri) was dissolved in 1.0 ml of 0.3M sodium bicarbonate. To this solution 0.1 ml 1% fluorodinitrobenzene in absolute ethanol was added and centrifuged to remove any precipitate that formed. After centrifugation the solution was mixed gently for 1 hr at room temperature, and 1.0 ml of 0.08M sodium periodate in distilled water was added. This solution was then mixed gently for 30 min. at room temperature. After mixing, 1.0 ml of 0.16M ethylene glycol in distilled water was added and again mixed for 1 hr at room temperature. This solution was dialyzed for 24 hrs against several changes of 0.01M carbonate buffer at pH 9.5. Five milligrams of antibody protein (gamma globulin fraction in carbonate buffer at pH 9.5) was added to 3 ml of the peroxidase-aldehyde solution, pH 9.5. This solution was mixed gently for 3 hrs at room temperature then centrifuged to remove any precipitate. After centrifugation, the solution was dialyzed overnight at $4^{o}C$ against 0.01M PBS pH 7.2. Conjugates were stored at $4^{o}C$.

Immunoelectrophoresis

Purity of the precipitated globulins was evaluated by immunoelectrophoresis. Globulin samples were added to wells cut in 1% agarose in barbital buffer and electrophoresed at 2 millamperes for 2 hours. Troughs were cut and then filled with goat anti-rabbit serum (Miles). The test agar was incubated in a humidity chamber for 6 hours at room temperature, then 24-48 hours at 4°C. Specificity of the rabbit antiserums was evaluated after electrophoresis of bovine serum in the wells and addition of rabbit anti-bovine IgM and IgG to the respective troughs.

Direct ELA

Specificity of conjugates was evaluated by a direct ELA procedure. Bovine IgM and IgG were purified by salting out (5) the globulins from pooled serum and subsequent separation of IgM and IgG on Sephadex G200 (Pharmacia) in a 3 x 45 cm column equilibrated with saline pH 8. Protein concentrations were determined by the Biuret procedure. IgM or IgG was diluted and then fixed to microtiter trays by placing 0.05 ml of each dilution in each well and allowing it to air dry. To each well 0.05 ml of 0.25% glutaraldehyde was added, allowed to stand for 30 minutes, washed 4 times with distilled water, and air dried. A 1:100 dilution of the

anti IgM, anti-IgG, and anti-immunoglubin conjugates was made, and 0.1 ml was added to each concentration of bovine IgM and IgG. The plates and contents were incubated 30 minutes and then washed 8 times with 0.5% Tween 80 in saline. To each well 0.05 ml of the substrate solution (described under indirect ELA procedure) and then incubated 10 minutes. The reaction was inhibited with 0.05 ml 1% sodium azide. The contents of each well was transferred to 0.9 ml of 0.005 N H_2SO_4, and the percent transmission was read in a Beckman DB-GT spectrophotometer at 560 nm. A blank containing 0.05 ml substrate solution and 0.05 ml sodium azide was used to set the spectrophotometer at 100% transmission. Results were expressed as 100 minus the percent transmission of each reading.

Indirect ELA for Sensitivity and Specificity Comparison of the Anti-IgG and Anti-IgM Conjugates

Sensitivity and specificity of the IgM and IgG conjugates was evaluated. This was accomplished by testing serums from 100 selected reactors and 100 non-reactor cattle with the indirect ELA procedure. Cattle selected for this study were considered reactors if the card test was positive, SAT 1:100 or greater, rivanol 1:25 or greater, and non-reactor if all three tests were negative.

Paired serum control (PSC) antigen plates were incorporated in the indirect ELA test protocol. Paired serum control plates were prepared by adding of 0.05 ml soluble B. abortus antigen (diluted 1:500 in distilled H_2O) to the odd numbered rows and 0.05 ml H_2O to the even numbered rows on the plates. The fluid within the wells was allowed to air dry. Dried antigen plates were sealed with tape to insure a longer shelf life. Stock antigen was furnished by the Veterinary Services Laboratory of the National Animal Diseases Center in Ames, Iowa. This antigen was prepared by propagating and harvesting Brucella abortus strain 1119-3 as described by Alton, G. G. et al. (2). Of this harvest, 50 gms were resuspended in 200 ml of sterile distilled water and shaken for two hours. This preparation was autoclaved at 121°C for 20 minutes. After a slow exhaust for 15 minutes, the suspension was centrifuged at 15,000 x g for 20 minutes at 4°C and the supernatant collected for the soluble antigen preparation.

Serums were diluted 1:20 in 0.5 M NaCl containing 1% Tween 80, 0.1% sodium azide and buffered at pH 7.4. Pooled samples of known positive and negative serum were diluted and tested with each series of forty six serums. From each diluted serum sample, 0.1 ml was added to the wells of the transfer plate so that each serum sample would be incubated in an antigen and a blank well.

The transfer plate was fitted over an antigen plate and prewetted with the ELA wash solution consisting of 0.85% NaCl in water containing 0.5% Tween 80. The transfer of the serum dilutions was accomplished using an air blower to force the fluid through the aperture of the transfer plate and into the antigen plate. The plate and contents were incubated for 10 minutes. ELA test plates were incubated at room temperature with gentle rocking. The wells were then washed 8 times with the ELA wash solution. After the excess fluid was removed, 0.05 ml of horseradish peroxidase-labeled rabbit anti-bovine IgG or anti-bovine IgM, diluted 1:100 in 0.5 M NaCl containing 1% Tween 80, was added and allowed to incubate 5 minutes. During this incubation, the substrate solution was prepared. A stock solution of color indicator was prepared by dissolving 225 mg of 5-amino-salicylic acid hydrochloride in a small amount of boiling water and adjusting the volume to 100 ml. This solution was stable for 2 weeks. To prepare for use, an aliquot of the stock solution was adjusted to pH 6.0 with 2% NaOH and then 0.05% H_2O_2 was added at the ratio of 0.1 ml per milliter of 5-aminosalicylic HCl, pH 6.0. After addition of the conjugate and incubation for 5 minutes, the plates were washed 8 times with the ELA wash solution and 0.05 ml of the substrate solution was added to each well and allowed to incubate for 10 minutes.

The reacting solution was then inhibited by addition of 0.05 ml of 1% sodium azide in distilled water to each well. The contents of each well were transferred to tubes containing 0.9 ml of 0.005 N H_2SO_4 in water. This solution was then transferred to a flow cuvette in a Beckman Model DB-G1 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA.) that was adjusted to 100% transmission with 0.005 N H_2SO_4 at 560 nm. The results were calculated as:

[(100)-(percent T of contents in antigen well)]

Minus

[(100)-(percent T of contents in blank well)]

Sensitivity and specificity of both conjugates were calculated according to the following equations:

$$\text{Sensitivity} = \frac{\text{true positives}}{\text{true positives + false negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{true negatives}}{\text{true negatives + false positives}} \times 100$$

Research Herds

Blood samples were collected from cattle at Bay City, Winnie, Litton Springs and DeKalb, Texas. The herd at Bay City, which consisted of approximately 450 registered Brahman cattle, had an estimated 10% herd infection. At Winnie the herd population was composed of approximately 260 commercial and registered Brahmans with a 25% herd

infection. The DeKalb herd consisted of approximately 860 Holstein dairy cows with an annual reactor rate of approximately 12%. At Litton Springs, there was an estimated herd infection of 10% in 85 commercial beef cattle.

Animals from the DeKalb and Winnie herds were inoculated subcutaneously with 3×10^9 organisms of Brucella abortus strain 19 vaccine. Cattle within these herds were grouped according to vaccination; 25% were adult vaccinated; 25%, calfhood vaccinated; 25%, calfhood and adult vaccinated; and 25%, non-vaccinated control animals.

Cattle within these herds were bled routinely, tested serologically, and the reactors were removed. Intervals between serologic tests varied between herds, but each herd was monitored for at least 26 months. Within this time, each animal was tested a minimum of 8 times.

All blood samples were allowed to clot, centrifuged, and the serums collected, and catalogued for storage at $-20^{\circ}C$. A sufficient volume of each serum was sent for testing to the Veterinary Services Laboratory of the Animal and Plant Health Inspection Services, United States Department of Agriculture, Austin, Texas. Serum samples were tested at Austin with the card, rivanol and SAT tests. All serums were tested with the indirect ELA

test, and serums from the DeKalb and Winnie herds were tested with the CF test in the Brucellosis Research Laboratory at Texas A&M University, College Station, Texas.

Culture results from cattle declared to be reactors by the conventional tests were also used in the evaluation of the ELA test. Cultures were taken from milk and supramammary, iliac and suprapharyngeal lymph nodes. Aborted fetuses and amniotic fluids were also cultured. Twenty milliliter aliquots of milk samples were centrifuged at 1,200 x g for 20 min and swabs of cream and sediment were streaked on Brucella agar (Difco Laboratories, Detroit, Michigan). Lymph node sections, held by forceps, were streaked on Brucella agar, and 0.1 ml aliquots of amniotic fluid were streaked with cotton swabs. Plates were allowed to incubate in the presence of 10% CO_2 for 5 days. Suspicious colonies were tested for agglutination with rabbit anti-Brucella abortus and rabbit anti-Brucella melitensis antiserums. Cells from colonies which agglutinated antiserums were streaked on Brucella agar slants and shipped to the Veterinary Services Laboratories, Ames, Iowa for type identification.

Indirect ELA for Comparison with Conventional Tests

Approximately 6,000 serum samples from cattle within herds were utilized for comparison of the indirect ELA test with the conventional tests.

Microtiter plates for the ELA test were prepared by drying 0.05 ml of soluble Brucella abortus antigen diluted 1:500 in distilled water in each well. Dried antigen plates were sealed with tape to insure a longer shelf life.

Serums were diluted 1:20 in 0.5 M NaCl containing 1% Tween 80, 0.1% sodium azide and buffered at pH 7.4. Pooled samples of known positive and negative serums were diluted and tested with each series of ninety three serums. One well did not have serum added and provided the blank for adjustment of the spectrophotometer. From each diluted serum sample, 0.1 ml was added to a transfer plate. The transfer plate was fitted over an antigen plate prewetted with the ELA wash solution consisting of 0.85% NaCl in water containing 0.5% Tween 80. The transfer of the serum dilutions was accomplished using an air blower to force the fluid through the aperture of the transfer plate and into the antigen plate. The indirect ELA procedure was as previously described, except for spectrophotometric readout. The spectrophotometer was set at 100% transmission with a blank containing 0.9 ml of 0.005 N H_2SO_4, 0.05 ml substrate, and 0.05 ml 1% sodium azide and set at 560 nm. The results were expressed as 100 minus percent transmission of each sample reading.

Comparative Sensitivity of the ELA Test with Conventional Tests

Sensitivity of the ELA test was compared to that of the card (29), SAT (28), rivanol (29), and CF (11) tests. Sensitivity was defined as the dilution of a Brucella reactor serum pool that gave a positive reaction with each test. Serial dilutions of a Brucella reactor serum pool was made in fetal calf serum and tests were run from each dilution. Positive test reactions were considered as 4 or greater with ELA, calculated as:

[(100) - (% T of content in antigen well)]

minus

[(100) - (% T of contents in blank well)]

A titer of 1:100 or greater with SAT, a titer of 1:50 or greater with rivanol, and a titer of 1:40 or greater with CF were considered positive with these conventional tests. The indirect ELA procedure was run as previously described except the paired serum control (PSC) method was added to the test protocol.

Early Detection of an Immunologic Response

The ability of the card and ELA tests to detect early infection was studied. Early detection was defined as sensitivity or the ability of the test to detect antibodies to Brucella antigens.

The ability of the ELA test to detect antibodies

to Brucella infection earlier than the card test was studied. The sensitivity of both tests was compared as to the detection of an immunologic response to Brucella infections within a five month period. Thirty animals were selected for study from the DeKalb herd and tested with the card test and the paired serum control ELA test. B. abortus type 4 was isolated from the tissues of all thirty animals. Many of these animals were serologically negative at the first bleeding. Fifteen animals were non-vaccinated and fifteen vaccinated. Of the fifteen vaccinates, ten animals were adult vaccinated after the first bleeding, four were calfhood vaccinates and adult vaccinated after the first bleeding and one animal was calfhood vaccinated only. Cattle were bled on days 1, 35, 63, and 127 during the study. Ten negative serums were added to the ELA plates along with the test samples to verify that ELA was not reacting non-specifically. These negative serums produce no reaction with any of the supplemental test methods.

All culture and test data received during this study were programmed and analyzed at the Data Processing Center, Texas A&M University.

RESULTS

Immunoelectrophoresis

Precipitated rabbit antiserum was electrophoresed and reacted with goat anti-rabbit serum. The immunoelectrophoretic pattern of antiserums used for conjugation showed two distinct lines of precipitation (Figure 1). These lines consisted of a dense band of high mobility and a lighter band of lesser mobility.

Specificity of the anti-IgG and anti-IgM antiserums was evaluated by immunoelectrophoresis against bovine serum (Figures 2,3). Specificity of rabbit anti-bovine IgG was indicated by the broad precipitin band in each side of the antiserum trough. A small precipitin band was evident which indicated the presence of a reacting immunoglobulin other than IgG. The precipitate apparent after electrophoretic separation of bovine serum and subsequent reaction with rabbit anti-bovine IgM showed a narrow, and less apparent area of precipitate, thus indicating that the concentration of the reacting antibodies and antigen was less than with the IgG system.

Direct ELA

Serologic activity of the anti-IgG, anti-IgM, and anti-immunoglobulin antiserums as determined by the direct ELA procedure was observed (Tables 1,2). After

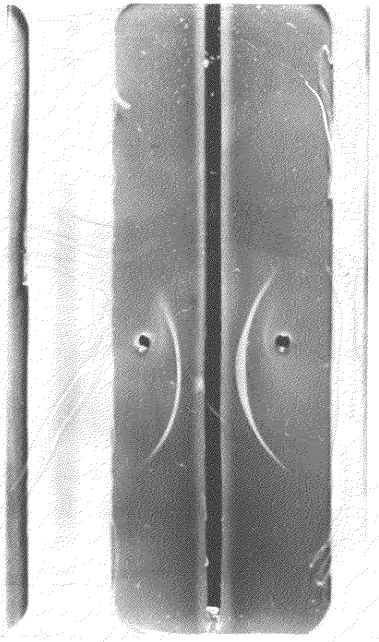

Figure 1. Immunoelectrophoretic pattern of ammonium sulfate precipitated rabbit globulin
right well - rabbit anti-bovine IgM
left well - rabbit anti-bovine IgG
trough - goat anti-rabbit serum

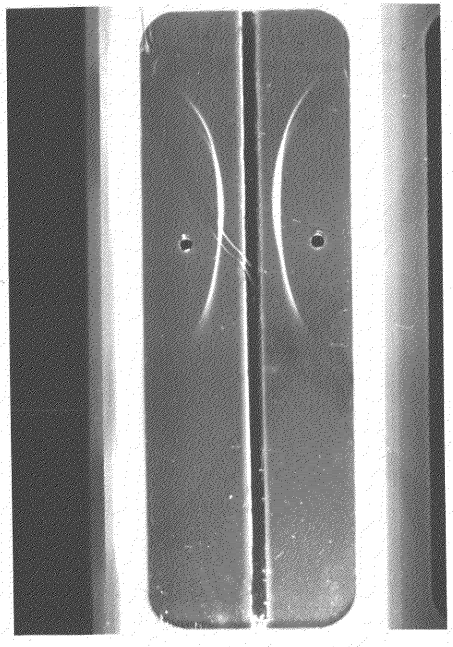

Figure 2. Specificity of rabbit anti-bovine IgG as shown by immunoelectrophoresis
right well - bovine serum
left well - bovine serum
trough - rabbit anti-bovine IgG

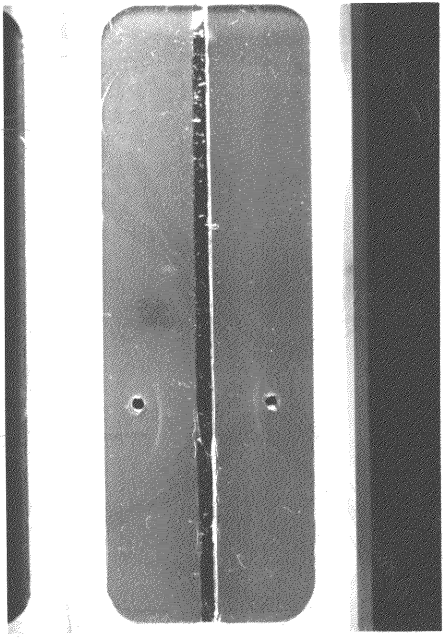

Figure 3. Specificity of rabbit anti-bovine IgM as shown by immunoelectrophoresis.
right well - bovine serum
left well - bovine serum
trough - rabbit anti-bovine IgM

Table 1. ELA test results[a] from reaction of anti-IgG, anti-IgM, and anti-gamma globulin conjugates with dilutions of bovine IgG

mg bovine IgG/well	anti-IgG	anti-IgM	anti-gamma globulin
6.25×10^{-3}	15	5	14
3.13×10^{-3}	16	6	14
1.56×10^{-3}	18	5	13
7.8×10^{-4}	18	4	10
3.9×10^{-4}	14	2	4
2.0×10^{-4}	9	1	2
9.7×10^{-5}	6	0	1
4.9×10^{-5}	5	0	1
2.4×10^{-5}	1	0	0
1.2×10^{-5}	1	0	0
6.0×10^{-6}	0	0	0

[a]Calculated as (100-percent transmission test well) - (100-percent transmission blank well)

Table 2. ELA test results[a] from reaction of anti-IgG, anti-IgM, and anti-gamma globulin conjugates with dilutions of bovine IgM

mg IgM/well	anti-IgG	anti-IgM	anti-gamma globulin
6.25×10^{-3}	16	17	16
3.13×10^{-3}	15	15	14
1.56×10^{-3}	14	14	10
7.8×10^{-4}	9	10	4
3.9×10^{-4}	6	7	2
2.0×10^{-4}	3	3	1
9.7×10^{-5}	2	3	1
4.9×10^{-5}	1	2	1
2.4×10^{-5}	0	1	0
1.2×10^{-5}	0	0	0
6.0×10^{-6}	0	0	0

[a]Calculated as (100-percent transmission test well) - (100-percent transmission blank well)

the three antiserums were separately conjugated to HRPO, they were diluted 1:100 and reacted with dilutions of IgG and IgM fixed in wells of polystyrene microtiter plates.

The anti-IgG, anti-IgM, and anti-immunoglobulin conjugates were reacted with decreasing concentrations of bovine IgG and the minimal concentration of antigen protein detected was 4.9×10^{-5} mg, 7.8×10^{-4} mg, and 3.9×10^{-4} mg, respectively. Detection was considered a reaction producing a 4 or greater spectrophotometric reading.

The anti-IgG, anti-IgM and anti-immunoglobulin conjugates were also reacted with decreasing concentrations of bovine IgM and minimal concentrations of antigen protein detected was 3.9×10^{-4}, 3.9×10^{-4}, and 7.8×10^{-4} mg, respectively.

Indirect ELA for Sensitivity and Specificity Comparison of the Anti-IgG and Anti-IgM Conjugates

The distribution of ELA results from testing serums from 100 reactor and 100 non-reactor cattle with the anti-IgG and anti-IgM conjugates were observed (Figures 4-10). Reaction of the anti-IgG conjugate with the 100 reactor cattle serums gave a range of readings 1.0 to 22.0 with a mean of 13.34 ± 3.38. Reaction of this conjugate with the 100 non-reactor

Figure 4. Frequency of ELA readings from 100 reactor cattle tested with the anti-IgG conjugate. Symbols: (•), total number of reactor cattle with equivalent ELA readings; (○), mean ELA reading from all reactors. Horizontal lines represent the standard deviation of the mean.

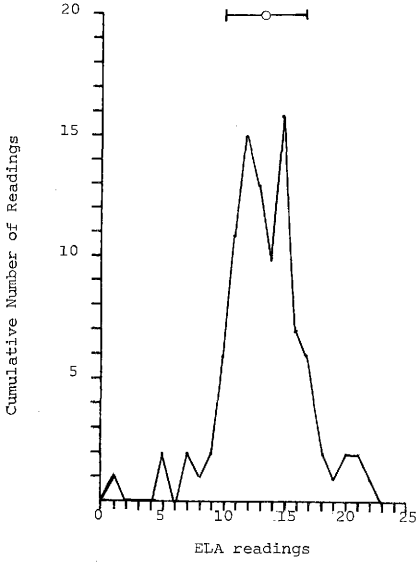
20
15
10
5
0
5
10
15
20
25
Cumulative Number of Readings
ELA readings

Figure 4

Figure 5. Frequency of ELA readings from 100 non-reactor cattle tested with the anti-IgG conjugate Symbols: (•), total number of non-reactor cattle with equivalent ELA readings; (○), mean ELA reading from all non-reactors. Horizontal lines represent the standard deviation of the mean.

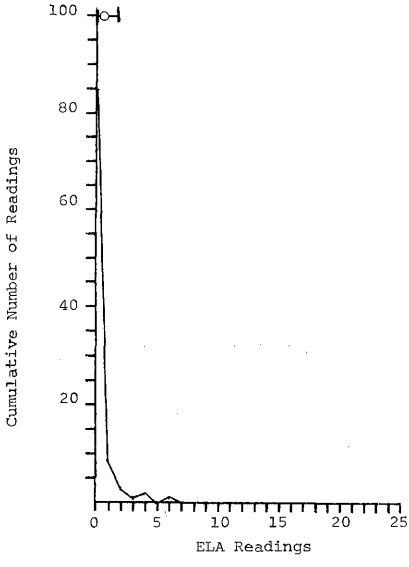
100
80
60
40
20
Cumulative Number of Readings
0
5
10
15
20
25
ELA Readings

Figure 5

Figure 6. Frequency of ELA readings from 100 reactor cattle tested with the anti-IgM conjugate. Symbols: (•), total number of reactor cattle with equivalent ELA readings, (○), mean ELA reading from all reactors. Horizontal lines represent the standard deviation of the mean.

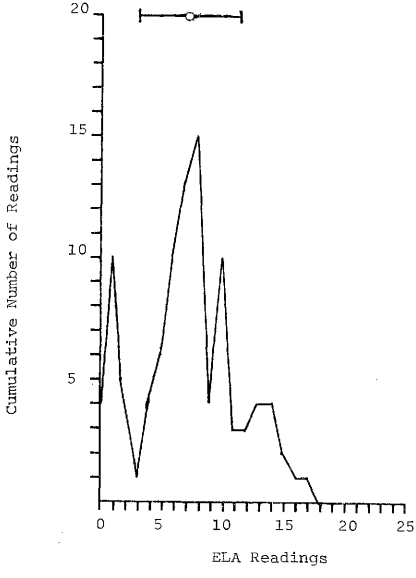
20
15
10
5
Cumulative Number of Readings
0
5
10
15
20
25
ELA Readings

Figure 6

Figure 7. Frequency of ELA readings from 100 non-reactor cattle tested with the anti-IgM conjugate. Symbols: (·), total number of non-reactor cattle with equivalent ELA readings; (○), mean ELA reading from all non-reactors. Horizontal lines represent the standard deviation of the mean.

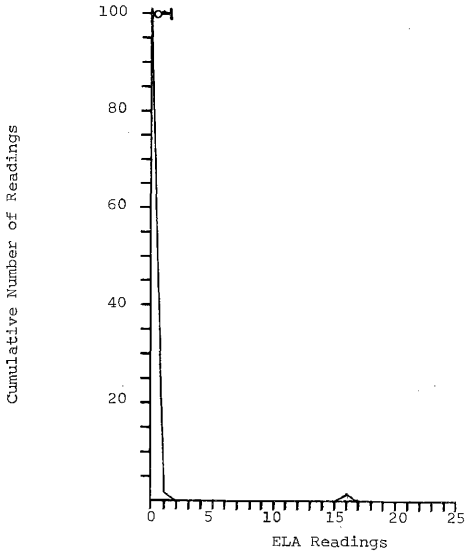
Cumulative Number of Readings
100
80
60
40
20
0
5
10
15
20
25
ELA Readings

Figure 7

cattle serums gave a range of readings 0 to 7.0 with a mean of 0.27 ± 1.01. The anti-IgM conjugate was reacted with the 100 reactor cattle serums which produced a range of readings 0 to 17 with a mean of 7.16 ± 4.09. This conjugate was also reacted with the 100 non-reactor cattle serums which gave a range of readings 0 to 16.0 with a mean of 0.17 ± 1.6.

The percent sensitivity and specificity of both conjugates was as follows:

Conjugate	Percent Sensitivity	Percent Specificity
anti-IgM	99.01	87.82
anti-IgG	97.09	99.01

Indirect ELA for Comparison with Conventional Tests

The results of card, SAT, rivanol, and CF tests were compared to the ELA test (Tables 3,4,5,6,) as to their overall agreement. Of the 5,118 card and ELA results compared (Table 3), agreement was evident in 4,599 (89.86%) serums and disagreement in 519 (10.14%). Of the 4,599 agreements, 1,131 (24.59%) serums were card positive, ELA positive and 3,468 (75.41%) were card negative, ELA negative. Of the 519 disagreements, 362 (69.75%) were card positive, ELA negative and 157 (30.25%) were card negative, ELA positive.

Table 3. Comparison of ELA and card test results

	Card positive	Card negative
ELA positive	1131 (22.10%)	157 (3.07%)
ELA negative	362 (7.07%)	3468 (67.76%)

SAT and ELA test results of 5,122 serums were compared (Table 4). Test results agreed in 4,683 (91.43%) comparisons and disagreed in 439 (8.57%). Of the 4,683 agreements, SAT positive, ELA positive results accounted for 1,065 (22.74%) and SAT negative, ELA negative, 3,618 (77.26%). Of the 439 disagreements, 215 (48.98%) were SAT positive, ELA negative and 224 (51.02%) were SAT negative, ELA positive.

Table 4. Comparison of ELA and standard agglutination tube test results

	SAT positive	SAT negative
ELA positive	1065 (20.79%)	224 (4.37%)
ELA negative	215 (4.2%)	3618 (70.64%)

Rivanol and ELA test results of 5,122 serums were compared (Table 5). Test results agreed in 4,716 (92.08%) comparisons and disagreed in 406 (7.92%). Of the 4,716 agreements, rivanol positive, ELA positive results accounted for 1,001 (21.23%) and rivanol negative, ELA negative, 3,715 (78.77%). Of the 406 disagreements, 118 (29.06%) were rivanol positive, ELA negative and 288 (70.94%) were rivanol negative, ELA positive.

Table 5. Comparison of ELA and rivanol test results

	Rivanol positive	Rivanol negative
ELA positive	1001 (19.54%)	288 (5.62%)
ELA negative	118 (2.3%)	3715 (72.54%)

CF and ELA test results of 5,122 serums were compared (Table 6). Test results agreed in 4,611 (90.02%) comparisons and disagreed in 511 (9.98%). Of the 4,611 agreements, CF positive, ELA positive results accounted for 954 (20.69%) and CF negative, ELA negative 3,657 (79.31). Of the 511 disagreements, 176 (34.44%) were CF positive, ELA negative and 335 (65.56%) were CF negative, ELA positive.

Table 6. Comparison of ELA and complement fixation test results

	CF positive	CF negative
ELA positive	954 (18.63%)	335 (6.54%)
ELA negative	176 (3.44%)	3657 (71.39%)

Distribution of ELA readings within SAT reactions was calculated from 5,430 tests (Figure 8), of which 2,506 (46.15%) were SAT negative, 888 (16.35%) had a 1:25 titer, 700 (12.89%) had a 1:50 titer, 347 (6.39%) had a 1:100 titer and 989 (18.22%) had a 1:200 SAT titer. The average ELA reading for a SAT negative reaction was 1.9 ± 2.02, 2.99 ± 3.09 for a 1:25 titer, 5.31 ± 4.95 for a 1:50 titer, 10.07 ± 5.61 for a 1:100 titer, and

Figure 8. The distribution of ELA readings within standard agglutination tube test reactions. Symbols: (·), mean ELA reading of serums with equivalent SAT reactions. Vertical lines through each point represent the standard deviation of the mean.

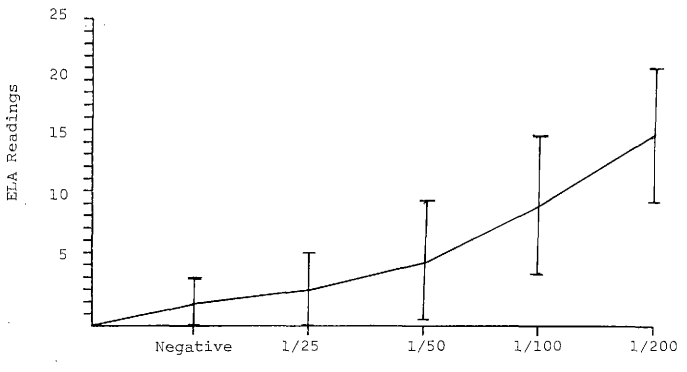
25
20
15
10
5
ELA Readings
Negative
1/25
1/50
1/100
1/200
Standard Agglutination Tube Test Reactions

Figure 8

15.7 ± 6.69 for a 1:200 titer.

A distribution of ELA readings within rivanol reactions was determined from the 5,675 tests (Figure 9), of which 4,196 (73.94%) were rivanol negative, 307 (5.41%) had a 1:25 titer, 267 (4.71%) had a 1:50 titer, 158 (2.78%) had a 1:100 titer, and 747 (13.16%) had a rivanol 1:200 titer. The average ELA reading for a rivanol negative reaction was 2.66 ± 3.08, 8.88 ± 5.15 for a 1:25 titer, 12.13 ± 4.56 for a 1:50 titer, 13.87 ± 5.96 for a 1:100 titer, and 18.10 ± 5.34 for a 1:200 titer.

The distribution of ELA readings within CF reactions was calculated from tests on 4,130 serums (Figure 10) of which 2,006 (48.57%) were CF negative, 771 (18.67%) had endpoint reactions at a 1:10 dilution of serum, 199 (4.82%) at a 1:20, 280 (6.78%) at a 1:40, and 874 (21.16%) at a 1:80. The average ELA reading for a CF negative reaction was 2.37 ± 2.58, 4.01 ± 3.6 for a 1:10 titer, 5.38 ± 3.59 for a 1:20 titer, 7.42 ± 4.04 for a 1:40 titer, and 14.53 ± 6.18 for a 1:80 CF titer.

Comparative Sensitivity of the ELA Test with Conventional Tests

Card, SAT, rivanol, CF and ELA tests were run on serial dilutions of a pooled reactor serum (Table 7). The last positive reaction of the card test was with the 1:6 dilution of serum. The SAT test was positive (1:100) with

Figure 9. The distribution of ELA readings within rivanol precipitation-plate agglutination test reactions. Symbols: (•), mean ELA reading of serums with equivalent rivanol reactions. Vertical lines through each point represent the standard deviation of the mean.

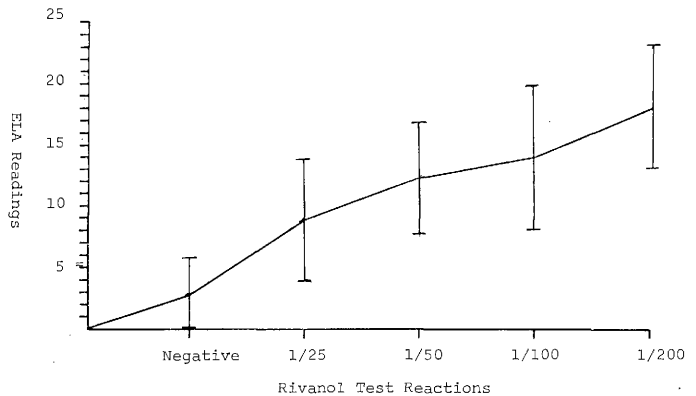
25
20
15
10
5
ELA Readings
Negative
1/25
1/50
1/100
1/200
Rivanol Test Reactions

Figure 9

Figure 10. The distribution of ELA readings within complement-fixation test reactions. Symbols: (•), mean ELA reading of serums with equivalent complement fixation reactions. Vertical lines through each point represent the standard deviation of the mean.

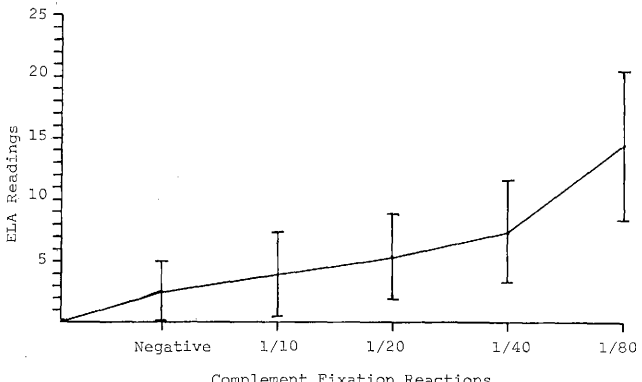
25
20
15
10
5
ELA Readings
Negative
1/10
1/20
1/40
1/80
Complement Fixation Reactions

Figure 10

Table 7. Sensitivity comparison of card, standard agglutination tube (SAT), rivanol precipitation-plate agglutination (rivanol), complement fixation (CF), and enzyme labeled antibody (ELA) tests.

Serum dilution	Card	SAT	Rivanol	CF	ELA
undilute	+	400	400	4 + 1:80	21
1:2	+	400	400	4 + 1:80	20
1:4	+	200	200	3 + 1:80	18
1:6	+	200	100	2 + 1:80	17
1:8	-	200	50	2 + 1:40	14
1:10	-	100	25	1 + 1:40	11
1:12	-	50	25	3 + 1:20	9
1:14	-	50	25	2 + 1:20	8
1:16	-	50	-	1 + 1:20	8
1:32	-	-	-	1 + 1:10	4
1:64	-	-	-	-	2
1:128	-	-	-	-	1
1:256	-	-	-	-	0
Fetal calf serum	-	-	-	-	0

serum diluted 1:10, rivanol positive (1:50) with the 1:8 dilution, and CF positive (1:40) with the 1:10 dilution. The ELA test was positive (4 or greater) at a 1:32 dilution of the pooled reactor serum.

Early Detection of Immunologic Response

Card and ELA tests were run on serum samples taken from 15 non-vaccinated and 15 vaccinated cattle within a five month period. B. abortus type 4 was isolated from tissues of these animals. In four instances, M42 (10-26-77), M320 (12-28-76), M17 (11-30-76), and Y448 (12-28-76), the ELA test gave positive readings one month before the card test. In no instance did the card test give a positive reaction before the ELA test.

DISCUSSION

The Immunoelectrophoretic pattern of precipitated rabbit serum (Figure 1) shows more than one immunoglobulin class to be present. Conjugates prepared from these fractions were utilized throughout this study and further purification of the IgG fraction, as prescribed by other protocols, was not justified.

The commercial anti-IgM and anti-IgG rabbit antiserums used for conjugation were heavy and light chain specific, however when evaluated for specificity by immunoelectrophoresis the antiserums appeared to be immunoglobulin class specific i.e. only one visible precipitation band. When evaluated by the direct ELA procedure, cross reactions of the anti-IgM conjugate with bovine IgG and the anti-IgG conjugate with bovine IgM was detected (Tables 1,2). This cross reactivity could result from homologous kappa and lambda light chains found on both IgG and IgM. The magnitude of the reaction detected by ELA compared to immunoelectrophoresis indicates a higher degree of sensitivity is inherent in the ELA test. The anti-IgG and the anti-gamma globulin conjugate was reactive against bovine IgG and IgM. The anti-IgG conjugate was almost as efficacious as the anti-IgM conjugate in detecting bovine IgM. This was probably the result of specific antibody concentration

of each conjugate. The concentration of specific antibody in rabbit anti-IgM serum was 1.9 mg/ml and 3.1 mg/ml for anti-IgG as determined by the commercial source. The conjugation procedure requires 1.66 mg/ml of total protein to be labeled with enzyme. This means that the ratio of labeled specific antibody to labeled non-specific antibody of the anti-IgG conjugate was greater than anti-IgM. It would seem that if conjugate concentration remains constant and immunoglobulin antigen concentration decreases, then the conjugate with the lower concentration of immunoglobulin would detect lower concentrations of serum antibody. With other in vitro serologic tests, "prozoning" phenomomen occurs when antigen or antibody is is excess, but here the opposite was observed in that the anti-IgG conjugate detected lesser concentrations of homologous antigen. Since the antigen was fixed to the bottom of the well it could have been that the conjugate with the greatest concentration of specific antibody would be more likely to come in contact with the surface during the short incubation period. Antigenic reactivity might also be a factor in that IgG could be more receptive to attachment by the anti-IgG conjugate during this brief incubation. If antigen detection by the direct ELA procedure was considered a reading of 4%T or greater, then the anti-IgG conjugate reaction detected at least 49

nanograms of IgG and the anti-IgM conjugate detected 390 nanograms of IgM. Further research into adjustments and refinements of conjugate dilutions, antigen fixatives, and test incubations might allow a more accurate estimation of detection levels.

The cross reaction of anti-immunoglobulin conjugates could be considered ideal for the indirect ELA procedure if group detection of all immunoglobulin classes is desired. If separate immunoglobulin class detection and quantitation is desired, then only class specific antiserums of high purity can be utilized for conjugate production.

The anti-IgG conjugate was more effective in distinguishing reactor from non-reactors than was the corresponding anti-IgM conjugate (Figures 7,8,9, and 10). The sensitivity of the anti-IgG conjugate (97%) was comparable to anti-IgM (99%). The specificity of the anti-IgG conjugate (99%) proved to be superior to anti-IgM (96%). As with other serologic procedures manipulation of variables, such as concentration of antigen, serum, or conjugate, to achieve increased sensitivity may lead to decreased specificity. The low specificity of the anti-IgM conjugate was probably the result of either low levels of IgM for detection or low concentrations of specific HRPO labeled anti-IgM. The high sensitivity of the anti-IgM conjugate seemed

to be the result of low specificity. Unlike the anti-IgM conjugate, the anti-IgG conjugate exhibited both high sensitivity and specificity. The most desirable characteristic of this conjugate was consistency in producing high readings with reactor and low readings with non-reactor serums. This increased the distance between the means of reactors (13.34) and non-reactors (0.25), which was 13.07. The distance between the mean readings of reactors (7.16) and non-reactors (0.17) tested with the anti-IgM conjugate was 6.89, considerably less than the anti-IgG results. The anti-IgM conjugate did not possess the specificity of the anti-IgG and should not be used for brucellosis serologic screening. However, if quality IgM heavy chain specific antisera can be obtained for conjugate production, then it would be a useful tool for research purposes.

Serums from one hundred reactor and 100 non-reactor cattle were tested with the anti-IgG and anti-IgM conjugates to determine the sensitivity and specificity of each conjugate. The percent sensitivity of the anti-IgG and anti-IgM conjugates was 97.09 and 99.01 and percent specificity 99.01 and 87.72, respectively. The anti-IgM conjugate did not possess the desirable detection proficiency necessary for brucellosis screening.

Comparisons of the ELA test with the card, SAT, rivanol, and CF (Tables 3,4,5,6,) showed that the

majority of test results agree, however, there were several disagreements within each comparison. The disagreement among tests probably resulted from the class of Brucella specific antibody, assayed for and detected by different serologic procedures utilized. The greatest agreement (92.08%) with ELA was the rivanol test followed by SAT (91.43%), CF (90.02%), and card (89.86%). No confident conclusions could be drawn from the disagreement in this study because there was no way to determine which test was indicative of infection. Accurate comparison and evaluation of serologic tests can only be achieved if the time of infection was known, as would be the case in experimentally infected animals.

The distribution of ELA readings within SAT, rivanol, and CF reactions (Figures 4,5, and 6) shows that as serum titers increase the ELA readings increase. There was, however, a wide deviation of readings for each test reaction which could have been due to the limitations of each procedure to quantitate total antibody. The ELA test measures total antibody concentration while the other tests measure only reaction at one of four dilutions of serum.

The basis for diagnosis of brucellosis with these standard serologic tests was detection of Brucella specific antibodies. Sensitivity was defined as the ability of each test to detect decreasing concentrations

of Brucella antibodies. The ELA test detected lower concentrations followed by CF, SAT, rivanol, and card, respectively. Hypothetically, if these thirteen dilutions were each a different serum sample of varying antibody concentration, and positive reactions were considered agglutination (+) with the card test, 1:100 with SAT, 1:50 with rivanol, 1:40 with CF, and 4 or greater with ELA, then the card test detected 4 of 13 (30.77%), SAT 6 of 13 (46.15%), rivanol 5 of 13 (38.46%), CF 6 of 13 (46.15%), and ELA 10 of 13 (76.92%).

The ELA and card test results from four bleedings of fifteen infected, vaccinated and fifteen non-vaccinated animals (Tables 8,9) further substantiates the sensitivity of ELA. Four animals, two non-vaccinated (M42, M320) and two vaccinated (M17, Y448) were ELA positive one bleeding before the card test. One of the non-vaccinated animals serum was ELA positive one month before the card test and the other animal's serum was positive by ELA two months before the corresponding card test. One of the vaccinated animal's serum (M17), was ELA positive the first month after vaccination. Poor antibody response of this animal to vaccination could account for the low positive reading of ELA and negative result of the card test. Animal Y448, the only calfhood vaccinate of the fifteen, was negative following the first and second

Table 8. Card and ELA test results of fifteen infected,[a] non-vaccinated cattle

Animal ID No.	10-26-76			11-30-76			12-28-76			Last Bleeding				Culture Date
	Card	ELA1[b]	ELA2[c]	Card	ELA1	ELA2	Card	ELA1	ELA2	Card	ELA1	ELA2	Date	
M40	-	1	-	-	0	-	-	0	-	+	28	+	3-1-77	3-15-77
M42	-	5	+	+	27	+	+	20	+	+	29	+	2-10-77	2-10-77
M82	-	1	-	-	1	-	-	2	-	+	25	+	3-1-77	3-15-77
M96	-	0	-	-	0	-	-	0	-	+	26	+	3-1-77	3-15-77
M116	-	0	-	-	0	-	-	0	-	+	31	+	3-1-77	3-14-77
M130	-	1	-	+	23	+	+	20	+	+	25	+	2-10-77	2-10-77
M142	-	1	-	-	0	-	-	0	-	+	29	+	3-1-77	3-14-77
M164	-	1	-	-	0	-	-	1	-	+	30	+	3-1-77	3-15-77
M180	-	1	-	-	1	-	-	1	-	+	20	+	3-1-77	3-26-77
M190	-	1	-	-	1	-	-	1	-	+	26	+	3-1-77	3-15-77
M202	-	0	-	-	0	-	+	10	+	+	25	+	2-10-77	2-10-77
M304	-	1	-	+	4	+	+	20	+	+	29	+	2-10-77	2-10-77
M308	-	2	-	-	1	-	+	17	+	+	28	+	2-10-77	2-10-77
M320	-	1	-	-	0	-	-	8	+	+	26	+	3-1-77	3-14-77
M478	-	2	-	-	0	-	-	3	-	+	29	+	3-1-77	3-14-77

[a] Brucella abortus type 4 isolated from tissues

[b] ELA reading

[c] ELA interpretation

Table 9. Card and ELA test results of fifteen infected,[a] vaccinated[b] cattle

Animal ID no.	10-26-76[c]			11-30-76			12-28-76			3-1-77			Culture date
	Card	ELA1[d]	ELA2[e]	Card	ELA1	ELA2	Card	ELA1	ELA2	Card	ELA1	ELA2	
M1	-	1	-	+	27	+	+	26	+	+	28	+	3-26-77
M17	-	0	-	-	4	+	+	24	+	+	24	+	3-26-77
M65	-	2	-	+	14	+	+	19	+	+	29	+	3-26-77
M133	-	1	-	-	2	-	-	2	-	+	29	+	3-26-77
M139	-	0	-	+	29	+	+	27	+	+	29	+	3-14-77
M151	+	7	+	+	29	+	+	23	+	+	23	+	3-26-77
M193	-	0	-	+	27	+	+	24	+	+	32	+	3-26-77
M311	-	3	-	+	23	+	+	16	+	+	27	+	3-26-77
M347	-	1	-	+	13	+	+	17	+	+	26	+	5-23-77
M527	-	0	-	+	9	+	+	8	+	+	29	+	4-30-77
Y423	-	1	-	+	16	+	+	18	+	+	24	+	5-23-77
Y448	-	1	-	-	0	-	-	8	+	+	17	+	5-12-77
Y459	-	1	-	+	14	+	+	9	+	+	14	+	5-11-77
Y469	-	1	-	+	29	+	+	12	+	+	20	+	4-30-77
Y507	-	2	-	+	19	+	+	15	+	+	29	+	3-14-77

[a] Brucella abortus type 4 isolated from tissues

[b] Calfhood (Y-even no.), adult (M-odd no.), calfhood and adult vaccinated (Y-odd no.)

[c] Date of adult vaccination

[d] ELA reading

[e] ELA interpretation

bleedings and detected positive by ELA after the third bleeding. Data from this investigation indicate that the paired serum control ELA test method may allow the detection of Brucella antibodies before the card test in serums from infected, non-vaccinated and vaccinated animals. The cases, where specific antibody concentrations were detected early by ELA, specific antibody was detected by both tests after the next bleeding. This was probably the result of the concentration differential evident following rising antibody levels. The value of early detection by ELA versus the card test as applies to simplicity and practicality must be considered in eradication of brucellosis.

During this study, several technical and mechanical problems arose. One of the technical problems associated with utilizing ELA for mass screening was the regulation of inherent variables. The ELA "sandwich" consists of an antigen-antibody-conjugate complex. Therefore, for maximum reaction and readout, the concentration of each entity must be optimal. Antigen and conjugate concentrations used for screening of test herds were adjusted so that maximum reaction was produced with reactor serum and minimum reaction with non-reactor serum. Other researchers (25) associated with ELA investigation have reported that higher concentrations of Brucella antigen markedly inhibit binding of Brucella

antibodies. By adjusting the protocol so that serums would be subject to different concentrations of antigen, low concentrations of serum antibody could be detected.

Mass screening of serums without automation produced technical problems associated with manipulation of the microsystem. Reagents must be prepared and dispensed with accuracy. Because of the short incubation periods and the number of serums tested per plate, reagents had to be added with speed to insure that all serums were tested uniformly. With increasing human involvement in ELA testing, decreasing accuracy may result, particularly if large numbers of animals need to be rapidly tested in a short period of time.

A few mechanical problems were encountered with the ELA test during this study. In some instances, bubbles were introduced into the sample addition line between samples. These small bubbles discretely lodged in the flow cuvette and produced higher readings by deflecting light transmittance. Initially, the presence of these bubbles was difficult to recognize and was probably responsible for the majority of the false positive reactions. This problem could be eliminated if flow cuvettes were designed so that the bubbles would rise instead of being trapped on the flat ceiling of the window. Washings between incubations had to be uniformly distributed and correctly pressured so that all unbound

reagents were eliminated and bound reagents were retained.

The paired serum control method was not added to the ELA protocol until late in the study. This method was adapted because non-specific color change of the 5-aminosalicylic acid was occurring with time in the wells. Sodium azide only inhibits the reaction in the wells; and with the time involved in transferring the contents to diluting tubes, a definite color change of 1-4% transmission was consistantly noted from the first to the last well. The 5-aminosalicylic acid was relatively stable after diluting. This non-specific color change caused higher readings in negative serums removed from wells at the last of a run. Light positives, removed at the beginning of a test run appeared negative. Using the paired serum control it was possible to subtract from each individual test its non-specific color change due to substrate solution over reaction, non-specific serum or conjugate attachment to the well, and non-specific catalysis of horseradish peroxidase by serum factors or hemoglobulin in abused or badly hemolyzed serums and eliminate this problem. This refinement produced consistantly low negatives which increased sensitivity by allowing detection of lower concentrations of Brucella antibodies.

Data obtained from this study indicate that the ELA technique is a highly sensitive and specific assay for detection of Brucella antibodies. The value of the ELA test for brucellosis screening must be considered. The impracticality of the ELA test outweighs the benefits for mass screening. ELA could be adapted to disease screening if proper automation and standardization were incorporated but this procedure has not as yet evolved to this point. Enzyme immunoassays possess high sensitivity and specificity and a wide applicability which should be directed towards brucellosis research.

The card test offers desirable practicality and sensitivity for brucellosis diagnosis and should continue to be utilized for routine screening in eradication programs.

SUMMARY

The data from this investigation indicate that peroxidase labeled rabbit anti-bovine IgG and rabbit anti-bovine IgM conjugates react with homologous antigen and cross react with bovine IgM and IgG respectively. It was suggested that cross reactions resulted from specific attachment of conjugate antibodies to kappa and lambda light chains present on both bovine IgG and IgM. This cross reaction was not detectable by immunoelectrophoresis. The anti-IgG conjugate proved to be more proficient in distinguishing reactor from non-reactor cattle in that it possessed 97.09% sensitivity and 99.01% specificity as opposed to 99.01% sensitivity and 87.72% specificity of the anti-IgM conjugate.

The overall agreement of the ELA results compared with buffered Brucella antigen (card), standard agglutination tube (SAT), rivanol precipitation-plate agglutination (rivanol), and complement fixation (CF) tests was 89.86%, 91.43%, 92.08%, and 90.02% respectively. These results were calculated from test comparisons of approximately 5,120 serum samples. Reasons for disagreement were not evaluated because time of infection was not known. It was concluded that accurate comparison and evaluation of ELA with other serologic

tests can only be achieved in experimentally infected animals.

A sensitivity comparison of card, SAT, rivanol, CF, and ELA tests showed the ELA test to be the most sensitive assay for detecting low concentrations of Brucella antibodies followed by CF, SAT, rivanol, and card.

The sensitivity of the ELA test was compared to that of the card test in detecting Brucella antibodies ellicited by 15 non-vaccinated and 15 vaccinated infected animals. Serums from four bleedings of the thirty animals over a five months period were utilized in this study. With serums from these animals, the ELA test became positive at least one bleeding before the card test.

Technical and mechanical problems associated with ELA mass screening operations were recognized.

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