CLASS AND SUBCLASS SPECIFIC
ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)
IN THE DIAGNOSIS OF BOVINE TUBERCULOSIS

by

SEIF EL-DIN AHMED MUMHED

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This thesis is dedicated to my parents, Asta Omer EL Voosh and Ahmed Mohamed Gamil, in appreciation for their continued support and encouragement.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature Review</td>
<td>3</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>18</td>
</tr>
<tr>
<td>Results</td>
<td>24</td>
</tr>
<tr>
<td>Discussion</td>
<td>31</td>
</tr>
<tr>
<td>Summary</td>
<td>38</td>
</tr>
<tr>
<td>Bibliography</td>
<td>40</td>
</tr>
<tr>
<td>Tables</td>
<td>53</td>
</tr>
<tr>
<td>Figures</td>
<td>57</td>
</tr>
</tbody>
</table>
SELECTED ABBREVIATIONS LIST

Ab/s  Antibody/s
Ag/s  Antigen/s
CFT  Complement Fixation Test
ELISA  Enzyme-Linked Immunosorbent Assay
ID  Immunodiffusion
IEP  Immunoelectrophoresis
Ig  Immunoglobulin
IHLT  Indirect Haemolysis Test
IHLT-1  IHLT (with only guinea pig complement)
IHLT-2  IHLT (with a mixture of equal parts of guinea pig and rabbit complement)
LPS  Lipopolysaccharide
MOD  Mean Optical Density
PBS  Phosphate Buffer Saline
RIA  Radioimmunoassay
RID  Radial Immunodiffusion Test
RIV  Rivarol Test
R-LPS  Rough-Lipopolysaccharide
SAT  Serum Tube Agglutination Test
S-LPS  Smooth-Lipopolysaccharide
SPT  Serum Plate Agglutination Test
INTRODUCTION
In the management of bovine brucellosis eradication programs, a herd history of recently introduced and rapidly spreading infection is often associated with epizootiological and immunological problems. The problems arise because infected cows, with titers below the reactor levels with standard diagnostic tests performed at frequent intervals, are usually left in the herd, and constitute a continuous reservoir. Later on they become reactors, but will already have served as a source of further transmission. Another problem is the residual antibody from vaccination which makes it difficult to diagnose and differentiate between infected and vaccinated animals.

The purposes of this study were to quantify and characterize the specific immunoglobulins produced in response to *Brucella abortus* in recently infected cows and vaccinated calves using different serological tests, with a view toward improving diagnostic efficiency.
Brucellosis is a contagious disease of cattle, sheep, goats, other wild and domesticated ruminants, swine, dogs and man. A variety of other wild animals can be infected including various species of rodents, hares, jack rabbits as well as several species of carnivores which are predators on the primary hosts. Soviet investigators have reported isolation of brucella organisms from ticks. Diagnosis in bovine brucellosis depends on isolation and identification of organisms or demonstration of specific antibody.

Vaccination vs Infection:

Buck (1956) reported the use of the attenuated smooth *Brucella abortus* strain 19 in vaccination of calves, sexually mature heifers and adult cows. The recommended age in calves for vaccination is 4 to 8 months of age. Jones and German (1975) reviewed the problem created by use of strain 19 vaccine in adult animals causing persistence of specific serum agglutinins titers which leads to confusion in the serological diagnosis of bovine brucellosis in adult vaccinated infected herds. Animals vaccinated with the rough killed strain 45/20 adjuvant vaccine produce a substantial amount of antibody directed against the protein and rough lipopolysaccharide (R-LPS), but not to the smooth lipopolysaccharide (S-LPS). The difficulty in differentiating between infected and vaccinated animals was described by numerous investigators since the development of these vaccines and their usage, and was reviewed by Rice et al. (1966), Hood and Corbel (1973) and Reh (1974).
addition, there are some animals which remain negative to all tests weeks after infection (Kepp (1938), Miller (1971) and Robertson (1971)). The incubation period varies in length and is sometimes shorter when infection has taken place late in pregnancy. Lapraik et al. (1975), Plaemmet et al. (1973) and Yilesmith et al. (1978) found that a small number of heifers had become serologically positive during or just after their first pregnancy although they harbored the organisms since their birth.

Antibody Isotypes in the Diagnosis of Bovine Brucellosis

The study of antibody isotypes is very important in the diagnosis of bovine brucellosis although there is disagreement on the function of antibody isotypes in different tests. Robjine et al. (1979) found that antibodies of both bovine immunoglobulin G subclasses (IgG1 and IgG2) fixed bovine complement while only IgG2 fixed guinea pig complement in vitro. Looking at the distribution of isotypes in sera from vaccinated and infected cows, high titers of IgG antibodies are produced during infection while vaccination titer residues are primarily IgM (Morgan (1969) and Elberg (1973)). In both vaccination and infection, the sequence of isotypes is the same, in that IgM is produced first followed by IgG.

It is well known that the serum tube agglutination test (SAT) is sensitive to the IgM class of immunoglobulin and since early vaccination titers are mainly IgM, this may lead to false positive results (non-infected animal giving a positive serological test) in
the SAT results (Kulkarni et al. (1973)). In a study on sera from vaccinated and infected animals, Levieux (1974) found that the differentiation of antibodies resulting from infection and those from vaccination was not possible by comparative studies of the IgM and IgG activities. He also found that IgG2 was active in normal agglutination but not in agglutination at pH 3.6 (Card and Rose Bengal Plate tests) or in the complement fixation test, while IgG1 was inactive in normal agglutination, but efficient in agglutination at pH 3.6 and it fixes complement. IgM was efficient in all these tests. According to Allan et al. (1976), the Rose Bengal Plate test (RBPT) and SAT are alike in that in both tests the antigen binds more to IgM than to the subclasses of IgG. Contrary to Allan et al. (1976), Connel (1972) stated that the RBPT detects only IgG and does not react with IgG1 or IgM. As in the SAT, positives found among sera when tested by RBPT are mainly due to prior vaccination titers, which means that RBPT can detect IgG as well as IgG1.

IgM at low pH (3.6 as in the card and RBPT) binds specifically, while heating of the serum removes some of the non-specific IgM from the reaction (Sutherland (1980)). Diaz and Levieux (1972) found that increased concentration of NaCl makes IgG1 more active in agglutination and precipitation in gel (Radial Immunodiffusion).

Sera from chronically infected cows have a very high content of IgG1 which cannot be missed by the RBPT (Miller et al. (1973), Welckett and Robertson (1973)). Reh (1974) in a quantitative study using the single radial immunodiffusion technique, found that 23% of the total
IgG₂ and 37% of the total IgM was specific antibody for *Brucella* in vaccinated animals at the peak of their immune response. In another study by Allan *et al.* (1976), it was found that IgM reacted more efficiently than IgG₁ and IgG₂ in both the RPT and the SAT while the complement fixation test (CFT) was more sensitive to IgM than to IgG₁ and did not react to IgG₂. They concluded that IgM fixed complement two times more efficiently as IgG₁ on a weight basis and ten times more efficiently on a molar basis.

McNaught *et al.* (1977) in a study of immunoglobulin concentration and the CFT, reported that the addition of *Brucella* specific IgG₂ to IgG₁ in sera and phosphate buffer solutions induced prozones, and that high concentrations of IgG₂ totally stopped the reaction. They concluded that high concentrations of specific IgG₂ relative to specific IgG₁ may lead to false negative CFT, and that prozones may be avoided by using a higher concentration of the antigen in the CFT. Plackett and Alton (1975) have also shown that non-complement fixing IgG₂ can block complement fixation by IgG₁ and IgM. Morgan (1969) stated that the CFT detects both IgG and IgM, and that IgG₂ is more efficient than the IgM as a complement fixing antibody. The fact that the antibodies for the CFT are of the IgG and IgM classes was confirmed by the work of Morgan (1969), Anderson *et al.* (1964), Gennoss *et al.* (1965), Schimmel and Erler (1967) while Curtain (1971), Cho and Ingram (1972) found that IgG₁ in general is the main subclass responsible for the CFT among the IgG subclasses.
Serological Diagnosis of Bovine Brucellosis:

One major problem in the diagnosis of bovine brucellosis arises from the fact that there is a long incubation period which varies widely among individuals. A large number of serological tests have been developed for the diagnosis of bovine brucellosis, as an approach to overcome the problem posed by the incubation period by increasing sensitivity without sacrificing specificity.

Qualitative Tests:

Among the qualitative tests used in the diagnosis of bovine brucellosis are the RBPT, card and P10 tests. The RBPT and the card test are agglutination tests with stained bacterial cell antigen buffered at pH 3.6. The tests may be used either for definitive diagnosis or as rapid screening tests, requiring confirmation, usually with the complement fixation test (Davies 1971). The basis for this antigen was the demonstration by Rose (1955), Rose and Roepke (1957) that non-specific agglutinins are prevented from reacting at low pH, whereas only specific, strong antibodies will give a positive reaction. The specificity and sensitivity of these qualitative serological tests depend on the prevalence of infection, or vaccination, or both in the population in which the test is to be used. Jones et al. (1930) in a study of the correlation between serological tests' results and isolation of Brucella abortus from 1,057 adult vaccinated cows in infected herds, found the card test to have 100% sensitivity and only 41.3% specificity. Nicoletti et al.
(1967) evaluated the card test as a specific test, but the herds in
that study were located in an area of low prevalence in which
vaccination had been confined to calves. Prior et al. (1975) using
the brucellosis card test for screening cattle in Saskatchewan
regarded the card test of low sensitivity.

Diaz et al. (1979) used a soluble Brucella antigen containing
polysaccharides but with no smooth lipopolysaccharide (S-LPS) in the
RID test to identify cattle infected with B. abortus in recently
vaccinated herds. The same test was used by Jones et al. (1980)
with a polysaccharide antigen (poly K) prepared from
B. melitensis strain 8615 and the test was compared to other
serological tests using sera from vaccinated and infected cattle. In
the latter study the RID showed a specificity of 80% and a
sensitivity of 87.8% compared to 66% specificity and sensitivity of
97.6% for the CFT. They suggested the use of the RID test with
another screening test, such as the card test, as inexpensive field
tests in infected herds in which vaccination with strain 19 had been
practiced.

Quantitative Tests:

The serum tube agglutination test (SAT) is widely used
throughout the world in control and eradication programs of bovine
brucellosis. The test has a problem of the presence of non-specific
reactions (Alton et al., 1975), which are encountered as false
positives and false negatives. Hess (1953), Rose and Roepke (1957)
pointed out that 60% of non-specific agglutinins in SAT in brucellosis free Minnesota herds were due to unrelated bacterial antigens. Alton et al. (1976) related non-specificity to two factors. The first was immunologically non-specific and due to other bacterial antigens and the second was immunologically specific but diagnostically non-specific, and primarily due to prior vaccination. Vaccination, especially with strain 19 attenuated vaccine, but also with the killed rough strain 45/20 in adjuvant, gives specific false positive reactions. Examples of non-specific false positive reactions had been described as occurring after inoculation with Pasteurella bacterin (Berman, 1956), Vibrio (Horse et al., 1953), Salmonella (Horse, 1953) and Versinia (Nurvell and Lindberg, 1973).

The complement fixation test is also widely used in quantitative serological diagnosis of bovine brucellosis. There are great variations of CFT methods among laboratories, including variations in antigen concentration, the use of either 50% or 100% haemolytic units of complement, time and temperature of the primary fixation reactions. There are fewer problems with prozones using cold fixation while warm fixation increases the number of sera showing prozones. Sutherland (1980) stated that the CFT was probably the most accurate serological test in wide spread use in the diagnosis of bovine brucellosis and he pointed out the drawbacks of the CFT as being unable to differentiate between animals recently vaccinated with strain 19 and infected ones. The test is subject to
that antibody to common determinants on rough and smooth strains may be involved in the IHLT. Hoffmann et al. (1980) examined the conditions for performing the IHLT, and found that optimal results were obtained when using bovine erythrocytes coated with alkali-treated dimethyl sulfoxide extracted antigen at a concentration of 800 µg/ml. Heat treatment at 58°C caused a reduction in titers of all sera tested. Jones et al. (1980) modified the original IHLT procedure and used alkali-treated S-LPS, obtained by phenol water extraction, at a concentration of 200 µg/ml to sensitize the cow erythrocytes. They found more reactions with the IHLT than with the CFT with sera from cattle given reduced doses of strain 19 vaccine and with sera from cattle experimentally inoculated with virulent B. abortus.

Quantitative Specific Binding Assays:

Quantitative specific antibody binding assays investigated in the diagnosis of bovine brucellosis are Radioimmunoassay (RIA) and Enzyme-Linked Immunosorbent Assays (ELISA).

RIA was developed originally by Person and Yalow (1959) to measure plasma insulin concentrations. In general, a radioactively labeled antigen (radioligand) is employed either directly or indirectly for the quantitative measurement of the unlabeled antigen by a competitive binding reaction to a specific antibody or other receptor system.

The principles of ELISA involve the adsorption of antigen to a
solid phase (polystyrene tubes or wells in microtiter plates) and incubation with antisera containing specific antibody to the antigen. Excess unreacted antibody is removed by washing. An enzyme-labeled preparation of anti-immunoglobulin is then added. The enzyme remaining in the tubes after washing provides a measure of the amount of specific antibodies attached to the wells.

Chappel et al. (1976) used a RIA for antibodies against Brucella abortus and found that it measured the amount of specific antibody of the IgG1 and IgG2 subclasses but was insensitive to IgM. On the basis of the proposition that most residual post-vaccinal antibody is of the IgM class, the RIA should be a more suitable test than the CFT or the SAT for distinguishing infected animals from those which have been vaccinated. The RIA is not subject to prozoneing and other disadvantages of the CFT. Chappel et al. (1978) confirmed the fact that SAT is less sensitive than the CFT and found SAT false positives which were totally negative to the CFT and RIA. They concluded that was attributable to antibody of the IgM class and that RIA is at least as sensitive as CFT, because SAT gave 35% false negative reactions and 5% false positives; the CFT gave 12% false negatives and 5% false positives while RIA gave 1% or 6% apparent false negative reactions depending on the minimum diagnostic value used.

Levioux (1975) used a solid-phase RIA for determining the quantities of specific antibody at repeated intervals from heifers experimentally infected with B. abortus during gestation and comparing the results with the CFT. The kinetics of appearance of
Brucella antibodies were very significantly correlated for the two tests and RIA was found to be 10,000 times more sensitive than CFT.

Another direct binding assay is enzyme-linked immunosorbent assay (ELISA) which was described first by Ingvall and Porhem (1972). ELISA is a sensitive and simple method for the quantitative determination of antibodies. Carlsson et al. (1976) found that an ELISA for bovine anti Brucella antibody was 10 to 100 fold more sensitive than the SAT, while being able to reveal antigenic differences between B. abortus and V. abysinica which were undetectable by the SAT and CFT. Saunders et al. (1977) applied ELISA to detect a number of animal diseases and found the test to be more sensitive than other tests currently in use to diagnose bovine brucellosis. Butler et al. (1978) studied five techniques for the quantitative measurement of specific antibody, the standard ELISA, amplified ELISA, Farr assay, precipitation and a double-antibody precipitin assay, and found the Farr assay to be a good indicator of total antibody as measured by double-antibody precipitation while both ELISA assays correlated better with changes in antibody affinity. The amplified-ELISA was comparatively less correlated with antibody affinity than the standard ELISA and only the amplified-ELISA was capable of detecting the low affinity antibody detectable by double-antibody precipitation and by Farr assay. Butler et al. (1981) stated that the measurement of IgG antibodies to Brucella antigen, using the amplified-ELISA, appears to provide a basis of distinguishing between cattle infected with vaccine or field strains.
of *R. abortus* and those that are exposed to these strains but that are not infected. Hock *et al.* (1979) found ELISA negative sera from non-infected animals (non-vaccinated or calfhood vaccinated) to agree 90 to 99% with the Card, Rhvano (RIV), SAT and CF tests.

There are major differences among ELISA's done at different laboratories due to differences in the nature of the antigens used. In some laboratories whole *Brucella* cell antigens are used while others employ various *Brucella* extracts. The nature of the anti-immunoglobulins, their dilutions, and the specificity of the different conjugates also vary among described procedures. Rynd *et al.* (1979) found ELISA to be 97% negative on sera designated disease-negative by conventional methods. Ruppenner *et al.* (1980) using the USDA tube agglutination antigen as was used by Rynd *et al.* (1979), found a CFT able to detect antibodies to *R. abortus* in a dairy herd at an earlier stage than SAT and ELISA. Using a soluble *R. abortus* antigen Hock *et al.* (1980) studied bovine sera classified as positive or negative to SAT and CFT, and concluded that ELISA absorbence values >0.08 should be considered negative and values >0.14 should be considered positive while those with intermediate values they declared as doubtful. Stearnsorn (1980) used a single dilution of serum on ELISA, which was found to be inadequate according to reactivity curves for quantitative tests (Builock and Walls 1977). Lamb *et al.* (1979) used several dilutions of sera from infected and normal animals. A competition or steric blocking among IgM, IgG1 and IgG2 for binding sites on S-LPS was shown to occur.
among some of the sera from infected cattle. They also observed non-specific binding of IgG to Brucella S-LPS with sera from uninfected animals.

Thoen et al. (1979) developed an enzyme immunoassay for detecting Brucella antibodies in milk of infected cattle using heat killed cells of strain 19 and suggested the automation of the method and its use as a screening test. Boraker et al. (1980) used the indirect enzyme antibody immunosorbent assay for the detection of Brucella antibody in cows' milk and found that the results correlate with positive Brucella milk ring tests and positive cultures. They concluded from the assays that indirect enzyme antibody immunosorbent assay eliminated false positives found in Brucella milk ring test reactions. The assay detected antibody in some samples which were milk ring test negatives, and they claimed it distinguished between vaccinated and infected animals. Teder and Hoffmann (1981) found the anti-immunoglobulin bound was a linear function of immunoglobulin concentration in an experiment to quantitate the immunoglobulin that binds to those R. abortus antigens absorbed to bovine red blood cells in the MHT. They also found the binding of bovine antibodies of the different immunoglobulin classes and subclasses to R. abortus antigens to be non-linear, and they suggested that the non-linearity was due to the competition between antibodies for antigen sites. Bruins et al. (1978) found ELISA to be affected by prions due to the competition for antigen by unexamined immunoglobulins when studied IgG antibody to rough mutants of Salmonella minnesota.
Prozone effect on ELISA were also seen by Vos et al. (1979) when they used the same technique to quantitate IgM and IgG antibodies to LPS.
MATERIALS AND METHODS
Sero logical Tests

The Standard Serum Tube Agglutination Test (SAT) (USDA) was performed as described by Alton et al. (1975) with the 1:200 dilution being the highest tested. Results were recorded as positive, incomplete or negative.

The Card Test (Buffered Brucella Antigen Test) was performed as described by Alton et al. (1975). Weak reactions on the card test were considered positive.

The Rivanos Test (USDA) was done according to the supplemental test procedures for the diagnosis of brucellosis, NAID Diagnostic Reagents, Manual 55E. USDA, NAID, Diagnostic Services, Diagnostic Reagents, Ames, Iowa. The maximum serum dilution used was 1:200.

The Complement Fixation Test (CFT) was a microtitration adaptation of the cold fixation method described by Jones et al. (1963). The standard R. abortus tube agglutination antigen was diluted 1:500. 1 + fixation (75% hemolysis) at 1:40 dilution of test serum or higher was considered positive.

The Indirect Hemolysis Test (IHT) was performed according to Jones et al. (1980). Lysis of 75% or more of the cells at a serum dilution of 1:10 or higher was considered positive.

The Radial Immunodiffusion Test (RID) was performed as described by Diaz et al. (1979) using a concentration of 200 μg/ml of polysaccharide B (poly B). The results were recorded every hour for the first 8 hours and left over night and read through the next day's working hours. The readings were based on a positive or negative
visual evaluation. Positive and negative controls were usually added each time the test was done.

Enzyme-Linked Immunosorbent Assay (ELISA) was performed as described by Lamb et al. (1979). In brief, Br. abortus crude S-LPS solution at 1 mg/ml in 0.06 M carbonate buffer (pH 9.6) were dispensed into disposable polystyrene tubes (12 by 77 mm; Falcon Plastics) in 1 ml volumes. The tubes were then incubated at 37°C for 3 hours. Brucella R-LPS solution at 1 mg/ml in barbital-acetate buffer, pH 4.6, was incubated in the tubes at 75°C for 3 hours. Tubes containing the LPS solutions were stored at 4°C until use.

LPS was removed by suction, and the tubes were washed 3 times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 to reduce non-specific binding and was used also to prepare 1:50, 1:100 and 1:250 serum dilutions. 1 ml of each dilution was added to triplicate tubes for each class and subclass-specific conjugate. Assay controls including tubes with fetal calf serum at 1:40 dilution, tubes without added serum, and tubes without fixed LPS. The tubes were incubated at room temperature for 5 hours. The contents were removed, and three washings were performed as before, after which 1 ml of enzyme conjugate was added. Rabbit anti bovine IgM, IgG, IgG1 and IgG2 were purchased from Miles, Elkhart, Indiana. The conjugates were prepared with these antisera according to the method of Nakane and Kawaoi (1974), modified to eliminate the final gel filtration step for separation of bound and unbound enzyme, as suggested by Saunders et al. (1977).
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Enzyme-Linked Immunosorbent Assay (ELISA) was performed as described by Lamb et al. (1973). In brief, *B. abortus* crude S-LPS solution at 1 μg/ml in 0.06 M carbonate buffer (pH 9.6) were dispensed into disposable polystyrene tubes (12 by 77 mm; Falcon Plastics) in 1 ml volumes. The tubes were then incubated at 37°C for 3 hours. *Brucella* R-LPS solution at 1 μg/ml in barbital-acetate buffer, pH 8.6, was incubated in the tubes at 75°C for 3 hours.

Tubes containing the LPS solutions were stored at 4°C until use. LPS was removed by suction, and the tubes were washed 3 14 mm with phosphate-buffered saline (pH 7.2) containing 0.05% tween 20 to reduce nonspecific binding and was used also to prepare 1:50, 1:100 and 1:250 serum dilutions. 1 ml of each dilution was added to triplicate tubes for each class and subclass-specific conjugate. Assay controls including tubes with fetal calf serum at 1:40 dilution, tubes without added serum, and tubes without fixed LPS. The tubes were incubated at room temperature for 5 hours. The contents were removed, and three washings were performed as before, after which 1 ml of enzyme conjugate was added.

Rabbit anti bovine IgM, IgG, IgG1, and IgG2 were purchased from Miles, Elkhart, Indiana. The conjugates were prepared with these antisera according to the method of Nakane and Kamao (1974), modified to eliminate the final gel filtration step for separation of bound and unbound enzyme, as suggested by Saunders et al. (1977).

against whole bovine serum using veronal buffer of pH 8.6.

**Bovine Sera**

Two groups of sera were collected at various times for the diagnostic tests. The first group consisted of 2F4 serum samples collected periodically from calves in the brucellosis free University of Wisconsin herds beginning 2-4 weeks, until 32 weeks after their
visual evaluation. Positive and negative controls were usually added each time the test was done.

Enzyme-Linked Immunosorbent Assay (ELISA) was performed as described by Lamb et al. (1979). In brief, R. abortus crude LPS solution at 1 μg/ml in 0.06 M carbonate buffer (pH 9.6) were dispensed into disposable polystyrene tubes (12 by 77 mm; Falcon Plastics) in 1 ml volumes. The tubes were then incubated at 37°C for 3 hours. Brucella LPS solution at 1 μg/ml in barbital-acetate buffer, pH 4.6, was incubated in the tubes at 75°C for 3 hours.

Tubes containing the LPS solutions were stored at 4°C until use. LPS was removed by suction, and the tubes were washed 3 times with phosphate-buffered saline (pH 7.2) containing 0.05%Tween 20 to reduce non-specific binding and was used also to prepare 1:50, 1:100 and 1:250 serum dilutions. 1 ml of each dilution was added to triplicate tubes for each class and subclass-specific conjugate. Assay controls including tubes with fetal calf serum at 1:40 dilution, tubes without added serum, and tubes without fixed LPS.

The tubes were incubated at room temperature for 5 hours. The contents were removed, and three washings were performed as before, after which 1 ml of enzyme conjugate was added.

Rabbit anti bovine IgM, IgG, IgG1 and IgG2 were purchased from Miles, Elkhart, Indiana. The conjugates were prepared with these antisera according to the method of Nakane and Kawaoi (1974), modified to eliminate the final gel filtration step for separation of bound and unbound enzyme, as suggested by Saunders et al. (1977).
The anti-IgM reagent was used at a concentration of 1:600 while the anti-IgG conjugate was used at a concentration of 1:750. Two concentrations were used for the anti-IgG1 which were 1:600 and 1:400. IgG2 concentrations were 1:250 and 1:100. All the conjugate dilutions were made in phosphate-buffered saline (pH 7.2) - 0.05% tween 20. After incubation at room temperature overnight, the tubes were again washed, and 1 ml of enzyme substrate was added. Working enzyme substrate was made up fresh and consisted of 1 ml of 2% 0-phenylenediamine (Eastman Kodak Co.) in methanol, 99 ml of distilled water and 0.1 ml of 3% H2O2. The stock 0-phenylenediamine solution could be kept at room temperature in the dark for up to 1 week. After incubation in the dark at room temperature for 30 minutes, 1 ml of 0.05 N H2SO4 was added to each tube to stop the reaction. The OD of the contents of each tube was measured at 490 nm.

Immunoelectrophoresis (IEP)

The rabbit anti-bovine IgM, IgG, IgA, and IgG2 were run in IEP against whole bovine serum using veronal buffer of pH 6.6.

Bovine Sera

Two groups of sera were collected at various times for the diagnostic tests. The first group consisted of 224 serum samples collected periodically from calves in the brucellosis free University of Wisconsin herds beginning 2-4 weeks, until 32 weeks after their insemination. There were mastitis problems at one time to another. Few records of health certificates were kept and when those animals were bled for the first time, some of them were found with ear tags from two states, one of which was Wisconsin. This shows clearly the difference in origin of these animals.

The first reactor was found on a market cattle test performed in March 1981. The serum sample was traced back to the herd of origin, the whole herd was tested and 26 cows were found to be reactors using
the card and the milk ring test. The results were confirmed in all these cases using the complement fixation test. The authorities decided to slaughter those reactors and retest the whole herd every month to see if they could eliminate infection. In April and May 1981, the number of reactors detected were 62 and 55, respectively. The authorities decided after that to retest every 15 days, but in June, July and August the reactors detected were 66, 130 and 90 respectively. The total number of reactors disclosed between March and August 1981 was 437. These results were based on card, serum tube agglutination, complement fixation and serum plate agglutination tests. Sera were available from animals which became reactors after being negative on the card and other standard tests or having titers below the diagnostic level on those tests. These sequential serum samples were used in class and subclass specific ELISA to evaluate and characterize the immunoglobulins produced in such sera in response to *Brucella abortus*. The isolation and identification of *Brucella abortus* from milk or tissue samples from this herd was done at the State Animal Health Laboratory.
RESULTS
Antibody Response After Calfohood Vaccination:

Two hundred-twenty four serum samples were collected from calves at intervals ranging from two to thirty-two weeks after they were vaccinated at three months of age with strain 19 vaccine. Results of these tests are given in Table 1. The sera from calves collected 2-4 weeks post vaccination gave strong positive reactions on SAT, RIV, CF, MHL and card tests.

The card test was positive on 42 out of 46 serum samples taken 5-8 weeks from vaccination while 21 serum samples were positive out of 34 taken at 9-12 weeks after vaccination. Only 3 sera out of 15 were positive on the card test 17-20 weeks after vaccination and the 17 sera tested 32 weeks after vaccination were found to be totally negative.

On SAT, of 46 sera taken 5-8 weeks after vaccination, 21 had titers of 1 at 1:200 or higher, 10 at + or I 1:100 and 15 at + 1:50 or below. Of 34 sera taken 9-12 weeks after vaccination, 6 had titers of 1 at 1:200 or higher and another 6 sera at + or I 1:100, while 22 sera had titers of + 1:50 or below. SAT was + 1:50 or below on 15 sera taken 17-20 weeks after vaccination.

On the RIV test done on 46 sera at 5-8 weeks after vaccination, 27 had titers of I 1:50 or higher, 16 sera at + 1:25 and 3 sera at I 1:25 or below. On 34 sera taken 9-12 weeks after vaccination, 14 had titers of I 1:50 or higher, 9 sera were + 1:25 while 11 were I 1:25 or negative. Out of 15 sera taken 17-20 weeks after vaccination, only 2 had titers of + 1:25 while the rest were I 1:25
or negative and all 17 sera obtained 32 weeks after vaccination were 1:125 or negative.

The indirect haemolysis test IHLT was conducted on all sera using guinea pig complement (IHLT-1) and in duplicate with a mixture of equal parts of guinea pig and rabbit complement (IHLT-2). With all 224 serum samples tested, IHLT-2 gave positive reactions at greater dilutions than IHLT-1. Of 46 sera obtained 5–8 weeks after vaccination, only 4 had titers of 3+ 1:16 or higher using IHLT-1 while 20 sera had the same titers using IHLT-2. The same titer was detected in only 10 sera using IHLT-1, while IHLT-2 detected 28 out of 58 sera tested 13–16 weeks after vaccination. None of the sera taken 17–20 weeks after vaccination was positive by either IHLT procedure.

Compared to the SAT, RIV, IHLT and card tests, the CFT was the first to become negative. Out of 46 sera taken 5–8 weeks post vaccination, 35 had titers of 1+ 1:20 or higher on CFT while 11 sera were below that titer and only 12 sera had titers of 1+ 1:20 or higher out of 34 sera taken 9–12 weeks after vaccination. Only 9 sera had titers of 1+ 1:20 or higher out of 58 sera taken 13–16 weeks after vaccination. Titers of all sera taken after 17 weeks from vaccination were below 1+ 1:20.

The radial immunodiffusion test was positive with only 11 sera out of 224 samples taken from calfhood vaccinated animals during the period from 2 to 32 weeks after vaccination, with some of these 11 sera giving weak reactions only. The RID test was negative on 12
serum samples which were taken 8 weeks to 9 months after vaccination.

**Antibody Response in Recently Infected Animals:**

These animals were from the large herd with a history of rapidly moving infection. Ninety five serum samples were tested using card, SAT, CFT, IHLT and RID tests. All of these sera were taken from animals which were identified as official reactors by card, SAT or CFT. The same animals had been tested 2-4 weeks earlier, and at that time were negative on the card test and had titers below the reactor levels on the CFT and SAT. The sera taken when the animals were identified as reactors, were tested by the card, SAT, CFT, IHLT-1, IHLT-2 and RID and the results are summarized in Table 2. Thirteen of these 95 reactor sera were negative on the card test but identified as reactors by the other tests. All of those which were missed by the card test were properly identified by the CFT or SAT. The CFT was negative with 9 of the 95 reactor sera. All 9 of these sera had titers below 1:50 on the SAT but none of them was completely negative. On IHLT-1, 14 serum samples were negative and a total of 45 serum samples had titers below 3+ 1:16 out of the 95 sera tested. Using IHLT-2 only 4 sera were negative and a total of 29 sera had titers below 3+ 1:16. In all 95 sera tested, IHLT-2 was more sensitive than IHLT-1, permitting the identification of 10 more sera as positive than IHLT-1. On these 95 early reactors the RID test was able to identify only 17 as reactors.

The strategy of this investigation was to look for a more
sensitive indicator of infection in recently infected animals. The need was demonstrated by the fact that 33 animals identified as card
and SAT or CFT positives, had been card test negative and had titers
below the reactor level on CFT and SAT 2-4 weeks earlier. The
highest SAT titer detected on them was 1:100 and the highest CFT
titers was below 1+ 1:20.

Class and subclass specific ELISA was done on all 33 sera when
they were showing titers below the reactor level. The same tests
were done on antigen and serum controls. Sera from reactor animals
with high titers on conventional tests were also included (Fig. 1 and
2).

The specificity of the rabbit anti bovine IgM, IgG, IgG1
and IgG2 used to prepare conjugates was tested by immuno-
electrophoresis and immunodiffusion. Both rabbit anti-bovine IgM and
IgG showed characteristic lines by both techniques while
IgG1 and IgG2 showed characteristic lines only by IEP and no 1:100 was
observed on ID.

The selection of appropriate dilutions of sera and conjugates
was based on preliminary tests using several dilutions of sera and
conjugates.

Results with sera from typical infected animals are shown in
Figs. 1 and 2 and a comparison with the other groups tested shown in
Tables 2 and 3. The mean optical densities in the tables show that
there was substantial specific binding to both S and R-LPS for all
classes and subclasses tested with sera from the infected animals.
Competition between immunoglobulin classes for antigen binding sites on smooth antigen was observed with some sera. As seen in Fig. 2, sera with high IgG1 binding, using concentrated sample (1:50 dilution) showed low OD values for IgG, IgG1 and IgG2. When more diluted sera were tested (1:100 and 1:250), there was an increase in the OD with the IgG, IgG1 and IgG2 conjugates. This may be due to more efficient specific binding by IgG which blocks the binding of the other immunoglobulin classes at high concentrations.

ELISA done on all 33 sera which were below the reactor levels according to the standard tests, showed elevated specific binding of IgM, IgG, IgG1 and IgG2 for both smooth and rough LPS. The results of ELISA with S-LPS for typical animals in the group are shown in Figs. 3-7. The ranges and mean OD's with both antigens are shown in Tables 2 and 3 for comparison with the other groups tested.

In this ELISA the optical density is proportional to the amount of conjugate bound to the antigen-antibody complexes and proportional to the amount of anti-lipopolysaccharide of that specific isotype. The OD reading using anti IgG2 conjugate at the 1:250 dilution, was low and the use of a more concentrated conjugate (1:100 dilution) gave a higher reading. Figs. 3, 4 and 5 show ELISA reactions of sera with SAT titers of + 1:25, + 1:50 and 1:100, respectively. From the three figures it is obvious that there is a progressive rise in all immunoglobulin classes and subclasses bound, with the increase in SAT titers with those sera.

Figure 6 shows ELISA reactions on a serum sample which was taken
DISCUSSION
Plackett et al. (1976) showed that the WLT had a disadvantage of being unable to detect recent infection. In this study, WLT was found to be entirely negative with 14 sera, which were identified as reactors by other methods (Table 2) and 29 other sera out of 95 showed titers below the level of 3 × 1:16, supporting the conclusions of Plackett et al. (1976). Comparing the results of card, SAT and CFT in 95 sera from recently infected animals, it was found that both SAT and CFT missed only 9 sera (false negatives) while the card test missed 14. A large number of other studies showed that there were more false negative reactions in other tests than the CFT (Altom et al. (1976), Hunter and Allen (1972), Morgan and Richards (1974), Mylroie and Fraser (1976) and Renoux et al. (1968)).

The instability of the conventional tests to identify recently infected animals accurately and early dictated the strategy of this investigation to look for a more sensitive indicator of infection in recently infected herds. The need was demonstrated by the fact that 33 animals which were identified as card and SAT or CFT positives, had been card test negative and had titers below the reactor levels on CFT and SAT two to four weeks earlier.

The use of class and subclass specific ELISA on sera from infected animals, with titers below the reactor level and negative controls (Tables 3 and 4), gave a clear indication of a rise in all classes and subclasses of specific anti Brucella abortus antibodies in both the infected group of sera and those which showed a titer below the reactor level according to the conventional tests used.
ELISA is apparently the most sensitive test available now to identify these infected animals approximately one month earlier than the conventional tests. The greater sensitivity of ELISA was demonstrated also by the studies of Carlsson et al. (1976), who found ELISA to be 10 to 100 fold more sensitive than SAT, as well as Saunders et al. (1977) who found it more sensitive than any other test currently in use to diagnose bovine brucellosis.

Figures 3, 4 and 5 show the parallel progressive increase in Ig bound in the ELISA with SAT titers of the 3 sera tested. Similar results were observed by Heck et al. (1979), who reported 98 to 99% agreement between the SAT test and their ELISA applied to sera from infected non-vaccinated animals.

There are major differences on ELISA done at different laboratories due to differences in the nature of the antigen used and the isotype tested in those studies. The nature of the antigens, their dilutions and the specificity of the different conjugates also vary among described procedures.

Byrd et al. (1979) found ELISA to be less sensitive than SAT on sera from infected animals and also Ruppanner et al. (1980) using USDA tube agglutination antigen, found a CFT able to detect antibodies to Brucella abortus at earlier stage than SAT and ELISA. These two reports contradict the results obtained (using the same antigen with differences in the sera and the concentrations of antigen and conjugates) by other researchers (Heck et al. 1980, Stenshorn 1980 and Bullock and Yalls 1977) who found the test to
be more sensitive than any other test now in use to diagnose bovine brucellosis.

Competition or steric blocking among different isotypes tested was reported by Lamb et al. (1979) and was confirmed by this study (Fig. 2). Tedder and Haffmann (1981) found the binding of specific bovine antibodies of the different immunoglobulin classes and subclasses to Brucella abortus antigens, non-linear and they suggested that the non-linearity due to the competition between antibodies for antigen sites. The competition was also observed by Bruins et al. (1976) on a work done on mutants of Salmonella minnesota and also by Yas et al. (1979) when they used ELISA to quantitate IgM and IgG antibodies to LPS.

The use of both S-LPS and R-LPS in this study showed that the smooth-LPS binds more antibodies of all isotypes in these sera than R-LPS (Tables 3 and 4), however there was a substantial amount of specific binding of all isotypes to the R-LPS. This is best explained on the basis of shared antigenic determinants between the S-LPS and the R-LPS. An ELISA that measures the response to more than one class or subclass of immunoglobulin as in this study and that by Lamb et al. (1979) gives a more complete understanding of the ELISA assay for bovine immunoglobulin response to Brucella abortus than the use of a single dilution or class of reagent as was done in other experiments.

I believe ELISA is capable of providing the best mean available now for identifying recently infected non-vaccinated animals.
Further studies in this area should include ELISA using sera from calfhood and adult vaccinated exposed and infected animals to fully evaluate the sensitivity and specificity of the assay.

The author would like here to suggest the standardization of procedures and reagents used in the diagnosis of bovine brucellosis in different laboratories as a step towards a better comparison of results of different experiments.
SUMMARY
Epizootiological and immunological problems arise in herds with a history of recently introduced and rapidly spreading bovine brucellosis since there are infected cows with titers below the reactor levels using the standard diagnostic tests at frequent intervals. A battery of these tests and class and subclass specific enzyme-linked immunosorbent assays (ELISA) were done on sera from such a herd. Conjugates of rabbit anti bovine IgG, IgG1, IgG2 and IgM were used in the ELISA.

Class and subclass specific ELISA was found to permit identification of such sera as positive approximately one month earlier than the conventional tests.

Also sera collected two to thirty-two weeks after calfhood vaccination and sera from recently infected animals were studied in another experiment using SAT, RIV, RID, GFT, IHLT-1, IHLT-2 and card tests. The relative sensitivities and specificities of these tests were discussed.
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immunoglobulin classes: Application to bovine Brucella abortus
properties of bovine IgG1 and IgG2 interaction with complement,
macrophages, neutrophils and skin. Immunology 36:249-256.
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Evaluation of a two-channel automated system for the sero
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<table>
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<tr>
<th>Animal Groups</th>
<th>Antigens</th>
<th>IgM 1:600</th>
<th>IgG 1:150</th>
<th>Conjugates</th>
<th>IgG1 1:600</th>
<th>IgG2 1:150</th>
<th>IgG2 1:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected (N=10)</td>
<td>S</td>
<td>1.408 (1.382-1.459)</td>
<td>1.643 (1.589-1.684)</td>
<td>1.293 (1.245-1.378)</td>
<td>1.01 (1.205-1.45)</td>
<td>1.194 (1.125-2.25)</td>
<td>1.01 (1.205-2.29)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.356 (1.296-1.389)</td>
<td>1.503 (1.459-1.550)</td>
<td>1.196 (1.135-1.276)</td>
<td>1.252 (1.206-1.298)</td>
<td>1.444 (1.199-1.90)</td>
<td>1.162 (1.047-1.78)</td>
</tr>
<tr>
<td>Dotted Reactor Level (N=53)</td>
<td>S</td>
<td>1.405 (1.347-1.496)</td>
<td>1.413 (1.353-1.576)</td>
<td>1.221 (1.168-1.397)</td>
<td>1.01 (1.097-1.291)</td>
<td>1.100 (1.055-1.321)</td>
<td>1.091 (1.073-1.218)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>1.306 (1.240-1.360)</td>
<td>1.320 (1.134-1.390)</td>
<td>1.172 (1.095-1.270)</td>
<td>1.277 (1.180-1.275)</td>
<td>1.089 (1.062-1.07)</td>
<td>1.135 (1.081-1.218)</td>
</tr>
<tr>
<td>Negative (N=610)</td>
<td>S</td>
<td>1.129 (1.091-1.159)</td>
<td>1.058 (1.041-1.062)</td>
<td>0.941 (0.929-0.957)</td>
<td>0.894 (0.931-0.960)</td>
<td>0.936 (0.926-0.951)</td>
<td>0.934 (0.931-0.951)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>0.921 (0.883-1.029)</td>
<td>0.951 (0.938-0.959)</td>
<td>0.836 (0.827-0.850)</td>
<td>0.940 (0.929-0.958)</td>
<td>0.951 (0.923-0.943)</td>
<td>0.937 (0.927-0.944)</td>
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</table>

N = numbers tested, R = Rough-LPS, S = Smooth LPS
(a) Numbers are mean and range of OD at 490 nm
(b) N = 10
(c) N = 53
(d) N = 610


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<table>
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<tr>
<th>Weeks after Vaccination</th>
<th>Number of BOP's Tested</th>
<th>SAT 4 or 100</th>
<th>SAT 100</th>
<th>SAT &lt;50</th>
<th>RIV 1:25</th>
<th>RIV 1:50</th>
<th>CF 1:10</th>
<th>CF 1:100</th>
<th>INF 1:10</th>
<th>INF 1:100</th>
<th>Card</th>
<th>RID</th>
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<td>2-4 weeks</td>
<td>54</td>
<td>40</td>
<td>9</td>
<td>5</td>
<td>59</td>
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<td>44</td>
<td>8</td>
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<td>30</td>
<td>52</td>
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<td>5-6</td>
<td>46</td>
<td>21</td>
<td>10</td>
<td>15</td>
<td>27</td>
<td>16</td>
<td>5</td>
<td>24</td>
<td>11</td>
<td>11</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>9-12</td>
<td>34</td>
<td>6</td>
<td>6</td>
<td>22</td>
<td>14</td>
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<td>13-16</td>
<td>58</td>
<td>3</td>
<td>11</td>
<td>42</td>
<td>10</td>
<td>13</td>
<td>35</td>
<td>-</td>
<td>9</td>
<td>49</td>
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<tr>
<td>17-20</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>2</td>
<td>13</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>17</td>
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</tbody>
</table>

(a) = only guinea pig complement
(b) = a mixture of equal parts of guinea pig and rabbit complement
Table 2: - Results of serological tests with sera from 95 recently infected cattle.

<table>
<thead>
<tr>
<th>Cord</th>
<th>SAT Negative</th>
<th>CFT 2+1:40 Negative</th>
<th>HLT-(a) 2+1:16 Negative</th>
<th>HLT-(b) 2+1:16 Negative</th>
<th>RID</th>
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<tbody>
<tr>
<td>62</td>
<td>13</td>
<td>86</td>
<td>86</td>
<td>52</td>
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<td>25</td>
<td>4</td>
<td>66</td>
<td>25</td>
<td>4</td>
<td>17</td>
</tr>
</tbody>
</table>

(a) = only guinea pig complement
(b) = a mixture of equal parts of guinea pig and rabbit complement
<table>
<thead>
<tr>
<th>Animal</th>
<th>Groups</th>
<th>Antigens</th>
<th>IgG 1:600</th>
<th>IgG 1:750</th>
<th>Conjugates</th>
<th>IgG1 1:600</th>
<th>IgG1 1:400</th>
<th>IgG2 1:250</th>
<th>IgG2 1:100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>S</td>
<td>1.33 (1.282-1.403)</td>
<td>0.52 (0.432-0.614)</td>
<td>0.267 (0.145-0.273)</td>
<td>0.305 (0.269-0.307)</td>
<td>0.104 (0.111-0.191)</td>
<td>0.200 (0.149-0.251)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N=10)</td>
<td>R</td>
<td>1.22 (1.188-1.250)</td>
<td>0.171 (0.279-0.452)</td>
<td>0.170 (0.121-0.194)</td>
<td>0.234 (0.224-0.244)</td>
<td>0.114 (0.092-0.143)</td>
<td>0.148 (0.113-0.184)</td>
<td></td>
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<tr>
<td>Below</td>
<td>S</td>
<td>0.25 (0.192-0.365)</td>
<td>0.28 (0.217-0.378)</td>
<td>0.145 (0.080-0.182)</td>
<td>0.095 (0.165-0.548)</td>
<td>0.061 (0.063-1.222)</td>
<td>0.131 (0.088-1.120)</td>
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<tr>
<td>Reactor</td>
<td>Level</td>
<td>(N=53)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(N=10)</td>
<td>R</td>
<td>0.167 (1.112-1.237)</td>
<td>0.204 (1.134-2.291)</td>
<td>0.097 (0.061-1.281)</td>
<td>0.169 (1.111-2.226)</td>
<td>0.071 (0.060-1.120)</td>
<td>0.102 (0.065-1.158)</td>
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<tr>
<td>Negative</td>
<td>S</td>
<td>0.102 (0.088-1.350)</td>
<td>0.052 (0.038-0.599)</td>
<td>0.038 (0.028-0.048)</td>
<td>0.040 (0.029-0.053)</td>
<td>0.05 (0.021-0.049)</td>
<td>0.093 (0.024-0.037)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N=10)</td>
<td>R</td>
<td>0.089 (0.087-1.121)</td>
<td>0.047 (0.037-0.045)</td>
<td>0.031 (0.029-0.041)</td>
<td>0.037 (0.025-0.045)</td>
<td>0.029 (0.020-0.039)</td>
<td>0.035 (0.020-0.041)</td>
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</table>

N = numbers tested, R = Rough LPS, S = Smooth LPS
(a) Numbers are mean and (range) of OD at 490 nm
(b) k = 2
(c) n = 10
(d) n = 4
Figure (1): Titration of IgM, IgG, IgG₁ and IgG₂ to Brucella S-LPS as measured by ELISA for a serum from an infected animal. The same serum showed SAT + 1:200, card positive, RID negative and INLT 2 + 1:32. The horizontal line shows the level of negative controls. OD at 490 nm.
Figure 1.
Figure (2): Titration of IgM, IgG, IgG₁ and IgG₂ to *Brucella* S-LPS as measured by ELISA for a serum from an infected animal. The same serum showed card test positive, CFT 4+ 1:80, SAT + 1:200, IHLT 3+ 1:128 and RID negative.
Figure 2.

IgG (1:750)

IgM (1:300)

IgG1 (1:400)

IgG1 (1:600)

IgG2 (1:100)

IgG2 (1:250)
Figure (3): - Titration of IgM, IgG, IgG₁ and IgG₂ to Brucella S-LPS as measured by ELISA for a serum which showed titer below the reactor level (SAT + 1:25), card negative and CFT negative) with the conventional tests. The animal was identified as a reactor 3 weeks later when the serum taken gave SPT + 1:200, card positive and CFT 4+ 1:160.
Figure 3.

- IgG (1:750)
- IgM (1:600)
- IgG1 (1:400)
- IgG1 (1:500)
- IgG2 (1:100)
- IgG2 (1:250)

50 100 250 1:ser.dil.
Figure 4: Titration of IgM, IgG, IgG1 and IgG2 to *Brucella* S-LPS as measured by ELISA for a serum which showed titers below the reactor level (SAT + 1:50, card negative and IMLT negative) with the conventional tests. The animal was identified as a reactor 30 days later when the serum taken gave card positive, CFT + 1:20, MRI negative and SPT + 1:200.
Figure (5): Titration of IgM, IgG, IgG\textsubscript{1} and IgG\textsubscript{2} to *Brucella* S-LPS as measured by ELISA for a serum which showed titers below the reactor level (card negative, IMiL negative, SAT 1:100 and CFT 2+ 1:10) with the standard tests. The animal was identified as a reactor 15 days later when the serum taken gave SPT + 1:200 and CFT 4+ 1:20 although the card test was still negative.
Figure 5.

- IgG (1:750)
- IgM (1:600)
- IgG1 (1:400)
- IgG1 (1:600)
- IgG2 (1:100)
- IgG2 (1:250)
Figure (6) - Titration of IgM, IgG, IgG1 and IgG2 to Brucella S-LPS as measured by ELISA for a serum which showed titers below the reactor level (SAT 1:25, card negative and CFT negative) with the standard tests. The animal was bled 2 weeks later and the serum showed SPT + 1:50, card negative, IMT 2+ 1:16 and ELISA as shown in figure (7). The animal was identified as a reactor 27 days after the first bleeding when the serum taken showed card positive, SAT + 1:200 and CFT 4+ 1:20.
Figure (7): Titration of IgM, IgG, IgA, and IgG2 to Brucella S-LPS as measured by ELISA for serum which showed titers below the reactor level (SPT + 1:50, card negative and IHLT 2+ 1:16) with the conventional tests. The animal was bled 2 weeks earlier and the serum showed SAT 125, card negative, CFT negative and ELISA as shown in figure (6). The animal was identified as a reactor 27 days after the first bleeding when the serum taken showed card positive, SAT + 1:200 and CFT 4+ 1:20.
Figure 7.