

## **A review of serological tests used in the diagnosis of Brucellosis: Usefulness and limitations**

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### **Abstract**

*Brucellosis is of serious economic importance in livestock and in humans. There are batteries of serological tests developed and in use for the diagnosis of brucellosis in human and livestock. Brief history, merits and demerits of some of these test are enumerated in this review. The purpose of the review is to bring together in one place different brucella tests from monographs, text books, occasional and conference papers and scientific journals to serve as a quick reference point for students and researchers.*

**Keywords:** *Brucellosis serological test, diagnosis, review*

### **Introduction**

Brucellosis is of great importance as a major and widespread disease of animals resulting in severe economic loss. It also occupies a significant importance as a zoonotic disease. It is transmitted to humans either through ingestion of contaminated milk or by contact with infected livestock or animal by-products (Alton *et al.*, 1975; Morgan *et al.*, 1978). Hence attention of many scientists was directed to finding an easy-to-perform, simple, accurate, sensitive and specific method for the diagnosis of Brucellosis.

Three important reasons for eradicating Brucellosis are: (a) Brucellosis is a zoonosis and is therefore a public health hazard (b) It is a cause of economic loss to farmers (c) There are means of eradicating the disease.

*Brucella* spp. is strongly antigenic and elicits a good antibody response (Alton *et al.*, 1975;

Morgan *et al.*, 1978), hence it is efficiently diagnosed by serological tests (Alton *et al.*, 1975; Morgan *et al.*, 1978). Some infected animals and humans with Brucellosis remain chronic carriers of infection even though they may have high levels of antibody in their serum (Alton *et al.*, 1975; Morgan *et al.*, 1978). Thus serological tests are therefore ideal for identifying infected livestock, poultry and humans.

There are several varieties of serological tests in use for the diagnosis of Brucellosis. Advantages and disadvantages of some of them are enumerated in this review.

#### *Serological tests*

The first series of serological tests were conducted by Wright and Semple in 1897 after which different researchers carried out modifications on the Wright and Semple tests or performed new sets of tests entirely.

Tube	1	2	3	4	5
Phenol saline (ml)	0.5	0.5	0.5	0.5	0.5
Serum (ml)	0.2				
	Mix and transfer	0.5	0.5	0.5	0.5 discarded 0.5 ml
Serum dilution	1:10	1:20	1:40	1:80	1:160

*Rapid plate agglutination test (Huddleson, 1920; Huddleson and Abel, 1928).*

Rapid plate agglutination test (RPAT) was first performed in 1924 and described in 1932 by Huddleson in the USA. The test will take about 15 minutes to perform and read. It is widely used as a routine test. It is applied singly or in conjunction with the tube test. Suspicious and positive sera to RPAT are further tested by the slow tube test.

#### *Procedure*

Deliver drops of 0.08, 0.04, 0.02 and 0.01ml serum sample with a graduated pipette on a sheet of glass marked out in squares. Place 0.03ml of the serum on the last square to serve as a control. These drops will give final dilutions equivalent to 1:25, 1:50, 1:100 and 1:200 respectively. Put one drop (0.03ml) of rapid reagent from the standard dropper on each of these drops and 0.03 ml of normal saline to the control square. Mix the serum and antigen with a glass rod and stir the mixture in a gentle circular motion for 8 minutes and then take note of the degree of agglutination.

#### *Interpretation*

The result is considered to be positive if agglutination of not less than ++ appeared in the squares with the 0.02 and 0.01ml serum doses. It is considered doubtful if there was agglutination only at a dose of 0.04ml of serum.

The advantages of the test are: The test establishes the presence of agglutinating antibodies. It is simple, rapid and inexpensive.

It needs no water-bath and virtually no apparatus except a pipette and glass plate or black tile to perform the test. It can be used to test animals vaccinated with *Brucella abortus* 45/20 vaccines. It can demonstrate infection at an early stage. It gives an indication as to whether the actual test titre will be high or low. It is not subject to the presence of "incomplete antibodies", prozone phenomenon and serum haemolysis (Cox and Lutner, 1950; Hall and Manion, 1953). It can be used in the field, market, farm or laboratory for survey, control and/or eradication campaigns, as well as in sampling to establish the prevalence of the disease.

The disadvantages of the test include: It is more liable to non-specific agglutinations, reactions tend to accelerate in hot environments and in environments favouring dehydration resulting in false positive reactions. It can be doubtful or negative with chronic infections. A positive result does not always guarantee a positive tube test.

*Serum agglutination test (Wright and Smith, 1987; Zammit, 1985).*

The SAT is an important test to which every eligible animal is subjected. It has been the basis of eradication schemes in many countries. Zammit (1985) probably performed the first SAT on goats and servicemen on the Island of Malta.

Tube	1	2	3	4	5
Phenol saline (9ml)	1.0	0.75	0.5	0.25	0.0
Antigen 1/2 (ml)	0.0	0.25	0.5	0.75	1.0
Representing	100	75	50	25	0
% agglutination (Degree of clearance)	(4+)	(3+)	(2+)	(1+)	(-)

Test result	AC result	Action
4/20	4/4	Retest on freshly collected sample.
4/20	4/2	The 4/20 result is accepted and action taken on the result
1/200	2/40	Retest on fresh sample.

Agglutination is the clumping of cells by an agglutinating antibody. Multivalent antibody molecules link with multivalent antigen on the particle surface to form a three-dimensional aggregate. When sufficient antigen particles are linked together the aggregate is clearly visible as a floccular precipitate which sinks to the bottom of the tube.

*Procedure*

Add 0.5 ml of phenol saline to five test tubes. Flush out the tuberculin syringe in phenol saline so that the 'dead space' tubing only is filled with saline. Draw up to the 0.3ml mark with serum sample. Transfer to the first test tube. Mix gently without frothing. Transfer 0.5ml to the next test tube and carry on to the last test tube and discard 0.5ml as shown below. Mix content of antigen bottle and add 0.5ml antigen to each tube and mix well again. Incubate at 37°C for 20 ± 1 hours.

Standards, positive and negative controls should be set up along with the test serum samples to check technique and antigen.

*Setting up of standards*

Mix 2ml antigen + 2ml phenol saline (antigen ½). The antigen is diluted ½ in phenol saline to bring it to the same opacity as in the test where it is diluted ½ with the serum dilutions. Other details are as shown below. Because the antigen consists of a suspension of organisms it possesses an opacity which will be reduced if

any of the cells are agglutinated and form a sediment.

*Reading the tests*

The degree of agglutination is read by comparing with the standard against a black background with a source of light (fluorescent strip light) coming from above and behind the tubes in a constructed illuminated reading box.

*End-point reading*

The titre of the serum is the highest dilution giving a definite 1 + reading or more. Traces or less than 1 + readings should be ignored. If two successive serum dilutions give a 1 + reading, e.g. 4+ 4+ 1+ 1+, the test on this serum should be repeated on the following day. If the same pattern is again found, the end point will be taken as the lower dilution giving 1+, in this case, 1/40, (the third tube).

*Recording of results*

The results must be recorded to show the degree of agglutination (numerator) and the dilution (denominator). Thus 3/40 is 3+ agglutination at a dilution of 1/40. If for any reason, a serum sample is retested, only the final titre should be recorded. Sera showing less than 40 iu pass the test, those showing more than 50iu fail the test.

Tube	1	2	3	4	5
Saline (ml)	0	0.5	0.5	0.5	0.5
Mucus (ml)	0.5	0.5	0.5	0.5	0.5
		mix and transfer			Discard 0.5ml.
Antigen (ml)	0.5	0.5	0.5	0.5	0.5
Dilution	1/30	1/60	1/120	1/240	1/480

#### Note

- The advantages of the test are similar to what has been enumerated for rapid plate test.
- Poor techniques, incorrect dilution and faulty observations are sources of errors in Performing the test.
- SAT gives negative results on sera from some animals from which *Brucella abortus* can be isolated (Alton *et al.*, 1975), yet false positive reactions can be caused by non-specific agglutinins in serum (Hess, 1953).
- SAT gives false negative reactions (Alton *et al.*, 1975) and false positive reactions, particularly as residual titres after *Brucella abortus* Strain 19 vaccination.
- Haemolysed serum samples are not good for slow agglutination in tube because of the interference of phenol with free haemoglobin which may cause "false agglutinations".
- For testing sheep and goats sera, the use of a 5% NaCl with 0.5% phenol is recommended both for the dilution of the sera and the concentrated antigens to prevent prozone effects.
- The result of SAT is also affected by vaccination of animals and as such as the result of test should be based on the vaccination status of herd or flock.
- In male animals low titres can not rule out the possibility of brucellosis. So other supplementary tests and test of the seminal plasma should therefore be conducted to establish proper diagnosis

- A strict diagnostic criterion should be applied in respect of the bulls whose semen is intended for artificial insemination.
- Human semen plasma can also be examined for brucellosis using the standard tube agglutination test.

#### Semen plasma agglutination test

This is a valuable test in examining bulls or men for brucellosis; the test is used when the bulls give inconclusive reactions to the blood tests.

To 1ml of semen add one drop of a 1% solution of sodium azide and leave at room temperature for 30 minutes. Centrifuge at 1000rpm for 10 minutes. Subject the supernatant fluid to the standard tube agglutination test.

#### Interpretation

Any degree of agglutination is considered as indicative of infection.

#### Vaginal mucus agglutination Test (Kerr, 1955)

The test is used to detect antibodies produced locally in the reproductive tract; these are invariably indicative of infection but false positive reactions may be obtained, for instance, if *Brucella abortus* Strain 19 vaccine has been used recently, or where mucus is collected by means likely to cause irritation of the genital tract e.g. tampons or where the mucus is mixed with blood as is the case immediately after an abortion. False negative reactions occur if the animal is not infected in the genital tract. The test is indicated, in the examination of 'normal' calvings in infected

heads and in cases of abortion; however, in cases of abortion, cultural examination of vaginal mucus using swabs is prefer.

#### Procedure

Put one part of mucus in a Griffith (or Hudson) tube and add 4 parts of phenol saline (=1/5 dilution) and emulsify. Leave the mixture at room temperature for one hour. Boil 3% Davis agar with 0.5% sodium chloride until it is melted. Place the melted agar in a water bath at 56°C. Put 2ml of the diluted mucus in a test tube. Put in the 56°C water bath for 10 minutes to bring it up to temperature and then add 2ml of the melted agar to the 2ml of the diluted mucus. Mix well and pour into a "mucus" pot. Allow the agar to set. Add 2ml of phenol saline (dilution now 1/15). Incubate overnight at 37°C and then subject to a routine standard tube agglutination test as shown below. Incubate at 37°C overnight and read.

#### Interpretation

Any reaction in the first tube or more is evidence of infection; the result must be interpreted along with those of other tests.

#### Note

- The vaginal mucus for the test should preferably be collected by pipette method as the sensitive swab method can induce non-specific reactions.
- This test is used to detect antibodies produced locally in the genital tract; such antibodies are considered indicative of infection. However, false positives may occur in recently vaccinated animals, or when methods that irritate the genital mucosa of the animal is used (e.g. by collecting the mucus with swab or severe irrigation of the genital swab).
- False negatives may also occur in infected animals when infection is not present in the genital tract.

- The test is valuable as supplementary to detect infected animal after normal parturition or some days following abortion (though bacteriological culture of mucus in the latter case is advocated).
- The test is affected by post-estral blood and abortion. Catarrhal or purulent mucus is also not adequate for the test. Trichomoniasis and Campylobacteriosis can also be studied simultaneously using this test.

**Complement fixation test** (Bordet and Gengon, 1901; McFadyean and Stockman, (1909).

Haemolytic Complement Fixation Test (CFT) was initially used in Denmark in 1900. The interest in the test was reviewed in the sixties after it had been demonstrated in the USSR (Yuskovets, 1956) that it could be used to distinguish serologic reactions produced by natural infection from those resulting from vaccination with *Brucella abortus* Strain 19 (S. 19). Before then, Blagoyesch chrenskaya (1954) observed that 98% of the animals became negative to complement fixing six months after vaccination. Since then, it has been widely used in Russia and Europe for the diagnosis of human and livestock brucellosis.

Bovine brucellosis has been eradicated from Denmark, Finland, Norway, Britain, USA, Sweden, Switzerland, Cuba, New Zealand, Israel etc through the use of the CFT among other procedures (Corbel, 1997).

CFT can be performed based on: (a) 100% haemolysis, which can be performed in tube (Kolmer's method, based on cold fixation, modified by Spaulding-Robinson, 1951) or in plastic plates (method developed by Hill in the Netherlands and modified in England by MacKinnon, (1963). (b) 50% haemolysis which can be performed as macro-or microtechnique. It is more accurate and gives excellent results.

but requires more time and highly trained personnel. It is a method of choice for research.  
(c) Automatic analysis equipment - applicable to test a large number of animals in control and eradication of brucellosis programs.

### *Procedure*

#### Primary and Secondary Dilutions

Place test tubes in rack. Using the tuberculin syringe dilute 0.4 ml of test serum with 0.4 ml of complement fixation (CF) diluent in a 74.5mm x 11.5mm test tube. Rinse syringe three times in a beaker of CF diluent reserved for washing and once in clean CF diluent. Proceed to the next serum. Place rack of tubes in a 58°C water bath for 50 minutes to inactivate native complement and destroy anti-complementary factors.

Each test consists of a row of 5 wells containing different serum dilutions in an 80-well perspex agglutination trays (henceforth called 'well'). Pick up 0.56ml of (½) primary dilution in a tuberculin syringe and add 0.02, 0.04, 0.1, 0.2 and 0.2 to the 4<sup>th</sup>, 3<sup>rd</sup>, 2<sup>nd</sup>, 1<sup>st</sup> and 5<sup>th</sup> well holes respectively to make serum dilutions of 1/20, 1/10, 1/4, 1/2, 1/2 and serum anti-complementary control respectively. Calibrate two automatic pipettes to deliver 0.1 and 0.17ml. Add 0.1 ml CF diluent to the second well and 0.2 to the fifth. Add 0.17ml to well holes 3 and 4. Calibrate an automatic pipette to deliver 0.2ml and add this volume of antigen to the first four wells (but not to the serum anti-complementary control).

#### *Addition of complement*

Add 0.2 and 1.25 unit complement to every well with a calibrated automatic pipette. Incubate plates in a 37°C water bath for 30 minutes. Mix together equal volumes of 3% sheep red blood cells' and 5 unit H18 30 minutes before they are required and leave at room temperature. Add 0.4ml of the mixture to every well with a calibrated pipette within a

few minutes after the first incubation period incubate plates in a 37°C water bath for a further 30 minutes.

### *Reading*

Assess the fixation in every well by observing the proportion of cells remaining unlysed (view the plate from above with a fluorescent light source beneath it). Express the degree of fixation as 0, 1, 2, 3 or 4 (0%, 25%, 50%, 75%, 100% cells unlysed) and record this as the numerator of a fraction. The denominator is the reciprocal of the serum dilution (1/2, 1/4, 1/10, 1/20). Record the end-point and any anti-complementary reaction (ignore traces of fixation in interpretation).

### *Controls*

Each batch of tests has the following controls:

(i) Serum anti-complementary control (AC) - Test every serum at a dilution of 1/2 but without antigen. This checks the ability of the serum to fix complement by factors other than a brucella antigen/antibody complex, i.e. the anti-complementary reaction.

It should be noted that (a) if the AC control shows only a trace, this can be ignored, (b) if the result of the CFT is "pass" (depending on the vaccination status) and the serum is AC at the 1/2 dilution, the AC effect can be ignored e.g. result 1/4; AC = 4/2; OV = poor NV = retest, (c) for all other sera (those that do not "pass" with an AC effect in the control) the serum must be retested using duplicate 8 dilutions for each serum (1/3 to 1/100). The first set of dilutions is set up as for the usual test but in the duplicate (second) set, antigen is left out, diluent buffer being used instead the second set then gives the titre if the AC reaction provided the first set shows a result which is at least 3 dilutions greater than the second (AC control) the AC effect may be ignored, e.g.

- (ii) Negative control - test a known brucella-negative serum in an identical way to test sera.
- (iii) Positive control dilute a known brucella-positive serum 1/75 in CF diluent and store in 1ml volumes at -20°C. For use in the test, melt 1ml and inactivate it. Thereafter treat as a test serum to give secondary dilutions of 1/75, 1/375, 1/750, 1/75 (serum anti-complementary control). This serum should have an end-point of 2/150.
- (iv) Complement titration controls - Add 0.2, 0.15, 0.1 and 0.05ml of complement to each of two rows of 4 wells. To the first row add 0.4, 0.45, 0.5, 0.55 and 0.6ml of diluent. To the second row add 0.2 ml of antigen to the first 4 wells. Incubate and add the haemolytic system. Readings in each case should be : -ve, tr, tr, 3.
- (v) Haemolytic system control - To one well add 0.6ml of CF diluent and 0.4 ml of haemolytic system. The cells should not lyse in the absence of complement.

All the controls should be performed in duplicate except the test serum anti-complementary control.

After each reagent has been added to the tests the volume remaining unused should be measured and recorded. This volume compared with the exact volume required provides a further check on the delivery of the automatic pipettes.

The advantages of CFT include: (a) The correlation between infection and positive reaction is greater with the CFT than with other agglutination tests (Morgan *et al.*, 1978); (b) Heterospecific antibodies are less problematic; (c) It can differentiate between natural infection and vaccination residual antibodies; (d) It can detect chronic infection when most sero-agglutination reactions will be negative; (e) In

man, it is a method of choice for both acute and chronic infections. The method of election is cold fixation at 4°C; (f) It is accurate and highly sensitive with fewest false positives; (g) It does not depend on the availability of known and defined antigen and antibodies. All that is necessary for the test to function is that antigen-antibody reaction should take place; (h) The test is applicable to several other disease conditions.

The disadvantages of CFT are: (a) It is cumbersome, expensive and more inconvenient to use compared with other serological tests; (b) Its uniformity is affected by myriad of variables and some of its components are unstable. Therefore adequate permanent control is essential; (c) The techniques for setting up the test vary from one country to another and from one laboratory to another. The sensitivity of the test depends upon the systems used; (d) The criteria for the interpretation of results and for the classification of animals into positive and suspicious also vary greatly; (e) It is highly influenced by vaccination with adjuvant and haemolysed serum; (f) It is affected by bacterial and chemical contaminants; (g) The test is complex, tedious and inconvenient to use. The complement is unstable and requires special storage and handling facilities; (h) It cannot be used to detect IgA and IgE which do not fix complement; It is not suitable to detect hypersensitivity or cell mediated reactions; not suitable where the antigen is a toxin which can lyse the RBCs of the complement; (i) Some avian sera do not fix complement; (j) Antigen, complement, haemolysin and RBC must be carefully prepared, titrated and standardized before the test is performed - this is time consuming (Morgan *et al.*, 1978); (k) The test does not detect reagents with incomplete antibodies and sometimes negative in early infection stages.

### **Conglutination complement absorption test (cent)**

Conglutination complement absorption test was originally described by Bordet and Strengé (1909); Stoker and Marmion (1950); Gulrajani (1951) who employed this test in the sero-diagnosis of influenza and swine influenza viruses respectively. Thereafter Englehard and Carlie (1955), Millian and Englehard (1961), and Sharma and Lohra (1966) employed this test in sero-diagnosis of brucellosis. It is not a popular test for brucellosis.

#### *Procedure*

The serum under test is incubated with brucella antigen, and a known amount of congrutinating complement (serum from horse or cat or pig; their minimum complementary doses are 1:80, 1:80 and 1:320 respectively). After 30 minutes incubation at room temperature (fixation period), and indicator system, consisting of a suspension of sheep red blood cells and heated bovine serum (containing congrutinin and an anti-sheep red blood cells antibody), is added to test for free complement. After incubation in a water bath at 37°C for 30 minutes, the tests are centrifuged at low speed and read. A known positive and a known negative serum should be used as controls.

#### *Interpretation*

If there is free complement present, that is no fixation and hence no antibody in the serum, the red blood cells will form large irregular clumps which sediment rapidly on resuspension indicating a negative result. In the absence of free complement, that is fixation and hence an antibody, the red blood cells will form an homogenous suspension on resuspension, indicating a positive result.

Advantages of the test are (a) congrutinin is not destroyed by heating the serum at 56°C for 30 minutes. (b) it gives good result comparable to CTF.

Disadvantages of the test are (a) the preservation of congrutinin is not easy and has been one of the drawbacks of the test, (b) partial congrutination is difficult to measure (c) the congrutinin is difficult to standardise (d) it is cumbersome and complex to perform.

### **Supplementary tests (2-mE, Rivanol, heat inactivation and acidified tests)**

"Supplementary test" or "Complementary test" have been developed to solve different diagnostic problems, such as; (a) Elimination or reduction of Heterospecific reactions; (b) Detection of "Incomplete" "Univalent antibodies"; (c) Correct diagnosis of the greatest possible number of cases, especially of chronic cases which remain hidden, or those of uncertain diagnosis; (d) Differentiation under specific conditions, of residual serologic titres resulting from the disease (e.g. *Vibria cholerae*, *Pasteurella* spp., *Francisella*, *Salmonella*, *Yersinia enterocolitica* IX or from vaccination (e.g. human cholera, S. 19 or Rev. 1 or S. 45/20 vaccine).

### **2-Mercaptoethanol test (Deutsh and Morton, 1957; Anderson *et al.*, 1964).**

2-Mercaptoethanol test (2-MET) is a selective and quantitative test that only detects the presence of IgG. The test is based on the premises that IgM antibodies with its pentamer structure are easily degraded into five similar antigenic subunits by the breaking of disulphide bonds due to the action of certain compounds containing the thiol radical, such as 2-mercaptoethanol, cysteine hydrochloride, dithiothreitol or Rivanol (Badnevic *et al* 1964). The test is therefore used as presumptive evidence for the presence of IgG antibodies.

The test is used as an adjunct to tube test and should be set up along side with Serum Agglutination Test. For 2-ME test phenol should not be used for the dilution of the antigen.

*Procedure*

A stock solution of 1 molar mercaptoethanol (ME) is made by adding 6.81 ml ME to 93.19 ml of distilled water. Store in a darkened bottle at 4°C to prevent decomposition. Make up a 0.2 molar solution of ME by diluting the stock solution 1 in 5 with distilled water before use. Set up tubes as for standard tube agglutination test (Morgan *et al.*, 1978). In the first tube place 0.1 ml of serum under test to 0.4 ml of 0.85% saline, 0.5 ml of 0.2 molar ME (dilution of the serum is thus 1:10), and in the remaining tubes add 0.5 ml of 0.85% saline. Incubate at 37°C for 1 hour.

Transfer 0.5 ml from the first tube into the second, mix and transfer 0.5 ml into the next tube and continue a serial dilution in this way, discard 0.5 ml from the last test tube. Add 0.5 ml *Brucella abortus* antigen to all tubes and incubate at 37°C for 20 ± 1 hours.

*Interpretation*

Record the degree of agglutination in each tube. A 4+ represents total agglutination 3+ equals 75% agglutination, 2+ is equivalent to 50% agglutination and a 1+ represents 25% agglutination. The titre of the serum is the highest dilution giving a definite reading of at least 1+, recorded as the degree of agglutination (numerator) and dilution (denominator). Thus, a titre of 3/80 is 3+ agglutination at a dilution of 1:80.

The test is usually interpreted on the basis of the differences between the titre of the serum agglutination test (SAT) and that of ME test. If the antibody level is lowered by the ME treatment, serological reaction is thought to be the result of S19 vaccination (mainly IgM produced). A titre of 1/80 before and 1/10 after MET treatment is evidence that the only immunoglobulin (Ig) present is IgM, this picture does not indicate infection with *Brucella abortus*. If the titre remains the unchanged or increased after treatment, this

indicates that the Ig present is IgG and indicative of infection.

Positive results to SAT and negative to ME test may mean heterospecific reactions, a recent infection in the incubation period or from a result of vaccination or of residual agglutinins following vaccination with S. 19. In such cases, test should be repeated after two to five months.

The test is of value in detecting chronic (human and animal) infections with possible low titres to the agglutination test, since the serum will contain either a high proportion or exclusively IgG antibodies.

Advantages of the test are: (a) It is based on simple modification of standard tube agglutination test, in which unspecific IgG antibodies are eliminated; (b) It can pick up chronic infections and advanced stage of infection; (c) It gives very few false positives.

The disadvantages of the test include: (a) It is a slow test to perform; (b) The ME is a chemical with an unpleasant smell; (c) The result of the test is affected by the serum sample quality.

**Heat inactivation test (HIT)**

Most serologic tests for infectious diseases are not completely accurate because of non-specific reactions. Non-specific reactions may be particularly responsible for suspect titers and, to a much lesser extent, for minimum reactors titers of the standard tube and plate agglutination tests (Hess and Roepke, 1951; Hess, 1953).

The possible use of heat for inactivating non-specific agglutinins had been recognised earlier Jones (1927) reported that antibody destruction progressed as temperature increased and that heating at 65°C for 20 minutes appreciably affected antibody activity. Hoerlein (1953) reported that serum of brucella-free swine, which reacted to the standard tube test, had

negative or much lower titres when incubated at 65°C for 16 hours. Morse *et al.*, (1955) found two non-specific Brucella agglutinins in bovine serum by using physicochemical methods; one agglutinin was heat-stable and the other was heat-labile at 65°C. Hess (1953) observed that the non-specific agglutinins of swine origin, associated with suspect *Brucella* titers, were inactivated when heated at 70°C for 10 minutes.

#### *Procedure*

Perform the dilutions of serum and antigen as was described for SAT. Also allow the antigen to reach room temperature before use. The serum-antigen mixtures were then immediately placed in a water bath at 65 ± 2°C for 15 minutes; time the incubation after an initial 3 minutes warming period. After incubation, remove the samples and immerse immediately in a cold water bath at 18°C for 3 minutes to terminate the incubation. Centrifuge the test samples at 2000 rpm for 5 minutes. Decant the supernatant fluid and replace with 2ml of sodium chloride solution and read the tests. Shake each tube gently and read the agglutination reaction with a titration illuminator.

#### *Interpretation*

Any agglutination in the 1:25 serum-antigen dilution is considered positive and specific. No visible agglutination should be considered negative or non-specific.

The film of agglutinated particles will not be dislodged after vigorous shaking in positive serum samples. In case of negative results; the unagglutinated bacterial antigen will produce a homogenous cloud when shaken. Grossly contaminated or haemolysed serum samples will produce a stringy, mucoid type of agglutination particle.

The advantages of heat inactivation test: (a) The test can be conducted at 65°C for bovine

sera (Amerault *et al.*, 1961); (b) at 56°C for swine sera (Hoerlein, 1935); (c) The test may be negative in the early stage of infection and in old long-standing chronic infections; (d) The test is designed to differentiate specific and non-specific agglutination reactions found in bovine/swine sera on the basis of the stability of sera at 65°C for 15 minutes; (e) The test reduces the number of non-specific reactions, but its efficacy to show infected cases is not greater than that of SAT. It is of value in detecting early stages of infection.

The main disadvantage of the test is the necessity for centrifuging the samples and resuspending the deposited cells. The test is also prone to show prozone phenomenon.

**The acid plate (mild acidified antigen) agglutination test** (Roepke *et al.*, 1949; Rose and Roepke, 1957).

Like the other supplementary tests it is also devised to eliminate or reduce heterospecific reactions. At pH 4.0 used for the serum-antigen mixtures, agglutinins and IgM are inhibited, whereas IgG retain their activity. The test is conducted by acidifying the antigen with acetic acid, lactic acid or tartaric acid and then mixing it with the serum which acts as a mild buffer system.

#### *Procedure*

The acidified antigen is prepared just before use by adding 0.18ml of lactic acid to 2ml of plate test antigen. One drop (0.03ml) of this antigen is added to 0.08, 0.04, 0.02 and 0.01ml of serum. Mix the serum and antigen with a glass rod and stir the mixture in a gentle circulation motion for 8 minutes and then take note of the degree of agglutination.

*Interpretation*

Any agglutination is taken as a positive reaction. The disadvantage of the test is that it is difficult to reproduce, thus not in use for routine purposes. The test is also cumbersome since it has to be performed at several pH values.

**Rose Bengal plate Test (RBPT) or card test or buffered antigen test** (Pietz, 1967; Nicoletti, 1967; Morgan *et al.*, 1969).

This is a quick qualitative test of macroscopic agglutination performed with only one dilution, and which mainly detects IgG, but not IgG<sup>2</sup> antibodies, though there are evidences that it could detect IgM as well (Levieux, 1974).

*Procedure for RBPT*

The test mainly consists of mixing equal volumes of antigen and serum and observing for agglutination after a stated period of time. The test is performed by placing one drop (0.03ml) of antigen on each square of enamel strip or test card or white tile. Place one drop (0.03ml) of the serum sample alongside (but not into) the antigen. Mix the antigen and serum thoroughly with tooth pick or sterile broom stick. Place the enamel strip or test on the rocking machine (use hand to rock the white tile) and mix for four minutes. Read the test by examining for agglutination in a good light. Reading is usually facilitated when the mixture is observed as it flows away from the operator. With some sera, agglutination takes place almost immediately after the serum and antigen have been mixed whereas, in other cases, agglutination is delayed until the end of the four minute period.

*Interpretation*

The results of the RBPT are interpreted as either: negative ( - ) or no agglutination; positive or any degree of agglutination. Positive reaction is also considered as either "weak" or "strong" according to the degree of agglutination.

A known positive and negative serum should be included in each day's tests. Wash the enamel strip and white tile well under running tap water, dry and rinse. The test cards should be discarded after use.

*Note*

\* RBPT may give false positive reactions (Alton *et al.*, 1975), but it is useful as a screening test because it is rapid and simple to perform.

\* RBPT give few false negative reactions but comparison with other serological tests suggests that it gives many false positives (Nicoletti, 1967; Morgan *et al.*, 1969). Residual antibodies after strain 19 vaccination are believed to cause these false positives.

*Cards test*

This utilises serum or plasma and a buffered antigen which reacts with IgG antibodies. An optional microbleeder containing phyto-hemagglutinins (lectins) or heparin is used to produce plasma or a Brewer Plasma Collection Card. All components of the test kit are disposable.

*Procedure*

Place approximately three to five drops of blood on to the Brewer plasma collection card. Collect the plasma produced by the agglutination of erythrocytes by a capillary pipette. 0.03 ml of plasma (or serum may also be used). Place 0.03 ml of plasma or serum or blood onto the "tear drop" area of the card and add two drops of antigen to it. Use the applicator stick provided in the kit to mix the plasma (serum or blood) add antigen and rock the card (on a rocking machine) or by hand, forward and backward continuously for four minutes.

The card test has been modified by Luchsinger (1972) by performing the test on a dense polystyrene plates divided into wells. Serum

(0.05ml) and antigen (0.025 ml) are allowed to mix thoroughly in the wells by shaking for 4 minutes at room temperature. Antigen is also prepared in buffered diluent to increase its strength. The modified test will also give false positives where animals are vaccinated.

#### *Interpretation*

Record the result as either (no agglutination), that is a uniform homogeneous distribution of antigen) or positive (presence of large or small-grained agglutination).

The advantages of the test are (a) It is handy, rapid and can be performed on slides, cards or white ceramic tile; (b) It can be used as screening or definitive test for brucellosis eradication or control program; (c) It is positive before other main or supplementary tests are conducted; (d) It detects the same antigen as complement fixation test; (e) The Rose Bengal stain does not interfere with the serological reaction. Stains such as acridine or tri-phenyl-methane can also be used (the specificity of the test pH of the antigen is the most critical); (f) A positive reaction to the buffered antigen indicates that; (i) IgG, are present in the serum or plasma; (ii) The animal is a potential shedder and is therefore dangerous to the health of the herd/flock; (iii) The reactor should be isolated or eliminated; (iv) The isolation of *Brucella* sp. from the secretions or tissues of the animal in question is highly probable.

The disadvantages of the test include; (a) There is no classification for suspicious cases; (b) The reading indicates mainly the presence or absence of brucella IgG agglutinins; (c) Animal vaccinated in adulthood reacts with the test resulting in over condemnation or false positives animals.

**Antiglobulin test (Coombs or ~~incomplete~~ antibodies or blocking antibodies test (Moreschi, 1911; Coombs, 1945).**

Extensive use of the anti-globulin test using a polyvalent anti-globulin reagent, has been made by Cunningham (1967, 1968, 1971) while investigating serological responses to *Brucella abortus* strain 19 and 45/20 vaccines. In man the anti-globulin test using mono-specific anti-immunological sera was used by Kerr *et al.* (1966a,b) in the diagnosis of chronic cases of human brucellosis. These workers found non-agglutinating antibody activity associated with IgG and IgA.

The principle for the reaction was described by Moreschi (1911) and developed by Coombs (1945) to demonstrate the presence of incomplete antibodies for erythrocytes.

Incomplete antibodies are mainly of classes IgA and IgG. Although these antibodies become attached to homologous cells, they do not agglutinate them. The two stages of the test are: (a) When the specific incomplete antibodies attach themselves to the antigen originating the primary complex, but the reaction is not visible. Antibodies remain attached to the membrane even after centrifugation and re-suspension (i.e. washing of the cell) to remove non-absorbed serum proteins; (b) Then the specific antibodies attached to the cells behave as antigens in the reaction with the specific antiglobulin serum for the species; this results in an agglutination observable reaction. The serum not reacting during this second phase is considered to be free from incomplete antibodies.

#### *Materials*

Anti-species gamma-globulin serum is prepared commercially by injecting rabbits with antigenic serum proteins of the species in question e.g. bovine, equine, porcine, human etc. Serum from these rabbits will contain the

required gamma-globulin, but it has to be standardized before use using chequer board titration against a high antiglobulin titred serum (with an SAT of 2/10). Ten dilutions of this serum are used between 1/10 and 1/5120 in 0.5 phenol saline and 6 dilutions of anti-species gamma-globulin between 1/50 and 1/600 in saline. The SAT is therefore repeated 6 times (as indicated below). A known negative serum is also used as a control.

The highest dilution of anti-species gamma-globulin to give a clear or sudden end-point reading is chosen as the working strength. An example is illustrated below from which it will be seen that the anti-species globulin should be used at 1/400

#### *Procedure*

Perform a serum agglutination test and record and test result in the usual way. Take the tube containing the highest dilution of serum showing agglutination and three subsequent tubes. These 4 tubes are used in the test, and the others are discarded. Centrifuge the tubes at 2000rpm for 30 minutes. Invert the tubes and discard the supernatant fluid and resuspend the sediment cells in 0.6ml saline and recentrifuge, delay may allowed the cells to be resuspended. Resuspend the remaining pellet in 1ml 0.86% saline using a vortex mixer. Repeat the process of centrifugation once more and resuspend the pellet in 0.5ml of anti-species of gamma globulin (e.g. if a bovine serum is under test, use anti-bovine gamma-globulin). Incubate for 20 hours at 37°C. Read the tests as described for the mercaptoethanol test.

Include a high anti-globulin titre bovine serum in each batch of tests on bovine samples as a positive control. After centrifuging and washing, the only soluble components left in the tube should be antibodies which remain attached to the antigen (Gell and Coombe, 1968). Unabsorbed proteins will be washed away in this process, leaving the malformed

antibodies to combine with the anti-species globulin.

Hadju (1958) in Czechoslovakia modified the test by inactivation of serum at 70°C for 10 minutes to increase its specificity and eliminate heterospecific thermolabile antibodies. The first phase incubation is also reduced to two hours. The disadvantages of this procedure of modified anti-globulin test is that it has a high specificity thus resulting in over condemnation of animals for slaughter.

Advantages of the test are: (a) It is sensitive and specific for chronic and acute cases of brucellosis in humans and animals (b) Recommended particularly for epidemiological surveys, since it can detect chronic infections and incomplete antibodies in endemic areas and in suspected cases of progressive brucellosis with negative reaction in sero-agglutination; (c) It produces few false negative results; (d) It is advisable for use where brucellosis incidence is chronic and vaccinations have not yet been performed.

Disadvantages of the test include: (a) It has a serious problem of interpretation in vaccinated animals (Weidmann, 1991); (b) The antiglobulin should be specific for the species of the problem serum (human and animal) and can be prepared in rabbit or goats; (c) The test is laborious. The first stage is performed in tube and the second stage either in tube or plate; (d) Careful washing of the primary antigen plus antibody complex is essential to eliminate all free, non combined proteins, which could block the reaction.

#### **Indirect haemagglutination test (IHT)**

Indirect haemagglutination has been applied for the detection of brucellosis in man and domestic animals by various investigators Brodhage and Fey, 1954; Freeman, *et al.* 1955; Becht, 1958; Baker and Wilson, 1963;

Vershilora, 1966, Diaz *et al.*, 1967; Le Garrec, *et al.*, 1968; Corbel and Day, 1973).

#### *Procedure*

Collect defibrinated sheep blood and wash three times with PBS (pH 7.2) at 4°C (2000 rpm for 15 minutes). Transfer 0.6ml of the washed cells into MacCartney bottles and resuspend in 10ml PBS and then add 10ml tannic acid solution (5mg/50ml in distilled water). Mix well and incubate at 37°C for 15 minutes in a water bath. Centrifuge the contents at 2000 rpm for 5 minutes at 4°C to pack the cells. Remove the supernatant fluids and resuspend the cells in 20ml PBS and rewash at 2000rpm for 15 minutes at 4°C. Remove the supernatant fluids and resuspend the packed cells in 10ml PBS. Set aside some bottles to provide uncoated cells for controls and absorptions. To the other bottles add another 10ml of PBS that is a total of 20ml containing the antigen (20mg/100ml of *Brucella* culture) and mix gently then incubate the contents at 37°C for 30 minutes in a water bath with frequent shaking of the bottle. After incubation, centrifuge all the bottles at 2000rpm at 4°C for 15 minutes. Remove the supernatant fluids and resuspend the packed cells in 20ml of PBS containing 1 per cent of inactivated and absorbed normal rabbit (normal rabbit serum is activated at 56°C for 30 minutes). To 2ml of the rabbit serum add 0.5ml of the washed sheep red blood cells that is cell absorbed with a quarter of its volume. Dilute the absorbed serum in 100 by adding 1ml serum to 99ml PBS and store at 4°C until ready for use. Rewash cells three times with the serum saline before use. Finally resuspend the cells in 50ml serum saline after the last wash to give a 1 per cent suspension ready for use in the test.

#### *Titration*

Any agglutinins against the sheep cells were absorbed to prevent error due to non-specific agglutination. This is done by diluting sera 1 in

10 with a 1 percent suspension of tanned but uncoated cells incubated at room temperature for 1 hour with frequent shaking before the cells were removed by centrifugation. Make double dilutions of the absorbed sera in 0.1ml volumes in WHO plastic plates. Prepare one extra well with the 1:10 dilution to serve as a control to which uncoated cells will be added to ensure that all non-specific agglutinins had been removed. To each of the other wells add 0.1 ml volumes of coated cells. Shake the plate gently, cover with another plate and place on a flat level table overnight before reading the plate.

#### *Interpretation*

The end point of the settling patterns is taken as a neat round button at the bottom of the well. The control well is used for comparison. The test is highly sensitive and usually gives higher titres than the other diagnostic techniques. False positive has been observed in non-infected animals vaccinated with Strain 19 or with vaccine 45/20 with oil adjuvants.

#### **Milk ring test (mrt) (Fleischauer, 1937)**

The test was devised to detect the presence of *Brucella* agglutinins in milk. Investigators in Denmark and Sweden succeeded in developing the test to its present level of sensitivity. It has been used for routine checking of certified brucella-free herds and as well as for detecting infected herds in the eradication programme.

Bendtsen and Wood (1950) developed vital-stained antigens for milk ring test. Roepke and Associates (1949) adapted the test to cream samples and in this way increased its range of usefulness for field examinations.

The antigen for the milk ring test has a packed cell volume four per cent and a pH of 4.0. It is stained with haematoxylin, which can be substituted by other tetrazolium salts, although the results are less satisfactory. The antigen has

a greater sensitivity due to its low cell concentration and has no buffering capacity.

The test is performed by adding a drop of antigen to 1ml of milk in a narrow test tube and incubate at 37°C for one hour. In the reaction, the haematoxylin stained *Brucella* cells are agglutinated by the milk brucella antibodies. The antigen-antibody complex collects on the surface of the fat droplets and is carried with them to the top, thereby producing a variably deep purple bluish cream line or ring according to the intensity of the reaction.

#### Procedure for MRT test

Milk samples to be used should be stored at 4°C for at least 12 hours post-collection. Remove the milk samples and antigen from the refrigerator and keep on the bench (at room temperature) for one hour before setting up the tests. Gently mix the milk in the sample tube to ensure homogeneity of the cream. Place 1.0ml of the milk in a narrow test tube to give a column of milk about 2cm high. Add 1 drop (0.03ml) of milk ring test antigen by holding the dropping pipette vertically above the test tube. Gently mix the contents within one minute of adding the antigen. Place in a 37°C incubator for 1 hour. Record readings as indicated below

#### A Typical Chequer-Board Titration

Anti-species globulin	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
Dilutions										
1/50	++++	+++	++	++	+					
1/100	++++	++++	++	++	+	+				
1/200	++++	++++	++++	+++	++	+				
1/400	++++	++++	++++	++++	++++	++++	Clear	Bad	None	
1/500	++++	++++	++++	+++	++					
1/600	++++	++++	+							

#### Interpretation

An assessment is made by comparing the intensity of the blue colouration in the cream with that in the milk. Thus a blue milk column and a blue cream ring a +++ positive. Between

- (i) Note should be made of any loss of blue colour from both the cream and milk column. If there is insufficient cream, as shown by the presence of a thin, blue line at the top of the milk column, the sample should be retested by resubmitting the sample to test as described above but add 4 to 5 drops of cream from a known MRT - negative milk sample just before adding the antigen. ("Negative" cream could be obtained by pooling MRT negative milk samples and collecting the gravity cream).
- (ii) The test can also be conducted using 250ml sample of well mixed milk on:
  - a) Bulk tank milk on the farm.
  - b) Can milk at creamery/receiving plant.
  - c) Can milk on the farm.

In testing the can milk on the farm, stir the milk vigorously with the sampling dipper at several depths for at least 10 seconds. Then plunge the dipper deeply up and down at least six times and then empty the content. Finally take sample from well below the surface of the well-mixed milk and transfer into the sample tube.

these two extremes are grades ±, + and ++. Any reading from ± to +++ is considered a reaction to the MRT. Only a negative (-) reading is considered a negative reaction to the MRT. For the purposes of the eradication

scheme any degree of positive reaction must be investigated further.

### Whey agglutination test

Centrifuge the milk sample at 1000rpm for 10 minutes and then collect about 5-6ml of the defatted milk under the cream layer. Add 1-2 drops of rennet to the defatted milk and stand the mixture overnight at room temperature or incubate at 37°C for 1-2 hours. Centrifuge the clotted milk at 2000rpm for 10 minutes and collect the clear whey. Subject the clear whey to a tube agglutination test as for serum using 5 percent saline as diluent in order to reduce prozone effect.

### Interpretation

Any reaction at 4/10 (20iu) or over is usually considered as indicative of infection. The test is important when dealing with problem animals. The test is less affected than the MRT by non-specific factors but it is also much less sensitive. The test is indicated as an adjunct to other milk tests (MRT, MRT dilution, Culture) or where, for instance in cases of mastitis, it is not possible to do an MRT.

The test is affected by: (a) The fat content of the milk and by the size of the fat globules as both influence the size of the fat layer after standing (Gill, 1970; Morgan *et al.*, 1978); (b) Incorrect sampling leading to excessive cream or insufficient cream content will affect ability to read the test (Roepke, 1950, Morgan *et al.*, 1978); Excessive shaking will adversely affect the creaming ability and hence the test (Morgan *et al.*, 1978); (d) Excessive heating e.g. temperature above 45°C for over five minutes, will lead to a decrease in brucella antibody content (Morgan *et al.*, 1978); (e) Time and temperature of storage Milk samples stored at 4°C are suitable for testing without much loss in titre for two weeks. Storage at higher temperatures for longer periods causes a serious loss of antibody (Morgan *et al.*, 1978); (f) Increased volume of antigen decreases the

sensitivity of the test and therefore accurate measurement of antigen is essential (Morgan *et al.*, 1978).

False positive reactions may be given:

a) From fresh milk tested on the day of collection which usually disappears after refrigeration (samples stored at -20°C or below also give false reactions) (Morgan *et al.*, 1978); (b) The presence of colostrum; (c) Milk from animals in the drying off period; (d) Milk from animal with mastitis; (e) Not every brucella infected animal has udder infections and this can lead to a false negative reaction, i.e. negative MRT but possible blood serology (Moore, 1951; Morgan *et al.*, 1978); (f) Vaccinated animals with S.19 (Gilman, 1950; Morgan *et al.*, 1978).

The advantages of the test: (a) The test is useful in locating infected or potentially infected herds as well as in ensuring that brucella-free herds remain so (i.e. it is a periodic screening test) (Morgan *et al.*, 1978); (b) Test could be performed on whole milk and whey or cream (Hunter, 1976; Farrell and Robertson, 1968); (c) It can detect milk-brucellae antibodies.

Disadvantages of the test include: (a) The test is only possible on lactating animals; (b) It is very uncertain at an individual animal level; (c) It is only applicable on entire herd, and it will yield a rough picture of the status of infection; (d) The test cannot be done on bulls, dry cows, heifers and beef herds for obvious reasons.

### The anamnestic test

The Anamnestic test for bovine brucellosis was developed by Tacken (1964) as a means to detect latent brucella infection in heifers that were serologically negative using conventional test. The basis of the test is that infected cattle will mount a secondary or anamnestic antibody response to the injection of brucella antigen, whereas non-infected cattle will develop a primary response. An anamnestic response is detected six to twelve weeks after vaccination.

is the antigen usually used in this test (Cunningham and O'Connor, 1971).

This test will be appropriate among Nigerian nomadic pastoralists where the application of conventional eradication methods will be extremely difficult because cattle cannot be regularly mustered for testing and therefore an alternative method has to be developed. The test if perfected will result in cost savings associated with avoidance of mustering and testing, the shorter time required to eradicate brucellosis and probably a reduction in the number of cattle slaughtered as reactors. The test will also identify infected cattle in all ages likely to be examined and in both pregnant and non-pregnant animals.

#### *Procedure*

Collect blood from animals to be inoculated prior to vaccination with 45/20 vaccine and again at 6, 44 and 68 weeks after vaccination. Then test the sera collected with Rose Bengal test, complement fixation test and the indirect haemolysis test. The principle of the test is that administration of the vaccine will stimulate the appearance of complement fixing antibodies in cattle infected with *Brucella* organisms that are CFT negative at the time of vaccination.

#### *Interpretation*

A positive result in the Rose Bengal test is any degree of agglutination or detectable 'rimming', in the CFT a titre greater than or equal to 4 and in the indirect haemolysis test a reaction greater than 25 percent lysis at a titre of 8.

Advantages of the test are: (a) It will detect false negatives; (b) It is used for antibody analysis following *Brucella abortus* 45/20 vaccination.

The disadvantages of the test include; (a) It may have a high false positive rate; (b) It can be performed routinely but only in use when

routine tests fail.

#### **Lymphocyte-stimulation recovery test**

*Brucella abortus* soluble antigen induced a significant lymphocyte-Stimulation Recovery (LSR) in lymphocytes from cattle infected with a field strain than in lymphocytes from cattle infected with *Brucella abortus* Strain 19, and this difference may be due to the pathogenicity of these strains. *Br abortus* S. 19 is a less virulent organisms than is the field strain. Therefore, it is possible that the field strain multiplies and sensitises a larger population of lymphocytes than does S. 19.

Strain 19 also multiplies but less so, and becomes localized in the udder in a few vaccinated cows and does not invade as many organisms as do field strains. It has been reported that residual localization of S. 19 organisms occurred after immunization of cattle, involving mammary glands mainly and did not spread or involve the reproductive tract.

#### *Procedure*

The difference in LSR (or in vitro (SK) test between cattle infected with field strain and those vaccinated with Strain 19 vaccine could be utilised as an aid to the diagnosis of brucellosis in cattle if properly studied.

The lymphocytes for the test are obtained by allowing the red cells to sediment in heparinized blood and then collecting the leucocyte-rich plasma. The lymphocytes are separated from this by very gentle centrifugation through a density gradient prepared from Ficoll. The lymphocytes are then cultured in the presence of phytohaemagglutinin (PHA, an extract from the beans of *Phaseolus vulgaris*, the runner bean, that also has the ability to agglutinate red blood cells) or the antigen being investigated.

### *Interpretation*

Assessment of the results can be made by staining and counting the number of cells that have transformed after three days in culture. A better method is to add tritiated ( $^3\text{H}$ ) thymidine to the tissue culture fluid. This radioactive DNA precursor is incorporated into cells when they prepare to divide their nucleus. After short incubation, the cells are separated from the culture medium and the amount of radioactivity in their nuclei is measured. A doubling of activity over that found in unstimulated control cells is considered to constitute a positive response.

### **Enzyme immunoassay (EIAS)**

With revolutionary trends in science and technology EIA was 'born' for both antibody and antigen detections. Enzyme immunoassays (EIA<sub>s</sub>) can be defined as quantitative immunological procedures in which the antigen-antibody reaction is monitored by enzyme measurements. The use of enzymes as immunochemical labels in place of radionuclides for use in competitive binding assays has reported EIA<sub>s</sub> are among the most rapidly developing of non-isotope methods employed in the research and clinical laboratory for diagnosis. There are two types of EIA<sub>s</sub> - heterogeneous and homogeneous. In the heterogeneous EIA<sub>s</sub>, the antigen-antibody interaction modulates the activity of the enzyme.

The classification of EIA<sub>s</sub> is based on: (a) which reactant is determined i.e. antigen or antibody; (b) which reactant is labelled; (c) whether competitive or non-competitive methods are used; (d) which method of separation of bound and free reactants is used.

The advantages of EIA<sub>s</sub> technique include: (a) It is highly specific and sensitive than the conventional methods; (b) Sensitive assays can be developed by the amplification effect of enzymes; (c) Reagents are relatively cheap and

can have a long shelf- life; (d) Multiple simultaneous assays can be developed; (e) A wide variety of assay configurations can be developed; (f) Inexpensive equipment are widely available; (g) No radiation hazards occur during labelling or disposal of wastes; (h) Adaptable automation can be developed; (i) It is very sensitive and good for detecting latent carriers and incomplete antibodies; (j) It can be used as control test in brucella-free areas and as survey test in areas where no vaccination have been performed.

The disadvantages of EIAs technique include: (a) The Measurement of enzyme activity can be more complex in some cases; (b) The enzyme activity may be affected by plasma constituents; (c) It is not as sensitive as radio-immunissay, (d) It requires complex immunochemical reagents for large protein molecules; (e) In some case the test is severely affected by vaccination (if ELISA, polyclonal antibodies is in use) but vaccines titre are detectable (if ELISA, monoclonal antibodies are in use); (f) The test can not be performed routinely.

### *Procedure*

A typical radioimmunoassay is set up as follows. Add 50ul of the reagent stated below to polystyrene tubes in the following order:

- 1) Albumin diluent - this is made by adding bovine albumin, to PBS at a concentration of 5mg/ml.
- 2) Serum (diluted 1/4 in PBS) or IgG<sub>1</sub> standard (diluted in PBS) or PBS. (IgG<sub>1</sub>, IgG<sub>2</sub> and IgM were purified from bovine sera positive to the CFT and their purity and identity established. Label the immunoglobulins with iodine - 125 by the lactoperoxidase method. Specific activities obtained will be in the range 0.4-1.4 uci/ug. The percentage of each immunoglobulin preparation directed specifically against *Brucella abortus* is

estimated by adsorbing radioiodinated antibody onto excess antigen. This is called the Brucella-specific percentage. Protein concentration is estimated by the method of Lowry *et al.* (1951) using a commercial preparation of bovine immunoglobulin as a standard. Using the protein concentration of Brucella-specific antibody in each preparation is then calculated

- 3) ( $^{125}$ I) IgG<sub>1</sub> (diluted in albumin diluent).
- 4) Wash antigen standardized Brucella abortus agglutination concentrate wash by centrifugation and diluted in sodium phosphate buffered saline (0.116M phosphate, 0.139M chloride), pH 7.2 (diluted in PBS) or PBS. Replicate standard curves four to six times, using typically 200  $\mu$ g. of specific ( $^{125}$ I) IgG<sub>1</sub> and add

antigen at a dilution of 1/300. Standards covered the approximate range 100-10,000 ng of specific IgG<sub>1</sub>. A zero point with no unlabelled antibody must be included. A zero point control, without antigen could be included but is not essential. Assay sera duplicate without controls.

Cap all tubes and incubate at 37°C overnight. To each tube add 1ml of albumin diluent between 0 and 4°C and centrifuge the tubes immediately at 10,000g for 10 minutes at 4°C. Then place the tubes in an ice bath and remove the supernatants as quickly as possible. Count precipitated radioactivity in a well-type scintillation counter.

#### Interpretation

Read the serum levels of IgG from the standard curve. The value obtained from a serum is accepted if the duplicate counts agreed within 10% of their mean, where both duplicates lay

outside the working range of the standard curve.

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