DIAGNOSIS OF BRUCELLOSIS: PROBLEMS AND PROSPECTS

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Introduction

Brucellosis remains a Zoonosis of worldwide public health and economic importance. The etiologic agents are the five species of *Brucella*, *viz.*, *Brucella abortus. B. melitensis, B. suis, B. ovis* and *B. canis*. The disease is prevalent worldwide and endemic in many countries especially underdeveloped and developing countries. It is estimated that there are more than 5 lakhs new cases of Brucellosis in man every year. In the last meeting of WHO/FAO expert committee on Brucellosis, described it as an ever increasing cause of concern to public health. In India, Brucellosis alone has been estimated to cause an annual loss of Rs.350 million in terms of food animals and man-days of labor.

Diagnosis of any infectious disease is made on the basis of certain clinical symptoms unique to the disease and can be confirmed by isolation of organism or detection of organism or its components in the clinical samples or by detection of antibodies produced (Alton *et al.*, 1975 and Anon, 2000). The "gold standard" for the diagnosis of Brucellosis is culturing of the bacteria from tissues from an infected animal. However, false negative culture results can occur for many reasons, including absence of the bacterium in the cultured tissues or insufficient numbers of the bacterium present to reproduce on growth media. Furthermore, some tests take days (*e.g.* Standard Tube Test) to weeks (*e.g.* microbiological culture) to produce a result, making them impractical for field testing or testing where livestock health authorities must make immediate decisions. These inadequacies can be largely overcome by use of many tests together, but they still often cause difficulties in test interpretation, especially when an immediate answer is needed. Consequently there is a constant quest for better tests. This review discuss in details about the conventional and newer techniques available for the diagnosis of Brucellosis.

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Detection of Brucella cells and components

Bacteriological isolation: The isolation of Brucella from tissue or fluid samples such as milk has traditionally provided the most accurate method for the detection of Brucella infection.

The most commonly used media for the isolation of Brucella are Serum Dextrose Agar, Serum Tryptose Agar and Serum Trypticase Soya Agar. A variety of selective media such as Farrel medium, Ryan's medium, Brodie-sinton's medium, Ewalt's medium containing antibiotics such as Bacitracin, Lincomycin, Polymyxin, Amphotrecin B, Nalidixic acid, Nystatin, Penicillin, Vancomycin *etc* has been developed for the isolation of Brucella from potentially contaminated tissue source or milk.

The type of tissue used for the isolation is very important. The supra-mammary lymph nodes are the best source for isolating Brucella in cattle, sheep and goats. In swine it is mandibular lymph nodes. For humans and dogs, blood is often the only available specimen.

Histological techniques: Brucella can be detected histologically in tissue by a variety of specific and non specific staining techniques. Immuno-staining methods can also be used for the detection, but the specificity of this test is dependent on the specificity of the primary anti-Brucella antibody. Immuno-gold labeling methods are used to label specifically Brucella antigen in tissues prepared for transmission electron microscopic examination.

Gene probes: Gene probes in a variety of hybridization formats have been used for the detection, identification and characterization of specific DNA segments. The primary principle upon which gene probe tests are based is that the complementary sequences of single stranded nucleic acid molecules can re-form the double helix structure under appropriate conditions. The target of gene probe hybridization assays is a particular region of DNA of the unknown organism, which is tested for the presence of a specific genetic sequence usually associated with a defined genetic function. The gene probe is an appropriately labeled nucleotide sequence, which can range in size from few bases to several thousand. The labels can vary widely and may be a reporter molecule, which can be directly detected (such as a radioactive moiety), or a modifying group (such as Biotin), which can be indirectly detected following the hybridization. These probes are useful in situations where the number of bacteria is reasonably large.

PCR and Nucleic Acid Amplification Methods:

The Polymerase Chain Reaction (PCR) is an in vitro method for replication of a defined target DNA sequence so that its amount is increased exponentially. PCR consists of repetitive cycles of DNA denaturation through melting at elevated temperature to convert double stranded DNA to single stranded DNA, annealing of oligonucleotide primers to the target DNA, and extension of the DNA by nucleotide addition from the primers by the action of

DNA polymerase. Specific primer sequences are available for the identification of the different species of Brucella as well to differentiate the Vaccine strain from the virulent Brucella (Bricker and Halling, 1995).

Molecular Fingerprinting of Brucella:

The identification of bacterial isolate to the species level, or even to the genus level, is sufficient in many situations. Bacterial sub typing is the differentiation of isolate below the species level. These methods include traditional methods like serotyping, phage typing, Biotyping and antimicrobial resistance typing and newer methods such as molecular sub typing or DNA fingerprinting. The sub typing may be important if the genotype is clinically significant. It is also extremely helpful in determining whether an infection is new or recurring. The most important use of this sub typing is the laboratory support of epidemiological investigation and tracing the source of infection. The common molecular fingerprinting method include PCR targeting specific genes, arbitrarily primed PCR, repetitive sequence typing and a variety of Restriction Fragment Length Polymorphism methods like Pulse Field Gel Electrophoresis (PFGE).

Serological tests

Serological tests are developed for two basic applications: screening and confirmation. Screening tests are 'first line assays which are generally rapid and cost effective and demonstrate high diagnostic sensitivity, usually at the expense of diagnostic specificity. Only the reactors, true and false positive alike, from the screening process are subjected to confirmatory testing. These assays demonstrate a higher diagnostic specificity and a relatively lower diagnostic sensitivity. To increase the diagnostic specificity, two (or more) conventional confirmatory tests may be applied. Only those animals which are sero-positive in both confirmatory assays are than culled.

Collection of samples:

Good blood sampling techniques reduces haemolysis and bacterial contamination. A clean glass tube without additives may be used for collecting blood. Clotting is aided by keeping the sample at 37°C for one to two hours. A fresh needle may be used for each animal to prevent the spread of blood borne pathogens. Blood samples can then be kept at 4°C until testing for period of about two weeks. For longer periods of storage serum may be removed form the clot as soon as possible and frozen at – 20°C or below.

Milk samples are collected in plastic containers. The teat should be thoroughly cleansed. The first milk striped out and examined for signs of mastitis before the sample is taken. Ensure that the milk from all quarters is included. If the milk is collected from the bulk tank the contents should be well mixed before the sample is taken. Excessive shaking, the presence of colostrum, milk from mastitic cows, incidence where a high proportion of animals are drying off or the testing of milk following pasteurization may produce false positive or negative reaction to Milk Ring Test (MRT). The samples should be allowed to stand overnight at 4°C before testing and then must be tested as soon as possible

False positive reaction: Nonspecific reaction to Brucella abortus may be shown by bovine sera due to the presence of natural antibodies, mainly IgM. This activity can be neutralized by the use of chelating agent Ethylene Diamine Tetra Acetic acid (EDTA) in the Serum Agglutination Test (SAT). In herds having known natural antibody activity, reaction to SAT may be interpreted in the light of supporting evidence provided by herd history and Complement Fixation Test (CFT).

Infection of animals with a number of bacterial species unrelated to the genus Brucella has been shown to provide an antibody response causing false positive reaction in serological tests using Brucella antigens. These bacterial species include *Salmonella urbana, Escherichia coli, Pseudomonas maitophilia and Yersinea enterocolitica*. Where the prevalence of Brucellosis is at a low level it is better to isolate and retest reactor animals as the titer of animals infected with cross reacting organism will usually fall dramatically after one or two months whereas those from Brucella infected animals will have remained high.

Vaccination and infection: Strain 19 is the calf hood vaccine used world wide for the control of Brucella. Sub-Cutaneous inoculation of a standard dose of S19 vaccine results in the appearance of an SAT response within 5-7 days reaching a peak of around 2000 IU at 2-3 weeks before declining at a rate largely dependent on the age of the animals. 80 % of the animals vaccinated at 6-9 months of age had diagnostically insignificant level within 12 months. But if vaccinated at more than 9 months of age 50 % will be serologically positive 12 months later.

Buffered Brucella antigen tests: The group of tests *viz.* Rose Bengal Plate Test (RBPT), Buffered plate agglutination test and the card test is based on the principle that the ability of naturally occurring IgM antibody to bind is markedly reduced in a low pH. RBPT is simple spot agglutination test that is effective in the diagnosis of Brucellosis in many species when used as a screening test. In humans the RBPT is effective as a rapid presumptive test and in pigs they are at least as efficient as any other established serological procedure. The sensitivity of serological reaction is greatly influenced by the temperature at which the reaction takes place. The reagents should be at ambient temperature; sera and antigen if used straight from the refrigerator will react poorly.

Serum agglutination tests (SAT):

The antigen used in the SAT is a smooth whole cell (strain 99 or strain 1119-3) and the antibodies detected are those directed against the surface molecules. In the standard procedure the test is conducted by making doubling dilution of serum in phenol saline in round bottomed tubes and adding an equal volume of the standard antigen. After mixing, the tubes are incubated overnight at 37°C and the degree of agglutination is then read by comparing the opacity against standards representing various degrees of agglutination. The resulting titer is then converted to international Units.

The EDTA modified SAT:

The nonspecific agglutination caused by non immune binding of bovine IgM to cells of *Brucella abortus* may be nullified by the use of chelating agents like EDTA to the standard SAT.

Complement fixation tests (CFT): CFT is considered a better indicator of infection than the SAT and the reaction to CFT receded sooner than those to the SAT test after S19 calf hood vaccination. CFT can be conducted using whole cells as antigen or using a soluble antigen, extracted from Brucella cells. The test is widely used in eradication programme and has reduced the time required

for eradication and the number of over condemnation. It has been found that CFT can correctly identify 98% of all culturally positive animals. CFT is an extremely valuable test, the best indicator of infection and is ranked next to the isolation of the causal organism.

Anti-globulin tests (AGT): the AGT detects the presence of non agglutinating antibodies. These incomplete agglutinins are detected by the use of an antibody directed against the IgG fraction of the species of animals being tested. The test is carried out as the continuation of the conventional SAT. The AGT is most useful in sera with inconclusive reaction to routine SAT and /or those giving consistently anti complementary reactions to the CFT.

Disulfide Bond reduction tests: Mercaptoethanol (ME) and Dithiothreitol (DTT) are substances that break down the disulfide bond of the pentameric structure of IgM leading to a loss of agglutinating activity. The test can be of value to resolve animals following vaccination with S19. The ME tube agglutination test has been used to resolve suspect SAT reactions in the diagnosis of *B. canis* infection in dogs.

Heat Inactivation tests: Heating sera to 56ÚC or 65°C for 15 minutes before conducting the SAT or the incubation of serum-antigen mixture at 56°C reduces non specific titers. The effect on titer is more pronounced with sera from vaccinated than sera from non vaccinated cattle

Milk ring tests: MRT can be used effectively as a screening test in dairy herds and is the most practical method for locating infected diary herds in many countries. This test can be used 3-4 times a year on pooled milk samples to detect most infected herds.

Enzyme Immuno Assays (EIA)**:**

EIA techniques offer a multitude of well known advantages over conventional and even other primary binding techniques (Nielsen *et.al.*, 1995 and Wright *et.al.*, 1997). The indirect ELISA is the most commonly used EIA technique for the detection of antibody. A wide variety of antigen preparation have been used in the indirect EIA, ranging from whole cells to crude and semi purified smooth lipopolysaccharides (SLPS) preparations to polysaccharides and protein extract. The antigens of the SLPS are likely the most immuno-dominant in all of these preparations.

The Competitive EIA is generally based on a competition between two antibody populations for a limited number of antigenic determinants. Thus, the most common type of assay involve the immobilization of antigen on a matrix and co incubation of test material (serum) and an antibody of known specificity and quantity which is conjugated with an enzyme. Displacement of known antibody would reflect the presence of antibody in the test sample and would ultimately result in less substrate utilization.

Standardization of ELISA :

The success of EIA depends on many factors like the Type of plates used for coating the antigen, nature of water used for the preparation of buffer, physical factors like temperature and purity of chemicals used. If SLPS is used as the antigen, a minimum requirement for the purity of SLPS should be established and reference standards should be available for comparison. The dilution of the test sample will have a dramatic effect on the diagnostic sensitivity and specificity.

Diagnostic validation and application:

Once a serological assay has been developed and standardized, it must be thoroughly validated before it can be effectively put to diagnostic use. The two principle parameters which must be defined are diagnostic specificity and diagnostic sensitivity. Both are estimates of the probability that the test results will truly reflect the infection status of the animal. It is important to establish the diagnostic objective of the new assay as related to screening or confirmation while developing a new assay. Only then the appropriate samples are selected for meaningful estimates of diagnostic specificity and sensitivity.

Automation of ELISA: The EIA is a labor intensive test requiring steps that involve liquid handling, reagent dispensing, Plate washing, incubation, shaking, and reading. The method also involves the manipulation of data generated from the Optical Density (OD) values. With advent of microcomputers all these methods has been automated. Now there are dedicated instruments designed to handle only one step in the assay like liquid dispensing. There are also integrated (all-in-one) instruments designed to take one micro plate at a time through several steps of an EIA procedure. Robotic arm systems designed to perform many EIA operations or functions on many plates in any order are now available.

Fluorescence based Brucellosis tests

Fluorescent Polarization Assay (FPA):

Fluorescence polarization immunoassays were first developed in the 1970's, and are based on measuring the polarization of light caused by changes in molecular size as a result of antigen-antibody reactions. The technology has long been used in human clinical applications. FPA is based on the rotational differences between a small fluorochrome labeled antigen molecule in solution and the antigen molecule complexed with its antibody. The theory is based on the fact that molecules naturally spin in a liquid medium. The rate of molecular spin is a function of the molecule's size. Larger molecules spin at a slower rate than smaller molecules do. A fluorescent dye label can be used to mark or tag a specific molecule. A beam of polarized light can determine quantitatively the rate of spin of the fluorescent molecule, and can detect any change in the rate of spin, and therefore the molecule's size. If the fluorescent dye tagged molecule (the reagent) finds and combines with the target molecule (antibody),

the antigen-antibody complex that forms, creates a larger molecule that spins at a slower rate. Fluorescence polarization therefore detects the binding of a tagged molecule to a target molecule.

The test is simple to perform, gives rapid results, is highly reproducible across laboratories and instruments, and reduces the human error and variability that occurs when reading agglutination tests such as the card test, the standard plate test, and other similar such test. The entire assay is done in solution, in a single tube with no precipitation or washing steps. Therefore, it is readily adaptable to field implementation. The FP has been shown to be a highly accurate assay for detection of antibodies to *B. abortus* in bovine sera without detecting vaccination-induced antibodies (Neilson *et al.*, 1996). The FP rarely misclassifies uninfected cattle as positive. Therefore, this test has a high degree of specificity. For the diagnosis of Brucellosis, a small molecular weight fragment (average 22 kD) of the OPS of *B. abortus* smooth LPS is labeled with fluorescein isothiocyanate and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes using a Fluorescence Polarisation Analyzer.

Allergic tests:

The delayed type hyper sensitivity (DTH) demonstrated in Brucella infected animals have been exploited for diagnosis to a greater extends in sheep and goat than in cattle. A protein allergen free from LPS (Brucellin) prepared from *B. melitensis* has been used for this purpose. Positive reaction to Brucellin is not seen in non-vaccinated, Brucellosis free flock. In general the allergic test is considered not suitable for diagnosis in individual sheep and goats, but is very practical for screening to detect infected flock.

Of late the test based on Interferon (IFN) to diagnose Brucellosis has been developed and extensively tested in animals. Antigens like Brucellergen have been used for this purpose. However, reports indicated that this test also can not rule out the infection caused by *Yersinia enterocolitica*.

Differentiation of Brucella species:

The differentiation of the Brucella species can be made by comparing many parameters. These include the colonial morphology, difference in the metabolic patterns, pattern of growth on appropriate concentration of the dyes, basic Fuchsin and thionin, need for CO_2 and serum, susceptibility to Bacteriophages and biological behavior in the nature.

Diagnosis of human Brucellosis:

Human Brucellosis is associated with a number of complications affecting many systems such as bones and joints, respiratory system, nervous system, Gastro intestinal system and reproductive system. Mortality due to Brucellosis is usually associated with endocarditis. This complication accounts for 80 % all death caused by Brucellosis. The SAT and plate agglutination test are useful in detecting the infection. Reactions are considered positive if the titer are more than or equal to 1: 160 or when a 4 fold rise in titer is demonstrated in convalescent sera. The Mercapto ethanol modified SAT and Coombs anti-globulin test are also useful in detection of infection. Cross reaction with *Afipia clevelendensis, Vibrio cholerae, Francicella tulerensis,* Salmonella species and *Yersinia enterocolitica* can cause false positive reaction. ELISA test has also been found suitable for diagnosing Brucella infection and is especially useful for the detection of neuro Brucellosis by CSF evaluation. In addition to these tests, Chest X-rays, Spinal Radiography and Radio nucleotide scintillography are also helpful in finding the chronic Brucellosis.

Canine Brucellosis:

The serodiagnosis of *B. canis* infection present difficulties for several reasons. All serological tests are unreliable during the initial four weeks of infection. Three serological methods commonly used in many veterinary laboratories are Rapid slide agglutination test, the tube agglutination test and the modified 2 Mercapto ethanol tube agglutination test. The SAT is performed using the *B. ovis* as antigen.

Swine Brucellosis:

Bacteriological isolation is the confirmative method for the diagnosis of swine Brucellosis. They grow well on usual Brucella media without additional CO_2. For serological diagnosis antigen containing smooth whole cells of *B. abortus* are generally used for the diagnosis of *B. suis*. The RBPT appears to be at least as effective as any of the tests for Brucellosis. The agglutination test, whether done in tubes or on a plate, is the test most often used, but deficiencies in both sensitivity and specificity prevent this test from being reliable in the diagnosis of Brucellosis in individual pigs. Serological cross reaction with *Yersinia enterocolitica, Salmonella urbana* and *Francicella tulerensis* are noticed in pigs.

OIE recommendations

The buffered Brucella antigen tests, *i.e.* RBPT and buffered plate agglutination test, ELISA or FPA are suitable tests for screening herds and individual animals. The reactivity of samples that are positive in screening tests should be retested using established confirmatory tests such as the CFT or ELISA. The SAT is inferior to other tests in specificity and sensitivity, and is not recommended if other procedures are available. The indirect ELISA or MRT performed on bulk milk samples are effective for screening and monitoring dairy cattle for Brucellosis, but the MRT is less reliable in large herds and is less sensitive with *B. melitensis*. Another immunological test is the Brucellin skin test, which can be used as a screening or as a confirmatory herd test when positive serological reactors occur in the absence of obvious risk factors in unvaccinated herds. (OIE, 2000).

Conclusion

The diagnosis of Brucellosis presents difficulties in spite of the fact that there is no other disease for which such a large number of tests are available. The cross reaction to other bacteria, complexity in the differentiation of vaccinal antibodies and lack of harmonization of tests in the international level are few problems in Brucella diagnosis. There is also a need for the characterization of better antigens for the use in these diagnostic assays. The efficacy and suitability of tests based on cell mediated immune response should also be explored.

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