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COMPARATIVE EVALUATION OF STANDARD TUBE TEST WITH ENZYME LINKED IMMUNOSORBANT ASSAY AND INDIRECT FLUORESCENT ASSAY USING *BRUCELLA ABORTUS* S 99 SONICATED AND HEAT EXTRACTED ANTIGEN.

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Abstract

Background: *Brucella sps are Gram negative, facultative intracellular bacteria causing brucellosis in humans and animals. Since it is a multi-organ disease and symptoms are nonspecific the diagnosis by clinical finding is difficult and can be missed .Many serological tests have been used form the diagnosis of human brucellosis. This study compared the diagnostic value of in-house ELISA and IFA using lipopolysaccharide heat extracted antigen (LPS-HE) and Lipopolysaccharide sonicated extracted antigen (LPS- SE) with STT.*

Methods*: The present study was carried out to evaluate the two different antigenic preparations from smooth strains of Brucella abortus S99 for standardising ELISA and IFA as the alternative test for STT. Standard Tube test antigen and Standard anti brucella serum was obtained from IVRI, Izatnagar were used as controls for standardizing ELISA and IFA. 81 human sera (cases) were collected from people working in organized farms including veterinary staff, 32 animal sera from organized farms mentioned above, 100 human sera (controls) collected from KIMS, Bangalore Blood bank. 100 WIDAL positive samples from Department of Microbiology used to check for cross reactivity. All the serum samples (cases, controls and WIDAL positive samples) were tested with STT.*

Result: *Out of 81 human sera 8 (9.87%) was found to be positive with SAT showing a titre of ≥ 1:80. Whereas by ELISA, 10 (12.34%) & 9 (11.11%) cases showed positive in LPS –SE and LPS –HE coated plates respectively. The sensitivity and specificity for IFA was 77 and 87.5.*

Conclusion: *It was found that ELISA was considered to be better test over IFA and STT. It is cheap and reproducible and a specific assay for the diagnosis of Brucellosis.*

Key words: *Lipopolysaccharide, ELISA, IFA, STT, brucellosis*

Background

Brucellosis is a disease caused by gram negative bacteria of the genus Brucella, a member of the alpha Proteobacteria class. Various species of Brucella cause disease in livestock with worldwide economic impact. Currently,six species of Brucella are formally recognized [1] *Brucella melitensis* , infecting goats and sheep; *B abortus* infecting cattle and bison; *B suis*, infecting primarly swine, but also hares, rodents and reindeer; *B ovis*, infecting sheep; *B canis*, infecting dogs; and *B neotomae,* infecting wood rats. However, not all Brucella species are strictly host specific and some species will cross host barriers naturally. Within the past decade discovery of Brucella bacteria in marine mammals has led to the proposal of two additional species.[2]

B cetaceae infecting cetaceans, and *B pinnipediae*, infecting pinnipeds.[3] Species of Brucella are typically pathogenic in their natural hosts, resulting in reproductive failure and /or infertility in the infected animal.[4]

Definitive identification of Brucella is key to the success of surveillance and eradication efforts. For brucellosis, two diagnostic approaches are currently used: 1) the serological screening of potential hosts as an indirect indicator of infection (e.g. Agglutination tests), 2) the isolation and identification of the pathogen from potentially infected hosts. Since a serologically positive response can occur in convalescent hosts or from antigenically cross-reactive bacteria, characterization of cultured bacteria remains the "gold standard". For Brucella, the identification of cultured organisms relies on an array of approximately 25 phenotypic traits, including serological typing for the A and M antigens, phage typing, requirement for elevated CO_2 atmosphere, and metabolic processes. However, there are some problems associated with these tests including the following: a) time: it takes approximately 10–14 days to culture the bacteria and complete the tests; b) biosafety: live organisms are required for testing, exposing the laboratory personnel to possible infection; c) training: the differential tests used are complex and require skilled technicians; d) limited subtypes: epidemiologists rely on unique strain markers for trace-back, but for Brucella species only a few subtypes are defined, and often a single subtype will dominate a geographic area e) ambiguous results: identification depends on the characterization of numerous traits, many of them defined in relative terms such as the rate of urease activity [5]. Atypical strains can exhibit a collection of traits that do not fit the description of any one species or subtype. As with any disease, control of brucellosis would benefit from new and improved diagnostic tests that address some of the problems encountered with current methods.

Recently, concern about intentional release of pathogens by bioterrorists and agriterrorists has fostered an additional sense of urgency for faster and more precise methods.[6] Diagnostic procedures need to address several features associated with the establishment and spread of the infectious agent. Rarely can a single test provide all the necessary information. Therefore, several assays may be needed in an eradication or surveillance program. The first aspect to consider is a screening test that could be used to detect a small number of affected animals within a larger population. In this capacity, sensitivity is slightly more important than specificity since it is better to examine a few false-positive reactors than to risk missing some infected animals. Ideally, an effective screening test should be quick, simple, inexpensive, sensitive, and robust. It should be impervious to uncontrollable conditions inherent in the sample, in the environment, or in the testing parameters. The initial screening test should, at a minimum, definitively identify the genus involved. In the case of brucellosis, this role is currently performed by serological tests that are genus but not species specific. However, issues regarding specificity and time are prompting investigators to consider alternatives such as PCR.

The second level of the diagnostic process is definitive confirmation of the disease agent. In most countries, the government response to brucellosis is dictated by policies specific for the species of Brucella involved. Therefore, the confirmatory diagnostic test must be able to differentiate Brucella strains at the species level so that the correct action is taken. It is also necessary to differentiate vaccine strains from wild type field strains.

PCR technology is well equipped to meet these needs and several promising PCR tests have already been developed.

In the third phase of the diagnostic process, the specific field strain needs to be characterised for epidemiological application. Once an outbreak has been identified and confirmed, the causative agent must be traced back to its original source and all potential opportunities for transmission between the point of origin and the epizootic location have to be identified to prevent additional spread of the disease. The epidemiology effort can be facilitated by methods which can positively link the epizootic strain to its point of origin via type-

specific markers. For years, finding unique, strain-specific markers has been the most challenging aspect of Brucella diagnostics. The remarkable genetic homogeneity within the genus [7] has made it difficult to find polymorphic genetic targets. Recently, progress in this facet of diagnostics has been advanced through PCR-based technologies.

PCR based assays are sensitive, specific, rapid assay which was reported by Mullis KB & Faloona FA[8]. Early PCR assays exploited single unique genetic targets that provided genus specific identification of Brucella. Fekete et al [9] published the first PCR –based targeted gene encoding a 43kDa outer membrane protein from Brucella. abortus S19. The first Brucella gene target to become widely used was 16 Sr DNA. Later a new genus specific assay that targets the gene coding BCSP 31 was detected. [10] Several studies on the virulence factors are directed at the main components of the outer membrane. The outer membrane contains Lipopolysaccharide (LPS), which is the major virulence factor of Brucella. Cross reactivity to lipopolysaccahride from non – Brucella bacteria is increasingly problematic. In addition to Yersinia enterocolitica, a number of other bacteria cross react with Brucella in standard agglutination tests. These include E coli O: 157 and O: 116, Salmonella spp with Kauffman White group N serotypes, Pseudomonas maltophilia11 Francisella tularensis12 & Vibrio cholera[13].

In India, the problem of Brucella infection remains in the back ground for the lack of awareness of the disease by the physicians. Mildness of the disease, keeps the problem in the background. [14] The present study aimed in establishing a simple, rapid and economical serological test for brucellosis

The Standard Tube Agglutination Test (SAT) developed by Wright and colleagues remains the most popular and easy test to perform. SAT can measure the total quantity of the agglutinating antibodies (IgG and IgM). The quantity of specific IgG is determined by treatment of the serum with 0.005M 2 mercaptoethanol (2ME), which inactivates the agglutinability of the IgM. However, many patients have low levels of agglutinating IgG antibodies and the results can easily be misinterpreted. SAT titers above 1: 160 are considered diagnostic in conjunction with a compatible clinical presentation, however, in endemic areas the titer of 1: 320 is taken as the cut off. Enzyme linked immunosorbant assay (ELISA) has become increasingly popular, as well as a standardized assay for brucellosis. It measures IgG, IgM, and IgA, which allows a better interpretation of the clinical situation. The specificity of ELISA, however seems to be less than that of the agglutination tests .As the diagnosis of Brucella is based on the detection of the antibodies to the smooth lipopolysaccharide, the cut off value needs to be adjusted, to optimize the specificity when this method is used in endemic areas. [15] The present study was undertaken to compare the STT with ELISA and IFA with in-house smooth lipopolysacharide antigen extracted by different methods and their use in serological diagnosis of brucellosis.

Materials and Methods

The study was conducted, from January 2005- Decemeber 2010 from in a tertiary hospital in Bangalore India.

1. 81 human sera was collected from people working in organized farms including veterinary staff from in and around Bangalore ,Karnataka India who were considered to be of high risk group.(cases)
2. 100 human sera were collected from KIMS, Bangalore, Blood Bank from normal healthy persons. (controls)
3. 25 samples which were WIDAL positive from Department of Microbiology were also collected to check for cross positivity.

A total number of 206 samples were collected which included cases and controls. The serum samples from cases and controls along with International standard antibrucella serum (ISAbs) which was procured from IVRI, Izatnagar, UP.
The antigen was procured from the Institute of Animal health and Veterinary Biologicals, Hebbal, Bangalore. A titre of 1:80 or greater was taken as significant.

Antigen extraction:

Smooth strain of B. abortus S99 obtained from IVRI, Izathnagar, UP, India was used to prepare various soluble antigens. The organisms were grown on Trypticase Soya Agar for 72hrs in Roux bottle flasks at 37°C with 5 % CO2. The culture was harvested in double distilled water and centrifuged at 500xg for 10min.The supernatant was then centrifuged at 7000g x 30 min at 4 °C and the deposit resuspended again in double distilled water so as to obtain a final concentration of 10 mg/ml (W/V) (Sutherland 1967). [16] The washed bacterial suspension was used for different antigen preparation. The entire procedure was carried out in class II biosafety cabinet.

Antigen extraction:

1. The lipopolysaccharide sonicated extract (LPS- SE) (Diaz 1967) [17]
   The bacterial suspension was sonicated and centrifuged at 7000g for 10 min at 4°C and the supernatant which was obtained after dialysis formed the LPS-SE.
2. The lipopolysacharide heat extract antigen (LPS-HE) (Taylor 1960)[18].
   For this, instead of using distilled water, physiological saline was used and it was heated for 1 hr at 100° C and centrifuged at 7000g for 10 min at 4°C. The supernatant which was obtained after dialysis was used as the LPS-HE antigen.
3. SDS-polyacrylamide gel electrophoresis of the antigens was done to characterize the extracted antigens. Protein estimation was carried out on the antigens which were extracted by the Biuret method.

SDS–Page:

The plates for casting the gel were assembled and they were held together tightly. It was ensured that this assembly was leak proof. 50 µl of ammonium persulphate (APS) was mixed thoroughly with 5 ml of separating gel. The gel solution was poured between the plates till the label was below 3-4 cm from the top of the notched plate. 200 – 250 µl of water was added to make the surface even. After the gel had set, the top of the separating gel was washed with distilled water and it was completely drained. 20 µl of APS solution was mixed with 2ml of the staking gel and this mixture was poured directly on the polymerized separating gel. A comb was inserted into the gel carefully without trapping air bubbles about 1 cm above the separating gel. This was allowed to set for 10 min.

50 µl of the test sample was mixed with 10 µl of the standard protein and 15µl of the loading buffer and this mixture was heated at 85ºC-95ºC for 1 min. After the stacking gel had set, the comb was carefully removed.

It was then placed in the PAGE apparatus with running buffer at the bottom of the reservoir. The samples were loaded, the 80lectrophoresis was started at 100v when the dye front reached to about 0.5 cm above the bottom of the gel and then the power was turned off. It was then transferred to a tray which contained 20 ml of Coomassie brilliant blue and was left to stain for 30-60 min. It was left overnight as the bands appeared light. Destaining was done with a destaining solution (200ml of methanol and 70 ml of glacial acetic acid and the volume was adjusted to 1 lt) and it was left for 24 hrs.

Standard tube test (STT):

The test was carried out with 81 samples (cases) and 100 (controls) samples from non-endemic healthy people and 25 WIDAL positive samples by using *B abortus* plain antigen procured from the Institute of Animal health and Veterinary Biologicals, Hebbal, Bangalore. A titre of 1:80 or greater was taken as significant.

Enzyme linked immunosorbant assay (ELISA):

The reagents for ELISA were commercially procured to develop the kit. The goat antihuman HRP conjugate, tetramethyl benzidine /H_2O_2 (Genie Lab, India) and 96 well ELISA plates (NUNC) were used. Positive serum samples from

confirmed cases of brucellosis (by culture) were obtained from IVRI, Izatnagar, as positive controls for standardization of the ELISA

The optimal working dilutions of the LPS-SE and the LPS-HE, as well as the conjugate, were found out by checker board titration for their use in ELISA.

2 sets of microtitre plates were then coated with the LPS-SE and the LPS –HE antigens of *B abortus* S99 by delivering 100µl/ well (1µg) of each in the antigen coating buffer (carbonate –bicarbonate buffer) pH 9.6 separately and they were incubated at 4°C over- night. The plates were then washed thrice with PBS-Tween. The remaining protein binding sites were blocked by adding 100µl of 5% skimmed milk with 0.1% Tween 20 respectively to all wells of the plate and the plates were incubated at 4°C for 1hr. The plates were then washed as has described above. The test sera and the control sera were diluted to 1:100 and they were added to the wells. The plates were then incubated at 37°C for 1 hr. The plates were washed thrice and then, the goat antihuman globulin in HRP (1:10000), which was diluted in the blocking buffer, was added to all the wells and the plates were incubated at 37°C for 1hr. The plates were washed thrice and they were treated with 100µl of TMB/H2O2 for 20 min. Finally, the reaction was stopped by adding 100µl of 1M H_2SO_4.

The readings were taken on a spectrophotometer at a wavelength of 450nm by using an ELISA microtitre plate reader (Teflon 96 microELISA plate reader)

Indirect Fluorescent Assay (IFA):

Reagents for IFA like goat anti human globulin with FITC were procured from Bangalore Genie lab India.

The test involves fixing (by acetone) a predetermined suspension of whole *B. abortus* cells (obtained from different commercial sources or reference laboratories) on acetone resistant slides. After the addition of doubling serum dilutions, incubated (30 min at 37 °C) and washing in phosphate-buffered saline.

Fluorescein labelled antihuman IgG is added to the designated circles on the slide, which were incubated (30minat37°C), repeatedly washed, and dried before being mounted .The slides are read using a fluorescence microscope to determine the titre that is the highest dilution showing positive fluorescence. Positive and negative control sera were also included in each run.

Statistics

Statistical software: The Statistical softwares, namely, SAS 9.2, SPSS 15.0, Stata 10.1, MedCalc 9.0.1, Systat 12.0 and R environ- ment ver. 2.11.1 were used for the analysis of the data and Microsoft Word and Excel were used to generate graphs, tables, etc.

Result

As per the SDS-PAGE profile of the Brucella S-LPS, two types of banding profiles which displayed diffuse and discrete bands had been described by earlier workers.

In the present study, the SDS-PAGE of the S-LPS of *B. abortus* S99 displayed diffuse bands from 25-43kDa and from 43-97 kDa.

The optimal cut off value for the ELISA was calculated by the mean + 3SD, and it was found to be 0.257 in LPS-SE and 0 .380 in LPS –HE. Eight (9.87%) sera gave a titre of ≥ 1:80 by STT, whereas by ELISA, 10(12.34%) and 9(11.11%) sera showed positivity for the LPS-SE and the LPS-HE antigens respectively. A correlation between the standard tube test and ELISA has been shown in [Table1].

Table 1:
Correlation of ELISA (Sonicated/LPS) with STT: An evaluation

	Sensitivity	Specificity	PPV	NPV	Accuracy	P value
ELISA (SE)	100.00	95.95	70.00	100.00	96.30	<0.001**
ELISA(HE)	100.00	97.30	77.78	100.00	97.53	<0.001**

The diagnostic potential of the test and its accuracy were determined by the Receiver operating Curve (ROC). This is always used to compare the different assays shows the ROC curve.

In case of IFA, 7 showed positivity with LPS-SE and LPS –HE antigens respectively.
A correlation between STT, ELISA and IFA has been shown in [Table 2].

Table 2:
Correlation of IFA with STT

	Sensitivity	Specificity	PPV	NPV	Accuracy	P value
IFA (SONICATED)	77.77	87.5	87.5	77	80.0	0.76
IFA(HEAT EXTRACTED)	77.77	87.5	87.5	77	80.0	0.76

Discussion

The smooth lipopolysaccharide (S-LPS) of Brucellae is the antigenic component which plays an important role in agglutination tests like STT and Rose Bengal test. Antibodies to S-LPS can be detected by variety of tests including ELISA, IHA and IFA. STT though is a standard test for diagnosis of brucellosis its specificity is low.[10] Thus the search for alternative screening test is recommended and ELISA being one of the best candidates.[11]

In the present study, the LPS were used for the development of ELISA for humans. Both the antigens ie the LPS-HE and sonicated extracted antigen seemed to be a good antigen for the detection.

According to table no 1 it was found that there were no false negatives with ELISA, but however there was 3 false positive when compared with STT with sensitivity being 100 and specificity of 95.95 and 97.30 with p value of ≤ 0.001.

In our study it was found that out of 81 human sera 7 (8.53%) was positive with STT and 10 (12.34%) were positive with sonicated extracted antigen and 9 (11.11%) were positive with lipopolysaccharide heat extracted antigen by ELISA. This study was in accordance with study done by S Isloor et al, were the overall seroprevelence was 15.69%.[19] In a similar study by Gad El Ram MO. ELISA was more sensitive, specific, accurate and reliable compared to agglutination tests and culture. ELISA showing greater sensitivity of 98%.[20]

Another study also showed that ELISA had a sensitivity of 100% and a specificity of 99.2 % when compared to STT. [21]

As compared to the SAT, ELISA was found to yield higher sensitivity and specificity [15].

Out of 81 human sera 8 (9.86) were found to be positive with IFA compared to 7 in STT but however there were 2 false positives. Sensitivity being 77 and specificity 87.5, accuracy 80.0 and p value being ≤ 0.76. IFA showed poor sensitivity when compared with IHA and ELISA though the specificity was 87.5 .These findings and the subjective reading of IFA limits its value in Brucella diagnosis when comparable with ELISA and IHA .This study was similar to that done by George F Arag etal, 1990. [22]

Conclusion

STT being cumbersome and time consuming procedure and as its titers lower than 1:80 do not exclude the existence of brucellosis, ELISA is better serological test as compared to STT and IFA. IFA showed a very low specificity as well as sensitivity.

Hence ELISA can be used for mass serological screening and can be considered as a better serological method for the diagnosis of brucellosis. It is a cheap, sensitive, reproducible and specific assay for diagnosis of brucellosis and the antigen coated plates can be kept for a long period.

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