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Original Article

Microbiology Section

Evaluation of Sonicated and Heat Extracted Lipopolysaccharide Brucella Abortus Antigens by an In House Enzyme Linked Immunosorbant Assay

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ABSTRACT

Aim: Brucellae are small gram negative coccobacilli that are known to cause brucellosis, the most common zoonotic disease world wide. The multisystem involvement and the protean and the unusual clinical presentation of the disease pose significant diagnostic challenges. Although the isolation of the causative organism is the definitive proof of the disease aetiology, practical difficulties are encountered. Hence, serological tests remain the most commonly used methods for its laboratory diagnosis. The standard tube agglutination test (STT) is the conventional serological test which is used.

Method: The present study was carried out to evaluate the two different antigenic preparations from the smooth stains of *B. abortus S99* for standardizing the enzyme linked immunosorbant assay (ELISA) as an alternative for STT. The standard tube agglutination test antigen and the standard antibrucella serum which were obtained from IVRI, Izathnagar, were used as the controls for the standardization of the ELISA.

A sonicated lipopolysaccharide antigen (LPS-SE) and a heat extracted lipopolysaccharide antigen (LPS-HE) were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and they were used to coat the micro titre plates for the enzyme linked immunosorbant assay.

81 human sera from people who were working in organized farms (cases), hundred human sera from apparently healthy persons (controls) and 100 Widal positive samples were selected to check for the crossreactivity for this study. All the serum samples (the cases , controls and the WIDAL positive samples) were tested by the standard tube agglutination test (STT).This study was conducted over a period of four years at a tertiary care hospital in India.

Results: Among the 81 cases, eight (9.87%) sera gave a titre of ≥ 1:80 by STT, whereas by ELISA, 10(12.34%) and 9 (11.11%) cases showed significant titres on the LPS-SE and the LPS-HE coated plates respectively. The accuracy of the ELISA by using both LPS-SE and LPS –HE was 93.83% and 95.86%, with a p value of > 0.001, as compared to STT.

Conclusion: The overall seroprevalence with ELISA was 12.34% and 11.11% with the LPS-SE and the LPS-HE antigens, whereas it was 9.87% with STT. Hence, ELISA can be considered as a better diagnostic serological test for the diagnosis of brucellosis. It is cheap and reproducible and the antigen coated plates can be stored for longer periods.

Key Words: Lipopolysaccharide sonicated extract, lipopolysaccharide heat extract, STT, ELISA, brucellosis

INTRODUCTION

Human brucellosis continues to be a major health problem worldwide. The endemicity is limited to some areas of the Mediterranean basin and the developing countries in Asia, Africa and Latin America. Sporadic cases may develop where the disease is nonendemic. At least half a million new cases of brucellosis annually are estimated by the World Health Organization to occur globally [1]. Thus, this illness is currently included among the travellers diseases [2]. One may also consider it to be an occupational, food borne or a laboratory acquired illness.

Phylogenetically, *Brucella* is classified within the α 2 subdivisions of the Proteobacterium, which includes Agrobacterium, Rickettsia, Rhodobacterium, and Rhizobium [3]. Establishing a relationship within the genus has been challenging, because of the relatively few genetic polymorphisms that distinguish each species [4]. Six species have been recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis,* and *B. neotomae*. This classification is based on the differences in the pathogenicity and the host preferences of the organism [5]. In recent times, two new species have been added to this genus, *B. cetaceae* and *B. pinnipediae,* which have been isolated from marine mammals, cetaceans, and pinnipeds [6].The *Brucella* genome consists of two circular chromosomes without plasmids, thus suggesting a remarkable difference as compared to the single chromosome of many bacteria. It is an infectious disease (International Classification of Diseases) ICD-9 023 or ICD-10 A23 that carries high morbidity and low mortality [7].

Brucellosis is a multisystem disease with a broad spectrum of clinical manifestations. The clinical findings of the disease are non specific and highly variable. The diagnosis depends on either the isolation of the bacteria from blood or tissue samples or on the demonstration of the presence of the *Brucella* antibodies by several serological tests.

The definite diagnosis of this disease is based on the isolation of the *Brucella* sps in blood cultures, but its sensitivity varies from 20 % to 70 %. PCR remains promising for the rapid diagnosis of acute

but not chronic brucellosis [8]. Thus, serological tests continue to play a relevant role in the diagnosis and the management of patients with brucellosis [9].

The most widely used serological techniques are the standard tube agglutination test (STT) and the Coombs anti-Brucella test, which can be used for detecting the antibodies against the smooth lipopolysaccharide antigen (S-LPS). S-LPS is the main antigenic and immunogenic structure on the surface of Brucella [10]. However, these techniques present some interpretation problems and the antibody titre can remain elevated over long periods even after the recovery of the disease [11]. This also causes difficulties in the diagnosis of a reccurrence /reinfection.

Enzyme linked immunosorbant assay (ELISA) has become an increasingly popular, as well as a standardized assay for the diagnosis of 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 against the smooth LPS, the cut-off value needs to be adjusted, to optimize the specificity, when this method is used in endemic areas [12].

In the present study, the LPS of *Brucella abortus* S99 was extracted by two classic and well known methods. (Diaz 1967, Taylor 1960) The diagnostic utility of the in house antigen coated plates in ELISA was standardized by using the standard antibrucella serum which was obtained from IVRI, Izathnagar, UP as the control.

This study was conducted from July 2005 to July 2010 at a tertiary hospital in India.

1. 81 blood samples were obtained from individuals who were working in organized farms in and around Bangalore, Karnataka, India, who were considered as the high risk group. A detailed history of these patients like the nature of their work, occupation, consumption of raw milk, history of fever and joint pain etc, was obtained (cases).
2. 100 blood samples were also collected from healthy individuals whose blood was sent for routine haematological investigations (controls).
3. 25 Widal positive samples were collected from the Kempegowda Institute of Medical Sciences, Bangalore, India, to check for false positivity. Several studies have shown that the outer membrane of Brucella contains Lipopolysaccharide (LPS), which is its major virulence factor which cross reacts with the lipopolysaccahride from non-Brucella bacteria. Apart from *Yersinia .enterocolitica*, a number of other bacteria cross react with *Brucella* in the standard agglutination tests and these include *E. coli* O: 157 and O:116, Salmonella spp with the Kauffman White group N serotypes, *Pseudomonas maltophilia* [13] *Francisella tularensis* [14] and *Vibrio cholerae* [15].

   A total number of 206 samples were included in the study, which included the cases and the controls.

   A drop of 1:10,000 mertiolate was added to all the serum samples and they were stored at -20°C till they were processed. The serum samples from the cases and the controls, along with the international standard antibrucella serum (ISAbs), were tested by the Standard tube agglutination test (STT) 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 a significant titre [16].

Antigen extraction

The smooth strain of *B. abortus S99* which was obtained from IVRI, Izathnagar , UP, India ,was used to prepare various soluble antigens .The organisms were grown on Trypticase Soya agar for 72 hrs in Roux bottle flasks at 37°C in 5 % CO_2. The culture was harvested in double distilled water and it was centrifuged at 500xg for 10min.The supernatant was then centrifuged at 7000g x 30 min at 4°C and the deposit was resuspended again in double distilled water, so as to obtain a final concentration of 10 mg/ml (W/V) (Sutherland 1967) [17]. The washed bacterial suspension was used for different antigen preparations. The entire procedure was carried out in a class II biosafety cabinet.

1. The lipopolysaccharide sonicated extract (LPS- SE) (Diaz 1967) [18].
   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) [19].
   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 elecrtophorosis 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 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 electrophorosis 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.

Enzyme linked immunosorbant assay

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 overnight. 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/H_2O_2 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)

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 environment 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 [Table/Fig-1]. A correlation between the standard tube test and ELISA has been shown in [Table/Fig-2 & 3].

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.

Later, according to the interpretation of the curve, the Confidence interval and the accuracy were found out.

These have been shown in [Table/Fig-4].

Results	STT		ELISA (Sonicated)		ELISA(LPS)	
	No	%	No	%	No	%
Positive	7	8.6	10	12.3	9	11.1
Negative	74	91.4	71	87.6	72	88.9
Total	81	100.0	81	100.0	81	100.0

[Table/Fig-1]: Frequency Distribution of Positivity/Negativity by STT and by Elisa Using Lps-Se and Lps-He Antigens

STT	Positive	Negative	Positive	Negative	
ELISA	Positive	Positive	Negative	Negative	Total
Sonicated	7	3	0	71	81
LPS	7	2	0	72	81

[Table/Fig-2]: Correlation of Elisa (Sonicated/Lps) with STT: An Observation

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

[Table/Fig-3]: Correlation of Elisa (Sonicated/Lps) with STT: An Evaluation

	AUC	SE	95%CI
ELISA (Sonicated)	1.000	0.00	0.95-1.00
ELISA (LPS)	1.000	0.00	0.95-1.00

[Table/Fig-4]: Roc Curve Analysis Elisa-Sonicated and Elisa LPS

DISCUSSION

This study showed that as compared to the alternative immunoassays, ELISA was the most versatile method and that its results were available within a short time. It also had the advantage of being readily automated, thus enabling its use as a screening test, with the results being numerically quantifiable. As compared to the SAT, ELISA was found to yield higher sensitivity and specificity [20].

There are many other techniques that increase either the specificity or the sensitivity of the ELISA, which include the use of various enzymes and washing methods. An important source of the non-specific background was the use of the phosphatase-conjugated second antibodies in ELISA. This non-specific background could be drastically reduced by using peroxidase-conjugated antibodies and by including skimmed milk as a blocking reagent. The background with the phosphatase assays easily arises from small contaminations, because phosphatases are ubiquitous enzymes which occur in all the body fluids, which includes the sweat on the finger tips, which was implemented in this study.

ELISA with purified S-LPS was developed from Brucella, basically for the serosurveillance of brucellosis in humans and this appeared to be a useful tool in its diagnosis. ELISA has several advantages over the other techniques e.g. the antibody being diluted, it reacts with the antigen without performing secondary functions such as agglutination, precipitation and activation of the compliments. Moreover the sera need not to be heat inactivated as is required for the CF test or pretreated as is required for the 2-mercaptoethanol test (2ME). The RBPT and SAT are subjected to prozoning and they detect the reactors as false negative.

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

According to [Table/Fig-3], it was found that there were no false negatives with ELISA, but however, there was 3 false positives when it was compared with STT, with their sensitivities being

100 and their specificities being of 95.95 and 97.30, with p value of 0.001.

In our study, it was found that out of the 81 human sera, 7 (8.53%) were positive by STT, 10 (12.34%) was positive with the sonicated extracted antigen and 9(11.11%) were positive with the lipolpolysaccahride heat extracted antigen [Table/Fig-1]. The Widal positive samples which were usedto check for the cross reactivity, showed negative results. No positives were seen either with STT or ELISA .

With reference to the ROC [Table/Fig-5] and [Table/Fig-4], ELISA seemed to be a good test as the AUC was 1.0 and as the 95% CI was 0.95-1.00. So, it can be considered as an excellent test. The results of this study were in accordance with those of a study which was done by S Isloor et al in 2007, where the overall seroprevalence was 15.69% [21].

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