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Comparative study on s-LPS and bp26 based iELISA for human brucellosis

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ABSTRACT

Brucellosis is an emerging zoonotic disease caused by members of the genus *Brucella* and remains a serious cause of human illness from livestock. Presently used sero-diagnostic tests depend upon the smooth lipopolysaccharide (sLPS), which cross react with other Gram–negative bacteria resulting in low specificity. To overcome this, several recombinant outer membrane proteins (OMP) have been tried as diagnostic antigen (s) in ELISA. In the present study, the ORF (753 bp product) of BP 26 protein was amplified from *Brucella suis* strain 1330, cloned in pET32a vector and expressed in BL21 *E.coli* hostcell. The expressed protein was purified by Ni-NTA column and characterized by SDS-PAGE and Western blot analysis. The purified recombinant protein (rbp26) antigen was tested in indirect ELISA (iELISA) and specificity was checked with *E. coli* (O157 H7), 17 salmonella and five *Yersinia entericolitica* reference sera. Further, rbp26 based standardized ELISA was evaluated with serum samples (n=626) collected from risk group individuals (veterinarians) using two conjugates IgM and IgG for diagnosis of brucellosis. Comparative evaluation of the developed assay with RBPT and sLPS based ELISA was carried out. In RBPT, 60 (9.5%) and in sLPS antigen based iELISA-IgM 36 (5.75%) and iELISA-IgG 122 (19.48%) were positive, respectively. Whereas in rbp26 based ELISA 18 (2.87%) and 66 (10.54%) were positive in IgM and IgG ELISA, respectively.

KEYWORDS

RBPT;
STAT;
IgM ELISA;
IgG ELISA;
Human;
Brucellosis;
Blood donors;
Risk group.

INTRODUCTION

Brucellosis is a zoonosis caused by facultative intracellular bacteria of the genus *Brucella*, which are capable of surviving and multiplying inside the cells of mononuclear phagocytic system and are widely distributed in both humans and animals[1]. Human brucellosis varies from an acute fabrile illness to chronic, low grade ill defined disease. It is a systemic disease characherized by pausity of signs accompanied with nocturnal sweating, malaise, fatigue and backache[2]. The disease can be a very debilitating, despite the fact that the fatality

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rate is generally low. It often becomes sub-clinical or chronic, especially if not diagnosed early and properly treated. The incidence in humans ranges widely between different regions, with values of up to 200 cases per 100,000 populations with high prevalence in Middle East, Mexico, Central and South America and the Indian subcontinents[2,3]. High-risk groups include those exposed through occupation in contexts where animal infection occurs, such as slaughterhouse workers, hunters, farmers and veterinarians.

The diagnosis of brucellosis can be challenging, and its diagnosis demands epidemimology, clinical and laboratory information. Its routine biochemical and hematological laboratory tests also overlap with those of many other pathogens such as *Salmonella*, *Yersinia*, and *Vibrio*[4]. Many tests are reported for diagnosis of *Brucella*, ranging from microbilogical culture to serodiagnostic tests such as slide or tube agglutination, indirect coombs test, enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent assays, to the recent molecular techniques such as polymerase chain reaction (PCR) are available.[5-7].

Isolation from blood, bone marrow and other tissues of suspect is classical diagnostic (gold standard) method for brucellosis. However, this microbiological technique is having the draw back of time consumption as the organism is having incubation period of 6 weeks and possibility of contamination to personnel cannot be avoided[8]. Rose Bengal Plate test (RBPT) is commonly used for the screening of brucellosis however results may at times inconclusive[9]. In standard tube agglutination test (STAT), interpretation of the result is difficult due to false positive reaction with *Salmonella*, *Yersinia* and *Vibrio* species. Further PCR is the molecular technique which is employed for the detection of brucellosis, but the technique is uneconomic and poorly suited for the laboratory with limited resources. In view of these limitations, robust, coast effective and rapid ELISA has been found an ideal tool for the diagnosis. Current serological tests based mainly on s-LPS antigen, chances in possible of false positivity due to cross-reactivity. In view of all these facts, the present study have been taken to develop recombinant antigen based ELISA for diagnosis of brucellosis in swine and humans. The recombinant outer-membrane protein (rOmp26) was purified from B.*suis* 1330 in pET-32a bacterial expression system and used in an indirect ELISA for the detection of Brucellosis. Comparisons of recombinant antigen based iELISA against RBPT, and s-LPS based iELISA.

In brucellosis, titre of IgM usually raises from day 5 to 7 with peak titre and IgG starts to appear from day 14 to 21, reaching peak during next 2 to 3 weeks in the infected individuals. Clinical applications of IgM and IgG ELISA in human disease have been reported[10,11]. This manuscript deals with the study on development and evaluation of recombinant bp26 antigen based indirect ELISA for the sero-diagnosis of *Brucella* infection in human beings and its comparison with laboratory standardized IgM and IgG ELISA protocol.

MATERIALS AND METHODS

Collection of sera samples

During the course of the study, 2 ml of blood samples without anticoagulant was collected aseptically in vaccutainers. The samples were sourced from risk group (veterinarians, para veterinarians, farm workers, animal-handlers and farmers), blood donors and patients with pyrexia of unknown origin (PUO). The pyrexia may be due to systemic cause of rheumatic fever, jaundice, C reactive protein, hepatitis etc., The samples were allowed to clot, transported to laboratory immediately at 4°C. The serum was separated by centrifuging the sample at 2500 r.p.m for 5 min and stored at –20°C for further use.

Rose bengal plate test (RBPT) and standard tube agglutination test (STAT)

Sera samples received were initially subjected to rapid screening RBPT according to standard procedures[12]. Briefly, for the RBPT, undiluted serum sample (30 µl) was mixed with an equal volume of colored antigen on a glass slide. The results were rated negative when agglutination was absent and 1+ to 4+ ratings as positive, according to the strength of the agglutination within 1 to 3 min.

Smooth lipopolysaccharide (sLPS) antigen extraction

Standard strain

Standard antigenic strain of *B.suis* 1330 procured

from Indian Veterinary Research Institute, Izatnagar, U.P., India and confirmed as *B.suis* 1330 by biochemical tests, PCR, cloning and sequencing in our was used for antigen extraction[15].

Large scale bacterial culturing

Freshly grown pure colonies were suspended in 10 ml of sterile PBS, after vortexing, the bacterial suspension was overlaid on Tryptose Agar (TA) in Roux flasks. Thirty flasks were simultaneously inoculated from the same master plate to provide the identical bacterial population originating from a single colony. After one-hour adsorption, Roux flasks were inverted and incubated for 72 hours at 37°C. The purity of the culture in every flask was confirmed by Gram's staining after 48 hours. To each flask, 30 ml of 2% phenol saline was added, gently agitated and incubated for 24 hours at 37°C. The suspensions were collected, pooled, centrifuged at 14,000 r.p.m at 4°C for 20 min. The centrifugation was repeated and pellets were carefully collected, weighed and used for antigen extraction.

Preparation sLPS antigen extraction

Wet cells of *Brucella* (5 gm) were suspended in 17 ml of distilled water and followed by the addition of 19 ml of 90% (v/v) phenol at 66°C. The mixture was stirred continuously at 66°C for 15 min, cooled and centrifuged at 10,000 rpm for 15 min at 4°C. The brownish phenol in the bottom layer was aspirated with a long micro tip and large cell debris was removed by filtration (using a Whatman No.1 filter). The sLPS was precipitated by the addition of 50 ml chilled methanol containing 0.5 ml methanol saturated with sodium acetate. After 2 hours incubation at 4°C, the precipitate was removed by centrifugation at 10,000 r.p.m for 10 min, stirred with 8 ml of distilled water for 18 hours and centrifuged at 10,000 r.p.m for 10 min. The collected supernatant solution was kept at 4°C and this step was repeated twice for the best recovery of antigen. Then, 0.8 g of trifluroacetic acid was added to the 16 ml of crude sLPS, stirred for 10 min and the precipitate was removed by centrifugation. The translucent supernatant solution was concentrated and dialyzed against distilled water (two changes of at least 4000 ml each) and then freeze dried to get the final yield of 10 ml of sLPS containing the antigen concentration of 3 mg/10ml.[16].

The optimum concentration of antigen for ELISA was standardized by checkerboard titration against 1:100 and 1:200 dilution of strong positive convalescent sera. The OD values were plotted on a graph and the point where there was sharp fall on the line graph was taken as the optimum dilution of antigen.

Standardized ELISA protocol

The polysorp micro titer plates (Nunc, Germany) were coated with 1:300 dilution of sLPS antigen at 100 µl per well in carbonate-bicarbonate buffer (pH 9.6) and incubated 4°C for overnight. Antigen coated plates were washed three times with PBST wash buffer (Phosphate buffered saline containing 0.05 % Tween 20) pH 7.2. Test and control sera diluted in PBST blocking buffer (1:100) containing 2% bovine gelatin was added to respective wells (100 µl) of the plates in duplicates (test sera) and quadruplicate (controls) and incubated at 37°C for 1hour. The plates were then washed as mentioned earlier. The anti-human IgG and IgM HRP conjugates (Pierce, Germany), diluted 1:8000 and 1:4000 respectively in PBST buffer were added to all the wells (100 µl) and incubated for 1 hour at 37°C on orbital shaker (300 r.p.m./min). After washing, freshly prepared o-Phenylenediaminedihydrochloride (OPD) (Sigma, Germany) solution containing 5 mg OPD tablet in 12.5 ml of distilled water and 50 µl of 3% H_2O_2 was added and kept for color development for 10 min. Enzyme-substrate reaction was stopped by adding 1M H_2SO_4 (50 µl) and color development was read at 492 nm using an ELISA micro plate reader (Biorad). The optical density (OD) obtained for the negative and positive samples were interpreted by cutoff values set at 3 standard deviations above the arithmetical mean of the OD obtained for the healthy controls[17].

Production and purification of recombinant BP26 protein

Standard antigenic strain, *Brucella suis* 1330 was procured from IVRI, Izatnagar. The organism was grown in brucella selective agar media at 37°C for 48 hrs at 10% CO_2. The organism was identified as Gram negative, coccobacillary rods on Gram's staining. The genomic DNA was extracted as per the standard protocol, using the "Bacterial genomic DNA spin" – QIAamp. The PCR amplification was carried out in 25µl reaction volume containing 12.5µl of 2X PCR master mix (4 mM $MgCl_2$; 0.4 mM of each deoxynucleotide triphos-

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phates (dNTPs); 0.5U of *Taq* DNA polymerase; 150 mM Tris-HCl PCR buffer), primers and 2.5µl of template DNA. The Thermal cycler conditions for BCSP31 are 93^{0}C for 5min 1 cycle; 35 cycles of 90^{0}C for 1min; 60 ^{0}C for 30sec and 72^{0}C for 1min with a final extension of 72^{0}C for 7 min. The PCR amplicons (5µl) were separated by electrophoresis in 1.5% agarose gel with TAE as running buffer and purified.

The purified Genomic DNA from B.*suis* 1330 was used for the amplification of the target gene rbp26 using published primer sequences. The cycling conditions followed were: preheating at 95^{0}C for 2min 1 cycle; 35 cycles of 94^{0}C for 45sec; 50 ^{0}C - 60 ^{0}C for 45sec and 72^{0}C for 2min with a final extension of 72^{0}C for 10 min. The products were analysed by agarose gel electrophoresis as mentioned before. The amplified product was purified from the gel using "MinElute gel extraction kit" from QIAGEN and employed for further sequencing and cloning[35,36].

Cloning and expression of rbp26 gene

The purified PCR product digested with EcoR1 and Not1 which was ligated with digested pGEMT vector, was transferred into E. coli Top10F competent cells and plated on LB amp plates, incubated overnight at 37^{o}C. Several ampicillin resistant colonies appeared and were streaked on LB amp plates for further recombinant clones for the presence of rbp26 gene. After an incubation of 16 hours at 37^{0}C, both blue (non recombinant) and white colonies (recombinant) were observed on LB Agar-amp supplemented with IPTC and X-gal plate and white colonies (recombinant clones) were purified, used as template and confirmed by Colony PCR. The amplified products were analyzed by agarose gel electrophoresis and further subjected to confirmation by restriction enzyme (RE) digestion[40].

The inserted rbp26 gene sequence is of 753bp nucleotides. The cloned bp26 gene was released from pGEMT bp26 by *Eco*R1 and *Not*1, digestion. The fragment was sub cloned into pET-32a vector and analyzed by agarose gel electrophoresis then it was digested with *Eco*R1 and *Not*1. After transformation of pETBP26 into *E. coli* BL21 competent cells several recombinant clones were formed on the LB plate supplemented with ampicilin. Plasmid DNA extracted from recombinant clone digested with which was ligated with digested pET-32a vector, was transferred into *Eco*R1 and *Not*1 released 753bp insert.

The transformed competent BL21 cells and pET-32a vector as a control and pETBP26 recombinant clones of interest were induced with 1mM IPTG and incubated for sixteen hours at 37^{0}C. The induced cell lysates were collected at one hour interval. The lysates of proteins were analyzed by 12 percent SDS PAGE and followed by western blotting. The size of the rbp26 gene is 753bp which corresponded to 42kDa protein. Thus along with fusion tag of 18kDa, the rbp26 protien showed in the gel as 42kDa band. No reaction was seen with respect to blots treated with Yersinia enterocolitica O:9, and Escherichia coli O:157 referral sera, This showed that the expressed protein was of *Brucella* specific epitope and there was no cross reaction with positive and negative sera.

Standardized ELISA protocol

The polysorp micro titer plates (Nunc, Germany) were coated with 200ng/well antigen at 100 µl per well in carbonate-bicarbonate buffer (pH 9.6) and incubated 4°C for overnight. Antigen coated plates were washed three times with PBST wash buffer (Phosphate buffered saline containing 0.05 % Tween 20) pH 7.2. Test and control sera diluted in PBST blocking buffer (1:100) containing 5% skim milk power was added to respective wells (100 µl) of the plates in duplicates (test sera) and quadruplicate (controls) and incubated at 37°C for 1hour. The plates were then washed as mentioned earlier. The anti-human IgG and IgM HRP conjugates (Pierce, Germany), diluted 1:5000 and 1:5000 respectively in PBST buffer were added to all the wells (100 µl) and incubated for 1 hour at 37°C on orbital shaker (300 r.p.m./min). After washing, freshly prepared o-Phenylenediaminedihydrochloride (OPD) (Sigma, Germany) solution containing 5 mg OPD tablet in 12.5 ml of distilled water and 50 µl of 3% H_2O_2 was added and kept for color development for 10 min. Enzyme-substrate reaction was stopped by adding 1M H_2SO_4 (50 µl) and color development was read at 492 nm using an ELISA micro plate reader (Biorad).

RESULTS

In ELISA, the 1 in 200 sLPS antigen concentration

was found optimum at serum concentration of 1 in 100 (Figure 1). Similarly, the conjugate dilutions were established by checkerboard titration and IgM conjugate of 1 in 4000 and IgG conjugate at 1 in 8000 were found optimum dilutions for the test (Figure 2). Similarly for rbp26 based ELISA antigen concentration was 200ng / well was found optimum at serum concentration of 1:32 and the conjugate dilutions were established by checkerboard titration at 1:5000 for both IgG and IgM.

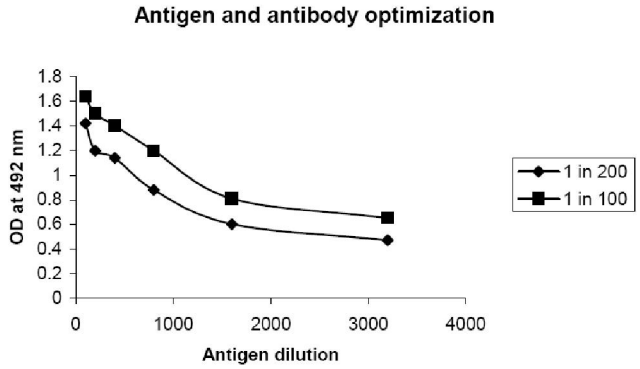

Figure 1 : Optimization of antigen versus serum concentration for ELISA (All the dilution are at the rate of 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1600 and 1in 3200 respectively)

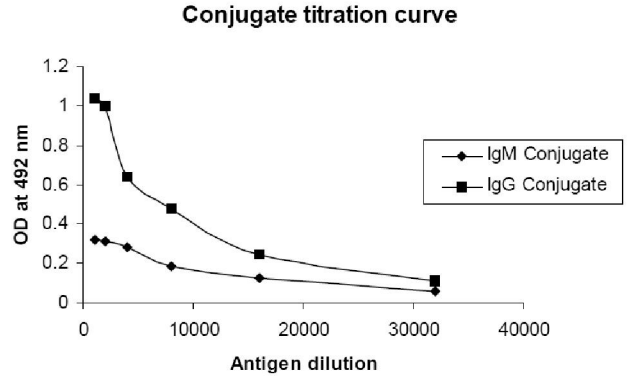

Figure 2 : Optimization of conjugates for ELISA (All the dilutions are at the rate of 1 in 1000, 1 in 2000, 1 in 4000, 1 in 8000, 1 in 16000 and 1 in 32000 respectively)

Among the 626 serum samples collected from risk group individuals (veterinarians) using two conjugates IgM and IgG for diagnosis of brucellosis. Comparative evaluation of the developed assay with RBPT and sLPS based ELISA was carried out. In RBPT, 60 (9.5%) and in sLPS antigen based iELISA-IgM 36 (5.75%) and iELISA-IgG 122 (19.48%) were positive, respectively (Figure 6). Whereas in rbp26 based ELISA 18 (2.87%) and 66 (10.54%) were positive in IgM and IgG ELISA respectively (Figure 5). Similarly comparative evaluation of the OD values of the both the assays were documented. (Figure 7,8)

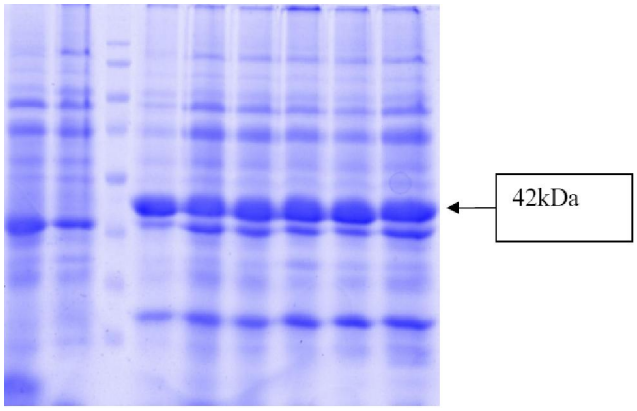

Figure 3 : SDS-PAGE analysis of sequential elutes of recombinant protein bp26 suis1330

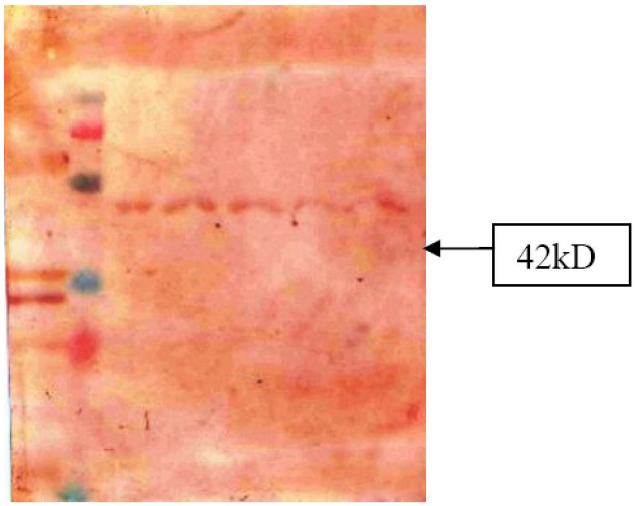

Figure 4: Western blot analysis with swine brucella positive and negative sera

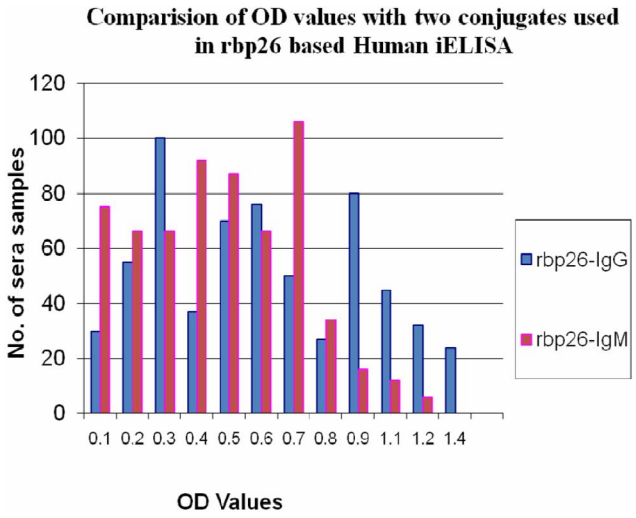

Figure 5 : Comparision of OD values with two conjugates used in rbp26 based human iELISA

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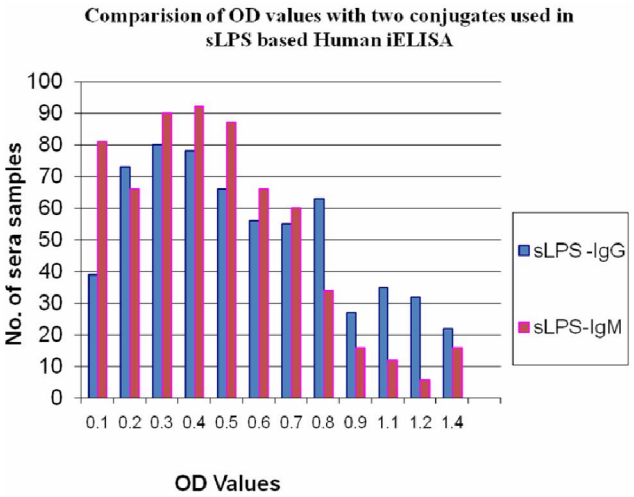

Figure 6 : Comparison of OD values with two conjugates used in sLPS based Human iELISA

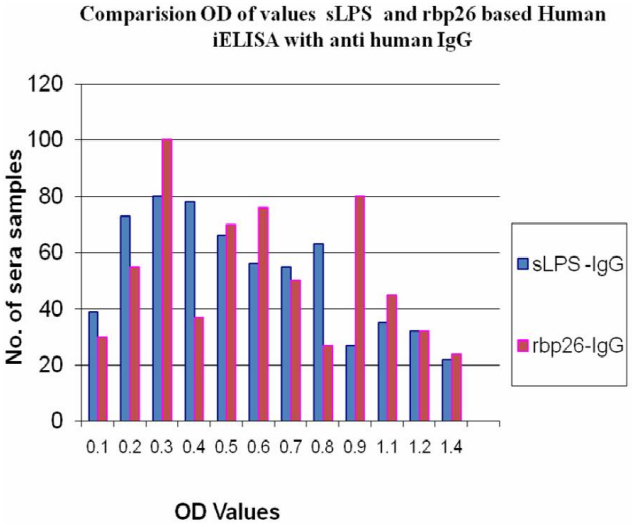

Figure 7 : Comparison OD of values sLPS and rbp26 based human iELISA with anti human IgG

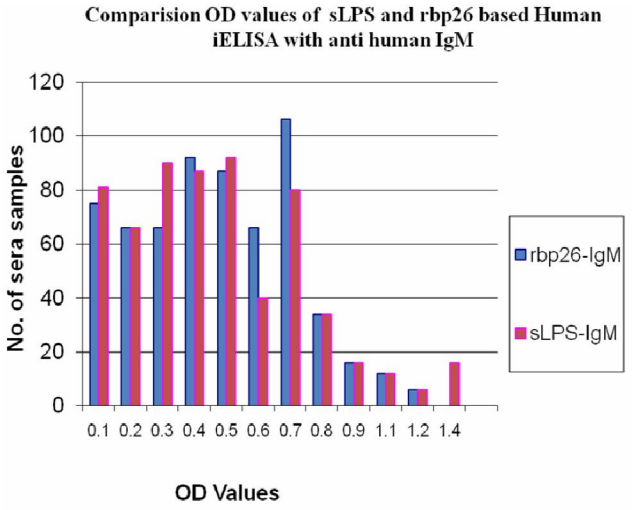

Figure 8 : Comparison OD of values sLPS and rbp26 based human iELISA with anti human IgM

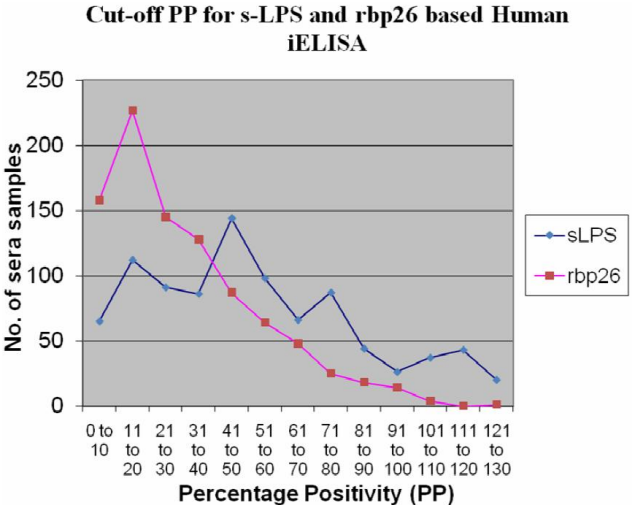

Figure 9 : Standardization and cut-off PP for s-LPS and rbp26 based human iELISA

DISCUSSION

The true incidence of human brucellosis however, is unknown for most countries and no data are available for many parts of India. It has been estimated that the true incidence may be 25 times higher than the reported incidence due to misdiagnosis and under-reporting. Several publications indicate that human brucellosis can be a common disease in India. The ELISA was first developed by Carlson et al, for the diagnosis of human brucellosis and since then, a large number of variations have been described[18]. ELISA have a distinct advantage over conventional serological tests in that, they are primary binding assays that do not rely on secondary properties of antibodies such as their ability to agglutinate or to fix complement. Secondly, ELISA can be tailored to be more specific by using highly purified reagents such as antigens and monoclonal antibodies.

The sLPS antigen of *Brucella* is considered the most important antigen during immune response and is the target for many serological and immunological studies. It gives better sensitivity and specificity with good reproducibility. It also possesses a convenient cut off value for diagnostic purposes. Finally, it is not restricted to bovines alone and can be adapted to different species of animals as well as to humans beings[3]. The sLPS antigen coated passively on to a polystyrene matrix is the method commonly employed in the ELISA[19]. The indirect ELISA and AB-ELISA have seen standard-

ized by several researchers using sLPS antigen from *B. abortus* S_{99} to screen the livestock and humans for brucellosis[15,19-21].

Similar antigenic studies has been taken on the bp26 by several researchers and previously identified *B.suis* 1330 bp26 immunodominant antigen[36] is nearly identical to the *B. abortus* BP26 antigen reported by Rossetti et al[35]. The name of BP26 should therefore be retained. The fact that two independent laboratories have identified the same immunodominant antigen either in the field of bovine or ovine[37,38,39] brucellosis emphasizes the importance of BP26 as diagnostic antigen[40].

The higher prevalence rates reported by various researchers are in accordance with our present findings in the high risk groups[21,25,30]. High sero prevalence in the risk group is attributed to constant exposure to infection due to contamination of hands and arm while handling animals and also human infection can occur through aerosol, occupational exposure of abattoir workers, veterinarians and laboratory technicians. In addition, consumption of infected raw milk, raw milk products and raw meat can result in infection[25].

The transmission of brucellosis to man is primarily by direct contact with infected animals or their products. However, the organisms can also be transmitted by transfusion of infected blood[31]. The blood donors tested in the study, showed 1.62% positivity by RBPT and 4.87%, by IgG ELISA. Two such similar reports from Karnataka, revealed the prevalence ranging from 1.8% (out of 26,948 adult donors)[25] to 14.7% (out of 353 donors) by RBPT[32]. These findings are relatively identical to our findings. This infection in the donors might be due to the exposure of the donors unintentionally to the animals or due to the consumption of raw milk, or may be due to the cross reacting antibodies such as *vibrio* or *yersinia*.

The basic knowledge of this study will help us for the development of indigenous ELISA kit for sero screening of the disease in humans and to identify active infection (IgM ELISA). The use of sLPS and rbp26 as antigen in the I-ELISA might be one of the reasons for higher sensitivity as the stronger immune responses are elicited against sLPS and found that the very specific immune response against rbp26 in infected individual. The advantage of using the indigenously developed kit/tests is that the large number of samples can be analyzed economically and it will also help to generate seroepidemiological data of the disease in the country. Screening of large number of sera samples and validation as per OIE guidelines is underway.

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