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Comparative Evaluation of Native Antigens for the Development of Brucellosis Antibody Detection System

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Comparative Evaluation of Native Antigens for the Development of Brucellosis Antibody Detection System

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Abstract
Brucellosis is a highly infectious zoonotic disease and an economically important infection of humans and livestock with a worldwide distribution. The main mode of transmission of this disease to humans is through the consumption of infected milk, milk products, and uncooked or raw meat. The present study was designed to prepare few native antigens, that is, sonicated antigen (SA), cell envelope (CE) antigen, and freeze and thaw (FT) antigen from *Brucella abortus* S99 culture and to test them in a highly sensitive and specific indirect enzyme-linked immunosorbent assay (I-ELISA) in both a microtiter plate and a dot-blot format for the development of field-based diagnosis. All 50 suspected bovine samples were tested by plate as well as in dot ELISA formats for all the three antigens prepared. The CE antigen was found to be more suitable as it had the maximum agreement with the Rose Bengal plate agglutination test results followed by the SA and the least agreement was found with that of the FT antigen. This detection system in microtiter plates and a dot-blot format will be useful for the rapid screening of samples for the disease surveillance and routine diagnosis.

Keywords: Brucellosis; *Brucella abortus* S99; ELISA; RBPT.

1. INTRODUCTION

Brucellosis is one of the world's major emerging zoonosis, caused by gram-negative, nonmotile, facultative (Edgardo *et al.*, 2002) intracellular bacteria of the genus *Brucella* and can be transferred from animal to humans (Christopher, 2004). Brucellosis in livestock is of great economic concern due to reduced productivity, increased numbers of abortions, and weak offspring. Human brucellosis is a severe debilitating disease that requires prolonged treatment with the use of several antibiotics. It may affect any organ and that reinforces the importance of brucellosis in differential diagnosis in endemic areas (Pappas *et al.*, 2005). This disease remains an uncontrolled problem mostly in highly endemic regions such as the Mediterranean, Middle East, Africa, Latin America, and parts of Asia, including India (Lopez, 1989; Corbel, 1997; Refai, 2002). Brucellosis is also known as Mediterranean fever, Malta fever, Gibraltar fever, Cyprus fever, Undulant fever, typhomalarial fever, intermittent typhoid, Bang's disease in cattle, contagious abortion, infection abortion, and epizootic abortion.

The isolation rate of *Brucella* is poor due to its slow growth rate, the low quantity of circulating viable bacteria, and problems with standardization of the culture medium and blood culture techniques employed, as well as the presence of antibiotics that inhibit growth (Yagupsky, 1999). The demonstration of antibodies generated against *Brucella* using serological tests remains a viable alternative to culture, and several serological tests, such as the Rose Bengal plate agglutination test (RBPT) and a standard tube agglutination test (STAT) are the most popular serological tests used in the field for the diagnosis of brucellosis (Morgan *et al.*, 1969; Ruiz-Mesa *et al.*, 2005; Gómez *et al.*, 2008; Chachra *et al.*,2009; Araj, 2010; Al Dahouk *et al.*, 2013). Enzyme-linked immunosorbent assay (ELISA) format offers high sensitivity and specificity in addition to a field-usable format (Jubier-Maurin *et al.*, 2001; Thavaselvam *et al.*, 2010). Several commercial indirect enzyme-linked immunosorbent assays (I-ELISAs) are available, which have been validated in extensive field trials and are in wide use (OIE, 2009). In the present study, efforts were made to prepare a few native antigens from *B. abortus* S99 culture and to test them in dot ELISA and plate ELISA format for the development of field-based diagnosis. The results of these two tests were compared with the standard RBPT. The development of these detection systems will be of great help in the endemic areas for the disease surveillance and routine diagnosis. It will be of help in the treatment of this disease as well as increase the productivity of livestock and thereby help in the improvement of rural economy.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strain

Brucella abortus strain S99 was routinely maintained in the laboratory on Brain Heart Infusion (BHI) Agar slants and fresh cultures were done from the stock onto BHI and Trypticase Soy Agar plates for further use in all experiments.

2.1.2. Clinical Samples

The sera samples were collected from animals with suspected brucellosis by Department of Veterinary Public Health, College of Veterinary Science and Animal Husbandry, Sardar Krushinagar Dantiwada Agricultural University, Sardar Krushinagar, Gujarat.

2.1.3. Buffers and Solutions

- Phosphate-buffered saline (PBS; pH 7.2–7.4) was used for harvesting of bacterial growth, for washing of bacterial pellets, and in enzyme immunoassay.
- PBS and Tween-20 (PBS-T), 1% bovine serum albumin (BSA), and carbonate bicarbonate buffer (0.05 M, pH 9.6) were used in enzyme immunoassay.
- Storage buffer (sodium azide, thimerosal, Phenylmethylsulfonyl Fluoride (PMSF), Tris; pH 7.2) was used for storage of antigens.
- Diaminobenzidine (DAB)–H_2O_2 and *ortho*-phenylene diamine (OPD)–H_2O_2 solutions were prepared for developing color reaction in dot ELISA and plate ELISA, respectively.
- 1N H_2SO_4 solution was used to stop an enzymatic reaction in plate ELISA.
- Tris-glycine buffer (pH 8.6) and blotting buffer with 20% methanol (pH 8.3) were also prepared for SDS–PAGE and Western blotting.
- 2× loading buffer was used for the preparation of protein samples.
- Alkaline reagent containing sodium hydroxide; sodium carbonate and copper sulfate (1%); and sodium potassium tartarate (2%) were mixed in appropriate ratio for protein estimation by Lowry's method.
- In the preparation of cell envelope (CE) antigen, the following three buffers were used:
  - ➢ *Buffer 1* containing Tris (pH 8), sucrose, Ethylenediaminetetraacetic Acid (EDTA), and lysozyme was used for resuspension of bacterial pellet.
  - ➢ *Buffer 2* containing Tris (pH 7.6), $MgCl_2$, and PMSF was used for sonication of pellet.
  - ➢ *Buffer 3* containing Tris (pH 7.6), $MgCl_2$, and PMSF was used for resuspension of pellet after ultracentrifugation.

2.2. Preparation of Antigens

2.2.1. Preparation of Sonicated Antigen (SA)

Brucella abortus strain S99 was subcultured from stock by inoculation into 5 mL BHI broth tubes and incubated in a shaker incubator (LABCON) overnight at 37°C and 180 rpm. The overnight culture was streaked onto BHI agar plate and incubated at 37°C overnight. A single colony was picked up with the help of sterile loop and spread on fresh sterile BHI agar plates and incubated in an incubator (JOUAN IG 150) overnight at 37°C. An overnight growth was harvested from the plates and suspended into sterile 10 mL 1× PBS buffer. One hundred microliters of formaldehyde (10 μL formaldehyde/mL of culture) was added to the bacterial suspension and kept at 4°C overnight to kill the bacteria. After inactivation, the suspension was again streaked onto fresh BHI agar plates to confirm complete inactivation. Then, bacterial suspension was sonicated by using Vibrocell (Sonics) sonicator. The bacterial suspension was placed in crushed ice to prevent degradation of protein. In all, five cycles of 5 min each with a pulse after every 8 s and an amplitude of 40 W were given for the complete sonication of bacterial cells. The suspension was centrifuged in SORVELL centrifuge RC5C at 10,000 rpm for 30 min at 4°C. The supernatant containing the soluble proteins was preserved and the pellet containing the insoluble proteins was discarded. The protein present in the supernatant was further purified by ammonium sulfate (80%) precipitation. The supernatant was stirred in a magnetic stirrer, and ammonium sulfate was added slowly so that the final concentration of the solution reached 80%. The supernatant was subjected to centrifugation at 10,000 rpm for 30 min at 4°C, and the precipitate was further washed twice with 80% ammonium sulfate solution, dissolved in sterile PBS, and stored at −20°C until further use.

2.2.2. Preparation of Cell Envelope (CE) Antigen

Three milliliters of overnight-grown culture of *Brucella abortus* strain S99 was inoculated into 300 mL of BHI broth and incubated in a shaker incubator at 37°C with constant shaking (180 rpm). Formaldehyde was added at a concentration of 10 μL per mL to kill the bacterial cells; after 1 h, the broth was centrifuged at 8,000*g* (10,000 rpm) at 25°C for 15 min. The bacterial pellet was washed twice with sterile PBS and resuspended in 100 mL of buffer 1. The bacterial suspension was incubated in a water bath at 47°C for 15 min and then centrifuged at 8,000*g* (10,000 rpm). The pellet was resuspended in 10–15 mL of buffer 2, chilled on ice, and sonicated as described previously. The sonicated suspension was centrifuged at 3,000*g* (6,000 rpm) for 30 min at 4°C, and the supernatant was subjected to ultracentrifugation at 43,900 rpm for 90 min at 4°C (Sorvall ultra centrifuge model). Before and after ultracentrifugation, 250 μL of sample was collected for SDS–PAGE analysis. The pellet was resuspended finally in 1.5 mL of buffer 3, centrifuged again at 10,000 rpm for 10 min, and the supernatant was stored at −20°C until further use.

2.2.3. Preparation of Freeze and Thaw (FT) Antigen

Brucella abortus S99 from the stock culture was inoculated into 5 mL of BHI broth and incubated overnight in a shaker incubator with constant shaking (180 rpm) at 37°C. Three milliliters of overnight-grown culture was inoculated into 300 mL of BHI broth and incubated at 37°C with constant shaking. The overnight-grown culture was inactivated by

adding formalin (10 µL/mL of culture) and left at 37°C for 1 h. The inactivated broth was centrifuged in SORVELL centrifuge RC5C at 10,000 rpm for 15 min at 20°C and the pellet was washed twice with sterile PBS. The pellet was resuspended in storage buffer (pH 7.4) and the FT cycles were started by immersion of the suspension in liquid nitrogen. After immersion, the suspension was allowed to thaw gently and again frozen by immersion in liquid nitrogen. The F–T cycle was repeated for 15 times for complete lysis of bacterial cells. The suspension was centrifuged at 10,000 rpm for 30 min at 4°C and after centrifugation the supernatant was stored at −20°C until further use.

2.3. Protein Estimation of Antigens

The protein concentration was estimated by the Lowry's (1951) method (Siddiqui and Ahmed, 2002). The optical density was measured at 700 nm. The total protein of the sonicated antigen (SA), CE antigen, and FT antigen prepared was estimated to be 5.19, 3.19, and 1.28 mg/mL, respectively. The antigen was suspended in a storage buffer, aliquoted into 0.5 mL each, and stored at −20°C until further use.

2.4. Plate ELISA for Antibody Detection

Ninety-six-well microtiter plates or 8-well immunomodules (Nunc, Denmark) were coated with different antigens in a carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The wells were washed thrice with PBS, the free sites were blocked by 1% BSA, and the wells were incubated overnight at 4°C. After incubation, the plates were washed with PBS-T. Bovine samples were added to each well at a 1:1,000 dilution in PBS and incubated for 1 h at 37°C. The plates were washed with PBS-T three times and incubated with polyclonal rabbit anti-cow immunoglobulin/Horseradish peroxidase (HRP) (Dako) at a 1:1,000 dilution in PBS at 37°C for 1 h. The enzyme activity was determined by adding freshly prepared PBS–OPD–H_2O_2 substrate solution and the plates were incubated at room temperature for 4–5 min in the dark. The reaction was stopped by adding 25 µL of 1N H_2SO_4 to each well. The optical density was taken at 490 nm in Biotek µQuant ELISA reader.

2.5. Dot ELISA for Antibody Detection

For the detection of positive and negative bovine sera samples, all the three antigens were suspended in 200 µL of carbonate–bicarbonate coating buffer and 2 µL of this antigen was dotted at the center of the nitrocellulose membrane fixed on plastic combs (MDI, Ambala, Haryana, India) and allowed to dry at 37°C for 1 h. Nonspecific sites were blocked by 1% BSA in PBS overnight at 4°C. Combs were washed thrice with PBS and incubated with different samples at 1:100 sera dilutions in PBS for 1 h at 37°C. Washed thrice with PBS, combs were incubated for 1 h at 37°C with polyclonal rabbit anti-cow immunoglobulin/HRP at a 1:500 dilution in PBS. After incubation of the conjugate, the strips were again washed thrice with PBS and the reaction was developed in PBS–DAB–H_2O_2 substrate solution. Rinsing the combs in distilled water stopped the reaction.

3. RESULTS AND DISCUSSION

3.1. Characterization of Antigens

The different banding patterns of protein in the prepared antigen were resolved by SDS–PAGE through the Laemmli (1970) method. The protein banding pattern was different for all these three antigens and all these three antigens were run simultaneously on a single gel for comparison (Figure1). Several major protein bands with the corresponding

Figure 1: Comparative protein profiles of all the three different antigens.

Lane 1, molecular weight marker; Lanes 2,3, F&T antigen; Lanes 4,5, CE antigen; Lanes 6,7, sonicated antigen; Lane 8, molecular weight marker. CE: Cell envelope; F&T: Freeze and thaw.

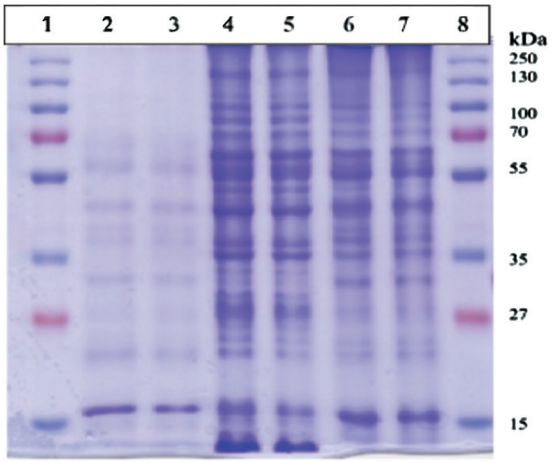

Figure 2: Immunoblot analysis of CE antigen of *Brucella abortus* S99 against positive and negative bovine sera samples.

molecular weight markers were observed for all three antigens. The resolved proteins were blotted onto nitrocellulose membranes, blocked with BSA, and probed with *Brucella* positive and negative sera to check for the reactivity of these proteins by Western blotting. Western blotting was done as described by Towbin and associates (1979) with some modifications. The prepared all three antigens reacted with the sera samples that are positive to *Brucella* antibodies as several bands were found to react with the major protein bands in the blots (Figure 2 shows the immunoblot analysis of CE antigen).

The blot was incubated in polyclonal rabbit anti-cow immunoglobulin/HRP (Dako) at a 1:500 dilution in PBS. The reaction was developed in 50 mM Tris (pH 7.6)–DAB–H_2O_2 substrate solution and stopped by rinsing in distilled water.

3.2. Standardization of Plate ELISA

Initially, different concentrations of the antigens were coated onto individual wells and reacted with different concentrations of positive and negative sera samples and a checkerboard titration was performed. The OD values of the positive and negative sera samples determined using checkerboard titration were compared and it was found that a concentration of 20 μg of SA per well, 22.5 μg of CE antigen, and 25 μg of FT antigen per mL were optimum.

3.3. Testing of Sera Samples by Plate ELISA

All the 50 bovine samples were tested by plate ELISA at a dilution of 1:1,000. All samples were run three times to confirm the reproducibility of the assay, and the mean values were used for calculations. The OD values observed at 490 nm are tabulated in Table 1. The negative cut-off value was worked out as 0.70. In comparison, the results showed that the CE antigen provided better results in comparison with RBPT and picked up more positive samples among the negative samples as plate ELISA is known to be more sensitive than RBPT (Table 2).

A true positive sample was defined as positive when results of both the tests were positive, and a true negative sample was defined as negative when results of both the tests were negative. False-negative samples were classified as those that were positive by the agglutination test but negative by ELISA, and false-positive samples were classified as those that were negative by the agglutination tests but positive by ELISA.

3.4. Standardization of Dot ELISA

Initially, dot ELISA with different concentrations of all the three antigens was performed on confirmed positive and negative sera samples. The optimum results were obtained with the coating of 0.625 μg of SA, 1.25 μg of CE antigen, and 0.600 μg of FT antigen in 2 μL of coating buffer per dot.

3.5. Testing of Sera Samples by Dot ELISA

The dot ELISA on all the 50 bovine samples was performed as described in the protocol. The samples were diluted at a concentration of 1:100 and the assay was performed at this concentration and the results tabulated were compared

Table 1: Plate ELISA OD at 490 nm against different *Brucella* antigens.

Sample no.	Sonicated antigen	Cell envelope antigen	Freeze and thaw antigen
1	3.086	1.308	2.075
2	2.402	1.796	1.561
3	2.027	1.011	1.065
4	1.762	1.108	1.582
5	0.931	0.697	0.922
6	1.945	1.191	1.311
7	2.110	1.203	1.179
8	2.694	1.689	1.727
9	0.972	0.746	1.132
10	0.410	0.453	0.459
11	0.467	0.424	0.423
12	2.042	0.787	0.823
13	0.020	0.853	0.775
14	0.423	0.252	0.179
15	1.332	1.248	1.277
16	1.347	0.980	1.115
17	1.014	1.199	1.398
18	0.786	1.200	1.132
19	0.697	0.272	0.572
20	0.519	0.509	0.827
21	0.644	0.359	0.474
22	0.931	0.810	0.537
23	0.598	0.883	0.974
24	0.476	0.855	0.933
25	0.696	0.587	0.348
26	2.344	1.652	2.659
27	0.567	0.569	0.595
28	0.987	0.692	0.725
29	0.549	0.399	0.520
30	1.770	1.496	1.657
31	1.108	0.793	0.948
32	0.628	0.668	0.517
33	1.731	1.880	2.326
34	0.693	0.642	0.485
35	1.163	1.692	1.724
36	0.330	0.294	0.148
37	0.392	0.558	0.458
38	0.554	0.837	0.625
39	0.438	0.655	0.812
40	1.337	1.229	0.660
41	1.809	1.969	2.346
42	0.126	0.497	0.347
43	0.227	0.544	0.614
44	0.0	0.0	0.031
45	1.376	1.974	2.406
46	0.422	0.608	0.378
47	0.768	1.238	0.606
48	0.791	1.093	0.531
49	1.101	0.797	0.418
50	0.845	0.980	0.420

OD: Optical density.

Table 2: Comparison between plate ELISA with different *Brucella* antigens and Rose Bengal plate agglutination test (RBPT) for the antibody detection against Brucellosis in bovine samples at a 1:1,000 dilution.

A. Sonicated antigen (SA)

	No. of samples
1. RBPT +ve plate ELISA (SA) +ve	21
2. RBPT +ve plate ELISA (SA) −ve	3
3. RBPT −ve plate ELISA (SA) +ve	7
4. RBPT −ve plate ELISA (SA) −ve	19

Negative cut-off OD <0.70.

B. Cell envelope (CE) antigen

	No. of samples
1. RBPT +ve plate ELISA (CE) +ve	22
2. RBPT +ve plate ELISA (CE) −ve	2
3. RBPT −ve plate ELISA (CE) +ve	8
4. RBPT −ve plate ELISA (CE) −ve	18

Negative cut-off OD <0.70.

C. Freeze and thaw (FT) antigen

	No. of samples
1. RBPT +ve plate ELISA (FT) +ve	19
2. RBPT +ve plate ELISA (FT) −ve	5
3. RBPT −ve plate ELISA (FT) +ve	8
4. RBPT −ve plate ELISA (FT) −ve	18

Negative cut-off OD <0.70.
OD: Optical density.

with the RBPT results in Table 3. In comparison, the dot ELISA results with the SA and CE antigens were comparable with the RPBT results than that of the FT antigen (Table 4).

Ever since Write and Smith developed the agglutination test for the detection of brucellosis in 1897, researchers are developing new methods and modifications to improve the sensitivity and specificity of this test. Several tests such as RBPT, serum (tube) agglutination test, and buffered antigen plate agglutination test were developed and tested. These tests use acidified antigens to reduce the agglutination by Immunoglobulin M (IgM), thereby reducing nonspecific false-positive reactions. RBPT is often used as a rapid screening test and is based on the agglutination of serum antibodies with a stained whole-cell preparation of heat-killed acidified bacteria. RBPT is performed by mixing on a glass plate a drop of RB reagent with an equal volume of serum and agglutination is read after 2–4 min. The sensitivity of RBPT is very high (>99%) but the specificity can be disappointingly low (Ruiz-Mesa *et al.*, 2005; Mantur *et al.*, 2006). So, the positive predictive value of the test is low and confirmation is needed by other specific tests. To increase the specificity and the positive predictive value of RBPT, the test may be applied to a serial dilution (1:2 through 1:64) of the serum sample. The specificity of the test increases when higher dilutions agglutinate and titers of 1:8 or 1:16 and above are considered positive, but this approach may result in a lower sensitivity.

The I-ELISA test has been used for some years with good results in terms of specificity and sensitivity in research (Diaz and Moriyon, 1989). In addition, when compared with the STAT, RBPT, Coombs test, and immunofluorescence assays, ELISAs were found to be simple, rapid, and reliable (Araj *et al.*, 2005). In the dot ELISA, the antigens coated on plate or on nitrocellulose in dot formats are developed and evaluated. In brucellosis, the IgM antibodies are present in high titers during the acute phase of the infection, whereas the IgG antibodies are present in high titers during the recovery phase of the infection. ELISA is used to differentiate between these two classes of antibodies and also to access the stage of illness. A serum that gives a positive result should be confirmed by a more specific test and the World Organisation for Animal Health (OIE, 2008) emphasizes that no single serological test is appropriate in all epidemiological situations. The sensitivities and specificities of indirect plate ELISAs as well as dot ELISA using native antigens were calculated. The sensitivities of the indirect plate ELISA were 87.50%, 91.67%, and 79.17% for SA, CE, and FT, respectively, whereas the specificities were 80%, 80%, and 74% for SA, CE, and FT, respectively. The sensitivities were 91.67%, 91.67%, and 87.50% and specificities were 88.46%, 92.31%, and 84.62% for indirect dot ELISA for SA, CE, and FT, respectively. Correlations of ELISAs with RBPT tests using all antigens were also calculated and are shown in Table 5.

Table 3: Dot ELISA results against different *Brucella* antigens.

Sample no.	Sonicated antigen	Cell envelope antigen	Freeze and thaw antigen
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	−-	−	−
6	+	+	+
7	+	+	+
8	+	+	+
9	−	−	−
10	−	−	−
11	−	−	−
12	+	+	+
13	−	−	−
14	−	−	−
15	+	+	+
16	+	+	+
17	+	+	+
18	+	+	+
19	−	−	−
20	−	−	−
21	−	−	−
22	−	−	−
23	+	+	+
24	−	−	−
25	−	−	−
26	+	+	+
27	−	−	−
28	−	−	+
29	−	−	−
30	+	+	+
31	+	−	+
32	−	−	−
33	+	+	+
34	−	−	−
35	+	+	+
36	−	−	−
37	−	−	−
38	−	−	−
39	−	−	−
40	+	+	−
41	+	+	+
42	−	−	−
43	−	−	−
44	−	−	−
45	+	+	+
46	−	−	−
47	+	+	+
48	+	+	+
49	+	+	+
50	+	+	+

Table 4: Comparison between dot ELISA with different *Brucella* antigens and Rose Bengal plate agglutination test (RBPT) for the antibody detection against Brucellosis in bovine samples at a 1:100 dilution.

A. Sonicated antigen (SA)

	No. of samples
1. RBPT +ve Dot ELISA (SA) +ve	22
2. RBPT +ve Dot ELISA (SA) −ve	2
3. RBPT −ve Dot ELISA (SA) +ve	3
4. RBPT −ve Dot ELISA (SA) −ve	23

B. Cell envelope (CE) antigen

	No. of samples
1. RBPT +ve Dot ELISA (CE) +ve	22
2. RBPT +ve Dot ELISA (CE) −ve	2
3. RBPT −ve Dot ELISA (CE) +ve	2
4. RBPT −ve Dot ELISA (CE) −ve	24

C. Freeze and thaw (FT) antigen

	No. of samples
1. RBPT +ve Dot ELISA (FT) +ve	21
2. RBPT +ve Dot ELISA (FT) −ve	3
3. RBPT −ve Dot ELISA (FT) +ve	4
4. RBPT −ve Dot ELISA (FT) −ve	22

Table 5: Comparison of sensitivities and specificities of plate enzyme-linked immunosorbent assay (ELISA) and dot ELISA using different native antigens with Rose Bengal plate agglutination tests (RBPTs) (at 95% confidence level).

	SA		CE		FT	
Calculations	**PlateELISA vs. RBPT**	**Dot ELISA vs. RBPT**	**Plate ELISA vs. RBPT**	**Dot ELISA vs. RBPT**	**Plate ELISA vs. RBPT**	**Dot ELISA vs. RBPT**
Sensitivity (%)[a]	87.50	91.67	91.67	91.67	79.17	87.50
Specificity (%)[b]	73.08	88.46	69.23	92.31	69.23	84.62
Correlation (%)[c]	80.00	90.00	80.00	92.00	74.00	86.00
Positive predictive value (%)[d]	75.00	88.00	73.33	91.67	70.37	84.00
Negative predictive value (%)[e]	86.36	92.00	90.00	92.31	78.26	88.00
Likelihood ratio for positive result[f]	3.25	7.94	2.98	11.92	2.57	5.69
Likelihood ratio for negative result[g]	0.17	0.09	0.12	0.09	0.30	0.15

SA: Sonicated antigen; CE: Cell envelope; FT: Freeze and thaw.
a: sensitivity (%) = [true positives/(true positives + false negatives)] × 100.
b: specificity (%) = [true negatives/(true negatives + false positives)] × 100.
c: correlation (%) = [(number of samples positive by both tests + number of samples negative by both tests)/total number of samples] × 100.
d: positive predictive value (%) = [true positives/(true positives + false positives)] × 100.
e: negative predictive value (%) = [true negatives/(true negatives + false negatives)] × 100.
f: likelihood ratio for positive result = sensitivity/1 – specificity.
g: likelihood ratio for negative result = specificity/1 – sensitivity.

4. CONCLUSION

Several ELISA systems were developed to improve the specificity, but at the same time high sensitivity should be maintained. I-ELISA was a better serological test compared with RBPT and STAT in the sense of sensitivity, specificity, and rapidity (Sadhu *et al.*, 2015). In the present study, all 50 bovine samples collected from suspected bovine animals were

tested by plate as well as in dot ELISA formats for all the three antigens prepared. The CE antigen was found to be more suitable overall as it had the maximum agreement with the RBPT results in both formats (plate ELISA and dot ELISA) followed by the SA and the least agreement was found with that of the FT antigen. When dot ELISA results were compared with those of plate ELISA, dot ELISA was found to be more sensitive as well as more specific than plate ELISA. In addition, ELISA is less time consuming and the results can be quantitatively analyzed using an ELISA reader. The dot-blot ELISA assay also had a high correlation, sensitivity, and specificity in comparison with RBPT and plate ELISA. The dot-blot format will be highly suitable for field use to provide a cost-effective test with prompt results where laboratory facilities and equipment such as ELISA readers are not available. Further results with these native antigens can be compared with several recombinant antigens mostly the outer membrane proteins as these recombinant proteins are known to have high specificity and will not cross-react with nonspecific antibodies. The development of antigen detection systems with the antibodies raised against these recombinant antigens will also be of help in the control of brucellosis. In conclusion, further studies with the recombinant *Brucella* antigens will further improve the sensitivity and specificity of this test system. It will help in the screening of clinical samples of human brucellosis as well as animal brucellosis.

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