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AN EVALUATION OF ELISA USING RECOMBINANT P17 ANTIGEN FOR CATTLE BRUCELLOSIS

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ABSTRACT

Brucellae are Gram-negative cocccobacilli, facultative intracellular bacterial pathogens of both humans and animals. Brucellosis is an important disease that is difficult to diagnose and treat that causes heavy economic losses and human suffering. Diagnosis of brucellosis plays a vital role for control and prevention of the disease. Lipopolysaccharide (LPS) based Enzyme Linked Immuno Sorbent Assay (ELISA) shows false positivity due to cross-reactivity with other gram-negative bacteria LPS. The present investigation was undertaken to assess the diagnostic potential of the recombinant P17 protein of *Brucella.* P17 gene of *Brucella abortus* (*B. abortus*) was amplified, cloned and subcloned into pQE 30 vector yielding high levels of protein expression. The purified recombinant P17 (rP17) protein was used to develop an indirect ELISA (i-ELISA) test for brucellosis. The rP17-ELISA was compared with RBPT (Rose Bengal Precipitation Test) and LPS-ELISA using 530 cattle sera. The concordance percentage and kappa statistics of P17-ELISA is greater in compression with LPS-ELISA. Relative sensitivity and relative specificity of P17-ELISA shows a positive trend with RBPT. The data suggest that P17-ELISA can be a useful method for *Brucella* diagnosis and recombinant P17 protein is a potential antigen for diagnosis of cattle brucellosis.

Keywords: Brucellosis, Recombinant P17 antigen, ELISA, Cattle, RBPT, Diagnosis

INTRODUCTION

Brucella is a facultative intracellular zoonotic bacterium causing chronic disease resulting in a substantial economic loss worldwide **(Im *et al*., 2016; Assenga *et al*., 2015).** *Brucella* infection arises from occupational and domestic contact with infected animals or their discharges. Brucellosis causes abortion in female livestock's (cattle, sheep, and pigs) and in males; it causes orchitis **(Neta *et al*., 2010)**. Although *brucellosis* occurs throughout the world, it is most prevalent in the Middle East, Africa, Russia, India, South America, and Southern Europe and Latin American countries. Among all the species of *Brucella*, *B. abortus* causes brucellosis in cattle commonly, whereas cattle kept in close association with sheep or goats gets brucellosis by *B. melitensis* **(Lopes *et al*., 2010).**
Twelve species of the genus *Brucella* have been identified using antigen (Ag) variation and primary host **(Sung *et al*., 2014).** Diagnosis of brucellosis is based on serological tests like Standard Tube Agglutination Test (STAT), Complement Fixation Test (CFT), the Coombs test etc. were appropriate for all situations. For animal screening Herds Rose Bengal Plate Test (RBPT) and Buffered Plate Agglutination Test (BPAT) are more suitable. Molecular techniques like the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) techniques are used to differentiate species and strains within the genus *Brucella* **(Yu *et al.*, 2010; Nielsen *et al.*, 1995).** The Enzyme-Linked Immunosorbent Assay (ELISA) can be used for screening and confirmation of brucellosis in one step while CFT is excellent in terms of sensitivity and specificity (**Nielsen *et al.*, 1995; Wright *et al.*, 1997)**. Antibodies produced against *Brucella* smooth- Lipopolysaccharides (LPS) antigen, which cross-reacts with LPS of *E. coli* O157 **(Stuart *et al*, 1982),** *Stenotrophomonas maltophilia* **(Corbel *et al.*, 1984),** and *Yersina enterocolitica* O:9, leading to false positivity. Various diagnostic proteins of *Brucella* has been known, which includes the 89 kDa protein, Outer membrane proteins (OMPs) like OMP36, 28 , 16, 10, Cell surface proteins like 31.6, 32.5, 58.5 and 14.7 kDa (**Kaushik *et al.*, 2009)**, periplasmic protein BP26 (Kumar *et al* ., 2008) and cytoplasmic proteins P39, P17 and P15 . P17 is a cytoplasmic protein and thought to be antigenic in nature and also a potential candidate for the diagnostic purpose **(Letesson *et al.*, 1997; Hemmen *et al*., 1995; Büyüktanir et *al*., 2011)**. The present work has been envisaged to express the P17 gene of *B. abortus* and to evaluate the diagnostic potential of recombinantP17 protein using indirect ELISA.

MATERIALS AND METHOD

Bacterial Strains, Growth Conditions, and Vectors

Escherichia coli DH5α as the host and pTZ57R/T cloning vector (Thermo Scientific, Fermentas) was used as the cloning vector for the cloning of the gene encodes P17 derived from genomic DNA of *B. abortus* (S19) from Indian Veterinary Research Institute (IVRI), izatnagar, India. The protein expression vector was pQE30 (Qiagen, USA), and *E. coli* Novablue (Novagen) strain was used as the screening host for pQE30 bearing inserts. Bacterial strain for expression of recombinant protein was *E. coli* BL21 (Novagen). All the *E. coli* bacterial cultures were grown at 37°C on Luria–Bertani (LB) agar plates or in LB medium. Where appropriate, media was amended with various substrates and 100 µg ampicillin/ml.

PCR amplification and cloning P17 F and P17 R

B. abortus strain (19) gene (which will encodes outer membrane protein MWt 17 kDa) was amplified by PCR, using a set of primers P17 F and P17 R (5'- CGG GGATCC ATGAACACTCTGGCTAGCAAT-3' and 5-CGGC AAGCTT TTACTTGATTTCAAAAACGAC-3') designed from the available nucleotide sequence accession number DQ437516.1. A high fidelity Novagen KOD XL DNA polymerase was used for DNA amplification, which was carried out with 30 cycles of denaturation (60 s at 94 °C), annealing (1 min at 55 °C) and extension (1 min at 72 °C), followed by 10 min of further extension at 72 °C. The PCR amplified product was separated by agarose gel, purified by gel elution and ligated into the pTZ57R/T cloning vector according to the manufacturer's instructions and then transformed into *E. coli* DH5α. The pTZ57R/T vector carrying the P17 gene was digested with the enzyme Bam H1 and Sal I, and the produced fragment was gel-purified before subcloning into an expression vector. The target DNA fragment was sub-cloned at the Bam H1 and Sal I site of pQE30vector, resulting in pQEP17. The vectors thus obtained were transformed into *E. coli* BL21 for fusion protein expression.

Induction, purification, and immunoblot assays of recombinant protein

E. coli cells harboring pQEP17 plasmid were grown in Luria-Bertani (LB) medium till the OD600nm reached to 0.5. The cells were then induced with 1 mM IPTG and allowed to grow further for 6h at 37^0C. Cells were harvested and analyzed using Sodium dodecyl sulfate Polyacrylamide gel electrophoresis (SDS-PAGE). Ni-NTA agarose purified protein was analyzed on SDS-PAGE and transferred to nitrocellulose membranes in a semi-dry transblot system (Atto, Japan). Transferred proteins were immunostained with field sera at a dilution of 1:200. Secondary antibodies conjugated to horseradish peroxidase (Sigma) and were used in the assays. The reactions were developed with Diamino Benzidine (Sigma) and Hydrogen peroxide (H_2O_2).

Bioinformatic analysis

The obtained DNA sequence was translated in EMBL database and obtained protein was analyzed in protean of DNASTAR.7.0.

Extraction and quantification of LPS

Extraction of *B. abortus* S19 Smooth-LPS was extracted by the hot phenol-water method. In brief, 50g wet weight of cells was suspended in 170ml of distilled water, followed by the addition of 190ml of 90% (v/v) hot phenol (66°C). After 30mins, the mixture was centrifuged and phenol layer was removed. The LPS in the resultant mixture was precipitated by cold methanol (4°C) and dissolved in 0.1M tris buffer. Proteinase K (50µg per 10mg protein) and then both DNase and RNase (50µg per 1mg nucleic acid) added to extracted samples to reduce protein and nucleic acid contaminations. The antigen was run in SDS-PAGE and stained using silver staining followed by quantification using the phenol- sulphuric acid method in microplate format **(Salmani et *al.*, 2008).**

Recombinant p17- ELISA

Clinical sera from cattle were analyzed by indirect ELISA using recombinant P17 as test antigen. The immunoassay plates (Maxisorp, Nunc, Denmark) were coated with purified recombinant P17 protein at a concentration of 100 ng per well, diluted in 0.1 M bicarbonate buffer (pH 9.0) and incubated at 4 ^{0}C, overnight. The wells were washed five times with phosphate buffer saline-Tween20 (PBST) and then blocked with 5% BSA. Immunoassay plates were charged with sera at a dilution of 1:100 and incubated at 37^0C for 1 h. After washing with PBST for five times the plates were incubated with HRPO conjugates for 1 h at 37^0C. After washing with PBST, the wells of immunoassay plates were fill with a substrate solution containing Ortho-phenyl diamine (OPD) and H2O2. Colour development was stopped by adding 2 M H2SO4, after 10 min of incubation of the plates in dark at room temperature. Absorbance was recorded at 490 nm wavelength in an ELISA reader.

Rose Bengal plate agglutination test

The RBPT was performed on a glass plate using 30µl of antigen and 30 µl of serum. Both serum and antigen are mixed thoroughly. Any degree of agglutination within 3 min was taken as positive. RBPT antigen was procured from Division of Biological products, Indian Veterinary Research Institute (IVRI), Izatnagar.

Evaluation of RBPT and rP17ELISA, and rP17 ELISA and LPS ELISA

The relative sensitivity, specificity, Positive predictive value (PPV), Negative predictive value (NPV) and accuracy of recombinant P17 ELISA for serodiagnosis of brucellosis were evaluated in comparison to RBPT as well as LPS ELISA as described below **(Chaudhuri *et al.*, 2010).,**

$$\text{Relative sensitivity} = a/(a+c) \times 100$$

$$\text{Relative specicity} = d/(b+d) \times 100$$

$$\text{Accuracy} = (a+d)/(a+b+c) \times 100$$

$$\text{PPV} = a/(a+b)$$

$$\text{NPV} = d/(c+d)$$

$$\text{Apparent prevalence} = a+b/N \times 100$$

$$\text{True prevalence} = a+c/N \times 100$$

$$\text{Accuracy} = a+d/N \times 100, \text{ where}$$

'a' is the number of sera positive by ELISA and RBPT
'b' is the number of sera negative by RBPT but positive by ELISA
'c' is the number of sera positive by RBPT but negative by ELISA
'd' is the number of sera negative by ELISA and RBPT
'N' is the total number of samples (a + b + c + d)

Statistical analysis of RBPT and ELISA

Concordance and Kappa statistics of rP17ELISA with a well as LPS ELISA were assessed as described as follows **(Gwida *et al.*, 2011)**

RESULTS

Construction and expression of P17 Gene.

The P17 gene was successfully amplified from the *B. abortus* S19 genomic DNA. The obtained PCR product of 442 bp was cloned to the and pTZ57R/T cloning vector for DNA sequence analysis and then *Bam* HI and *Sal* I digested P17 fragment was subcloned at *Bam* HI and *Sal* I cleaved pQE30 expression vector. In this recombinant plasmid, the P17 sequence was fused with 6x His tag and was tightly regulated by a T7 promoter. All constructs were confirmed by restriction analysis and DNA sequencing. The nucleotide sequence reported in this paper has been deposited in the GenBank DNA databases with the Genbank Id: KJ702467.1. The resulting clone was named pQEP17 recombinant plasmid.

Analysis of recombinant P17 protein by SDS-PAGE and Western Blot

E. coli cells harboring the recombinant plasmid pQEP17 was induced with 1M IPTG for overnight produced a predominant band similar to 17kDa protein on 15% SDS-PAGE (Fig 1, Lane-2). No such protein band was observed in uninduced *E. coli* cells harboring recombinant plasmid pQEP17 (Fig 1, Lane-1). Histidine-tagged expressed recombinant protein was purified using Ni-NTA beads (affinity chromatography) produced a single band of 17kDa protein on SDS-PAGE (Fig 1, Lane-3).
The Polyclonal serum was raised in rabbits, by injecting the purified rp17 protein with 2 to 3 boosters. Polyclonal serum against rP17 protein could bind to expressed pQEP17 protein (Fig 2, Lane-2), as well as purified E. coli, expressed 17kDa protein (Fig 2, Lane-1), as detected by Western blot analysis.

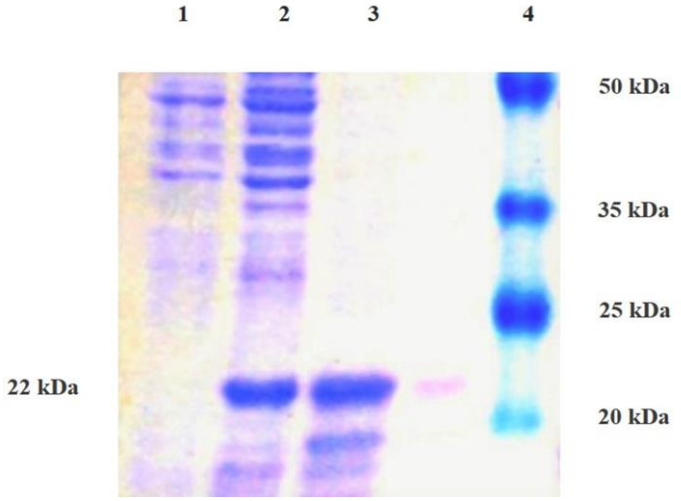

Figure 1SDS–PAGE analysis of recombinant P17 kDa expressed protein in *E. coli*
Lane 1, Uninduced *E. coli* cells containing recombinant pQEP17 insert
Lane 2, Induced *E. coli* cells containing recombinant pQEP17 insert
Lane 3, Purified P17 kDa protein
Lane 4, Pre-stained protein Marker

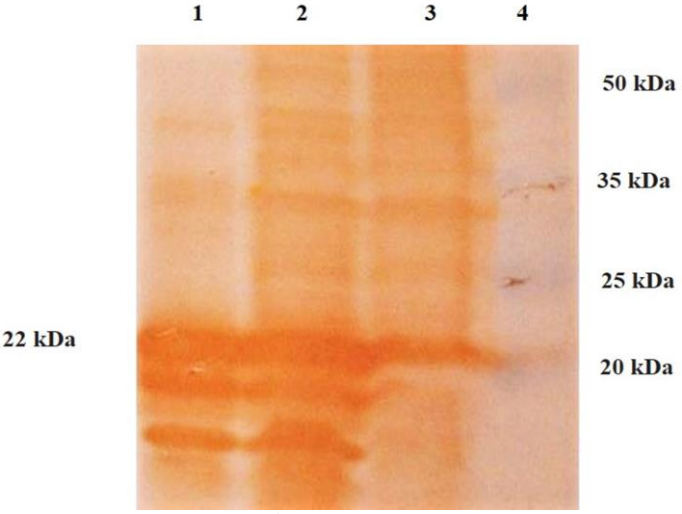

Figure 2 Western Blot analysis of recombinant P17 kDa protein with anti-P17 specific serum
Lane 1, Purified P17 kDa protein
Lane 2, Induced *E. coli* cells containing recombinant pQEP17 insert
Lane 3, Uninduced *E. coli* cells containing recombinant pQEP17 insert
Lane 4, Pre-stained protein Marker

rP17Elisa standardization and comparison with RBPT, LPS ELISA

The recombinant protein and LPS were used at the concentration of 62.5 ng and 125 ng respectively per well, and at a serum dilution of 1:100, the test got standardized **(Tiwari *et al*., 2013)**. Twenty known positives and twenty-five known negative samples were taken as gold standard and recombinant P17-ELISA (rP17-ELISA) and LPS-ELISA was performed (Table 1). The mean value of sera at OD490nm + 3 SD (standard deviation) was considered as cut-off value for declaring a serum as positive or negative for brucellosis. The ELISA test results of rP17, RBPT, LPS were shown same results with gold standard samples indicates that the rP17 ELISA is accurate in compression with the other methods.

Table 1Comparison of rP17 ELISA with RBPT and LPS ELISA

	Known Positives (20 nos)	Known Negative (25 nos)
rP17 ELISA	20	25
RBPT ELISA	20	25
LPS ELISA	20	25

Evaluation of RBPT and rP17ELISA, and rP17 ELISA and LPS ELISA

The study has been conducted with 530 unknown samples to evaluate the rP17 ELISA sensitivity and specificity with the RBPT and LPS ELISA. The rP17-ELISA results showed (86.06%) relative sensitivity and (93.62%) relative specificity on comparison with RBPT (Table 2). Negative predictive value was (95.02%), while positive predictive value was (80.15%) (Table 2) between RBPT and rP17ELISA. The accuracy of prediction was 91.88% (Table 2) between RBPT and rP17-ELISA. rP17-ELISA showed (84.28%) relative sensitivity and (93.60%) on comparison with LPS-ELISA (Table 3). The accuracy of prediction was 91.32% between rP17-ELISA and LPS-ELISA. Negative predictive value was (94.32%), while positive predictive value was (83.09%) (Table 3). RBPT and LPS- ELISA showed the sensitivity of (84.28%) and specificity of (93.60%). (Table 4)

Table 2 Comparison of RBPT and P17-ELISA in diagnosis of cattle brucellosis

Serological test		RBPT		
		Positive	Negative	Total
P17-ELISA	Positive	105 (a)	25 (b)	130 (a+b)
	Negative	20 (c)	380 (d)	400 (c+d)

Relative Sensitivity $= a/a+c\times100 = 86.06\%$

Relative Specificity $= d/b+d\times100 = 93.62\%$

Positive Predictivevalue $= a/a+b\times100 = 80.15\%$

Negative Predictivevalue $= d/c+d\times100 = 95.02\%$

Apparent Prevalence $= a+b/N\times100 = 24.71\%$

True Prevalence $= a+c/N\times100 = 23.01\%$

Accuracy of prediction $= a+d/N\times = 91.88\%$

Table 3 Comparison of LPS-ELISA and P17-ELISA in diagnosis of cattle brucellosis

Serological test		LPS-ELISA		
		Positive	Negative	Total
P17-ELISA	Positive	118 (a)	24 (b)	142 (a+b)
	Negative	22(c)	366 (d)	388(c+d)

Relative Sensitivity $= a/a+c\times100 = 84.28\%$

Relative Specificity $= d/b+d\times100 = 93.60\%$

Positive Predictivevalue $= a/a+b\times100 = 83.09\%$

Negative Predictivevalue $= d/c+d\times100 = 94.32\%$

Apparent Prevalence $= a+b/N\times100 = 26.79\%$

True Prevalence $= a+c/N\times100 = 23.41\%$

Accuracy of prediction $= a+d/N\times = 91.32\%$

Table 4 Comparison of RBPT and LPS -ELISA in diagnosis of cattle brucellosis

Serological test		RBPT		
		Positive	Negative	Total
LPS-ELISA	Positive	113(a)	22(b)	135 (a+b)
	Negative	6(c)	389(d)	395 (c+d)

Relative Sensitivity $= a/a+c\times100 = 94.95\%$

Relative Specificity $= d/b+d\times100 = 94.64\%$

Positive Predictivevalue $= a/a+b\times100 = 94.95\%$

Negative Predictivevalue $= d/c+d\times100 = 98.48\%$

Apparent Prevalence $= a+b/N\times100 = 25.47\%$

True Prevalence $= a+c/N\times100 = 22.45\%$

Accuracy of prediction $= a+d/N\times = 94.71\%$

Statistical analysis of RBPT and ELISA'S

Concordance was maximum between P17-ELISA and LPS- ELISA, which was (94.71%), followed by RBPT and P17-ELISA test with a concordance of (91.40%). Minimum concordance of (91.32%) was observed between RBPT and LPS-ELISA. In Kappa statistics, LPS- ELISA and P17-ELISA showed almost perfect agreement as the value observed was .833. (Table 4)

Table 4 Concordance and Kappa statistics between different tests for cattle brucellosis

Test 1	Test 2	Concordance (%)	Kappa value
RBPT	**P17-ELISA**	91.40	0.778
RBPT	**LPS-ELISA**	91.32	0.871
P17-ELISA	**LPS-ELISA**	94.71	0.833

DISCUSSION

Brucella diagnosis is based on the detection of antibodies generated against immunodominant antigenic molecules using serological methods. The RBPT is the commonly used test at field levels, based on the agglutination of antibodies. Although RBPT sensitivity is high but has low specificity also. *E. coli* O: 159, *Y. enterocolitica* O: *Vibrio cholera*, and *Salmonella sp*. show cross-reactivity with other clinically important bacterias leading to false positive results in various tests (**Im *et al*., 2016)**. Different ELISA models and FPA have been developed using LPS and O-polysaccharide as diagnostic antigen **(Nielsen *et al*., 1997; Genc *et al*., 2011; Saegerman *et al*., 2004; Munoz *et al*., 2005).** Similar epitope sharing between LPS of *Brucella* and that of other Gram-negative bacteria is the major cause of cross-reactivity in brucellosis infection (**Kaltungo *et al*., 2014; Godfroid ., 2010).**

To overcome this problem, efforts have been made to improve the serodiagnosis of brucellosis by replacing the native antigens with highly purified specific recombinant antigens. It is expected that the recombinant protein will have less cross-reactivity and might act as better potential diagnostic antigens in animal brucellosis **(Thavaselvam, *et al*., 2010)**. The recombinant-based serological test showed high sensitivity and specificity owing to the high concentration of immunoreactive antigens and lack of non-specific molecules present in the whole cell preparation. Few cytoplasmic Ags of *Brucella* P17, P15, and P19 has shown to be antigenic in nature **(Letesson *et al*., 1997)**. Hence, in the present study, the rP17 protein was expressed, purified and rP17 ELISA was standardized and compared with LPS ELISA and RBPT. DNA encoding the P17 protein was cloned, sequenced and the amino acid sequence of rP17 was deduced. The obtained accession number is KJ702467.1. The blast results in NCBI matched with lumazine synthase of *Brucella*, a cytoplasmic protein **(Berguer *et al*., 2012)**. Tthe sequence was analyzed using Protean in DNA Star program showed that the protein has 6.933 isoelectric Point and negatively charged at pH-7.0. The P17 protein had both 4 alpha helix and 1 beta regions, the alpha region could play an important role in antigenicity. Molecular Weight of the protein was predicted as 17354.92 Daltons containing 158 amino acids. The protein had 15 Strongly Basic(+) Amino Acids (K,R), 17 Strongly Acidic(-) Amino Acids (D,E), 71 Hydrophobic Amino Acids (A,I,L,F,W,V) and 27 Polar Amino Acids (N,C,Q,S,T,Y) **(Goldbaum *et al* 1999; Bagath *et al*., 2015).**

The P17 protein was expressed in the form of inclusion bodies, which is a common feature when proteins are expressed at very high level. Nickel –NTA agarose resin, was used to purify the expressed P17 protein having Histidine tag at the N-terminal end which is similar to other recombinant *Brucella* protein

purification **(Letesson *et al*., 1997)**. Reactivity of cattle sera with rP17 in western blotting indicated the similarity of reports published by Hemmen *et al.*, and Letesson *et al.*, in the year 1995 and 1997 respectively**.** ELISA is more sensitive than conventional tests, hence it is regarded as meritorious over RBPT in the diagnosis of brucellosis. However, RBPT has less specificity leading to false positivity **(Chaudhuri *et al*., 2010).** Moreover, ELISA assays were reported to be more sensitive, to give positive results sooner after infection in non-vaccinated animals and to be more persistent than the traditional serological tests in both experimentally and naturally infected animals **(Saegerman *et al*., 2004)**.

Comparison of rP17-ELISA with LPS-ELISA and RBPT, rP17-ELISA showed (24.52%) seropositivity while LPS-ELISA showed (27.73%) seropositivity, for a total of 530 sera samples. The reason why LPS-ELISA showed more seropositivity is due to the presence of high cross-reacting antibody produced against LPS with other gram-negative bacteria **(Tiwari *et al*., 2011)**. RBPT also showed more seropositivity when compared with rP17-ELISA, due to the use of the whole cell antigen for agglutination reaction **(Tiwari *et al*., 2011)**. The RBPT should be used in combination with iELISA as rapid, simple and easy methods for screening (**Ahmed *et al*., 2016)**. Concordance between rP17-ELISA and RBPT was higher than concordance between LPS-ELISA and RBPT. In laboratories, the iELISA for brucellosis diagnosis could also be used as a single diagnostic test, where the ELISA technique is already used for the diagnosis of other diseases. The agreement between rP17-ELISA and LPS-ELISA was significant, as determined by the Kappa index of concordance of 0.83 between tests, a similar finding was observed by Gwida in the year 2011. Further, P17 coated in the ELISA plates and was able to react even after 6 months of storage at 4^0C, which showed the stability of the protein to sustain for a longer period of time at lower temperature. In the present study, we have used 17 kDa proteins (P17) of *B. abortus* strain (19) for identification of anti-*Brucella* antibodies in the sera collected from different parts of the country. Our result was in accordance with previous reports describing the usefulness of this protein for detecting anti-*Brucella* antibodies to cattle **(Kumar *et al*., 2008)**. Both the Kappa value and the Concordance percentage of rP17-ELISA with LPS-ELISA were almost perfect and had maximum agreement respectively. Relative sensitivity and relative specificity of rP17-ELISA was 86.06 % and 93.62%, respectively, when compared with LPS-ELISA. Relative sensitivity and relative specificity of P17-ELISA was also on a positive trend with RBPT.

In conclusion, it can be inferred that the recombinant P17 protein was successfully expressed in *E. coli*. The yield of recombinant P17 protein was high, which will make the test cost effective. The tendency of the protein to remain stable for months makes P17 a suitable candidate in the field. Both Kappa value and concordance percentage of P17-ELISA with LPS-ELISA were perfect and had maximum agreement respectively. Relative sensitivity and relative specificity of P17-ELISA was 86.06 % and 93.62% respectively when compared with LPS-ELISA. Based on the results obtained, it can be concluded that recombinant P17 protein is a potential candidate antigen for serodiagnosis of cattle brucellosis.

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Conflict of interest: The authors of this paper declare that they have no conflict of interest.

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