



## Determination of Herd Prevalence of Brucellosis using Rose Bengal Plate Test and Indirect ELISA

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### ABSTARCT

The present study was aimed at diagnosis of brucellosis in cattle and buffalo using RBPT and iELISA and comparing the two tests. The animals belonged to a cattle shelter house (Gaushala). Out of 303 sera samples collected, 125 (41.25 %) were positive by RBPT and among 125 positive samples, 2 were of male and other 123 were of female animals. On the other hand the results of ELISA were tripartite and 142 (46.86%), 22 (07.26 %) and 139 (45.54%) samples were found as positive, moderately positive and negative, respectively. Overall by combining the both type of positive results, 164 (54.12%) samples were found positive by iELISA. On herd level, this was much higher than reports from most of earlier workers. On taking iELISA as reference test, the sensitivity and specificity of RBPT were calculated as 80.78% and 100%, respectively and the level of agreement between two tests was 0.871. But three categories of variations were observed between two tests, *i.e.* eighteen samples were positive in RBPT but negative in ELISA, 49 were negative in RBPT but positive in ELISA and 12 were negative in RBPT but moderately positive in ELISA.

**Keywords:** Brucellosis, RBPT, ELISA, Bovines, Sensitivity.

Brucellosis is a major reproductive affliction of cattle and buffalo caused by biovars of *Brucella abortus* and sometimes by biovars of *Brucella melitensis* (Jimenez *et al.*, 1991). Bacteria remain usually asymptomatic in nonpregnant female but infection with *B. abortus* or *B. melitensis*, pregnant adult females result in abortion due to development of a placentitis. Even in the absence of abortion, profuse excretion of organisms occur in the placenta, fetal fluids and vaginal discharges. As WHO classified Brucellosis as a category III risk organism, it is readily transmissible to humans and veterinarians are particularly vulnerable due to their frequent exposure with bovine abortion cases (OIE, 2012).

The clinical picture of brucellosis is not pathognomonic, and unequivocal diagnosis of *Brucella* infections can be made only by the isolation and identification of *Brucella*, but due to risk of laboratory acquired infection bacteriological examination is discouraged and the diagnosis is established

using serological methods (Yohannes *et al.*, 2012). As per description of OIE manual for the control of brucellosis at the national or local level, the buffered *Brucella* antigen tests (BBATs), *i.e.* the Rose Bengal test (RBPT) and the buffered plate agglutination test (BPAT), as well as the ELISA and the FPA, are suitable screening tests. RBPT is though a simple and economical test, it is generally considered to be less sensitive than other serological tests like standard tube agglutination test (STAT), complement fixation test (CFT) and enzyme linked immunosorbant assay (ELISA). So, an adjunct test is necessary to affirm the diagnosis and out of many possible alternatives, use of ELISA has been claimed to be a good screening test, particularly, in combination with the RBPT (Jacques *et al.*, 1998).

In India, the sero prevalence of the disease has been described as 8.8 per cent whereas in Gujarat state, the location of present studies, it was found at 8.7 per cent



(Renukaradhya *et al.*, 2002). Unlike the developed countries this figure is very high, primarily due to economical and religious constraints over culling of affected animals. Hence routine surveillance of brucellosis is of great importance from economical as well as public health point of view.

Considering the importance of brucellosis and complexity of diagnosis through serological testing, use of RBPT and ELISA is described for detection of anti-*Brucella* antibodies along with their relative efficacies upon each other.

**Table 1.** Summary of tests applied for Brucellosis Detection (n=303)

S. No.	Test applied	Negative	Moderately Positive	Positive	Total
1	RBPT	178 (58.75 %)	-	125 (41.25 %)	303 (100 %)
2	iELISA	139 (45.87 %)	22 (07.26%)	142 (46.86 %)	303 (100 %)

## MATERIALS AND METHODS

### Sample Collection

A total number of 303 cattle and buffalo sera were collected from adult animals which belonged to a cattle shelter house (locally called panjarapole or gaushala), where animals of uncertain production values were kept. There was no record of *Brucella* S-19 vaccination in the herd. Out of 303 samples, 14 were from male animals and rest 289 were of female animals.

### Rose Bengal Plate Test (RBPT)

RBPT was carried out as per technique described in OIE terrestrial manual with slight modifications (OIE, 2012). RBPT antigen and brucellosis positive serum were procured from Biological Product division, Indian Veterinary Research Institute, Izatnagar and used to set positive control. The test reactions were carried out on 12 wells cavity slide where equal volume of antigen and test serum ( 30 µl each ) were placed as separate drop

and mixed with a tooth pick. Development of visible clumps was considered as positive reaction. Grading of agglutination was not attempted.

**Table 2.** Variations of results between RBPT and ELISA

S. No	Category of Results	Number of Samples
1	Positive in RBPT and Negative in ELISA	18
2	Negative in RBPT and Positive in ELISA	49
3	Negative in RBPT but Moderately Positive in ELISA	12

### Indirect Enzyme Linked Immuno Sorbent Assay ( iELISA )

iELISA kit was obtained from National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru and used as per instructions supplied along with the kit . Briefly, after reconstitution in coating buffer, 100 µl of recombinant antigen was dispensed in each well of 96 well microtitre plate (Nunc, Denmark and supplied with the kit) and incubated at 4°C overnight. Wells were blocked with gelatin buffer for one hour at room temperature, after washing and removal of unbound antigen. Then plate was washed again thrice and used on the same day. Control and test sera were diluted as per instruction and put in to respective well as quadruplicate and duplicate, respectively. After one hour incubation at room temperature, again three washings were given and appropriately diluted conjugate was applied to each well. Next to this step, 1 hour incubation at room temperature was provided and plates were washed three times to remove any unbound conjugate. Substrate (3% H<sub>2</sub>O<sub>2</sub>) and chromogen (5 mg OPD, supplied as tablet) were mixed in 25 ml DW and used at the volume of 100 µl. After 10 min, the reaction was stopped with 2M sulphuric acid and absorbance was read at 492 nm using TEKAN micro plate reader. The per cent positivity (PP) was calculated as per formula given by manufacturer.

$$PP = (\text{Average OD value of test serum} / \text{Median OD of C++ wells}) \times 100$$

Three categories negative, moderately positive and positive were decided upon cut off values provided by manufacturer of kit.

### Analysis of results

The samples found positive in both RBPT and ELISA were considered positive and those which were positive in ELISA and negative in RBPT were also declared positive. While samples showing positive results in RBPT and negative in ELISA were retested and then repetition of same results were considered as positive. The sensitivity and specificity of RBPT was calculated using ELISA as reference test. The level of agreement between two tests was calculated as  $(a + d)/n$ , where  $a$  is the number of samples positive by both RBPT and ELISA,  $d$  is the number of samples negative by both methods, and  $n$  is the total number of samples under examination (Martin *et al.*, 1997).

### RESULTS AND DISCUSSION

A total number of 303 samples were collected and out of them, 125 (41.25%) were found positive using RBPT and rest of samples *i.e.* 178 (58.75%) were negative for presence of agglutinating antibodies. Out of 125 positive samples, 2 were from male and other 123 were of female animals. All samples were tested with indirect ELISA test using smooth O antigen. Here results obtained were fall into three categories *i.e.* negative, moderately positive and positive. Out of 303 samples, 142 (46.86%), 22 (07.26%) and 139 (45.87%) samples were found as positive, moderately positive and negative, respectively (Table 1). If positive and moderately positive results were clubbed together, then it can be inferred that 164 (54.12%) animals were having brucellosis antibodies. As two tests did not provide unequivocal results, the variations between two tests were carefully analyzed and shown with the Table 2.

Brucellosis is a prevalent disease among the bovines at the tune of 8.8% and 8.7% at Indian and Gujarat level, respectively (Renukaradhya *et al.*, 2002). Considering the population prevalence, this was a significantly high percentage of positive animals at herd level. Contrary to this Varasada (2003) studied overall seroprevalence study of brucellosis in cattle and buffaloes of central Gujarat, and observed 16.80% and 14.03% of animals were positive by RBPT and STAT, respectively. Patel (2007) obtained 7.79% and 18.61% seropositivity among cattle and buffaloes by RBPT and STAT, respectively. Even Ghodasara *et al.* (2010) found it at much lower level (maximum 25 % positive with iELISA) while using

samples from aborted cows and retention of placenta cases. Similar works from other parts of country like by Sharma and Saini (1995) reported 8.69% and 14.61% brucellosis prevalence in cattle and buffaloes, respectively.

But similar or higher level of reactors were reported by Barbudde *et al.* (2004) who revealed that 37.38% and 36.45% buffaloes were positive for *Brucella* antibodies by RBPT and STAT, respectively in Goa, and Genc *et al.* (2005) who detected *Brucella abortus* antibodies at rate of 58.9% and 55.2% by RBPT and STAT, respectively.

Though bacteriological, serological and molecular diagnostic methods had been employed by various workers (Rekha *et al.*, 2013; Pathak *et al.*, 2014) serological methods are widely used due to safety, operational ease and economics. Earlier the use of three tests viz. RBPT, STAT and ELISA (dot ELISA version) has been documented (Chachra *et al.*, 2009) but in their own study STAT was showing false negative result. Due to this and other limitation OIE recently limits the use of STAT (OIE, 2012) and recommends RBPT as qualitative and ELISA as quantitative test for detection of *Brucella* antibodies.

In the present study, ELISA could detect *Brucella* antibodies in slightly higher number of animals (54.12%) than RBPT (41.25). The results corroborated to other studies, where RBPT was found lesser sensitive than iELISA such as 50% vs 100% (Chachra *et al.*, 2009), 10.56% vs 25% (Ghodasara *et al.*, 2010) and 6.5% vs 45% (Rekha *et al.*, 2013) for RBPT vs STAT. Due to which,, ELISA may be used as a screening test for brucellosis diagnosis (Nitu *et al.*, 2013). On herd level, sensitivity and specificity of RBPT have been calculated as 80.78% and 100%, respectively and level of agreement between RBPT and ELISA was at 0.871. On the same line, Gall and Nielsen (2004) made a very elaborative comparison between various serological tests and found the superiority of ELISA over RBPT.

But for individual samples, it becomes very difficult to reach on definitive conclusion about positivity or negativity of sample due to differences in test results. Three categories of non agreement of results have been observed in present study (Table 2). As ELISA is considered more sensitive test than RBPT (Gall and Nielsen, 2004; Chachra *et al.*, 2009) it can be understand that it is able to detect the much lower level of antibody amount than RBPT. But the reverse situation, where samples were found positive in



RBPT and negative in ELISA, may point for two possible causes, one is vaccination (OIE, 2012) and other is cross reactivity with *Yersinia enterocolitica*, *E. coli* O: 157 and other organism's antibodies (Kittelberger *et al.*, 1995) in RBPT which was ruled out by ELISA. Though, it cannot be conclusively proven due to lack of other supportive evidences like vaccination and abortion history.

Overall conclusion can be made on the line of Yohannes *et al.* (2012) that no test is perfect, and the clinical history coupled with a combination of two or more tests will reduce diagnostic errors and application of bacteriological and molecular techniques can be employed as well (Al-Bayatti and Al-Thwani, 2009 and Rekha *et al.*, 2013), particularly for culling decision of valuable animals.

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