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Comparative efficacy of rose bengal plate test, standard tube agglutination test, microagglutination test and Indirect enzyme linked immunosorbent assay for the diagnosis of bovine brucellosis

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Abstract: The present study was carried out to evaluate the comparative efficacy of four different serological techniques (Rose bengal plate test- RBPT, Standard Tube Agglutination Test- STAT, Microagglutination Test- MAT and Indirect enzyme linked immunosorbent assay- I-ELISA) for diagnosis of bovine brucellosis. A total of 92 serum samples (75 samples from cattle and 17 from buffaloes) were tested for the presence of *Brucella* antibodies. Out of a total of 92 serum samples tested, 35 (38.04%) samples were positive by RBPT, 45 (48.9%) by STAT, 42 (45.6%) by MAT and 53 (57.6%) samples were positive by I-ELISA. I-ELISA was found to have 97.25% sensitivity and 67.85% specificity when compared to RBPT, 93.7% sensitivity and 81.8% specificity in comparison to STAT and 95.45% sensitivity and 77% specificity in comparison to MAT.

Key words: *Brucella*, Cattle, Buffaloes, RBPT, STAT, MAT, I-ELISA

Introduction

Bovine brucellosis is an important zoonotic disease characterized by abortion and reduced fertility in cattle and buffaloes. The disease in cattle and buffaloes is caused almost exclusively by *Brucella abortus* - a small, gram negative, non motile, non spore forming coccobacilli arranged singly or in pairs or short chains. The organism causes great economic losses to the livestock industry in the form of abortion, infertility, increased calving interval and reduced milk yield (Franco *et al.,* 2007; Islam *et al.,* 2009). Diagnosis of brucellosis is made from the history, time of abortion and appearance of the aborted fetus. Diagnosis based on clinical signs cannot be generalised to all age group animals especially the non pregnant animals, heifers and bulls since abortion is the only chief symptom of this infection. Isolation of *Brucella* remains the "gold standard" in diagnosis but it has reduced sensitivity in chronic infections and is unsuitable for use in large animal populations. Serological tests viz. Rose Bengal plate test (RBPT), Complement fixation test, Enzyme linked immunosorbent assay (ELISA) or the Fluorescence polarisation assay are suitable tests for screening of herds and individual animals (OIE 2009). ELISA has been found to be a good screening test whether used alone or in combination with RBPT (Jacques *et al*., 1998) and can detect antibodies against *Brucella* with high sensitivity and accuracy (Nielsen *et al*., 2006). However, there is lack of information pertaining to the comparative sensitivity of the four tests. Particularly, in case of samples declared negative by RBPT or standard tube agglutination test (STAT), such samples need to be confirmed by ELISA so as to prevent wrong diagnosis because of false negative reactions by these tests. The present study was therefore undertaken with the objective to compare the four tests in terms of sensitivity and specificity for detection of *Brucella* antibodies in serum of cattle and buffaloes.

Materials and Methods

Collection of serum samples: Blood samples (10 ml) were collected from the jugular vein of cattle and buffaloes from various organised and unorganised farms of different districts of Punjab after obtaining the consent of the farmers. There was no history of vaccination of the animals. Serum was separated from the blood samples and stored at -20^{0}C till use.

RBPT: Serum samples and RBPT antigen were brought to the room temperature and then one drop (0.03 ml) of serum was taken on a clean, dry and non greasy glass slide by micropipette. The antigen bottle was shaken well to ensure homogenous suspension and then one drop (0.03 ml) of the antigen was added. The antigen and serum were mixed thoroughly for four min. and analyzed for clumping/ agglutination. Definite clumping/agglutination was considered as positive reaction, whereas no clumping/agglutination was considered as negative. In each test, a positive and a negative control was run using known positive and known negative serum.

STAT: Brucella abortus S99 plain antgen (IVRI, Izatnagar) was used as per the method of Alton *et al* (1975). Two fold serial dilutions of the sera were prepared in phenol saline and equal quantity (0.5ml) of the antigen was added in each tube. The tubes were then incubated at 37^{0}C for 24 hrs. A titer of 1:40 or above was considered a positive reaction and the results were compared with the antigen control tube showing 50% agglutination.

Microagglutination test (MAT): MAT was performed on 96 well microtitre plate. 80 µl of phenol saline was taken in the first well and 50 µl in rest of the wells. 20 µl of serum sample was added in the first well, mixed well and 50 µl of diluted serum was transferred to the second well. The process was continued up to the 12th well. After mixing, 50 µl of antigen was added to each wells, mixed thoroughly and the microtiter plate was incubated at 37ºC for 20 h.The degree of agglutination was judged by opacity of the supernatant fluid. The highest serum dilution showing 50 per cent or more agglutination (50 % clearing) was considered as the titre of the serum. The titre so obtained was expressed in unit system by doubling of the serum titre as International Unit (I.U.) per ml of serum.80 I.U. per ml or above was considered positive for brucellosis. A control well was set by mixing 50 µl of antigen with 50 µl ml of 0.5 % phenol saline.

Indirect Enzyme linked immunosorbent assay (I-ELISA): I-ELISA was performed using commercially available kit (IDEXX

CHEKIT-BRUCELLOSE SERUM- *Brucella abortus* antibody test kit- Netherlands) as per the manufacturers instructions. The samples were analysed in relation to the positive and negative controls as per the formula.

Value (%)= (OD samples - OD negative control / OD positive control - OD negative control) x100

Samples scoring less than 80% were assigned negative and greater tthan or equal to 80% as positive.

Results and Discussion

In the present study, RBPT, STAT, MAT and I-ELISA were used for detection of antibodies to *Brucella* in cattle and buffaloes. Out of a total of 92 serum samples (75 samples from cattle and 17 from buffaloes), 35 (38.04%) samples were positive by RBPT, 45 (48.9%) by STAT, 42 (45.6%) by MAT and 53 (57.6%) samples were positive by I-ELISA. The results are summarized in Table 1,2,3 and 4. Indirect-ELISA was found to have 97.25% sensitivity and 67.85% specificity when compared to RBPT. However, I-ELISA exhibited 93.7% and 95.45% sensitivity in comparison to STAT and MAT respectively, whereas specificity was found to be 81.8% and 77.0% for STAT and MAT respectively. In the present study, I-ELISA failed to detect antibodies to *Brucella* in serum samples (1, 3 and 2) which were otherwise positive by RBPT, STAT and MAT, respectively. 19 samples negative by RBPT were positive by one or the other serological tests used in the present study. It is pertinent to mention that the titre of such samples negative by RBPT was high in MAT and or STAT and the corresponding OD values in I-ELISA was also high.

Isolation remains the gold standard for diagnsosis of brucellosis, however, inherent problems associated with isolation of *Brucella* sp.viz requirement of trained personnel, cost, potential health hazard for workers, lower number of viable organisms in the sample and other factors, make isolation difficult (Nielsen and Yu, 2010). Several agglutination based tests such as RBPT, MAT, STAT and ELISA in combination have been used for the detection of *Brucella* specific antibody response in livestock as no single test can detect all positive reactors (Radulescu *et al.,* 2007; Junaidu *et al.,* 2008). In the present study, I-ELISA detected maximum number of positive samples (57.6%) followed by STAT, MAT and RBPT. The results are in accordance with findings of many other workers who have reported I-ELISA to be a highly sensitive and specific diagnostic assay with minimal false positive samples (Senthil and Narayanan 2013; Islam *et al*., 2013; Chachra *et al.*, 2009). Out of a total of 92 serum samples, STAT detected 45 positive samples whereas RBPT detected 35 in the present study. Similar findings have been reported by Otlu *et al* (2008) who reported 32.9% and 34.65% samples positive by RBPT and STAT, respectively. However, in contrast, Chakraborthy *et al* (2000) observed that RBPT had higher sensitivity in comparison to STAT as it detected more number of samples as positive.

19 samples negative by RBPT were positive by one or the other serological tests used in the present study. It is pertinent to mention that the titre of such samples negative by RBPT was high in MAT and or STAT and the corresponding OD values in I-ELISA was also high. This discrepancy could be due to false negative reactions in RBPT against high antibody titres observed due to a prozone phenomenon (OIE 2009).

The present study retrieates the findings of other workers, that commonly used conventional serodiagnostic tests viz. RBPT, MAT and STAT when used alone may not be absolutely reliable diagnostic assays (Chachra *et al.,* 2009). ELISA based diagnostics have been found to be more reliable with greater sensistivity and specificity which is also evident from the present study. However, to arrrive at an accurate diagnosis a combination of STAT and I-ELISA and or RBPT and i-ELISA is suggested to rule out false negative samples by other tests.

Table-1: Detection of *Brucella* specific antibodies by different serological tests

Result	RBPT	MAT	STAT	i-ELISA
Positive	35	42	45	53
Negative	57	50	47	39
Total	92	92	92	92

Table-2: Sensitivity and specificity analysis between RBPT and I-ELISA

Test	Result	RBPT -ve	RBPT +ve	Total by (i-ELISA)
I-ELISA	+ve	18	35	53
	-ve	38	1	39
	Total	56	36	92

Table-3: Sensitivity and specificity analysis between STAT and I-ELISA

Test	Result	STAT -ve	STAT +ve	Total by (i-ELISA)
I-ELISA	+ve	8	45	53
	-ve	36	3	39
	Total	44	48	92

Table-4: Sensitivity and specificity analysis between MAT and I-ELISA

Test	Result	MAT -ve	MAT +ve	Total by (i-ELISA)
I-ELISA	+ve	11	42	53
	-ve	37	2	39
	Total	48	44	92

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