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Comparative Evaluation of Serological Tests for Diagnosis of Brucellosis in Bovines with History of Abortion in Punjab

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ABSTRACT

Brucellosis is highly infectious zoonotic disease that causes huge economic losses to livestock farmers by affecting the reproductive potential of animals causing last trimester abortions and infertility. In the present study evaluation of different serological tests to diagnose the seroprevalence of brucellosis in bovines with history of abortion using various serological tests [Rose Bengal Plate Test (RBPT), modified rose bengal plate test (mRBPT), microtitre plate agglutination test (MAT) and indirect enzyme linked immunosorbent assay (i-ELISA)] was carried out. A total of 134 blood samples of cattle and buffalo with history of abortion were collected from organized and unorganized farms. Seroprevalence by mRBPT, RBPT, MAT and i-ELISA was 75.37%, 67.91%, 72.38% and 72.38%, respectively. In organized farms, prevalence of 78.12%, 81.25%, 78.12% and 81.25% while in unorganized farms prevalence of 64.70%, 73.52%, 70.58% and 69.60% was reported by RBPT, mRBPT, MAT and i-ELISA, respectively. The sensitivity and specificity of serological tests by keeping i-ELISA as gold standard were also calculated and the results revealed that sensitivities of RBPT, mRBPT and MAT were 91.75%, 97.94% and 96.91%, respectively, whereas specificities were 94.59%, 83.78% and 91.89%, respectively.

Keywords: Bovine Brucellosis, i-ELISA, MAT, mRBPT, RBPT

Bovine brucellosis is a contagious zoonotic disease of livestock and is a major threat to dairy industry. The causative agent of this disease belongs to the genus *Brucella* comprising of different species each with preferential natural hosts (Olsen and Palmer, 2014). Bovine brucellosis is mainly caused by *Brucella abortus*, less frequently by *B. melitensis*. *Brucella* is gram negative, non-motile, non-spore forming *cocobacilli* bacterium (WHO, 2006). Brucellosis causes huge economic losses to farmers as it adversely affects the productivity and reproductive potential of bovines and can lead to storm of abortions in pregnant animals. The disease mainly affects sexually mature animals causing abortion in last trimester in females, birth of weak calves or stillborn and infertility in both sexes (OIE, 2012). Brucellosis is characterized by inflammatory changes in the foetal membranes which leads to premature expulsion of the foetus (Kungu *et al.,* 2010). The clinical diagnosis is difficult due to a lack of obvious observable symptoms except for last trimester abortion in pregnant animal (Minda and Gezahegne, 2016).

Bovine brucellosis is endemic and prevalent in 13% of world's total bovine population (Anon, 2012) including India. The disease is widely prevalent throughout India among the bovine population causing economic losses to the tune of ₹ 350 million (PD_ADMAS, 2012).

The diagnosis of brucellosis is based on culture, serological and molecular methods. Isolation and identification of *Brucella* organisms is considered as gold standard for diagnosis (OIE, 2012). However, during isolation *Brucella* can be contaminated by other microbes resulting in a reduced sensitivity (Matope *et al*., 2011). The bacterial

culture and isolation procedure can be hazardious for the laboratory technicians (Nielsen and Yu, 2010; Traxler *et al*., 2013). The technique requires advanced BSL3 (biosafety level 3) laboratory facilities (Ahasan *et al*., 2017). Thus, for routine screening of livestock for brucellosis, serological tests are often relied upon and considered safer for laboratory personnel. Various serological tests used for screening of brucellosis include Rose Bengal Plate Test (RBPT), modifiedRose Bengal Plate Test (mRBPT), Microtitre Plate Agglutination Test (MAT), Standard Tube Agglutination Test (STAT), indirect ELISA (i-ELISA) and Competitive ELISA (c-ELISA). Each test has its own limitations and therefore a battery of serological tests are used in the confirmatory diagnosis of brucellosis. The present study was aimed to investigate the prevalence of brucellosis in bovine with the history of abortions and further compare the serological tests for sensitivity and specificity in diagnosis of the diseases.

MATERIALS AND METHODS

Punjab is the north-western state of India bordering with Pakistan on the west and situated between the 29.30° N to 32.32° N latitude and 73.55° E to 76.50° E longitude. The study was conducted in organized and unorganized farms in and around Ludhiana, Punjab. Total of 134 bovine sera samples were collected from four organized farms and 15 unorganized farms from animals with history of abortion. Out of 134 sera samples, 90 were from cattle and 44 from buffalo. Thirty two samples were taken from organized farms while 102 samples were from unorganized farms. Approximately 20 ml of blood was collected aseptically from the jugular vein of dairy animals. The serum was separated and stored at -20°C until further testing.

Serological testing

Rose bengal plate test (RBPT)

The antigen used for RBPT was procured from Punjab Veterinary Vaccine Institute, Ludhiana. The sera samples collected were tested for the presence of antibodies against brucellosis as per protocol of the OIE (OIE, 2012). The antigen were mixed properly before use to ensure the homogeneous suspension of antigen. The test serum sample (40µl) was placed on a clean glass slide and equal volume of RBPT coloured antigen was added. With the help of a clean sterile toothpick both the serum and coloured antigen were mix properly. Positive and negative control was also used to perform RBPT. The slide was observed for 4 minutes for the formation of clumps. The formation of clear clumps was considered as positive test while the absence of clear clumps was considered as negative reaction.

Modified rose bengal plate test (Super agglutination)

The test was performed as per the technique of Saxena *et al.* (2015). Equal volumes (2.5 ul each) of RBPT coloured antigen, 2.5µl of test serum was mixed with 1 µl 0.1% Commassie Blue dye. Out of this mixture, 2.5µl test serum stained with 0.1% Commassie Blue dye was mixed with 1 µl of biotinylated anti-bovine IgG (1:100, Gene tex) and 1 µl streptavidin (1:100, SRL) on a clean glass slide thoroughly in the above mentioned sequence. The slide was observed for 4 mins for formation of clumps. Ordinary hand lens was used occasionally for better visibility. Along with the sera samples, positive and negative controls were also used. The slides were viewed under microscope to confirm the clumping. Formation of clear clumps which were of blue colour (due to the Coomassie blue dye staining the serum antibodies) and pink colour (due to the Rose Bengal dye stained RBPT coloured antigen) were considered as positive, while absence of clear clumps was considered as negative.

Microtitre plate agglutination test (MAT)

The Microtitre plate agglutination test (MAT) was performed by using commercially available *Brucella* plain antigen procured from Punjab Veterinary Vaccine Institute, Ludhiana. The test was performed in microtitre plates with 12 × 8 U-shape or round bottom wells. *Brucella abortus* plain antigen at 1:2 working dilution was used for the test. Normal saline (80µl) was taken in all the wells of the first row and 50µl in rest of the wells. 20µl of serum sample was added in the well in the first row, mixed well and 50µl of diluted serum was transferred from first well in the column to the second well in the column. The process was repeated and 50µl of the diluted serum sample from second well was added to the third well and so on. Finally 50µl of the sample was discarded from the 8th well. After

mixing, 50µl of *Brucella* plain antigen was added to each well and mixed thoroughly. The positive and negative controls were also taken for the performance of the test. The plate was sealed and incubated at 37ºC for 24 hrs. The formation of matt signified agglutination which was indicative of positive reaction while button formation was indicative of a negative reaction. Titres (log 10 values) were recorded as the reciprocal of the highest dilution of the serum givingat least 50 percent agglutination.

Indirect enzyme linked immunosorbent assay (i-ELISA)

Indirect ELISA was performed using the commercial available ELISA kit (Svanovir® *Brucella*- Ab I-ELISA) following the manufacturers protocol. Briefly, positive and negative controls (10 µl each) were added in duplicates in the wells coated with *Brucella abortus* antigen. Serum samples (10 µl each) were added in duplicate in the wells. The plate was sealed and incubated at 37°C for 1 hour in the shaker incubator.

After incubation, the plate was washed three times with PBS-Tween Buffer followed by addition of HRP Conjugate (100 µl) to each well. Again the plate was sealed and incubated at 37° C for 1 hour, followed by washing three times with PBS-Tween Buffer. 100 µl substrate solutions (TMB) was added to each well and incubated for 10 minutes at room temperature. Reaction was stopped by adding 50µl stop solution to each well and mixed thoroughly. Optical density (OD) of the controls and samples was measured at 450 nm in microplate photometer within 15 minutes of addition of stopping solution. The optical density was used to calculate percent positivity (PP) as per the manufacturer's protocol. The test sera were categorized as positive or negative as per percent positivity value. If PP value less than 60 of the test sample it indicated negative result and PP value equal to or greater than 60 indicated positive result.

Statistical analysis

Statistical analysis was performed using SPSS software. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy were calculated.

Kappa-value was estimated and strength of agreement was predicted as given in Table 1.

Table 1: Correlation between value of kappa and the strength of agreement

Value of kappa	Strength of agreement
<0	Bad
0.01-0.20	Poor
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Strong
0.81-1.00	Almost perfect

RESULTS AND DISCUSSION

Seroprevalence of brucellosis was studied on 134 sera samples from animals with history of abortion. Various serological tests such as mRBPT, RBPT, MAT and i-ELISA were carried out for the diagnosis of disease. In the present study, an overall of 101 (75.37%) animals were positive for brucellosis by various serological tests. Seroprevalence of 101 (75.37%), 91 (67.97%), 97 (72.38%) and 97(72.38%) were estimated by mRBPT, RBPT, MAT and i-ELISA, respectively. In cattle, prevalence by mRBPT, RBPT, MAT and i-ELISA were 73 (81.1%), 64 (71.11%), 70 (77.77%) and 70 (77.77%) respectively while in buffalo the seroprevalence was 28 (63.63%), 27 (61.36%), 27 (61.36%) and 27 (61.36%) by mRBPT, RBPT, MAT and i-ELISA, respectively (Table 2). The seroprevalence of brucellosis 26 (81.25%), 25 (78.12%), 25 (78.12%) and 26 (81.25%) by mRBPT, RBPT, MAT and i-ELISA in organized farms while in unorganized farms, it was 75 (73.52%), 66 (64.70%), 72 (70.58%) and 71 (69.60%) respectively (Table 3).

In the present study, seroprevalence of brucellosis was reported as 64% to 75% using various serological tests. The results of our study were similar to the studies carried out by various workers on the sera samples of animals having history of abortion. Trangadia and Patel (2018) reported the seroprevalence of 72.00% (18/25) by RBPT, Islam *et al.* (2018) reported the seroprevalence of 76.91% (16/21) by i-ELISA, Gurbilek *et al.* (2017) reported 81.3% and 83.7% seroprevalence by RBPT and i-ELISA, respectively. Zadon and Sharma (2015) reported the seroprevalence of the disease as 75% by RBPT and 58.33% by MAT in cattle while in buffaloes 88% by RBPT and 80% by MAT.

Table 2: Seroprevalence of brucellosis in bovines having history of abortion by different serological tests

Test	Cattle (n= 90)		Buffalo (n= 44)		Total (n= 134)	
	Positive	Negative	Positive	Negative	Positive	Negative
mRBPT	73 (81.1%)	17 (18.88%)	28 (63.63%)	16 (36.36%)	101 (75,37%)	33 (24.62%)
RBPT	64 (71.11%)	26 (28.88%)	27 (61.36%)	17 (38.63%)	91 (67.97%)	43 (32.08%)
MAT	70 (77.77%)	20 (22.22%)	27 (61.36%)	17 (38.63%)	97 (72.38%)	37 (27.61%)
i-ELISA	70 (77.77%)	20 (22.22%)	27 (61.36%)	17 (38.63%)	97 (72.38%)	37 (27.61%)

Table 3: Seroprevalence of brucellosis in bovines having history of abortion in organized and unorganized farms

	mRBPT		RBPT		MAT		ELISA	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Organized farms	26 (81.25%)	6 (18.75%)	25 (78.12%)	7 (21.87%)	25 (78.12%)	7 (21.87%)	26 (81.25%)	6 (18.75%)
Unorganized farms	75 (73.52%)	27 (26.47%)	66 (64.70%)	36 (35.29%)	72 (70.58%)	30 (29.41%)	71 (69.60%)	31 (30.39%)

Table 4: Comparison of RBPT, mRBPT and MAT keeping i-ELISA as gold standard

	RBPT		MAT		mRBPT	
	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	91.75%	84.39% - 96.37%	96.91%	91.23% - 99.36%	97.94%	92.75% -99.75%
Specificity	94.59%	81.81% - 99.34%	91.89%	78.09% - 98.30%	83.78%	67.99% - 93.81%
Positive predictive value	97.80%	92.03% - 99.42%	96.91%	91.37% - 98.93%	94.06%	88.38% - 97.05%
Negative predictive value	81.40%	69.16% - 89.51%	91.89%	78.75% -97.20%	93.94%	79.61% - 98.40%
Accuracy	92.54%	86.70% - 96.36%	95.52%	90.51% - 98.34%	94.03%	88.58% - 97.39%

In the present study, comparative analysis (sensitivity, specificity, Positive predictive value and Negative predictive value) of the serological tests by keeping i-ELISA as gold standard were conducted (Table 4). Modified RBPT showed 97.94% relative sensitivity and 83.78% relative specificity, 94.06% PPV and 93.94% NPV. These results are similar to those reported by Saxena *et al.* (2015) who reported the sensitivity of 95.88% and specificity of 89.32% by mRBPT. RBPT showed the 91.75% relative sensitivity, 94.59% relative specificity, 97.80% PPV and 81.40% NPV. MAT showed the 96.91%, relative sensitivity, 91.89% relative specificity with 96.91% PPV and 91.89% NPV. The comparison of i-ELISA showed almost perfect agreement with RBPT, MAT and mRBPT with kappa index of 0.84, 0.90 and 0.87 at $p< 0.05$, respectively (Table 5).

The results in the present study are similar to those of other workers who calculated sensitivity and specificity by keeping i-ELISA as gold standard. The sensitivity of 85.71% and specificity of 100% by MAT was reported by Mishra *et al.* (2016). The sensitivity of 88.46% and specificity of 97.75% by RBPT was reported (Singh *et al.,* 2004) while Neha *et al.* (2017) reported specificity of 88.89% by RBPT. To eliminate false positive results among RBPT positive sera and to have better results for diagnosis of brucellosis in cattle, ELISA is recommended over RBPT as chances of non-detection of an infected animal in ELISA are much lower (Erdenebaatar *et al*., 2004; Chand and Sharma, 2004).

In the present study, 86 samples were found to be positive and 30 negative by all the four serological tests. Seven samples were detected positive by ELISA, MAT and mRBPT. Two samples were positive only by RBPT. On the other hand, two samples were positive by both RBPT and ELISA but these were negative by MAT and mRBPT. Five samples were found to be positive by mRBPT while these were negative by RBPT, MAT and ELISA. One sample which was positive by mRBPT and ELISA but it was found to be negative by other two tests (Table 6). False positive results may arise due to immune response of

Table 5: Inter-agreement between serological tests keeping i-ELISA as gold standard

Test		I-ELISA		
mRBPT		**Positive**	**Negative**	**Total**
	Positive	a=95	c= 6	a + c = 101
	Negative	b= 2	d =31	b + d = 33
	Total	a + b = 97	c + d = 37	134
Kappa	k= 0.87			
		Positive	**Negative**	**Total**
MAT	Positive	a=94	c=3	a+c=97
	Negative	b=3	d=34	b+d=37
	Total	a+b=97	c+d=37	134
Kappa	k=0.90			
		Positive	**Negative**	**Total**
RBPT	Positive	a=89	c=2	a+c=91
	Negative	b=8	d=35	b+d=43
	Total	a+b=97	c+d=37	134
Kappa	k=0.84			

Table 6: Comparison of serological tests in diagnosis of brucellosis in bovines having history of abortion

Number of samples	RBPT	MAT	i-ELISA	mRBPT
86	+	+	+	+
30	-	-	-	-
1	+	+	+	-
0	+	+	-	-
2	+	-	-	-
1	-	+	-	-
0	-	+	+	-
2	-	+	-	+
5	-	-	-	+
1	-	-	+	+
7	-	+	+	+
0	+	-	-	+
2	+	-	+	-
Total	**91**	**97**	**97**	**101**

an animal to other microorganisms (Corbel, 1985). Seven samples which showed negative results in RBPT while positive in other tests may be due to prozoning that causes false negative reaction in RBPT. Two samples which were positive only by RBPT may be due to false positive reactions due to nonspecific agglutinins (OIE, 2012).

In order to avoid the false reactions, modifications in the RBPT (mRBPT) have been developed in which test serum is stained with the Commassie Blue dye (Saxena *et al.,* 2015). In mRBPT, biotinylated anti-bovine IgG and streptavidin are used to enhance the clump size by cross-linking the antibody molecules thereby minimizing the false negative results. Staining of serum with dye help in differentiation of specific aggregates of both antigen and antibody from that of non-specific aggregates of antigen alone which leads to false positive results. Thus, in mRBPT, the true two (blue and pink) colored agglutinates could be easily differentiated from non-specific one

colored aggregates. The antigen and antibody which did not participate in the agglutination reaction could be seen as either blue or pink particles alone. In present study, mRBPT detected the positive samples that were not detected by other serological tests. mRBPT showed higher sensitivity and negative predictive value than RBPT and MAT on taking iELISA as gold standard.

CONCLUSION

From the present study, brucellosis was diagnosed in bovines having history of abortion using multiple serological tests. mRBPT showed higher sensitivity than RBPT and MAT indicating its usefulness as a pen side diagnostic test for screening of animals in large scale. Indirect-ELISA also detected higher positive animals than RBPT and MAT.

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