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Comparative evaluation of Serum Plate Agglutination Test (SPAT) and Rose Bengal Plate Test (RBPT) for diagnosis of *Brucella abortus* in sera of cattle and human

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Abstract

To diagnose bovine brucellosis, various conventional and advance molecular techniques are in practice. No single serological test is appropriate in all epidemiological circumstances; each of them has a number of restrictions predominantly for screening individual animals and human. The performance characteristics of Serum Plate Agglutination Test (SPAT) and Rose Bengal Plate Test (RBPT) for diagnosis of *Brucella abortus* were evaluated by using Indirect Enzyme Linked Immunosorbant Assay (i-ELISA) as a Gold Standard. A total of 410 human and 202 cattle blood sera were screened. In human sera, RBPT detected 75 positive samples, while SPAT detected 78 positive samples. In cattle sera, RBPT detected 29 positive samples, while SPAT detected 32 positive samples. In cattle sera, RBPT showed high sensitivity 82.60%, specificity 94.72%, positive predictive value 76% and negative predictive value 96.41% as compared to SPAT which showed 81.15% sensitivity, 93.54% specificity, 71.79% positive predictive value and 96.08% negative predictive value. Similarly, in human sera RBPT showed high sensitivity 76%, specificity 94.35%, positive predictive value 65.51% and negative predictive value 96.53%, while SPAT showed 72% sensitivity, 92.09% specificity, 56.25% positive predictive value and 95.88%negative predictive. In the present study, although RBPT showed comparatively better result than SPAT but still its sensitivity and specificity is low, so it can be used as a screening test but cannot be used as a confirmatory test.

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Introduction

To control and eridicate brucellosis at local or national level, it is extremely important to diagnose it urgently and accurately. Various conventional and advance molecular techniques are in practice for diagnosis of brucellosis. No single serological test is appropriate in all epidemiological circumstances, each of them has a number of restrictions predominantly for screening individual animals. All aspects should be under deliberations that have an impact on the test results and method. The most appropriate screening tests are the Rose Bengal test (RBT), the Buffered Plate Agglutination test (BPAT), Enzyme Linked Immunosorbent assay (ELISA) and the Fluorescence Polarization Assay (FPA). Some ELISAs and FPA have similar or better diagnostic performance as compared to complement fixation test (CFT) because they are simple, easy to perform, sensitive and preferred to use (OIE, 2009).

Serum plate agglutination test (PAT) is unmistakable and rapid as compared to Tube agglutination test (TAT) but influenced by the environmental conditions. The sensitivity and specificity of SPAT is similar to those of TAT and is mostly used when serum quality is low (Weidmann, 1991). The Rose Bengal Plate Test (RBPT) is used as a diagnostic test for screening individual animal of herds. It is conventionally though-about that both SPAT and RBPT have poor specificity, in animal and humans which are already immunized with strain 19. Therefore positive blood sample should be confirmed by definitive test (Weidmann, 1991).

An easy, rapid and economical serological test that will diagnose infected animals and humans in acute and chronic form of bovine brucellosis and that does not detect anti brucella antibodies in vaccinated animals is still a great challenge. However, a great deal of development was accomplished by the introduction of enzyme immunoassays (Wright *et al.,* 1990).

As RBPT and SPAT gives more false positive, false negative and doubtful results, due to which i-ELISA is recommended for screening cattle as well as humans due to its more sensitivity and specificity (Agasthya *et al.,* 2007; Hussain *et al.,* 2008 and Elsheikh *et al.,* 2012).

The present study was designed to compare the sensitivity and specificity of Rose Bengal Plate Test (RBPT) and Serum Plate Agglutination Test (SPAT).

Materials and methods

Collection of blood samples

A total of 410 human and 202 cattle blood samples were collected aseptically in vacutainer tubes. After proper labeling and sealing, samples were transported to Disease Investigation Laboratory, Livestock and Dairy Development Department, Peshawar. The samples were centrifugated for 3minutes at 400 rpm to separate the serum. Sera samples were stored in ependorf tubes at -20^{o}C.

Serological Tests

Rose Bengal Plate Test (RBPT)

All serum samples were screened through Rose Bengal Plate Test (RBPT) according to procedure already described (OIE manual 2009). Briefly, 30 µl of serum was mixed with equal amount of Brucella antigen obtained from Veterinary Research Institute (VRI) Lahore, Pakistan on a slide to create a circular or an oval zone approximately 2cm in diameter. The mixture was shacked gently for 4 minutes at room temperature. Complete agglutination was considered as positive while, partial and no agglutination was considered as doubtful and negative, respectively.

Serum Plate Agglutination Test (SPAT)

The test was performed according to the procedure provided with the antigen (Catalog # S-277) by Laboratory Diagnostics Co., Inc. Morganville, N.J. 07751 USA. Briefly, the serum samples and antigen were brought to room temperature. Then 80, 40, 20, 10 and 5 µl serum samples were placed on 4 cm^{2} area on glass plate. To each square area 30 µl of antigen was added. Antigen and serum were mixed with applicator stick in circles having 2 cm diameter except for 1:20 dilution. The plate was rotated after 4 minutes for proper mixing and was kept for 8 minutes at room temperature. After 8 minutes, level of agglutination was examined by slanting the plate to let the flow of mixture in a good source of light adjacent to black background. The dilutions represent 1:20, 1:40 1:80, 1:160 and 1:320 dilution of Tube Agglutination test (TAT).

A titer of 50% up to 1:80 was considered as positive, and 1:40 was classified as doubtful and 1:20 was recorded as negative. Confirmed positive and negative serum of known titer was included as control.

Indirect Enzyme Linked Immunosorbant Assay (i-ELISA)

All serum samples were confirmed by Indirect Enzyme Linked Immunosorbant Assay (i-ELISA). Bovine brucella antibody (IgG) ELISA kit having catalog number (CSB-E13061B) were used provided by Cusabio Biotech Co., Ltd. The assay was carried out in the following steps:

Sample Preparation

Serum samples were diluted by adding 10 µl sample to 490 µl of sample diluent to obtain 50 fold dilutions.

Reagent Preparation

To prepare 200 ml of wash buffer (1x), 20 ml of wash buffer concentrate (10x) was diluted into 180 ml of distilled water. HRP-conjugate (1x) 10 ml was prepared by diluting 0.1 ml of HRP-conjugate (100x) into 9.9 ml wash buffer (1x). In distilled water 30% H_2O_2 was diluted with 3% H_2O_2. Substrate (40x) 0.1 ml was diluted into 3.9 ml substrate diluent. Then 3% H_2O_2 was added (1/100). To obtain 50 fold dilution of negative and positive control, 10 µl of negative and positive control were added to 490 µl of sample diluent.

Assay Procedure

All the reagents and serum samples were brought to room temperature for at least 1 hour before use and were prepared as directed by user manual. Before starting all the wells were aspirated and 100 µl of diluted negative and positive control or sample were added per well. After 30 minutes incubation at 20-30°C temperature each well were aspirated and washed 3 times by filling each well with 300 ul wash buffer using auto washer. Then 100 µl of HRP-conjugate (1x) was added to each well and incubated at 20-30°C for 30 minutes. The aspiration and wash process was repeated for three times as done previously. After washing 100 µl of substrate was added to each well and were incubated for 10 minutes at 37°C.

At the end 100 µl of stop solution was added to each well, and was sealed to ensure proper mixing. Optical density of each well was determined by microplate reader set to 405 nm within 5 minutes.

The valence of *Brucella abortus* antibody (IgG) was calculated by comparing the sample well with control well according to the kit user manual in the following range.

1) $OD_{negative} < 0.2$
2) $0.80 < OD_{positive} < 2.5$

Evaluation of Serological Test for diagnosis of bovine brucellosis

The performance characteristics of Serum Plate Agglutination Test and Rose Bengal Plate Test were evaluated by using two by two tables with i-ELISA (Gold Standard).

		i-ELISA		
		Positive	Negative	
RBPT/SPAT	Positive	(a) True Positive	(b) False Positive	a + b
	Negative	(c) False Negative	(d) True Negative	c + d
		a + c	b + d	

RBPT and SPAT were compared with i-ELISA according to the following formulas (Smith, 1995).

Sensitivity = a / a + c
Specificity = d / b + d
Positive predictive value = a / a + b
Negative predictive value = d / c + d
Test accuracy = a + d / a + b + c + d

Results

The performance characteristics of SPAT and RBPT were evaluated by using two by two tables with i-ELISA (Gold Standard). Comparatively RBPT showed high sensitivity, specificity, positive predictive value, negative predictive value and accuracy than SPAT both in cattle and human as shown in table 1.

A total of 410 human and 202 cattle blood sera were screened. In human sera, RBPT detected 75 samples as positive and 335 samples as negative, while SPAT detected 78 samples as positive and 332 samples as negative. In cattle sera, RBPT detected 29 samples as positive and 173 samples as negative, while SPAT detected 32 samples as positive while 170 samples as negative.

Table 1. Performance characteristics of SPAT and RBPT for diagnosis of *Brucella abortus* in cattle and human sera.

S. No	Specie	Test Type	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
1	Cattle	RBPT	82.60	94.72	76.00	96.41	92.68
		SPAT	81.15	93.54	71.79	96.08	91.46
2	Human	RBPT	76	94.35	65.51	96.53	92.07
		SPAT	72	92.09	56.25	95.88	89.60

SPAT, Serum Plate Agglutination Test; RBPT, Rose Bengal Plate Test; PPV, Positive Predictive Value; NPV, Negative Predictive Value.

In cattle RBPT showed high sensitivity 82.60%, specificity 94.72%, positive predictive value 76% and negative predictive value 96.41% as compared to SPAT which showed 81.15% sensitivity, 93.54% specificity, 71.79% positive predictive value and 96.08% negative predictive value.

In human RBPT also showed high sensitivity 76%, specificity 94.35%, positive predictive value 65.51% and negative predictive value 96.53%, while SPAT showed sensitivity 72%, specificity 92.09%, positive predictive value 56.25% and negative predictive 95.88%.

Discussion

An easy, rapid and economical serological test that will diagnose infected animals and humans in acute and chronic form of bovine brucellosis and that does not detect anti brucella antibodies in vaccinated animals is still a great challenge. However, a great deal of development was accomplished by the introduction of enzyme immunoassays (Wright *et al.,* 1990). For diagnosis of brucellosis various conventional and advance molecular techniques are in practice. No single serological test is appropriate in all epidemiological circumstances. All aspects should be under deliberations that have impact on the test results and method. The most appropriate screening tests are the RBPT and the BPAT, ELISA and FPA (OIE, 2009). In the present study, the performance characteristics of SPAT and RBPT were compared with i-ELISA (Gold Standard) for diagnosis of bovine brucellosis in cattle and humans. Comparatively, RBPT showed high sensitivity and specificity than SPAT both in cattle and human. In most of the countries, the RBPT is mostly used as a screening test, followed by the Complement Fixation Test (CFT) as a confirmatory test for diagnosis of brucellosis (Glynn & Lynn 2008).

In many studies, the RBPT showed more sensitivity as well specificity as compared Serum tube agglutination test (Stemshorn *et al.* 1985; Chachra *et al.*2009). The false negative results showed by RBPT may be due to prozoning in acidified antigens in RBPT. While, the false positive results might be due to cross reaction of antibodies in RBPT (Nielsen 2002).

As RBPT and SPAT gives more false positive, false negative and doubtful results, i-ELISA is recommended for screening cattle as well as humans due to its more sensitivity and specificity (Agasthya *et al.,* 2007; Hussain *et al.,* 2008 and Elsheikh *et al.,* 2012).

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