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# Comparative Assessment of Rose Bengal Plate Test (RBPT) for the Diagnosis of Brucellosis in Pakistan

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**Brucellosis is a zoonotic bacterial infection caused by *Brucella* spp. and is more prevalent in the rural areas of Pakistan. Serological tests' including Rose Bengal Plate Test (RBPT) is gold standard method for the diagnosis of Brucellosis. In this study the RBPT antigen was prepared, and its efficiency was compared with the local and imported RBPT antigens. A total of 288 serum samples from different animals were collected. In-house RBPT antigen was prepared, and its efficiency was compared with the local and imported RBPT antigens using serum agglutination test (SAT) and competitive enzyme linked immune sorbent assay (cELISA). The levels of concordance (288 sera) of in-house with local and imported RBPT antigens were 80.2% and 80.5% respectively. A total 174 sera were tested by cELISA which showed 82.7% concordance between cELISA and in-house RBPT antigen. The concordance level of cELISA with local and imported antigen was 77% and 72.9% respectively. The relative sensitivities by local and imported RBPT antigens were 66.1% and 62.3% and relative specificities were 81.3% and 78.1% respectively. The relative sensitivity (84.9%) and specificity (82.1%) of in-house RBPT antigen was found higher as compared to other available antigens. The local, imported and in-house RBPT showed 74.2 (95% CI, 65.8 – 82.6), 70.2 (95% CI, 61.4 – 78.9) and 83.9 (95% CI, 77.1 – 90.7) area under the receiver operating characteristic (AUROC) curve. The newly prepared RBPT antigen is more efficient in terms of sensitivity and specificity for brucellosis diagnosis.**

**Key words**: Pakistan, Brucellosis, RBPT, bacterial infection.

Brucellosis is a contagious zoonotic bacterial disease caused by *Brucella* spp. *Brucella melitensis, Brucella abortus* and *Brucella suis* are the etiological agents of brucellosis for small ruminants, cattle and pigs, respectively, and among these, *Brucella melitensis* is an important species that causes infection in humans[1, 2]. The disease causes abortion and infertility in animals[3]. In humans, the disease onset occurs with nonspecific signs and symptoms such as fever, headache, myalgia, night sweating, and arthralgia[4]. Brucellosis is diagnosed both by *in vitro* culturing, and detection of *Brucella* antigen using serological tests[4-6]. Cultural methods are time consuming, hazardous, and also not feasible for mass scale diagnosis[4, 7]. Thus, laboratory diagnosis of brucellosis is made primarily by serological tests[5] such as RBPT, complement fixation test (CFT) and SAT which are also recommended by Office International des Epizooties[8]. The ELISA is widely used as confirmatory test for the disease diagnosis

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because it is more specific and sensitive in results as compared to other serological tests[9] .

Pakistan is an agricultural country and about 70% of the population resides in rural areas which depend on agriculture and livestock for their living [10]. Due to greater exposure of people to livestock, they are more prone to zoonotic infection primarily and particularly brucellosis. To reduce the risk of getting brucellosis, a proper prevention and control measures are mandatory; to achieve this goal a thorough screening is needed. The eradication strategy would therefore require production and availability of large quantity of RBPT antigen of good quality for quick diagnosis on a mass scale. At present antigens which are being produced for the diagnosis of animal brucellosis is neither enough and nor of good quality as contradictory results have been reported by testing sera of various animals using both the locally produced and imported antigens[10]. The present study prepared RBPT antigen from *Brucella abortus* strain 99 for the effective diagnosis of brucellosis, and also compared its specific reactivity with available local and imported RBPT antigens to conclude its efficiency.

## MATERIALS AND METHODS

### Samples collection

A total of 288 serum samples including 155, 58, 40 and 35 samples from buffaloes, cattle, sheep and goats respectively, were randomly collected. These sera were stored at - 20°C and thawed immediately before use.

### Preparation of in-house RBPT antigen

For antigen production, seed culture of *Brucella abortus* S99 was obtained from Veterinary Research Institute (VRI), Lahore, Pakistan. The antigen was prepared as per prescribed standards of[8] and level III safety. Trypticase soya agar (0.1% yeast extract) was used for propagation of the seed culture, and mass culture was obtained by inoculation of culture in roux flask incubated at 37°C for 48 hours. The organisms were harvested in 0.5% phenol saline and were killed by heating in water bath at 80°C for 90 minutes. After viability testing packed cell volume was determined by centrifugation at 3000 rpm for 75 minutes. The antigen produced was stored at 4°C for further processing.

### Comparison of the efficiency of RBPT antigens

The efficiency of freshly prepared RBPT antigen was checked by comparing it with the locally produced RBPT antigen (VRI, Lahore, Pakistan) and imported brands of RBPT antigens (Institute POURQUIER, Spain). The efficiency of freshly prepared antigen was evaluated by using SAT and also the cELISA.

### Rose Bengal Plate Test (RBPT)

Serum (30µl) was mixed with RBPT antigen (30µl) at room temperature on a glass plate and shaken thoroughly for 4 minutes. Simultaneous procedure was done for all the three RBPT antigens and the formation of clumps were considered as positive result.

### Serum agglutination test (SAT)

The serum samples were diluted as 1/10, 1/20, 1/40, and up to 1/320. SAT antigen was provided by VRI, Lahore, Pakistan. SAT is used as a confirmatory test for RBPT diagnosis for the brucellosis[11, 12]. The highest dilutions with agglutination were recorded as titre in the procedure.

### Competitive enzyme linked immune sorbent assay (cELISA)

For cELISA the dilution of the control and samples were prepared by adding 45µl of sample dilution buffer to each well of the plate. Then 5µl of serum controls (positive, weak positive and negative) and test samples were mixed with 45µl sample dilution buffer in their respective wells. It was followed by the addition of 50µl of monoclonal antibodies solution to all the wells. The plate was then sealed and was then incubated at room temperature for 30 minutes. The plate was rinsed with phosphate buffer saline-Tween buffer followed by the addition of the conjugate solution. After incubation the plate was rinsed, and then 100µl of Tween buffer substrate solution was added. Finally the reaction was ended by adding 50µl of stop solution ($H_2SO_4$). Optical densities of control and samples were measured at 450nm.

### Evaluation of newly prepared in-house RBPT antigen

Efficiency of the freshly prepared in-house RBPT antigen was evaluated by calculating the values of relative sensitivities and specificities for all the three brands of RBPT antigens. The levels of agreement between the RBPT antigens and cELISA were calculated by Kappa test. Area under

the receiver operating characteristic (AUROC) curve was determined by using statistical software of Win Episcope (version 2.0) and SPSS (version 16.0) respectively.

## RESULTS AND DISCUSSION

### Comparison with local and imported antigen by RBPT and SAT

A total of 288 serum samples were tested for comparative analysis using in-house, the locally produced and imported RBPT antigens. The recorded concordance level between the local and in-house RBPT was 80.2%, while the disconcordance was 19.7% between both the tests (Table 1). The level of agreement (*K* values) between in-house and local RBPT antigens was found as 0.49 (95% CI, 0.38 – 0.61). Both the in-house and imported antigens showed 80.5% of concordance and 19.4 of disconcordance (Table 1), while the level of agreement was 0.51 (95% CI, 0.39 – 0.62). The serum samples which showed disconcordance between different antigens types for the detection of *Brucella* antibodies were further tested by SAT and compared with the results obtained with RBPT antigens (Table 2).

### Comparison of in-house RBPT with competitive cELISA

To evaluate the efficiency of the RBPT antigens, the concordance of their results were measured with the cELISA which was used as a reference test. The obtained level between cELISA and in-house RBPT antigen was 82.7%, and the recorded 77% and 72.9% concordance with the local and imported RBPT respectively (Table 3). The kappa value between cELISA vs. in-house RBPT antigen was found to be 0.62, showing a good level of agreement. The kappa value between cELISA vs. local and imported RBPT antigens were 0.45 and 0.39 showing moderate and fair level of agreements respectively. The relative sensitivities and specificities of all the three brands of antigens were calculated using cELISA as a reference test. Relatively the sensitivity and specificity of in-house RBPT antigen was found to be higher as compared to that of local and imported brands of antigens (Table 4). The newly prepared in-house RBPT antigen yielded large AUC 83.9 (95% CI, 77.1 – 90.7) followed by RBPT (local) 74.2 (95% CI, 65.8 – 82.6) and RBPT (imported) 70.2 (95% CI, 61.4 -78.9)

**Table 1.** Comparison between in-house, locally produced, and imported RBPT antigens for the detection of *Brucella* antibodies

| | | Different brands of RBPT antigens | | | | | |
|---|---|---|---|---|---|---|---|
| | | Local RBPT | | | Imported RBPT | | |
| | | Positive | Negative | Total | Positive | Negative | Total |
| In-House RBPT | Positive | 49 | 36 | 85 | 50 | 34 | 84 |
| | Negative | 21 | 182 | 203 | 22 | 182 | 204 |
| | Total | 70 | 218 | 288 | 72 | 216 | 288 |
| | Concordance =80.2 % | | | | Concordance =80.5 % | | |
| | Disconcordance =19.7 % | | | | Disconcordance =19.4 % | | |

**Table 2.** Comparison between SAT and various brands of RBPT antigens on the samples showing disconcordance with RBPT for the diagnosis of brucellosis

| | | Different brands of RBPT antigens | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | In-house RBPT | | | Local RBPT | | | Imported RBPT | | |
| | | Positive | Negative | Total | Positive | Negative | Total | Positive | Negative | Total |
| SAT | Positive | 21 | 10 | 31 | 17 | 14 | 31 | 18 | 13 | 31 |
| | Negative | 1 | 03 | 04 | 03 | 01 | 04 | 02 | 02 | 04 |
| | Total | 22 | 13 | 35 | 20 | 15 | 35 | 20 | 15 | 35 |
| | Concordance = 68.5 % | | | Concordance =51.4 % | | | Concordance =57.1 % | | | |
| | Disconcordance =31.4 % | | | Disconcordance =48.5 % | | | Disconcordance =42.9 % | | | |

(Table 5). Brucellosis is important bacterial zoonotic disease caused by *Brucella* spp[3, 13]. This disease has profound global economic impact on livestock industries and public health sector[14]. In different areas of Pakistan the prevalence of animal brucellosis ranged between 28 to 31% [15, 16], and warrants a comprehensive country wide survey for observing actual disease burden.

A study on in-house RBPT antigen was carried out in Malaysia by[17] by the procedure described by OIE, and similar outcomes as adopted in the present study were observed. It was established that in-house RBPT antigen was quite efficient for the diagnosis and screening, and the results were confirmatory on revalidation. All the results of in-house RBPT antigen were in concordance with local and imported antigens and the levels of concordance were not significantly different from each other. Though it is quite clear that no single test provides absolute efficiency of the RBPT antigen; however SAT was recommended as confirmatory test for RBPT by[8]. The present study showed that the detection of *Brucella* antibodies by newly prepared antigen were more in agreement with SAT which declared in-house RBPT antigen as more efficient antigen as compared to local and imported antigens. Similarly SAT has been used as a confirmatory test for RBPT detection for the brucellosis by other researchers and showed comparable agreement of the results[11, 12]. The samples which were negative with RBPT and positive by cELISA were considered as positive. It was found that RBPT antigens were not able to detect the antibodies in these positive serum samples and produced false negative results. It could be due to low titer of antibodies which might not be detected by RBPT antigens. The method of ELISA efficiently detects very low titer of antibodies[4] and has more sensitivity than other serological methods such as SAT[18]. Previously, comparative efficiencies of RBPT, SAT and Dot ELISA were checked, and was observed that all the samples were showing *Brucella* infection with ELISA[19]. The results were not in line with the present study which might be due to the differences of ELISA kit findings (competitive vs. Dot). Secondly, in the previous study[20] the tested samples were all from suspected cases of brucellosis whereas in the present study, samples were randomly collected from different categories

**Table 3.** Comparison of cELISA and different brands of RBPT antigens for the diagnosis of brucellosis

| | | Different brands of RBPT antigens | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | In-house RBPT | | | Local RBPT | | | Imported RBPT | | |
| | | Positive | Negative | Total | Positive | Negative | Total | Positive | Negative | Total |
| cELISA | Positive | 45 | 22 | 67 | 36 | 23 | 59 | 33 | 27 | 60 |
| | Negative | 8 | 99 | 107 | 17 | 98 | 115 | 20 | 94 | 114 |
| | Total | 53 | 121 | 174 | 53 | 121 | 174 | 53 | 121 | 174 |
| | Concordance =82.7% | | | | Concordance =77% | | | Concordance =72.9% | | |
| | Disconcordance =17.2 % | | | | Disconcordance =22.9 % | | | Disconcordance =27.01 % | | |

**Table 4.** The relative sensitivity and specificity values of all the RBPT antigens for the diagnosis of brucellosis

| RBPT Antigens | Relative Sensitivity (%)95 % CI | Relative Specificity (%)95 % CI |
|---|---|---|
| In-house | 84.9 (75.3 - 94.5) | 82.1 (75.3 - 88.9) |
| Local | 66.1 (53.3 - 78.8) | 81.3 (74.4 - 88.2) |
| Imported | 62.3 (49.2 - 75.3) | 78.1 (70.7 - 85.4) |

**Table 5.** Values of area under curve of the three RBPT antigens using cELISA as a reference test

| Pair of Serological Test | AUC | 95 % CI |
|---|---|---|
| In-House RBPT vs. cELISA | 83.9 | 77.1 – 90.7 |
| Local RBPT vs. cELISA | 74.2 | 65.8 – 82.6 |
| Imported RBPT vs. cELISA | 70.2 | 61.4 - 78.9 |

of animals. The relative sensitivities and specificities values of RBPT antigens were calculated considering cELISA as a reference test. The results revealed that in-house RBPT antigen was relatively more sensitive [84.9% (95% CI, 75.3 – 94.5)] as well as more specific (82.1%) in the diagnosis of brucellosis as compared to local (81.3%) and imported antigens (78.1%). Results of this study were in line with a previous study reported by[20, 21].

The AUROC curve analysis of the test for all the three antigens were ranged between 70 to 90%. The highest AUC values of in-house RBPT indicated its good efficiency in the diagnosis of brucellosis. The results obtained (94.3%) by [20] for RBPT with ROC analysis was found higher than the values obtained in the present study 83.9% (95% CI, 77.1 – 90.7). Such variations might be due to the reason that in the present study the serum samples were collected from different animal categories while in the former study the serum samples were collected from suspected humans[20]. The levels of agreement of the entire three antigens were measured with ELISA as reference test. Good level of agreement was observed for the newly prepared in-house RBPT (0.62), while local and imported RBPT antigen revealed moderate (0.45) and fair (0.39) level of agreements, respectively with similar outcomes in interpretations[22].

It is concluded that the newly prepared RBPT antigen was more efficient in terms of sensitivity and specificity for detection of brucellosis, suggesting it an accurate epidemiological and surveillance test. Findings from the current study could be helpful in brucellosis control program and adoption of prompt eradication strategies for the disease prevention.

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