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Research Article

Comparative evaluation of new and conventional serological assays for diagnosis of brucellosis in buffaloes

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Abstract

Brucellosis, a major zoonotic disease, is prevalent in most parts of the world and is mainly diagnosed by serological tests. The present study aimed to assess the diagnostic efficacy of RBPT, MAT, mMAT and I-ELISA, in order to identify an optimal combination for enhanced and precise diagnosis of brucellosis in buffaloes. A total of 450 buffalo serum samples were analysed against these tests. The data were analysed in Winepiscope-2 software for determining the degree of agreement between test combinations. Out of these, 138 (30.66%) tested positive in I-ELISA, while 72 (16%), 93 (20.66%), and 84 (18.66%) were positive in RBPT, MAT, and mMAT, respectively. I-ELISA detected the highest number of positive samples, with RBPT exhibiting the lowest positivity rate. Across all four serological assays, 296 samples tested negative and 63 tested positive. I-ELISA identified 49 samples as positive that were negative in the other tests. Nine samples positive in RBPT were negative in rest of the three tests. RBPT identified 21 samples as negative, all of which yielded positive results in the remaining three serological tests. 12 samples positive in MAT were negative in rest of the sero-assays. MAT exhibited the highest sensitivity (75.85%), followed by mMAT (72.16%), with RBPT having the lowest (69.89%), all relative to I-ELISA, which had 100% sensitivity. In terms of specificity, RBPT and MAT recorded 97.44%, while mMAT had the highest at 98.53%. A substantial degree of agreement was observed between the test combinations. It may be concluded that I-ELISA is a highly accurate and precise test and can be used for routine sero-diagnosis of brucellosis in buffaloes, in combination with mMAT to supplement the diagnosis, so as to reduce the likelihood of false test outcomes.

Keywords: Brucellosis, Buffaloes, Conventional Serological assays, I-ELISA

Highlights

- Percent positivity of I-ELISA was more compared to the other tests
- RBPT exhibiting the lowest positivity rate
- A Substantial degree of agreement was observed among all the three test combination

INTRODUCTION

Brucellosis, a prominent zoonotic disease is prevalent in most parts of the world including India (Refai, 2002). Bovine brucellosis is mostly caused by *Brucella abortus* and less frequently by *B. melitensis* (OIE, 2018). Though *Brucella* spp may enter into the host through conjunctiva, mucus membranes, lungs or abraded skin, however, oral route is the most common portal of entry of this agent (Ferrero *et al*., 2014). The disease in India leads to significant economic losses in ruminants (Singh *et al*., 2015; Shome *et al*., 2021). Data suggests that 95.6% of the total economic losses attributed to brucellosis in livestock populations occur in cattle and buffaloes (Singh *et al*., 2015). The chief clinical feature of brucellosis includes abortion, retained placenta and still birth in females and orchitis in males (McDermott *et al*., 2013). The chronic nature of disease and its asymptomatic course in heifers and young animals, poses a significant challenge in diagnosing the infection before it is clinically manifested (Malik *et al*., 2014; Khan and Zahoor, 2018). Isolation (the gold standard) involves culturing the agent from uterine discharge, aborted materials, milk or blood (Ali *et al*., 2014). Though, isolation of the causative agent is indisputable, in its diagnostic accuracy; however, it is cumbersome, requires a biosafety level three laboratory setup, trained personnel, and poses a serious threat to lab workers. Additionally, the isolation of the organism is not always feasible due to low bacterial count in the sample, loss of viability of organisms, presence of contaminants, and inhibitory factors (Sari *et al*., 2008; De Miguel

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et al., 2011). Lack of sensitivity of isolation and zoonotic nature of the organism makes serological assays more preferable. Conventional sero-assays like, Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (SAT) and Complement Fixation Test (CFT) are commonly used for diagnosis of this infection in buffaloes (Malik *et al*., 2014). Although sensitive, these serological tests can yield false-positive results, particularly in cases of prior vaccination or exposure to cross-reacting bacteria (Bonfini *et al*., 2018). These cross reactions primarily arise due to the antigenic similarities between *Brucella* species and other microorganisms and are more prevalent in regions where these organisms are endemic (Nielsen and Yu, 2010). To improve the accuracy and reliability, modifications to traditional serological assays are often introduced to minimize the likelihood of false test outcomes (Nielsen and Yu, 2010). Conducting parallel testing of serum samples is an additional approach to augment the accuracy and reliability of diagnostic assays (Nielsen, 2002). New serological assay like ELISA is widely considered for diagnosis of brucellosis in animals. This method is recognized for its robustness, reliability and sensitivity and plays a crucial role in the early diagnosis of brucellosis in animals; which is crucial for prompt implementation of control measures, minimizing economic losses, protecting public health, and facilitating disease monitoring and management (Godfroid *et al*., 2010; Nicoletti, 2013). Numerous studies have emphasized the utility and efficacy of ELISA in this context, showcasing its effectiveness in identifying specific antibodies indicative of brucellosis in animals (Rahman *et al*., 2016; Islam *et al*., 2018; Deka *et al*., 2021; Sarangi *et al*., 2022). Most of these studies have documented the use of RBPT, SAT, CFT, or ELISA alone or in combination in cattle, however limited data is available on the comparative performance of traditional and new serological assays in buffaloes from Punjab region of India. Keeping in view the inherent limitations of traditional serological tests and the limited data available on the synergistic application of new and traditional sero-assays in buffaloes from Punjab region of India, the present study aims to assess the diagnostic efficacy of RBPT, Micro-plate agglutination test (MAT), modified Micro-plate-agglutination Test (mMAT), and Indirect ELISA (I-ELISA) in order to identify an optimal combination for enhanced and precise serodiagnosis of brucellosis in buffaloes.

MATERIALS AND METHODS

Selection of animals: The sample details, processing, and storage under laboratory conditions are outlined elsewhere (Islam *et al*., 2018). A total of 450 samples were drawn randomly from the entire collection using table of random numbers. Samples drawn for evaluation were exclusively obtained from animals aged one year and older, with no vaccination history against brucellosis.

Serological analysis: Four serological assays, I-ELISA, RBPT, MAT (which is actually a miniaturization of Standard Tube Agglutination Test) and its modification (mMAT) were used to analyse the samples. Both MAT and mMAT were carried out in U bottom 96 micro-titre plates. Antigen for RBPT and MAT were obtained from Biological Product Section of DAH Punjab. RBPT and MAT were performed as per the method described by Alton *et al*. (1988). mMAT was performed as per the method of Nasir *et al*., (2005) and OIE (2018). Modification involved addition of 0.5% EDTA- a chelating agent, to each serum dilution, which helps in reduction of nonspecific agglutinins. Commercially available I-ELISA kit was obtained from Bionote (2-9, Seogu-dong, Hwaseong-si, Gyeonggi-do, Korea 445-170). I-ELISA was performed as per manufacturer's instructions. The optical density (OD) values were used to calculate the percent positivity as shown in the equation. The test sera were categorised as positive or negative based up on the percent positivity value. Samples having percent positivity value 25 or above (%P≥ 25) were categorised as positive and below 25 as negative (%P ≤ 25).

$$\% \text{ Positivity} = \frac{\text{OD of Sample}}{\text{Average OD of standard strong positive control}}$$

Statistical analysis: In estimating the relative sensitivity and specificity, I-ELISA was chosen as the reference test, against which RBPT, MAT and mMAT were cross classified in 2 × 2 contingency table. The tests were pair compared with each for calculating the degree of agreement. The data were analysed in Winepiscope-2 software for determining the degree of agreement between test combinations. Arbitrary benchmarks for observed kappa values as described by Thrusfield (2005) were used for evaluating observed kappa values.

RESULTS

This study aimed to compare the test outcomes of I-ELISA with those of traditional diagnostic tests. Among 450 serum samples, 138 (30.66%) tested positive in I-ELISA, while 72 (16%), 93 (20.66%), and

84 (18.66%) were positive in RBPT, MAT and mMAT respectively. Notably, I-ELISA detected the highest number of positive samples, followed by MAT, with RBPT exhibiting the lowest positivity rate (Table 1).

Table 1. Test results of the serum samples

Sero-assay / Test Outcome	I-ELISA	RBPT	MAT	mMAT
Positive	138	72	93	84
Negative	312	378	357	366
% positivity	30.66	16.00	20.66	18.66
%Sensitivity*	100	69.89	75.85	72.16
%Specificity*	97.9	97.44	97.44	98.53

*Sensitivity and specificity of traditional tests relative to I-ELISA

Across all four serological assays 296 samples tested negative and 63 tested positive. I-ELISA identified 49 samples as positive that were negative in the other tests. Nine samples positive in RBPT were negative in rest of the three tests. RBPT identified 21 samples as negative, all of which yielded positive results in the remaining three serological tests. 12 samples positive in MAT produced negative outcomes in rest of the three serological tests (Table 2).

Table 2. Comparison of serological tests

I-ELISA	RBPT	MAT	mMAT	No of samples
-	-	-	-	296
+	-	-	-	49
-	+	-	-	9
-	-	+	-	12
+	-	+	+	21
+	+	+	+	63

The observed proportion of agreement and kappa values between I-ELISA and traditional serological tests is depicted in Table 3. A Substantial degree of agreement was observed among all the three test combination (Table 3).

Table 3. Observed proportion of agreement between the tests

Test combination	OPA*	kappa value (k)	Degree of Agreement
I-ELISA+ RBPT	0.86	0.71	substantial
I-ELISA + MAT	0.89	0.76	substantial
I-ELISA + mMAT	0.88	0.74	substantial

*Observed proportion of agreement

DISCUSSION

Brucellosis poses a significant zoonotic challenge globally and is endemic in India (OIE, 2018). There has been a staggering increase in the prevalence of the disease, owing to lack of sanitary practices, hygiene, bio-security measures and increased stocking density of animals (Deka *et al*., 2021). The complexity arises from the unrestricted movement of animals, particularly through livestock markets (Mandi), from one region to another, desperate sale of cattle testing positive on screening and lack of compensation for disposing of these positive animals due to religious issues, quarantine facilities at state borders and coordinated vaccination programme further tend to aggravate the disease scenario (Behera *et al*., 2020).

Serological tests for diagnosis of bovine brucellosis have been more useful when compared with gold standard (Andrade *et al*., 2024), as diagnosis is complicated in sero-positive animals without an active infection (Goldbaum *et al*., 1993). By comparing the test results, it is evident that I-ELISA gave the highest positive test outcomes and least by RBPT. Little difference in percent positivity was observed among RBPT, MAT and mMAT, but significant difference was observed between I-ELISA and other three tests. This is due to the fact that I-ELISA is more sensitive compared to the traditional tests. I-ELISA kit used in the present study had sensitivity and specificity of 100% and 97.9%, respectively. I-ELISA has undergone extensive validation and standardization for the sero-diagnosis of brucellosis in various animal species. It has high sensitivity, specificity and throughput, making it a valuable tool for screening and surveillance programmes (Legesse *et al*., 2023). This inherent feature of ELISA enables it to detect very low levels of antibodies present in the early stage of infection, which RBPT and SAT fail to detect (Guarino *et al*., 2001). Paweska *et al*. (2002) proposed that I-ELISA could not only replace the confirmatory CFT but also serve as a substitute for routine screening tests like RBPT and STAT. Gall and Nielsen, (2004), in their review of various serological tests, acknowledged that no single test is flawless but emphasized that errors can be minimized by using the most reliable test available. Chand and Sharma, (2004) recommended the use of ELISA over RBPT and STAT in evaluating brucellosis in cattle, as it yields better results with minimal chances of non-detection of anti *Brucella* antibodies. Gorsich *et al*. (2015) reported that I-ELISA test exhibited a sensitivity of 92.8% and specificity of 87.0% for diagnosing brucellosis in African buffaloes. Mantur *et al*. (2010) reported the sensitivity and specificity of ELISA was 100% and 71.31%, respectively in diagnosing

human brucellosis. Xu *et al*. (2020) concluded that ELISA is the most sensitive and specific method to detect human brucellosis in endemic areas and is superior to culture and agglutination test. I-ELISA is more sensitive and specific compared to RBPT and SAT and could significantly reduce non-specific reaction by improving the diagnostic specificity for an accurate brucellosis diagnosis in endemic areas (Alamian *et al*., 2023). As per Islam *et al*. (2013) and OIE (2018), I-ELISA is recommended primarily as a screening test and is advocated for routine use in the sero-diagnosis of brucellosis in animals.

RBPT is a suitable individual screening test. The low pH (3.65) of the RBPT antigen helps in reduction of nonspecific reactions, which are mainly due to IgM (Allan *et al*., 1976). Though RBPT is easy to perform, inexpensive, has high throughput, yet low sensitivity in chronic cases and low specificity in endemic areas compared to other assays results in false test outcomes (Greiner and Gardner, 2000). It is plausible that nine samples, exclusively positive in RBPT, may be attributed to false positive reactions. These false positive reactions are mostly due to immune response of an animal to cross reacting micro-organisms (Bonfini *et al*., 2018). Presence of nonspecific agglutinins in serum of some animals without any history of brucellosis can also contribute to false test outcomes in RBPT (Falade *et al*., 1978). RBPT can give false negative outcomes also, a phenomenon attributed to either presence of below detectable range antibody levels; often seen in incubatory phase of disease, incomplete antibodies, nonspecific agglutinins and inhibitors (Lamees *et al*., 2020). Additionally, serum samples from animals classified as high responders can have exceptionally high antibody titres, leading to pro-zoning in RBPT (OIE, 2018). This phenomenon can contribute to false-negative test outcomes, as observed in 21 samples that exclusively tested negative in RBPT. The storage of serum samples and fluctuations of temperature can affect the test outcomes, as improperly stored samples can lead to loss of integrity and function of immunoglobulins. However, long term storage under deep freezing conditions, typically -20°C or lower does not affect the structure of antibodies or outcome of serological assays, if proper storage conditions are maintained (Coffey *et al*., 2020). Available information suggests that immunoglobulins can remain stable for years when properly stored under deep-freezing conditions (Lewis *et al*., 2016). These particular samples exhibited exceptionally high antibody titers in both MAT and mMAT, necessitating dilutions beyond 1:1280 to reach the titre endpoint. Non-responsiveness of an animal to the antigenic challenge resulting in an inability to mount a detectable immune response can also contribute to false negative reactions (Islam *et al*., 2013). Likelihood of false test outcome in traditional sero-assays has been reported to increase in endemic areas and chronic cases (Greiner and Gardner, 2000). Ta *et al*. (2022) reported that false negative rate of RBPT in human brucellosis cases increases with the course of disease and constituent ratio of false negative reactions was 20% after 180 days.

The neutral pH of SAT antigen makes the assay susceptible to false test outcomes. The susceptibility of SAT under neutral pH environment is attributed mostly to cross-reacting antibodies, especially IgM isotype, which is most active at neutral pH (Nielsen, 2002). This results in decreased assay sensitivity and specificity. It is plausible that 12 samples exclusively positive in MAT might be due to cross-reacting antibodies, which are active at neutral pH. Due to this reason a large number of modifications have been done to the original SAT like chelating by divalent cations such as ethylene diamine tetraacetic acid (EDTA), additions of reducing agents (Alton *et al*., 1988), which reduces cross reactions due to nonspecific agglutinins, mainly attributed to IgM (Nielsen *et al*., 1979), thereby resulting in increase in specificity of SAT. The underlying mechanism is not understood, however, Alton *et al*. (1988) are of the opinion that it eliminates the attachment of immunoglobulin to *Brucella* cell wall via Fc region. Although SAT is still used for diagnosis of brucellosis, it is laborious, time-consuming and requires more antigen and other inputs, which reduces its throughput. The test is usually carried out in glass test tubes. SAT and its modified formats can be carried out in U bottom 96 well micro-titre plate i.e., miniaturization (MAT), which saves time, reagents and enhances throughput (Emmerzaal *et al*., 2002). MAT shows significant correlation with SAT and ELISA and can potentially be automated for its inclusion as supplementary test in diagnosis and control of brucellosis (Baum *et al*., 1995; Park *et al*., 2012). A substantial degree of agreement between I-ELISA and rest of the sero-assays indicates significant level of consistency between the test combination. Suggesting that I-ELISA can be used alone for routine sero-diagnosis of brucellosis in buffaloes.

Though the study provides valuable insights into comparative performance of traditional and new serological assays however, a larger sample size and inclusions of a confirmatory test or gold standard could improve the statistical power of findings, so that the generalization of results is enhanced.

Keeping in view the findings of the present study, it may be concluded that I-ELISA is a highly sensitive and specific test and can be used for more accurate and precise sero-diagnosis of brucellosis in buffaloes, in combination with mMAT to complement and supplement the diagnosis, so as to reduce the likely hood of false test outcomes.

Conflict of interest: The authors have no conflict of interest.

Author's contribution: MRUI: Involved in investigation, data generation, preparing original draft, statistical analysis; GF: Engaged in conceptualization, data curation, supervision and final editing; MPG: Editing of the manuscript, preparation of final draft and proofreading.

Data availability statement: The data placed in this article will be available from the corresponding author on request.

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