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Comparative evaluation of indirect enzyme linked immunosorbent assay, rose bengal plate test, microagglutination test, and polymerase chain reaction for diagnosis of brucellosis in buffaloes

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Abstract: In the present study, 178 blood samples from buffaloes were tested against indirect enzyme linked immunosorbent assay (I-ELISA), rose bengal plate test (RBPT), microagglutination test (MAT), modified microagglutination test (mMAT), and polymerase chain reaction (PCR) to select the most suitable test for efficient and effective diagnosis of bovine brucellosis. Win Episcope 2 software was used to determine the agreement between tests (kappa values at 95% confidence interval). I-ELISA was pair compared with all the other tests. Out of 178 samples, 102 were found positive by I-ELISA, 81 by RBPT, 85 by MAT, 79 by mMAT, and 68 by PCR. Substantial agreement was observed between I-ELISA and RBPT ($\kappa = 0.72$), I-ELISA and MAT ($\kappa = 0.65$), and I-ELISA and mMAT ($\kappa = 0.67$). The least degree of agreement was observed between I-ELISA and PCR ($\kappa = 0.15$). I-ELISA detected more samples as positive among these tests. The results of the present study indicate that I-ELISA can be used for routine sero-diagnosis of *Brucella* infection in buffaloes. Furthermore, PCR can be used in combination with I-ELISA to complement the serological diagnosis, especially in the initial phase when the immune response of the animal is not detectable.

Key words: Brucellosis, I-ELISA, RBPT, MAT, mMAT, PCR

1. Introduction

Brucellosis is caused by gram-negative bacteria of the genus *Brucella*, which are facultative intracellular coccobacilli that belong to the family α2-Proteobacteriacea (1). The genus *Brucella* has been subdivided into 6 classical *Brucella* species, namely *Brucella abortus* (cattle and buffaloes), *B. melitensis* (goats), *B. suis* (pigs, reindeer), *B. ovis* (sheep), *B. neotomae* (desert wood rats), and *B. canis* (dogs), based on strong affiliation to specific natural hosts (2). In addition to the classical *Brucella* spp., the genus has recently been expanded to include marine isolates, which have been divided into 2 species, *Brucella ceti* and *Brucella pinnipedialis*, based on their preferential hosts, i.e. cetaceans and pinnipeds, respectively (3).

Outbreaks of bovine brucellosis are associated with abortion during the last trimester of gestation, production of weak newborn calves, and infertility in cows and bulls (4). Diagnosis based on clinical signs cannot be generalized to all age groups, especially in nonpregnant heifers and males, as abortion is the only chief clinical feature of this infection. Therefore, a definitive diagnosis must be supported by laboratory tests, including serological assays or direct diagnostic tests, i.e. isolation and biochemical characterization of the organism. As isolation of this organism is laborious and poses a potential public health threat to laboratory workers, an alternative is the use of polymerase chain reaction (PCR)-based methods for detecting *Brucella* genomic DNA (5,6). Serological assays are based on the fact that *B. abortus*, as well as other smooth *Brucella*, has the O polysaccharide, which induces a humoral response with an initial production of IgM followed by IgG1 and IgG2/IgA (7). Screening tests include the buffered acidified plate antigen test and the milk ring test, both of which have high sensitivity. These tests can be complemented by confirmatory tests like the complement fixation test. Indirect or competitive ELISA and fluorescent polarization assay are also employed as confirmatory tests (8). There is a lack of information pertaining to the comparative serological diagnosis of brucellosis in buffaloes and it is likely that different animal

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species may react differently to the infectious agent owing to genetic variability of individual animals, resulting in different test results. The present study was envisaged to compare serological tests and PCR for diagnosis of bovine brucellosis, so as to select the most suitable test for its efficient and effective diagnosis.

2. Materials and methods

2.1. Selection of animals

A total of 178 animals were selected from an organized buffalo herd of 215 animals. Animals less than 1 year old were excluded. None of the animals were vaccinated against brucellosis. There were 54 animals that had aborted over a period of 4 years. Most of these abortions (48) had taken place in the last trimester of gestation, while 6 abortions were recorded in the second trimester.

2.2. Sample collection

Blood samples were aseptically collected from the selected 178 animals by jugular vein-puncture. About 5–10 mL of blood was collected in plain tubes without any anticoagulant. The blood samples were put on ice immediately and kept on it until transportation to the laboratory. Serum was separated from clotted blood by centrifugation at 3000 rpm for 5 min and stored at –20 °C until further use.

2.3. Serological analysis

Five different diagnostic techniques, i.e. indirect enzyme linked immunosorbent assay (I-ELISA), rose bengal plate test (RBPT), microagglutination test (MAT; which is actually a miniaturization of the standard tube agglutination test), modified microagglutination test (mMAT), and PCR, were comparatively evaluated for detection of brucellosis. RBPT and MAT were performed as per the method described by Alton et al. (9). mMAT was performed as per the method of Nasir et al. and OIE (10,11). *Brucella abortus* (strain 99) colored and plain antigens were obtained from the Punjab Veterinary Vaccine Institute, Ludhiana, India.

2.3.1. Rose bengal plate test (RBPT)

Thirty microliters of serum was mixed with an equal volume of rose bengal antigen on a clean grease-free slide to produce a zone approximately 2 cm in diameter. After that, both drops were mixed by a disposable stirring stick, spreading them over the full surface of the circle. The slide was rotated manually for 4 min and analyzed for the presence or absence of any degree of agglutination. Controls were run using known positive and known negative sera.

2.3.2. Microagglutination test (MAT)

All the serum samples were tested with a minimum of 8 dilutions. A microtiter plate was appropriately labeled and 80 µL of 0.85% normal saline was added to the first row and 50 µL to the rest of the rows. To each well of the first row was added 20 µL of a particular serum sample. The contents in the first row, i.e. the serum and saline, were thoroughly mixed and 50 µL of this mixture was transferred to the corresponding well in the second row. The process was repeated until the last row. From the last row 50 µL of the mixed contents was discarded. This was followed by addition of 50 µL of plain antigen to each well. The microtiter plate was incubated at 37 °C for 24 h before the results were read. Controls were run using known positive and known negative sera. Interpretation of the results was based on the formation of an agglutination matrix (mat formation) or button formation at the bottom of the well. The titer so obtained was expressed in the unit system by doubling of the serum titer as International Units (I.U.) per milliliter of serum; 80 I.U. (≥1:40) or above was considered positive for brucellosis as per the recommendations of OIE (11).

2.3.3. Modified microagglutination test (mMAT)

The test was performed in a fashion similar to that of MAT except for the buffer used, which was phosphate buffer saline (PBS) containing 10 mmol of EDTA. Controls were run using known positive and known negative sera. Results were also read in the same fashion as those of MAT.

2.3.4. I-ELISA

A commercially available ELISA kit was obtained from BioNote (Korea) (catalogue no. EB 43-01). I-ELISA was performed as per the manufacturer's instructions. The optical density (OD) values were used to calculate the percent positivity as shown in the equation below. The test sera were categorized as positive or negative based upon the percent positivity value. Samples having percent positivity values of 25 or above (%P ≥ 25) were categorized as positive and below 25 as negative (%P ≤ 25).

$$\% \text{ Positivity} = \frac{\text{OD of sample}}{\text{Average OD of standard strong positive control}} \times 100$$

2.3.5. Polymerase chain reaction (PCR)

For PCR analysis, DNA was extracted from 100 µL of serum using the DNeasy blood kit (Qiagen, Germany) according to the manufacturer's instructions and was eluted in 100 µL of the elution buffer supplied with the kit. DNA amplification using primers (JPF -GCGCTCAGGCTGCCGACGCAA & JPR - ACCAGCCATTGCGGTCGGTA) originally described by Leal-Klevezas et al. (5) was performed on 8 µL of DNA sample in a 25 µL reaction mixture containing 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 200 µmol/L of each dNTP, 50 pmol/µL of each primer, and 2.5 units of Taq polymerase. After an initial denaturation step at 94 °C for 4 min, 35 amplification cycles

were performed, each consisting of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C and followed by a final extension step at 72 °C for 3 min. All PCRs were performed with the appropriate inclusion of positive and negative controls. Eight microlitres of the amplification reaction was taken and resolved on 1.5% agarose gel containing 1 × TBE, stained with an ethidium bromide solution and visualized under ultraviolet light.

2.4. Statistical analysis

I-ELISA was pair compared with RBPT, MAT, mMAT, and PCR. The data were analyzed in Win Episcope 2 software to test their agreement. Arbitrary benchmarks for observed kappa values as described by Thrushfield (12) were used for evaluating observed kappa values.

3. Results

Out of 178 samples, 102 were positive by I-ELISA, 81 by RBPT, 85 by MAT, and 79 by mMAT, and amplicons of 193 bp (Figure) were detected in 68 samples by PCR (Table 1). Forty-nine samples were negative in all the tests. I-ELISA detected 10 samples as positive that were negative in the rest of the tests. There were 5 samples that were positive by I-ELISA and RBPT but negative in the rest of the tests. Six samples that were negative by RBPT and PCR were positive in the other 3 tests. Thirty-four samples negative by PCR were positive in the serological tests. Twenty samples that were negative by serological tests were positive in PCR. There were 6 I-ELISA and PCR positive samples that were negative in the other 3 tests. Only 3 samples tested positive against I-ELISA, RBPT, and PCR. Thirty-six samples were positive in all the tests.

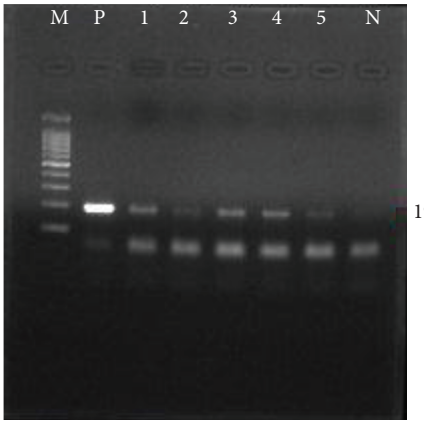

Figure. Amplified products of 193 bp.
Lane M: molecular lane marker; P: positive control; 1–5: samples; N: negative control.

The agreement of I-ELISA with the 4 other tests along with OPA is depicted in Table 2. There was substantial agreement between I-ELISA and RBPT ($\kappa = 0.72$ at $P < 0.05$), between I-ELISA and MAT ($\kappa = 0.65$ at $P < 0.05$), and between I-ELISA and mMAT ($\kappa = 0.67$ at $P < 0.05$), while the least degree of agreement was observed between I-ELISA and PCR ($\kappa = 0.15$ at $P < 0.05$).

4. Discussion

Achievement of an infallible diagnosis of brucellosis is a tedious process, since isolation is influenced by a number of factors, such as highly fastidious growth requirements, a lower number of viable organisms in the sample, and delay in sample transportation to the laboratory, and it is a potential health hazard for laboratory workers.

Table 1. Outcome of individual tests.

Test	I-ELISA	RBPT	MAT	mMAT	PCR
Positive	102	81	85	79	68
Negative	76	97	93	99	110

Table 2. Comparison of I-ELISA with other tests.

Test combination	Observed proportion of agreement and proportion of agreement beyond chance (κ value)*
I-ELISA/RBPT	0.86, $\kappa = 0.72$
I-ELISA/MAT	0.82, $\kappa = 0.65$
I-ELISA/mMAT	0.83, $\kappa = 0.67$
I-ELISA/PCR	0.56, $\kappa = 0.15$

*Statistical features calculated from category-wise comparison of results using Win Episcope 2 software with 95% confidence level.

RBPT has been widely used as a screening test. All the results in the present study were read within a specified time period (4 min), so as to avoid false positive reactions that may arise due to formation of fibrin clots. The acid pH further diminishes agglutination by IgM but encourages agglutination by IgG1, thereby reducing cross reactions (13). Most of the false positive results, but not all, may arise due to immune response of an animal to other microorganisms that share epitopes with *Brucella* species (14). Thirty samples that failed to yield a positive outcome in RBPT were positive in the other 3 serological tests. In I-ELISA these samples exhibited high OD and in some cases it was beyond the readable range. These samples had very high titers (above 1:1280) in MAT and mMAT. This may be due to prozoning leading to a false negative reaction in RBPT when sera of high antibody titers are tested against it (15). Nine samples that were positive in RBPT were negative in mMAT, which may be due to false positive reactions. False positive reactions can happen due to naturally occurring nonspecific agglutinins, which may occur in some animals. These agglutinins are EDTA-labile and can be differentiated from agglutinating antibodies by the addition of EDTA to the diluent used in the standard serum agglutination test, which may explain why these 9 samples were negative by mMAT. Given that we obtained a substantial agreement in classifying sera as positive or negative (except for the I-ELISA/PCR combination), it is likely that the bias of nonspecific serological reactions was minimized using mMAT. Therefore, the serological reactions were likely to be true reflections of the field *Brucella* sero-conversion status of the individual animals. Further, the study animals had never been vaccinated against brucellosis.

In the present study, I-ELISA was found to be more sensitive, which is in concurrence with the reports by Chachra et al. (16), Ruppanne et al. (17), Kerby et al. (18), Rao et al. (19), and Paweska et al. (20). This finding differs from that of Mittal et al. (21), who reported that RBPT is more sensitive, followed by STAT and ELISA, when applied to buffalo sera. Erdenebaatar et al. (22) reported that ELISA can be used to eliminate false positive results amongst RBPT positive sera. Chand and Sharma (23) recommended the use of ELISA over RBPT and STAT for assessing the situation of brucellosis in cattle to have better results because the chances of nondetection of an infected animal in ELISA are much lower.

PCR was found to be the least sensitive of the serological tests, therefore giving negative results in a good percentage of samples that were positive by serological tests. Nevertheless, PCR detected 20 samples as positive that were negative in the serological tests. This may be due to the fact that in the early phase of infection bacteremia may be present before the production of circulating antibodies, which may explain why these 20 samples were negative in the serological tests. Some of the animals may even fail to produce detectable levels of antibodies, yet at the same time harbor the organism in their circulation for quite some time without manifesting the disease; such animals will be classified in routine serological assays as negative. Moreover, the weak humoral response elicited against *Brucella* infection may explain the failure of serological tests. Since *Brucellae* are facultative intracellular organisms, fluctuations in the antibody titers in the presence or absence of bacteremia could also be an explanation as reported by Johnson and Walker (24). PCR may be an indispensable tool for identification of such animals.

I-ELISA is a robust test with high throughput and sensitivity. From the results of the present study, it may be concluded that I-ELISA can be routinely used for an accurate and efficient diagnosis of *Brucella* infection, because the chances of nondetection of an infected animal in I-ELISA are minimal. Further, PCR can be used in combination with I-ELISA to complement the serological diagnosis of brucellosis, especially in the initial phase when the immune response of the animal is not detectable.

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