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Short Paper

Comparative efficacy of serological diagnostic methods and evaluation of polymerase chain reaction for diagnosis of bovine brucellosis

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Summary

The aim of present study was to compare the diagnostic performance of the different *Brucella abortus* antigen based serological and molecular tests such as Rose Bengal Plate Test (RBPT), indirect enzyme linked immunosorbent assay (I-ELISA) and polymerase chain reaction (PCR). Out of 89 samples, 44 were positive by I-ELISA, 18 by RBPT and 21 by PCR. Substantial agreement was observed between PCR and I-ELISA ($\kappa=0.48$). A slight degree of agreement was observed between RBPT and I-ELISA and PCR ($\kappa=0.18$) and RBPT and PCR ($\kappa=0.11$). Indirect enzyme linked immunosorbent assay detected more samples as positive among these tests. In conclusion, I-ELISA can be routinely used for an accurate and efficient diagnosis of *Brucella* infection, because the chances of non-detection of an infected animal in I-ELISA are minimal. However, PCR could be used as a supplement and complement test along with I-ELISA for identification and differentiation of bovine brucellosis.

Key words: Bovine Brucellosis, I-ELISA, PCR, RBPT

Introduction

Brucellosis, caused by *Brucella* species, is a serious zoonosis affecting a wide range of domestic and wild animals all over the world (Kumar *et al.*, 2009, 2016; Neha *et al.*, 2014; Verma *et al.*, 2014). At present, brucellosis is usually diagnosed based on serological and microbiological tests. Serological methods such as Rose Bengal Plate Test (RBPT) and enzyme linked immunosorbent assay (ELISA) are not always sensitive or specific due to cross-reactivity with other bacterial antigens (OIE, 2012). Isolation of etiological agent is considered as gold standard test in diagnosis of disease, but isolation of *Brucella* spp. is tedious, time consuming and difficult due to the intra-cellular and fastidious nature of the bacteria and is relatively difficult under field conditions due to its zoonotic nature (Kaynak-Onurdag *et al.*, 2016). Recently, new molecular techniques like the polymerase chain reaction (PCR) have been developed for the detection of *Brucella* DNA in body fluids having low number of non-viable *Brucella* organisms (Çiftci *et al.*, 2017). Therefore, the present study was conducted to evaluate PCR assay for the detection of *Brucella* DNA in serum and from bovine and to compare its performance with serological

methods.

Materials and Methods

Sample collection

The present cross-sectional study was carried out in different districts viz., Agra, Amroha, Baghpat, Bulandshahr, Etawah, Firozabad, Ghaziabad, Hapur, Mainpuri and Mathura of the western part of Uttar Pradesh, India during 2014-2015 using a convenient sampling procedure with records of animal determinants. All the animals were handled as per the guidelines of Ethical Committee. 3-5 ml blood samples were aseptically collected from the selected 89 animals by jugular vein-puncture using vacutainers (BD, USA) and serum was separated by centrifugation and stored at -20°C till tested. A total of 89 animals were selected showing reproductive disorders like abortion, repeat breeding, anoestrous, pyometra, metritis, retention of placenta. No animal had the history of vaccination against brucellosis.

Serological analysis

Three different diagnostic techniques i.e. RBPT,

indirect enzyme linked immunosorbent assay (I-ELISA) and PCR were comparatively evaluated for diagnosis of bovine brucellosis. Rose Bengal Plate Test was conducted as per standard procedure (Alton *et al.*, 1975). Indirect enzyme linked immunosorbent assay was performed as per the manufacturer's instructions, using the kit procured from Svanova (Biotech-AB), Uppasala, Sweden. For PCR analysis, the genomic DNA of *Brucella* spp. was extracted from sera samples. Briefly, 500 μ L of sera samples were placed in a 1.8 ml Eppendorf tube and centrifuged for 15 min at 15,000 \times g. The pellet was resuspended in 200 μ L of nuclease free water and processed for extraction of DNA using phenol-chloroform method (Sambrook and Russel, 2001). DNA amplification was performed using primers (5'-GAC GAA CGG AAT TTT TCC AAT CCC-3' and 3'-TGC CGA TCA CTT AAG GGC CTT CAT-5') (Bricker and Halling, 1994).

Statistical analysis

Serological and molecular tests were compared with each other. Sensitivity, specificity, concordance percentage and the agreement between the tests (kappa statistic) were evaluated (Thrusfield, 2008). Arbitrary benchmarks for observed kappa values as described by Thrusfield (2008) were used for evaluating observed kappa values.

Results

Out of 89 samples, 44 were positive by I-ELISA, 18 by RBPT and 21 by PCR (Table 1). Of 21 PCR positive sera samples, 47.73% were obtained from seropositive animals, while one serum sample that was negative by the serological tests but its serum was positive by PCR. The agreement of I-ELISA with RBPT and PCR is depicted in Table 2. There was fair agreement between PCR and ELISA ($\kappa=0.48$), while a slight agreement was observed between RBPT and ELISA ($\kappa=0.18$); and RBPT and PCR ($\kappa=0.11$). Analysis of concordance percentage indicated a higher concordance percentage of 74.16% between PCR and I-ELISA. Different tests viz., RBPT and PCR were compared and sensitivity, specificity, predictive value (positive) and predictive value (negative) of different tests when compared with I-

Table 1: Outcome of individual tests

Test	RBPT	I-ELISA	PCR
Positive	18	44	21
Negative	71	45	68

Table 2: Agreement between RBPT, ELISA and PCR for diagnosis of bovine brucellosis

Combination of tests	Kappa value	0.95 confidence interval		Concordance percentage (%)
		Lower limit	Upper limit	
RBPT and ELISA	0.1856*	0.0483	0.3229	59.55
PCR and ELISA	0.4801 ⁺	0.3238	0.6364	74.16
RBPT and PCR	0.1149*	0	0.338	69.66

* Slight agreement, and ⁺ moderate agreement

Table 3: Sensitivity and specificity of RBPT for diagnosis of bovine brucellosis in comparison to I-ELISA

Statistics	Value (%)	95% CI
Sensitivity	29.55	16.76 to 45.20%
Specificity	88.89	75.95 to 96.29%
Positive predictive value	72.22	46.52 to 90.31%
Negative predictive value	56.34	44.05 to 68.09%

Table 4: Sensitivity and specificity of PCR for diagnosis of bovine brucellosis in comparison to I-ELISA

Statistics	Value (%)	95% CI
Sensitivity	47.73	32.46 to 63.31%
Specificity	100.00	92.13 to 100.00%
Positive predictive value	100.00	83.89 to 100.00%
Negative predictive value	66.18	53.68 to 77.21%

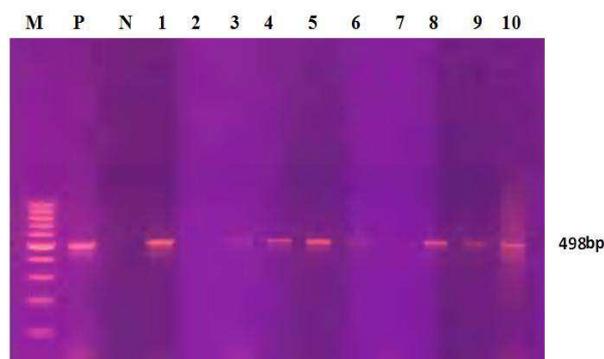


Fig. 1: PCR assay for detection of brucellosis in dairy animals. Lane M: 100 bp DNA ladder, Lane P: Positive control (*Brucella abortus* vaccine), Lane N: Negative control (foetal calf serum), and Lane 1-10: Cell lysate (serum samples)

ELISA considering it as “golden standard technique” were calculated (Tables 3 and 4). Sensitivity of PCR was the higher (47.73%) than that of RBPT (29.55%). Specificity of PCR was 100%, while the specificity of RBPT was 88.89%.

Discussion

Due to difficulty in bacterial isolation, serological tests viz., RBPT, complement fixation test, buffered plate antigen test, I-ELISA are routinely used for its diagnosis. Thirty-one samples that failed to yield a positive reaction in RBPT were positive in the other serological test (I-ELISA), where these samples showed high optical density (OD). Therefore, false negative reaction in RBPT might be due to prozoning effect, when sera of high antibody titers are tested against it (OIE, 2012).

Out of 89 samples, 44 were positive by I-ELISA, 18 by RBPT and amplicons of 498 bp (Fig. 1) were detected in 21 samples by PCR (Table 1). The wide variation in number of samples detected as positive by RBPT (18), I-ELISA (44) and PCR (21) could be because of many factors. PCR detects DNA, which may be in low quantity in serum samples even though the antibody titre is quite high. Alternatively, the anti-*Brucella* antibodies titre of serum may be below detectable level but a sufficient DNA quantity for PCR detection as it has been reported that PCR could detect 5 fg of DNA (Kaushik *et al.*, 2006). In present study, the serum sample was preferred over whole blood for nucleic acid amplification because of easier extraction of DNA; and no inhibition of amplification by anticoagulants, hemoglobin and host DNA (Zerva *et al.*, 2001; Al-Garadia *et al.*, 2011). As far as the presence of *Brucella* DNA in serum sample is concerned, these are breakdown products of *Brucella* organism during bacteremia (Zerva *et al.*, 2001) or before settlement to its preferred location in the host, organisms remain in blood circulation for some time.

In the present study, 21 PCR positive sera samples, 47.73% were obtained from seropositive animals, while one serum sample was negative by the serological tests but its serum was positive by PCR. These findings indicate that PCR can be valuable for laboratory diagnosis of chronic infections or very early stage when antibodies could not be diagnosed (Ghorbani *et al.*, 2013). In the present study, I-ELISA was found to be more sensitive, which is in concurrence with the previous study (Ul Islam *et al.*, 2013). On the contrary, Mittal *et al.*, (2005) reported that RBPT is more sensitive than ELISA, when applied to buffalo sera. In conclusion, I-ELISA can be routinely used for an accurate and efficient diagnosis of *Brucella* infection, because the chances of non-detection of an infected animal in I-ELISA are minimal. However, PCR could be used as a supplement and complement test along with I-ELISA for identification and differentiation of bovine brucellosis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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