IMMUNOLOGICAL AND MOLECULAR DETECTION OF *Brucella abortus* IN BUFFALOES (*Bubalus bubalis*)

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ABSTRACT

Brucellosis is a zoonotic disease of livestock species, wild animals and human beings which causes birth of unthrifty newborn and orchitis-epididymitis with frequent sterility in affected animals and undulant fever in human beings. In the present study a total of 73 serum samples were taken from buffaloes and bulls from organized as well as rural areas, eighteen of which had tested previously as positive samples by AB-ELISA. Out of these eighteen, four (22.22%) were found to be positive using dot-ELISA, while none of these were found to be positive with RBPT. For rest of the 55 samples both assays (RBPT and dot-ELISA) gave the same results: 12.72% of the samples were found to be positive; however, positivity was higher in case of dot-ELISA. In three (5.45%) out of seven positive samples Brucella specific DNA was detected in blood using PCR while in milk samples, no positive results were observed. The same results were observed through serological testing.

Keywords: *Brucella abortus*, buffalo, dot-ELISA, PCR, RBPT

INTRODUCTION

Brucellosis is a widespread infectious disease of livestock and causes significant health hazards in human beings. The disease is manifested by abortions, birth of unthrifty calves and retained placenta in female animals. Lesions in males are largely confined to the genital organs including testicles, seminal vesicles and epididymes (Morgan and Mac Kinnon, 1979). Infection can occur in cattle of all ages but persist most commonly in sexually mature animals. The disease in different animals is caused by different species of Brucella like: *Brucella melitensis* (goats), *B. abortus* (cattle), *B. suis* (swine), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (wood rats). Brucella organisms are small, non motile, gram negative, coccobacilli. They cause infection by entering the host cells. Transmission of the disease is by both direct or indirect contact with infective excretors. The organisms are commonly present in the seminal fluid and may be transmitted to susceptible females through artificial insemination (AI) (Campero *et al.*, 1990). The cryopreservation of semen increases the national as well as the international distribution of semen and at the same

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time, it also increases the possibility of spreading diseases transmitted through semen like brucellosis among animal populations (Philpott, 1994). High rates of incidence of brucellosis are observed in areas where animals are bred by AI (Sarumathi *et al.*, 2003; Mittal *et al.*, 2005); thus, there is a need to screen semen before AI. Correct and prompt diagnosis is important in controlling and eradicating the disease in animals. The disease can also be serologically diagnosed by the Rose Bengal plate test (RBPT), serum agglutination test (SAT), complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA). While polymerase chain reaction (PCR) can directly detect DNA of the disease causing agent and therefore could be used for establishing current infection status. This assay is not only sensitive and fast but also safe for human handling. The present study was undertaken to detect the seroprevalence of brucellosis and to detect the presence of Brucella organisms by molecular assays. The seroprevalence study was done by RBPT and dot-ELISA. To detect the presence of Brucella organisms in blood and milk samples, PCR methods were used. A comparison was also made among serological and molecular methods for detecting Brucella infection in *Bubalus bubalis.*

MATERIALS AND METHODS

A total of 73 serum samples were taken from buffaloes and bulls from organized as well as rural areas, 18 of which had been tested previously as positive samples by AB-ELISA.

Serological testing

Blood was collected aseptically from the jugular vein of buffaloes and bulls and was dispensed into two aliquots; one was not treated with anticoagulants such as EDTA, and was used to obtain sera. Serological examination of animals was conducted by RBPT and dot- ELISA.

DNA Extraction

DNA extraction from blood

EDTA was added to blood samples that were obtained, and DNA was extracted by using QIAamp DNA Mini Kit. Qiagen proteinase K in 20 µl was mixed with 200 µl of sample and an equal volume of lysis buffer (AL), ethanol was also added. Bound DNA was washed with 500 µl of washing buffer (AW1) and 500 µl of another washing buffer (AW2). Elution of DNA was carried out by 50 µl of elution buffer (AE) eluted DNA was stored at -20ºC.

DNA extraction from milk samples

DNA extraction was done by using the CTAB-NaCl method (Leal-klevezas *et al.*, 1995; Romero *et al.*, 1999) with slight modifications. To 500 µl of cream 100 µl of NET buffer (NaCl 50 mM, EDTA 25 mM, Tris HCl (pH 7.6) 50 mM), 100 µl of 24% sodium dodecyl sulphate (SDS), 12 µl of Proteinase K (20 mg/ml), 2.5 µl of DNase free RNase A was added and incubation was carried out at 56^{o}C for 2 h. To this, 100 µl of 5 M NaCl, and 80 µl of CTAB-NaCl, and an equal volume of a saturated phenol: chloroform: isoamyl alcohol mixture (PCI) in the ratio of 25:24:1 were added and repeated until the interface was clear. A further equal volume of phenol: chloroform (PC) in ratio of 24:1 was added. Later on, 800 µl of pre-chilled isoproponal was added and DNA pellets were washed with pre-chilled 100% ethanol, and the DNA was then dissolved in 50 µl of nuclease free water (NFW) and the aliquots were stored at -20 ^{o}C for future use.

DNA amplification and detection of PCR product

Fekete *et al.* (1990) used PCR for the first time for the diagnosis of brucellosis, targeting a 43KDa outer membrane protein gene of *B. abortus* S19. PCR was carried out using primers flanking the nucleotide sequence of the gene encoding Brucella cell surface protein (BCSP-31) (Table 1).

The PCR reaction was set up in 25µl volume. The PCR reaction was initially optimized by using varying concentrations of molecular biological chemicals and varying cycling conditions - 4.5 µl nucleic acid free water (Fermentas), 2.5 µl 10x PCR buffer, 1.5 µl 25 mM magnesium chloride, 1 µl 10 mM dNTPs, 0.5µl of Taq polymerase (5 U/µl) (Fermentas), 5 µl of 10 µMol forward and reverse primer. The reaction mixture was mixed gently by vortexing and spinning. In the end, 5 µl of DNA extracted from a different sample was added. The cycling condition included initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, final extension at 72°C for 10 minutes, and finally hold at 4°C. The same cycle was repeated for 40 cycles. Further, amplification products were separated through electrophoresis, agarose (1.5% w/v) gel containing 0.5% ethidium bromide and were visualized under a UV transilluminator.

RESULTS AND DISCUSSION

A total of s73 samples were taken in the present study including 18 positive serum samples from rural areas of Haryana state which had previously tested positive by AB-ELISA. Various serological tests like Rose Bengal plate test (RBPT), dot-ELISA were employed to detect antibodies against *Brucella* sp. in serum while PCR was used for molecular detection of Brucella organisms by amplifying the conserved fragment of an outer membrane protein gene. The efficacy of different immunological and molecular assays for detection of *Brucella* sp. in *Bubalus bubalis* was also evaluated.

RBPT and dot-ELISA, both differed in positivity of seven (12.72%) positive samples (Figure 1). Positivity of samples lies in between two to four and the maximum positivity i.e. four was observed using dot-ELISA. Both the serological tests were found to differ in their positivity (Table 2). Dot- ELISA was found to be more accurate and gave high strength signals in comparison to RBPT tests.

The PCR product of one of the blood sample yielded, three bands i.e. a 223 bp band along with two extra PCR products, one was observed between 300-400 bp and second was between 100-200 bp, but in another sample only one expected product at 223 bp was observed (Figure 2). This may be because of either gene heterogeneity or genomic contamination.

Out of 55 animals, only three i.e. 5.45% of the animals' blood samples were found to be positive while in case of milk samples none were found to be positive. Similar false negative results with milk sample PCR were observed by Romero *et al.* (1995). These false negative results were correlated with the presence of various inhibitory factors such as lactoferrin in milk which may interfere with the PCR reaction (Al-Sound and Radstrom, 2001) and thus, interfere with the detection of Brucella DNA by PCR as compared to detection in uterine discharge.

The serological statuses of all animals were determined by RBPT and dot-ELISA followed by PCR assay. By using RBPT and dot-ELISA, a

Table 1. Nucleotide sequence of the gene encoding Brucella cell surface protein (BCSP-31).

Gene	Primer	Sequence
BCSP-31	Forward Primer	5'- TGG CTC GGT TGC CAA TAT CAA- 3'
	Reverse Primer	5' –CGC GCT TGC CTT TCA GGT CTG-3'

Table 2. Positivity of samples tested by RBPT and dot-ELISA.

Sample No.	RBPT	dot-ELISA
1	+ + +	+ + + +
2	+ +	+ ++
3	+ +	+ + ++
4	+ +	+ +
5	+ +	+++
6	+ + +	++ +
7	+ + +	+ ++

'+' in numbers signifies the strength and signal of test.

Table 3. Detection of Brucellosis by different tests.

Test	Kind of samples	Positive samples	Negative samples	Total no of animals tested
RBPT	Serum	7(12.72%)	48(87.27%)	55
dot-ELISA	Serum	7(12.72%)	48(87.27%)	55
PCR	Blood	3(5.45%)	52(94.54%)	55

Table 4. Sensitivity and specificity of different tests used for detection of Brucellosis.

Tests		**PCR**		**Total**	**Sensitivity (%)**	**Specificity (%)**	**Overall agreement (%)**
		+	-				
RBPT	+	3	4	7	100	92.30	92.72
	-	0	48	48			
	Total	3	52	55			
dot-ELISA	+	3	4	7	100	92.30	92.72
	-	0	48	48			
	Total	3	52	55			
		dot-ELISA					
		+	-				
RBPT	+	7	0	7	100	100	100
	-	0	48	48			
	Total	7	48	55			
PCR	+	3	0	3	42.85	100	92.72
	-	4	48	52			
	Total	7	48	55			
		RBPT					
		+	-				
dot-ELISA	+	7	0	7	100	100	100
	-	0	48	48			
	Total	7	48	55			
PCR	+	3	0	3	42.85	100	92.72
	-	4	48	52			
	Total	7	48	55			

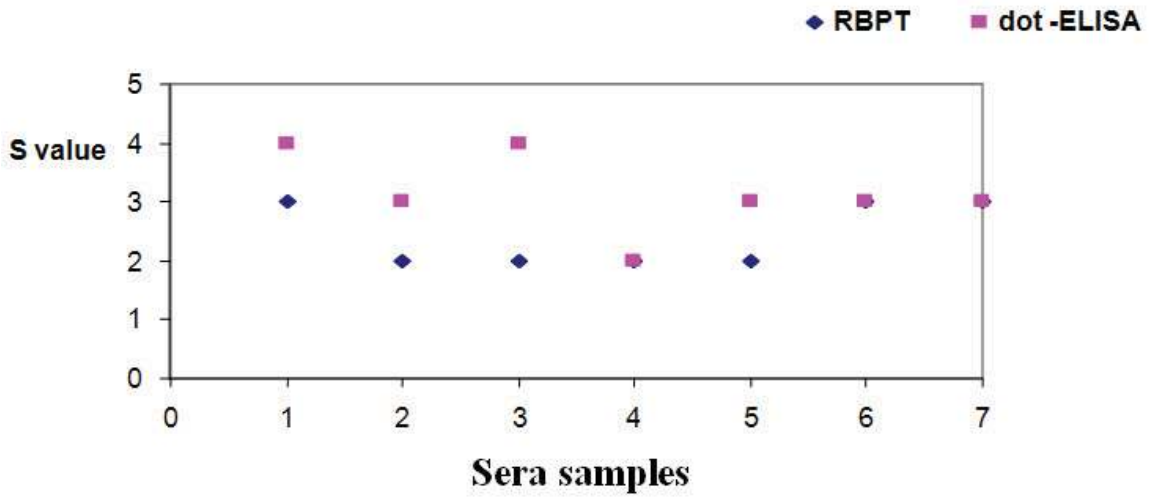

Figure 1. Positivity of samples for Brucellosis as determined by RBPT and dot-ELISA.

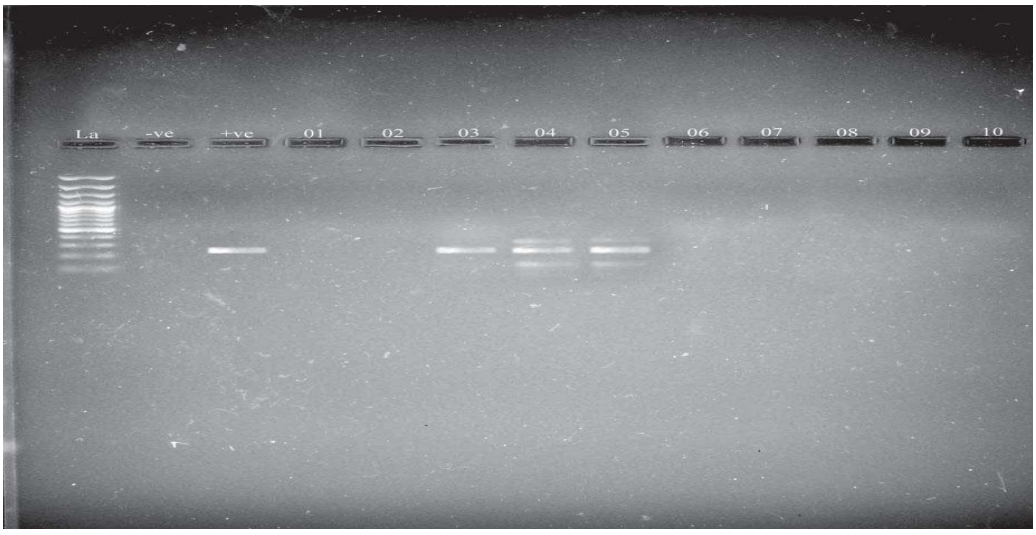

Figure 2. Agarose gel electrophoresis pattern of *Brucella BCSP*-31 gene, specific PCR product amplified with primer B4/B5.
La: DNA molecular weight ladder 100 bp
+ve: positive control
-ve: negative control
01 to 10: samples

total of seven (12.72%) samples were found to be positive but only 5.45% were found to be positive using PCR (Table 3). When both blood and milk samples were compared, only blood samples gave positive results while milk samples gave negative results.

Comparing the sensitivity of RBPT and PCR considering dot-ELISA and RBPT as standard tests, RBPT and dot-ELISA were found to be 100% sensitive and PCR 42.85%. While the sensitivity of RBPT and dot-ELISA came out to be 100%, when PCR was used as the standard test and specificity was found to be 92.30%. The overall agreement of RBPT and dot-ELISA with PCR was found to be 92.70% (Table 4).

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