Detection of *Brucella abortus* in Buffalo Blood and Milk by IS711 PCR Assay

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**ABSTRACT**

Efficacy of *Brucella abortus*-specific IS711 PCR assay in direct detection of *B. abortus* from blood and milk samples of naturally infected buffaloes and from blood and milk spiked with *B. abortus* was evaluated. The PCR assay amplifying IS711 genetic element of *Brucella* genome was optimised using standard *B. abortus* 544 strain. Examination of blood samples of 20 animals including 9 serologically positive animals derived from the herds with the history of abortions revealed *B. abortus* specific amplification product of 498 bp in 11 (55%) blood samples. However, none of the milk samples from serologically positive as well as negative animals generated *B. abortus* specific amplification product. The minimum limit of detection of *B. abortus* from spiked blood sample by IS711 PCR was found to be 20,000 CFU/ml, while that from milk samples was 1000 CFU/ml.

**Keywords:** Blood, *Brucella abortus*, IS711 PCR, milk.

**Introduction**

Brucellosis, an infectious disease affecting many different vertebrate species attributes to serious economic losses to livestock industry through its adverse effects on the reproductive and productive potential of the affected animals. Serological tests that have been widely employed for the detection of brucellosis in animals lack adequate specificity (Debeaumont et al., 2005). Isolation of the causative agent has been the most accepted tool for confirmatory diagnosis of brucellosis; however, the procedure is time consuming and the culture materials need to be handled carefully since the organism is categorized as class III pathogen. Moreover, the technique has a reduced sensitivity in chronic infection (Alton et al., 1988). The nucleic acid-based detection methods developed in the recent past have been found to be sensitive, specific and quick in detection of brucellosis. Moreover, these techniques do not require handling of live organism thereby reducing the safety concerns. A PCR assay based on the polymorphism arising from species-specific localization of the genetic element IS711 has been developed and can identify selected biovars of four species of *Brucella* (Bricker and Halling, 1994). In the present investigation, we evaluated the efficacy of IS 711 PCR assay in direct detection of *Brucella* from milk and blood samples of the naturally infected animals and from spiked milk and blood samples.

**Materials and Methods**

**Specimens**

Thirty eight clinical specimens including 20 whole blood samples in EDTA and 18 milk samples collected from a herd with the history of abortions due to brucellosis were included in the study.
Bacterial strains

*Brucella abortus* 544 a reference strain and field isolates of *Brucella abortus* maintained in the Department of Microbiology, Bombay Veterinary College, Mumbai were used for standardization of IS711 PCR assay.

Spiking of blood and milk samples with *Brucella abortus* 544

The blood and milk samples collected from a healthy, serologically negative buffalo were spiked with different concentrations of *B. abortus* 544 growing in its exponential phase. Equal volumes of blood / milk and bacterial suspensions adjusted to concentrations of 500, 1000, 2000, 4000, 20000, 30000 and 40000 CFU/ml were mixed and incubated at 37°C for 30 min. The blood and milk samples so spiked were further processed for extraction of DNA.

Extraction of *Brucella* DNA from blood samples

The extraction of genomic DNA of *B. abortus* from both naturally infected and spiked blood samples was carried out as per the protocol described by Leal-Klevezas et al. (1995). Briefly, 500 µl blood was centrifuged at 12000 rpm for 8 min at 4°C. Cell pellet was suspended in RBC lysis buffer (140 mM ammonium chloride, 17 mM Tris HCl pH 7.6) and centrifuged at 12000 rpm for 8 min at 4°C; the cycle was repeated two to three times until the red colour due to the erythrocytes was minimal. The pellet was then treated with leucocyte lysis buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100mM NaCl, 10 mM Tris/HCl, pH 8.0) and digested with 10 µl proteinase K (20 mg/ml) for 40 min at 50°C. Following enzymic digestion, the samples were extracted with phenol/chloroform and genomic DNA was precipitated, dried, suspended in 50 µl nuclease free water and stored at -20°C.

Extraction of *Brucella* DNA from milk samples

Extraction of *Brucella* DNA from milk was accomplished by the procedure described by Romero and Lopez-Goni (1999). Briefly, 500 µl of the sample was mixed with 100 µl of NET buffer (50 mM NaCl-125 mM EDTA-50 mM Tris-HCl (NET)) (pH 7.6) and 85 µl of 24% SDS. The preparation was thoroughly mixed by vortexing and kept at 80°C for 10 min followed by cooling on ice for 10 min. Twelve µl of proteinase K (20 mg/ml) was then added to the mixture and incubated at 50°C for 2 h. Following enzymic digestion, the samples were subjected to phenol/chloroform extraction and genomic DNA was precipitated, dried, suspended in 50 µl nuclease free water and stored at -20°C.

*B. abortus* specific IS 711 PCR assay

The PCR amplification of 498 bp region of IS711 genetic element of *B. abortus* was carried out using forward primer IS711 (5'-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT- 3') and reverse primer AB (5'-GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC-3') as described by Bricker and Halling (1994). The oligos were manufactured and supplied by M/s Bangalore Genei, Bangalore (India). The PCR was set in a final volume of 25 µl consisting 10x PCR buffer (2.5 µl), 200 µM dNTPs (0.50 µl), 2mM MgCl₂ (2 µl), 10 mM each primer (0.50 µl), 500 ng template DNA (3 µl) and 1.25 units Taq polymerase (0.25 µl) and sterile nuclease free water (15.75 µl). The cycling parameters used were initial denaturation at 94°C for 3 min.; 35 cycles of denaturation at 95°C for 1 min., annealing at 60°C for 45 s and extension at 72°C for 1 min. The PCR products were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and visualized automatic gel documentation system (UVitech).

Results and Discussion

A definitive and early diagnosis of brucellosis is of prime importance in order to initiate appropriate control measures. Its detection by conventional culture and serology has disadvantages like lack of sensitivity and specificity. The DNA based techniques developed in the recent times are suggested to overcome the disadvantages of conventional methods. In the present investigation we evaluated the efficacy of IS711 PCR assay in direct detection of *B. abortus* from blood and milk samples of...
Detection of *Brucella abortus* in buffalo blood and milk

Table 1: Detection of *B. abortus* in spiked blood and milk samples by IS711 PCR

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of <em>B. abortus</em> (CFU/ml)</th>
<th>Amplification product of 498 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blood</td>
</tr>
<tr>
<td>1</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1000</td>
<td>-</td>
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<tr>
<td>3</td>
<td>2000</td>
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<tr>
<td>4</td>
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<td>20000</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>30000</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>40000</td>
<td>+</td>
</tr>
</tbody>
</table>

naturally infected animals. A total of 20 whole blood samples of animals derived from the herds with the history of abortions were processed for molecular detection of *Brucella abortus* by IS711 PCR. Nine out of these 20 samples were serologically positive for the presence of *Brucella* antibodies. In IS711 PCR assay, *B. abortus* specific amplification product of 498 bp was observed in 11 (55%) samples, while the remaining 9 blood samples proved negative (Fig 1). The IS 711 PCR assay thus, could detect greater number of positive animals than serology. Similarly, the milk samples of all the animals were also processed for the detection of *B. abortus* by IS711 PCR, however, none of the milk samples yielded amplification product specific of *B. abortus*. Application of PCR for direct detection of *Brucella* spp. from whole blood samples and other clinical specimens of animals has been attempted by many researchers with varying degrees of success. Leal-Klevezas *et al.* (1995) developed a PCR targeting *omp2* gene and suggested the method to be useful in detection of pathogen from milk and blood. In contrast to this, O'Leary *et al.* (2006) employed IS711, 31kDa and 16S rRNA PCR assays for detection of *B. abortus* from blood, milk and lymphoid tissues of infected animals and found that the PCR could detect presence of organisms in milk and lymphoid tissues, but not in blood. Mukherjee *et al.* (2007) compared the efficacy of *bcsp* and *omp2* PCR assays in detection of *Brucella* spp. from bovine blood and found the former to be more sensitive. It can, therefore, be inferred from the results of the present study that the IS711 PCR assay is effective in direct detection of *Brucella* spp. from the blood samples and could be of value when used in conjunction with serology especially in eliminating false positive reactions due to cross-reacting antibodies. None of the milk samples from both serologically positive and negative animals was found positive for brucellosis by PCR. De *et al.* (1989) reported failure in isolation of *Brucella* from milk, vaginal discharge and cervical swabs of 10 seropositive cows. It is well known that the *Brucella*...
organisms localize in the udder and supramammary lymph nodes in the infected animals and are excreted periodically in the milk in quantities ranging from few hundreds to 2,000,000 organisms per ml of milk (Corbel, 1988). Patel et al. (2008) while comparing the efficacy of indirect-ELISA and PCR in detection of Brucella spp. in milk found PCR to be less effective than the I – ELISA.

In order to assess the efficacy of PCR assay in detecting minimum number of organisms, blood and milk samples spiked with different concentrations of B. abortus were subjected to IS711 PCR assay (Table 1). It is evident that no amplification was found at a concentration of 4000 CFU/ml of blood, while specific amplification product of 498 bp was observed in blood sample spiked at the concentration of 20,000 CFU/ml of blood (Fig. 2). The minimum limit of detection of B. abortus in blood by IS711 PCR, thus was 20,000 CFU/ml. The limit of detection of B. abortus from spiked milk samples by IS711 PCR assay was found to be better than that of blood. The assay could detect 1000 CFU/ml of B. abortus from spiked milk sample (Fig. 3). On reviewing the available literature it appears that attempts for detecting the limit of detection of B. abortus from spiked blood samples have not so far been done. However, limits of detection of B. abortus from milk samples have been studied by a few workers. Romero and Lopez-Goni (1999) while improvising the methods of DNA extraction and purification from milk samples reported the limit of detection of Brucella of 5 to 50 CFU/ml. A higher limit of detection of 2.8x10⁵ CFU/ml was observed by Rijpens et al. (1996) with the PCR performed with primers BRU-P5 and BRU-P8.

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References


