Isolation, Biochemical Characterization, Antibiogram Pattern and PCR Based Confirmation of *Brucella* from Cows and Buffaloes

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**Abstract**

Brucellosis is a worldwide zoonotic disease that still constitutes a major public health problem in India. In the current study, *Brucella* were isolated from aborted samples and typed by both molecular and conventional techniques. A total 114 aborted samples were collected from cows and buffaloes in and around Anand district. Aborted samples were cultured on the *Brucella* agar medium and incubated for 24–48 h. Three samples from cow were found to be positive for *Brucella*. All the isolates were positive for catalase, oxidase and nitrate reduction while negative for urease reaction, indole test, VP test, motility examination and production of H₂S. For the detection of *Brucella* DNA by PCR, three different genus-specific primer pairs viz., B4/B5, JPF/JPR, and F4/R2 were used. All three *Brucella* isolates were positive by B4/B5 and F4/R2, while two isolates were positive for JPF/JPR. For species-level identification of *Brucella* isolates were subjected to AMOS PCR and Bruce-ladder PCR, and were found to be *B. abortus*.

**Keywords:** AMOS-PCR, *Brucella*, Buffalo, Bruce-ladder, Cattle, PCR.

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**Introduction**

The term brucellosis is applied to a group of closely related infectious diseases, all caused by Gram-negative bacterial pathogens from the genus *Brucella*. Phenotypic characteristics, antigenic variation, and prevalence of infection in different animal hosts have resulted in the initial recognition of six species: *Brucell (B.) melitensis, B. suis, B. abortus, B. canis, B. ovis* and *B. neotomae* (Vizcaino et al., 2004). In addition, in the 1990s, new *Brucellae* have been isolated from marine mammals, and a new species, *Brucella marins* was proposed (Nymo et al., 2011). Manifestations of the disease may range from abortion in the cow to orchitis or epididymitis in the bull (Dougherty et al., 2013). This disease is transmitted by direct or indirect contact with infected excreta. The most important routes of transmission are the oral and venereal ones.

The economic importance of brucellosis requires the use of sensitive and rapid diagnostic methods. At present, the diagnosis of brucellosis in live dairy cattle involves either the isolation of *Brucella* from various samples like milk, placenta, cotyledons, and fetal stomach contents or samples the detection of anti-*Brucella* antibodies in milk (Hamdy et al., 2002). Recently, polymerase chain reaction (PCR)-based detection of organisms has been found to be more convenient as compared to cultural isolation. PCR is an option for diagnosis of brucellosis. AMOS (from the initial letters of abortus, melitensis, ovis, and suis) PCR assay can identify *B. abortus, B. suis, B. melitensis, B. ovis* (Bricker et al., 2003). The present study was carried out on isolation, antibiogram, and PCR based identification of *Brucella* species from samples of reproductive disorders in cows and buffaloes.

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an atmosphere of 5% CO₂ in a CO₂ incubator for minimum 15 days. The plates were observed every 24 hours for the growth. The suspected colonies of *Brucella* were picked up and transferred to another BAM plates and incubated under 5% CO₂ tension to obtain a pure culture.

**Identification of Brucella Isolates**

Cultural and biochemical tests like oxidase, catalase production, nitrate reduction, urease, indole, VP, H₂S production, motility test, and agglutination reaction with anti-*B. abortus* serum were carried out for the identification of *Brucella* isolates (Parlak et al., 2013).

**Reference Bacterial Strain**

The vaccine strain *Brucella abortus* cotton strain19 (IIL, Hyderabad, India) was used as reference bacterial strain for cultural and molecular work.

**Antibiogram Pattern**

*In vitro*, antibiotic sensitivity patterns of the isolates were conducted as per the method of Bauer et al. (1966). Antibiotics disc (Hi-Media Ltd., Mumbai, India) used in the present study were Streptomycin (10 mcg), Tetracycline (30 mcg), Amikacin (30 mcg), Erythromycin (15 mcg), Pefloxacin (5 mcg), Amoxyclov (30 mcg), Spectinomycin (100 mcg), Norfloxacin (10 mcg) and Ampicillin (10 mcg). Diameters of the clear zone of inhibition were measured, and the interpretation of the results was made in accordance with the instructions supplied by the manufacturer (Hi-Media Ltd., Mumbai, India).

**Bacterial DNA Extraction**

Suspected colonies from BAM plates were streaked on BAM slants. Slants were incubated at 37°C for 4 to 5 days at 5% CO₂ tension. Bacterial colonies were picked and suspended in 100 μl DNAase free mili Q water. The colonies were boiled for 15 min, cell debris were removed by centrifugation, and 3 μl of the supernatant was used as a template.

**Polymerase Chain Reaction:** The extracted DNA preparations were screened with genus-specific *Brucella* PCR using B4/B5, JPF/JPR and F4/R2 primers (Table 1). For all PCR reactions 3μl (90 ng) template DNA was taken and added to the reaction

| Name of primers                  | Sequence 5’—3’                                           | Product length (bp) | References                |
|----------------------------------|----------------------------------------------------------|---------------------|---------------------------|
| *Brucella* genus-specific primers|                                                          |                     |
| B4 (F)                           | TGG CTC GGT TGC CAA TAT CAA                              | 223                 | Bailey et al. (1992)      |
| B5 (R)                           | CGC GCT TGC CTT TCA GGT CTG                              |                     |
| JPF (F)                          | GCG CTC AGG CTG CCG AGC CAA                              | 193                 | Leal-Klevezas et al. (1995)|
| JPR (R)                          | ACC AGC CAT TGC GGT CGG TA                               |                     |
| F4 (F)                           | TCG AGC GCC CGC AAG GGG                                  | 905                 | Romero et al. (1995)      |
| R2 (R)                           | AAC CAT AGT GTC TCC ACT AA                               |                     |
| AMOS-PCR primers                 |                                                          |                     |
| IS711-specific (F)               | TGC CGA TCA CTT AAG GGC CTT CAT                          | 498                 |                            |
| *B. abortus* specific            | GAC GAA CGG AAT TTT TCA AAT CCC                         |                     | Bricker and Halling (1994) |
| *B. melitensis* specific         | AAA TCG CCT TGG TGG TGG TGG GA                          | 731                 |                            |
| *B. ovis* specific               | CGG GTT CTG GCA CCA TCG TCG                             | 976                 |                            |
| *B. suis* specific               | GCG CGG TTT TCT GAA GGT TCA GG                          | 285                 |                            |
| Bruce-ladder PCR primers         |                                                          |                     |
| BMEI0998f                        | ATC CTA TTG CCC CGA TAA GG                               | 1682                |                            |
| BMEI0997r                        | GCT TCG CAT TTT CAC TGT AGC                              |                     |
| BMEI0535f                        | GCG CAT TCT CGG TGT ATG AA                               | 450                 |                            |
| BMEI0536r                        | CGC AGG CGA AAA CAG CTA TAA                              |                     |
| BMEI0843f                        | TTT ACA CAG CCA ATC CAG CA                               | 1071                |                            |
| BMEI0844r                        | GCG TCC AGT TGT TGT TGA TG                               |                     |
| BMEI1436f                        | ACG CAG ACG ACC TTC GGT AT                               | 794                 | Garcia-Yoldiet et al. (2006) |
| BMEI1435r                        | TTT ATC CAT CGG CCT GTC AC                               |                     |
| BMEI0428f                        | GCC GCT ATT ATG TGG ACT GG                               | 587                 |                            |
| BMEI0428r                        | AAT GAC TTC ACG GTC TGT CG                               |                     |
| BR0953f                          | GGA ACA CTA CGC CAC CTG GT                              | 272                 |                            |
| BR0953r                          | GAT GGA GCA AAC GCT GAA G                               |                     |
| BMEI0752f                        | CAG GCA AAC CCT CAG AAG C                               | 218                 |                            |
| BMEI0752r                        | GAT GTG GTA ACG CAC ACC AA                               |                     |
| BMEI0987f                        | CGC AGA CAG TGA CCA TCA AA                               | 152                 |                            |
| BMEI0987r                        | GTC TTC AGC CCC CGT TAC CT                               |                     |
mixture (22 µL) containing 1 µL of each forward and reverse primer pair (MWG, Biotech, Germany) in a 10 pmol/µL primary concentration, 12.5 µL of PCR 2x PCR Master mix (MBI, Fermentas) containing 0.05U/µL TaqDNA polymerase in reaction buffer, MgCl2 (4 mM) and dNTPs (0.4 mM of each) and 7.5µL of molecular grade nuclease-free water. The PCR reactions were performed in a thermocycler (Veriti Thermal Cycler, Applied Bioscience, USA) as per the method described by Baily et al. (1992) for B4/B5, Leal-Klevezas et al. (1995) for JPF/JPR and Romero et al. (1995) for F4/R2 primers. Positive samples were subjected to AMOS PCR for species identification using primers (Table 1) as per Bricker and Halling (1994). The Bruce-ladder PCR was carried out as per methods described by Garcia–Yoldi et al. (2006). The PCR product was run on a 1.5 % agarose gel along with DNA ladder for 90 min at 105 V, stained with ethidium bromide (1 mg/mL), and visualized under UV light using a gel documentation system.

Results and Discussion

Out of 114 samples processed from vaginal swabs, aborted materials and milk, three (all from cows) isolates were recovered on BAM and were presumed to be of Brucella. All, three isolates of Brucella were from abortion cases in cows. All three isolates (named as C1, C2, and C3) were Gram-negative and MZN positive coccobacilli. Biochemical tests showed that these isolates were positive for oxidase, catalase production and nitrate reduction. While negative for urease, indole, VP, H₂S production, and motility. All the isolates showed B. suis DNA polymerase B. abortus. (1995) also used this cotton strain 19) from milk and bull semen, respectively. Kotadiya et al. (2012) reported that all the Brucella isolates were sensitive to all the antibiotics tested. JPF/JPR primer pair generated a 193bp (Fig. 1) amplicon from reference strains as well as from two isolates of Brucella but failed in C2. Leal-Klevezas et al. (1995) also used this primer homologous to regions of the gene coding for an omp2 for the detection of Brucella in blood and milk of the infected animals and obtained promising results. Navarro et al. (2002) and Kanani et al. (2008) also used the same primer for the detection of Brucella in infected human blood and bull semen. Patel et al. (2008) carried out PCR based detection for Brucella organisms in 53 milk samples collected from normal milk cattle by Brucella genus-specific primer pairs, and one isolate was positive by JPF/JPR primer pair.

B4/B5 primer pair generated a 223bp (Fig. 2) amplicon size from reference strains as well as, all the three isolates presumed to be Brucella. This bscp31 gene based primer has also been successfully used by Kanani et al. (2008) for detection of Brucella DNA bull semen. Similar results were also reported by Morata et al. (2001), Navarro et al. (2002) and Boeri et al. (2018) using same primer pair for diagnosis of brucellosis.

F4/R2 primer pair generated a 905bp (Fig. 3) from reference strains as well as all the three isolates of Brucella. Romero et al. (1995) applied this primer pair to DNA extracted from all of the representative strains of the species, biovars of Brucella and from 23 different Brucella isolates and amplified 905bp fragment. Similar amplicon size were obtained from milk and lymph tissues by Kanani et al. (2008) from bull semen and Patel et al. (2008) from milk by using same primer.

AMOS PCR assay is a multiplex primer assay that uses a five-primer cocktail. One primer anneals to the IS711 element. As designed, B. abortus amplifies a 498 bp product, B. melitensis amplifies a 731 bp product, B. ovis amplifies 976 bp product and B. suis amplifies a 285 bp product. AMOS PCR assay was developed to differentiate between field strains.
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The vaccine strain S19 and RB51. The product size of 498bp was amplified for all the three isolate as well as *B. abortus* strain 19 using AMOS primers cocktail indicating our isolates belonging to *B. abortus* species (Fig. 4). Similarly, Matope et al. (2009) also detected *Brucella* isolates from cattle and goat as *B. abortus* and *B. melitensis*, respectively. In agreement with the present study, Shahzad et al. (2013) recovered thirty *Brucella* isolates and identified as *B. abortus* by AMOS PCR. In a similar study, Pathak et al. (2016) recovered eight *Brucella* isolates from cattle demonstrated amplification of *B. abortus* specific primer by AMOS PCR indicating isolates to be of either *B. abortus* biotype 1, 2 or 4. Ledwaba et al. (2019) identified as *B. ovis*, *B. abortus*, *B. canis*, *B. suis*, and *B. canis* species in Zimbabwe.

In the present study, all the three isolates as well as the reference *B. abortus* cotton strain 19 could amplify products of 1682bp, 794bp, 587bp, 450bp and 152bp using cocktail of 8 pairs of primer pair and the product size were specific for *B. abortus* using Bruce ladder (multiplex) PCR technique (Fig. 5). Results showed that microbiological typing and multiplex Bruce-ladder amplification were identical for all *Brucella* isolates tested. In a similar study, Lopes et al. (2014) found that all field strains identified to the species level by biochemical and physiological tests were confirmed by the genus-specific PCR and by the Bruce-Ladder PCR. None of the field strains of *B. abortus* presented the profile expected for vaccine strains S19 and RB51.

**Conclusion**

Among the three different genus-specific primer pairs used (B4/B5, F4/R2 and JPF/JPR) for identification of *Brucella* organisms, B4/B5 and F4/R2 primer pairs were found to be more sensitive for identification of *Brucella* organisms. For the species identification, multiplex PCR named AMOS PCR and Bruce-ladder could identify *B. abortus*.

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