RESEARCH ARTICLE

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 H_2S. 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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Materials and Methods

A total of 114 samples comprised of vaginal swabs, aborted material, milk, and placenta were aseptically collected from cows (98) and buffaloes (16) in and around Anand district.

Bacterial Culture

Isolation of *Brucella* organisms from the vaginal swabs, aborted material, milk, and placenta from cow and buffalo was carried out after collection in transport swab (Hi-media transport swab w/Amies medium w/o charcoal in polystyrene tube). Each swab collected from an animal was separately streaked on *Brucella* agar medium (BAM) plates in duplicates. One plate was incubated aerobically in an incubator at 37°C (without CO_2), and the other incubated at 37°C aerobically in

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an atmosphere of 5% CO_2 in a CO_2 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% CO_2 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 stain 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 (15mcg), Pefloxacin (5 mcg), Amoxyclav (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% CO_2 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

Table 1: List of Primers

Name of primers	*Sequence 5′— 3′*	*Product length (bp)*	*References*
	Brucella genus-specific primers		
B4 (F)	TGG CTC GGT TGC CAA TAT CAA	223	Baily *et al*. (1992)
B5 (R)	CGC GCT TGC CTT TCA GGT CTG		
JPF (F)	GCG CTC AGG CTG CCG ACG 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	Bricker and Halling (1994)
B. abortus specific	GAC GAA CGG AAT TTT TCC AAT CCC		
B. melitensis specific	AAA TCG CGT CCT TGC TGG TCT 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	Garcia–Yoldi*et al.* (2006)
BMEI0997r	GCT TCG CAT TTT CAC TGT AGC		
BMEI0535f	GCG CAT TCT TCG GTT ATG AA	450	
BMEI0536r	CGC AGG CGA AAA CAG CTA TAA		
BMEII0843f	TTT ACA CAG GCA ATC CAG CA	1071	
BMEII0844r	GCG TCC AGT TGT TGT TGA TG		
BMEI1436f	ACG CAG ACG ACC TTC GGT AT	794	
BMEI1435r	TTT ATC CAT CGC CCT GTC AC		
BMEII0428f	GCC GCT ATT ATG TGG ACT GG	587	
BMEII0428r	AAT GAC TTC ACG GTC GTT CG		
BR0953f	GGA ACA CTA CGC CAC CTT 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		
BMEII0987f	CGC AGA CAG TGA CCA TCA AA	152	
BMEII0987r	GTA 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 *Taq*DNA 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, H_2S production, and motility. All the isolates showed agglutination with *Brucella*-mono specific antiserum. Based on cultural and biochemical tests, the isolates were confirmed to be *B. abortus.* The overall prevalence of *Brucella* infection, as detected in the present study by cultural isolation, was 2.63% (03/114). Pal and Jain (1985) obtained 20.93% *Brucella* from aborted buffaloes. Similarly, Patel *et al.* (2008) and Kanani *et al.* (2008) obtained 7.56% and 7.92% isolates of *Brucella* from milk and bull semen, respectively. Kotadiya *et al*. (2014) recovered 3.92% *Brucella* from sheep.

In the present study, all the isolates of *Brucella* were found to be 100% sensitive to Streptomycin, Tetracycline, Amikacin, Erythromycin, Pefloxacin, Amoxyclav, Spectinomycin, and Norfloxacin. At the same time, all the isolates were found resistant to Ampicillin. Ghodasara *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 milch 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 bcsp31 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,

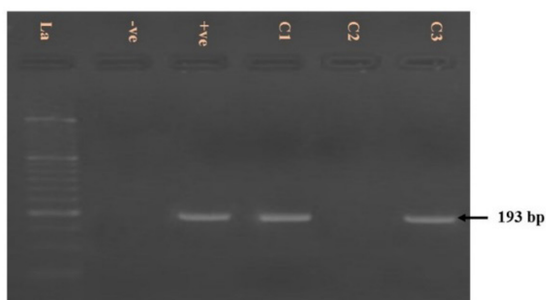

Fig 1: PCR showing amplified product of 193bp for omp2 gene by using primer pair JPF/JPR
La: DNA molecular weight ladder
-ve: Negative control
+ve: Positive control (*B. abortus* cotton strain 19)
C1, C2, C3 : Field isolates

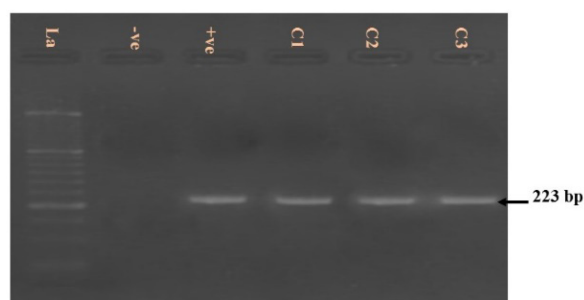

Fig 2: PCR showing amplified product of 223bp for BCSP31 gene by using primer pair B4/B5
La: DNA molecular weight ladder
-ve: Negative control
+ve: Positive control (*B. abortus* cotton strain 19)
C1, C2, C3 : Field isolates

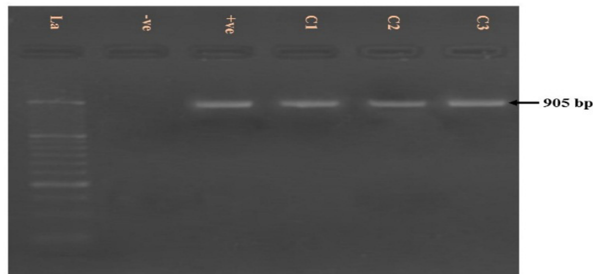

Fig 3: PCR showing amplified product of 905bp for 16SrRNA gene by using primer pair F4/R2
La: DNA molecular weight ladder
-ve: Negative control
+ve: Positive control (*B. abortus* cotton strain 19)
C1, C2, C3 : Field isolates

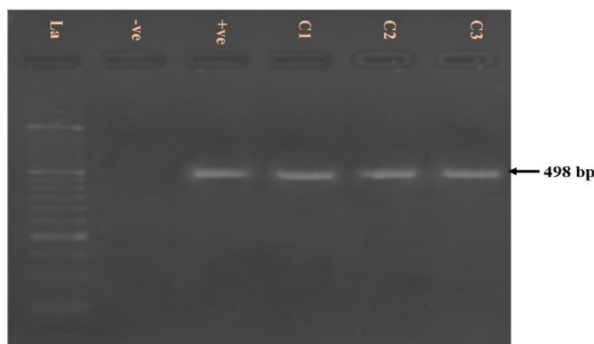

Fig 4: PCR showing amplified product of 498bp for IS711 *B. abortus* specific gene by cocktail of 5 primers
La: DNA molecular weight ladder
-ve: Negative control
+ve: Positive control (*B. abortus* cotton strain 19)
C1, C2, C3 : Field isolates

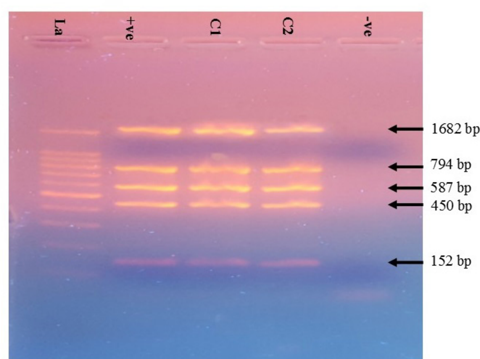

Fig 5: PCR showing amplified product of 1682bp, 794bp, 587bp, 450bp, 152bp for *B. abortus* field strains.
La: DNA molecular weight ladder
-ve: Negative control
+ve: Positive control (*B. abortus* cotton strain 19)
C1 & C2: Representative field isolates

vaccine strain S19 and RB51. The product size of 498bp was amplified for all the three isolate as well as *B. abortus* cotton 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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