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Isolation, Identification and Molecular Detection of *Brucella abortus* from Buffaloes in Gujarat, India

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A B S T R A C T

Brucellosis is an infectious disease, has a considerable impact on animal health as well as socioeconomic impacts. It causes significant reproductive losses in sexually mature animals. The present study reports the isolation, identification and molecular detection of *Brucella abortus.* A total of 192 buffalo clinical samples were processed by cultural isolation on BBL Brucella agar plate with selective antibiotic supplements and direct PCR. Out of 192 clinical samples, 7 samples growth yielded on BBL *Brucella* agar plate and identify *Brucella* organism by colony character, Gram's staining, MZN staining, Catalase, KOH and Triple Sugar Iron Agar (TSI) test. Moreover, the confirmation of these isolate as *Brucella abortus* was carried out by genus specific PCR using B4/B5 (223bp), species specific PCR using +IS711 (498bp), and SYBR green based real time PCR. In direct PCR out of 192 clinical samples, 12 samples detected positive by genus specific PCR using B4/B5 (223bp). Genus specific PCR positive 12 samples conform *Brucella abortus* by species specific PCR using +IS711 (498bp), and SYBR green based real time PCR. In this study 7 clinical samples positive for *B. abortus* by culture isolation as well as direct PCR. However, 5 clinical samples positive by direct PCR but could not be growth yielded on BBL *Brucella* agar plate.

Introduction

Brucellosis is caused by various species of the genus *Brucella,* which is the second most widely spread zoonosis worldwide (Dawood, 2008). It is one of the infectious diseases, which poses major constraint for animal production. The disease is an important public health problem in many parts of the world including India (Pal, 2007; Hadush and Pal, 2013). The disease is manifested by late term abortions, weak calves, still births, infertility and characterized mainly by placentitis, epididymitis and orchitis, with excretion of the organisms in uterine discharges and milk (England *et al.,* 2004). In addition to its direct effects on animals, brucellosis causes economic losses through abortions, stillbirths or the death of young stock. The disease can also have a blow on exports and have negative impact on the efforts to improve breeding. Brucellosis has a considerable impact on animal and human health, as well as wide socio-economic impacts, especially in countries in which rural income relies largely on livestock breeding and dairy products

(Maadi *et al.*, 2011). As signs and symptoms of brucellosis are unspecific so, culture isolation andserology are necessary for diagnosis (Colmenero *et al.*, 1996). Cultural isolation and identification of the agent is the gold standard test for *Brucella* diagnosis, although, limitations associated with cultural isolation and identification of the *Brucella* from clinical samples, the only unequivocal method for the diagnosis of brucellosis is based on the isolation of *Brucella* organisms (Alton *et al.*, 1988). To surmount the problems associated with cultural isolation. Nucleic acid amplification has been explored for the rapid detection and confirmation of *Brucella*. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus specific PCR assays, including 16S rRNA (Romero *et al.*, 1995), IS711 genetic element, omp2 (Leal-Klevezas *et al.*, 1995) and bcsp31.

Materials and Methods

Collection of sample

A total of 192various clinical samples of buffaloes were collected in BBL broth from different district of Gujarat.

Isolation

Each sample collected from an animal was separately streaked on BBL *Brucella* agar medium with selective antibiotic supplements and incubated at 37^{o}C anaerobically in an atmosphere of 5 per cent CO_2 in CO_2 incubator for minimum of 15 days. The plates were observed at every 24 hours interval for the growth.

Identification

The isolates suspected to be of *Brucella* were subjected to Gram staining and Modified Ziehl-Neelsen (MZN) staining for confirming the purity of cultures and morphological characters, identification of *Brucella* organism by agglutination and biochemical test.

Rapid slide agglutination test

One drop (0.03 ml) of known *Brucella* positive serum (I.V.R.I., Izatnagar) was taken on a glass slide by micropipette. A loopful culture from suspected single colony was mixed thoroughly with the spreader and then the slide was rotated for four min. The result was read immediately. Definite clumping/agglutination was considered as positive reaction, whereas no clumping/agglutination was considered as negative.

Biochemical characterization of isolates

Oxidase test

Standard oxidase discs (HiMedia Laboratories Ltd., Mumbai) containing 1% NNN'N' – tetramethyl- p- phenylene diamine dihydrochoride were used to perform the test. The loopful of culture from single colony was just touched on the disc. Development of blue colour within 10 seconds was considered as positive test.

Catalase test

This test was performed by taking 2-3 drops of 3% H_2O_2 on clean grease-free sterile glass slide and single colony from BAM plate was mixed with the help of a wire loop. Immediate development of gas bubbles was considered as positive test.

Triple Sugar Iron Agar (TSI) test

In Triple Sugar Iron Agar test, a test colony was taken with a sterilized straight inoculation needle and inoculated first by

stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant. Then tube with loose cap was incubated at 37^0C for 18 to 24 hours and observed for colour changes and gas production.

Molecular detection of *Brucella abortus*

DNA extraction

DNA extraction was carried out from clinical samples and colony using DN easy Blood and Tissue Kit (Qiagen) following manufacturers protocols.

Detection of *Brucella abortus* using genus-specific B4/B5 primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5'TGG CTC GGT TGC CAA TAT CAA3') and reverse (5'CGC GCT TGC CTT TCA GGT CTG3')(Bailey *et al.*, 1992)primers each 1 µl, Template DNA 2 µl and nuclease free water up to 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 93ºC for 5 min, followed by 35 cycles at 90ºC for 60 s, 64ºC for 30 s and 72ºC for 60 s. Final extension was carried out at 72ºC for 10 min. The amplified product (223 bp) was electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiS Bio Imaging System).

Detection of *Brucella* using species-specific *B. abortus*+ IS711primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2 x PCR Master mixture, 10 pmol of forward (5' GAC GAA CGG AAT TTT TCC AAT CCC 3') and reverse (5' TGC CGA TCA CTT AAG GGC CTT CAT 3') (Bricker and Halling, 1994) primers each 1 µl, Template DNA 2 µl and nuclease free water up to 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany). with initial denaturation at 95ºC for 5 min, followed by 35 cycles at 95ºC for 90 s, 57ºC for 120 s and 72ºC for 120 s. Final extension was carried out at 72ºC for 5 min. The amplified product (498 bp) was electrophoresed in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and image was documented by gel documentation system (Mini BiS Bio Imaging System).

SYBR green based real time PCR using *B. abortus*+ IS711primer

A PCR was standardized in a total reaction volume of 25 µl, containing 12.5 µl of 2X SYBR green PCR Master mixture, 10 pmol of forward (5' GAC GAA CGG AAT TTT TCC AAT CCC 3') and reverse (5' TGC CGA TCA CTT AAG GGC CTT CAT 3') primers each 1 µl, Template DNA 2 µl and nuclease free water up to 25 µl. The reaction was standardized in a thermal cycler (Eppendorf, Germany) with initial denaturation at 95ºC for 5 min, followed by 40 cycles at 90ºC for 60 sec, 64ºC for 30 sec and 72ºC for 60 sec and final Melting curve analysis was carried out at 95ºC for 15 sec, 60ºC for 1 min and 95ºC for 30 sec.

Results and Discussion

Isolation

Out of 192 clinical samples, 07(3.64%) samples produce round, glistening and smooth or mucoid colonies on *Brucella* agar medium (Fig. 1, Table 1). In the present finding was in agreement with earlier studies which reported 4% to 8% overall isolation rate (Ghodasara, 2008; Kanani, 2007).

However, in contrast to these findings overall isolation rate between 20 to 39 % (Das, 1990; Pal and Jain, 1985).

Identification

Morphological and staining characters of isolates

The all 7 isolates were subjected to Gram's staining and Modified Ziehl-Neelsen's (MZN) staining. In Gram's staining pink, gram negative, coccobacillary rods were observed (Fig. 2). While in MZN staining they appeared to be red coccobacillary organisms (Fig. 3). Similar morphology of organism was observed by Alton (1998), Ghodasara (2008) and Kanani (2007).

Rapid slide agglutination test

All the colonies presumed to be of *Brucella* organism were tested for agglutinatibility with known positive anti *Brucella* serum. All the isolates revealed clear agglutination, indicative of *Brucella*.

Biochemical characterization of isolates

All these 07 isolates gaved positive reaction in catalase (Fig. 4) and oxidase test (Fig. 5). On TSI slant, organism showed reaction as slant (yellow), butt (black) indicative as *Brucella abortus* (Fig. 6). Pal and Jain (1985) and Rhyan *et al.,* (1994) reported catalase and oxidase positive for *B. abortus*.

Table.1 Molecular characterization of *Brucella abortus* from clinical samples

Type of sample	No. of tested	No. of sample positive in direct PCR from clinical samples	No. of isolate obtained from clinical samples
Blood	99	00	00
Vaginal swab	46	00	**00**
Vaginal discharge	05	**03**	**01**
Milk	08	00	00
Placenta	09	**02**	**01**
Placental fluid	02	**01**	00
Hygroma fluid	01	00	00
Amniotic fluid	02	00	00
Foetal intestine fluid	01	00	00
Foetal lung	04	**01**	**01**
Foetal liver	04	**01**	**01**
Foetal stomach content	01	**01**	**01**
Foetal heart blood	04	**01**	**01**
Cotyledon	04	**01**	00
Foetal heart	02	**01**	**01**
Total	**192**	**12 sample**	**07 isolate**

Fig.1 Growth on BBL AGAR

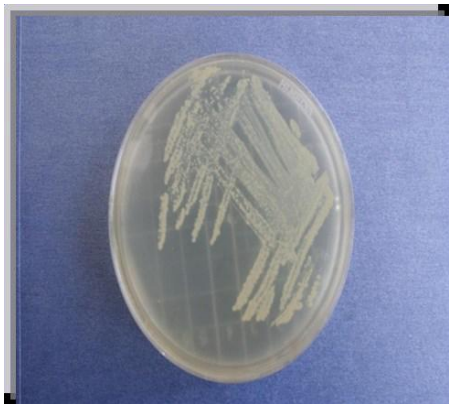


Fig.2 Gram –ve coco bacilli

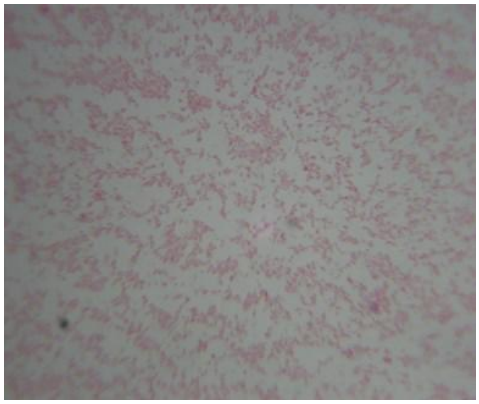


Fig.3 MZN positive

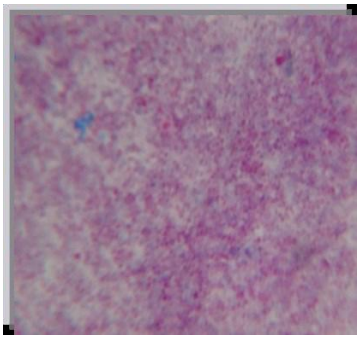
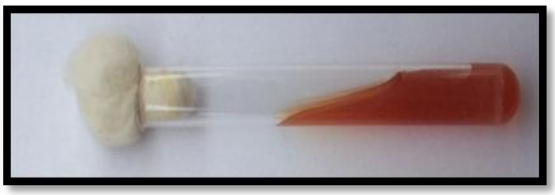


Fig.4 Catalase test

Fig.5 Oxidase test

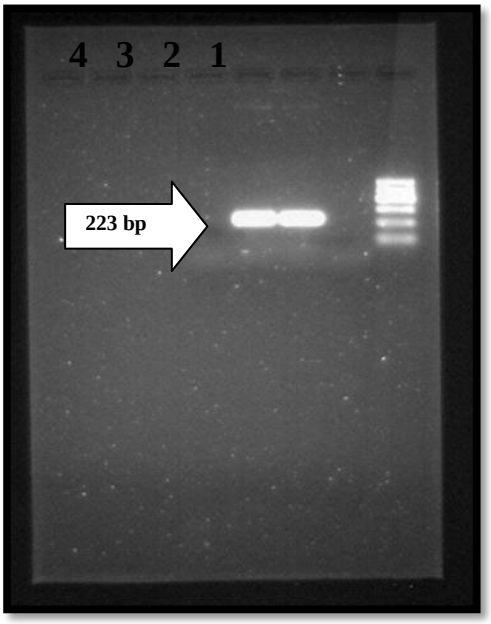
Fig.6 Triple Sugar Iron Agar (TSI) Test

Control



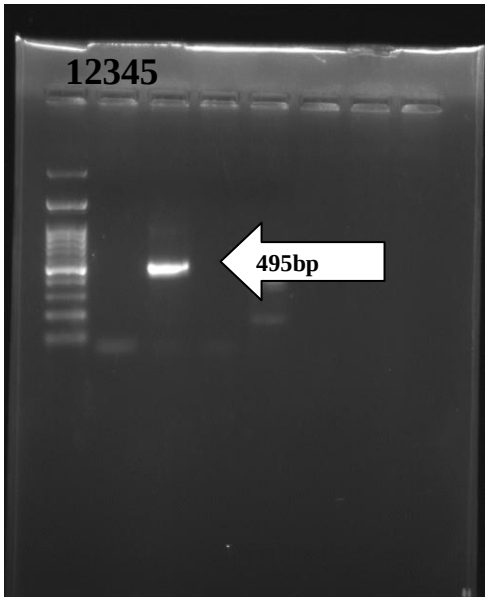
TSI: +Ve

Fig.7 Agarose Gel electrophoresis of 223bp PCR products with *bcsp31* primers



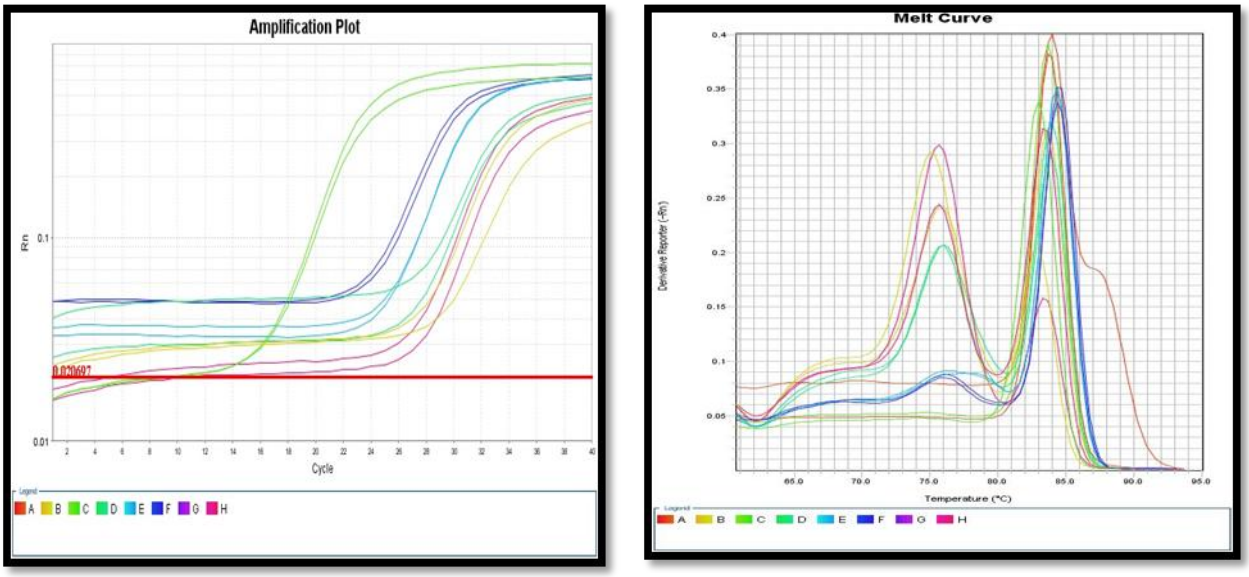
1- Ladder
2- NTC
3- Sample (positive)
4- Sample (positive)

Fig.8 Agarose Gel electrophoresis of 495 bp PCR product with primer IS711



1- Ladder
2- NTC
3- Sample (positive)
4- Sample (Negative)
5- Sample (Negative)

Fig.9 SYBR green based Real time PCR amplification plot and Melt curve



Molecular detection of *Brucella abortus*

In PCR study targeting 16S rRNA gene, Out of 192 clinical samples, 12 clinical samples (Table 1) and 7 culture isolated colonies were found positive to give specific amplicon of 223bp region of the sequence encoding a 31 kDa immunogenic bcsp31 by *Brucella* genus specific primer pairs B4/B5 (Fig. 7). All genus specific PCR positive 12 samples and 7 cultural colony yielded an amplicon of 498bp in +IS711 primers indicate species as *Brucella abortus* (Fig. 8). Similarly, Kanani (2007) and Jung *et al.,* (1998) detection of *Brucella* by using bcsp31 gene based B4/B5 primer. Navarro *et al.,* (2002) and Varasada (2003) used same primer pair for diagnosis of human brucellosis. Earlier Navarro *et al.,* (2002), Kanani (2007) and Patel (2007) used same three primer pairs for molecular detection of *Brucella abortus.* Patel *et al.,* (2015) and Karthik *et al.,* (2014) used species specific +IS711 primers for detection of *Brucella abortus* and they yielding 498 bp band when electrophoresed through 2 per cent agarose gel. SYBR green based real time PCR used for detection of *Brucella abortus* species by IS711primer. All genus specific positive 12 samples and 7 cultural colony were processed by SYBR green based real time PCR. After the complete cycling parameters as described in Material and methods data analysis was done based on amplification curves obtained (Fig. 9).

The specificity of the amplified PCR products was assessed by performing a melting curve analysis. The samples which were detected positive in conventional species specific PCR also detected positive for *Brucella abortus* as it is matching the Tm values of the *Brucella abortus* control sample.

In conclusion, Molecular detection of *Brucella abortus* from clinical samples is more sensitive and rapid method than culture isolation. The use of the Polymerase Chain Reaction (PCR) to identify *Brucella* DNA at genus and species levels has becoming extended to improve diagnostic tests. The Molecular detection results showed the presence of *B. abortus* in clinical samples which is of public health importance because it is zoonotic disease.

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