

Original Research Article

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## Isolation, Identification and Molecular Detection of *Brucella abortus* from Cattle and Buffalo

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### ABSTRACT

Bovine brucellosis is one of the most wide-spread zoonosis accounting for enormous economic losses through abortion, stillbirths, reduction of milk yield and infertility. Brucellosis has wide socioeconomic impacts, especially in countries in which rural income relies on livestock breeding and dairy products. So early and accurate diagnosis is essential to avoid the losses occurred due to brucellosis. The present study reports the isolation, identification and molecular detection of *Brucella abortus*. We have collected 610 serum samples, 5 foetal abomasal contents and 5 foetal tissues from dairy farms of cattle and buffalo in and around Mumbai and Pune region. Out of 610 sera samples, 135 were detected positive by RBPT. These sero positive animals (n=135) were then subjected to blood collection and DNA isolation. Similarly vaginal swabs were collected from the seropositive females (n=92). Vaginal swabs, abomasal contents and foetal tissues were processed by cultural isolation on Brucella Agar Media with selective antibiotic supplements. We have successfully recovered 14 bacterial isolates on cultural isolation, identified as *Brucella* by colony character, Gram's staining, MZN staining and biochemical tests. The recovered bacterial cultures and *Brucella* reference strains (*B. abortus*S19, *B. abortus*544 and *B.melitensis* Rev1) were further processed for DNA isolation. The DNA isolated from seropositive animals, bacterial cultures and the reference strains were subjected to genus (*Brucella*) specific PCR using BCSP 31 B4/B5 primer pair and species (*B. abortus*) specific PCR using IS711/AB primers. As expected, the PCR analysis of reference strains revealed genus specific amplicon (223bp) in all the three strains, and only *B. abortus* S19 and *B. abortus*544 exhibited species specific amplicon (498 bp). All the 14 cultural isolates and 83 seropositive animals were positive for genus specific PCR. While in species specific PCR, 10 cultural isolates and 29 seropositive animals were confirmed as *B. abortus*. The PCR products of five representative field isolates were sequenced directly and analysed by bio informatic tools. The obtained nucleotide sequence exhibited 100% homology with other strains of *B. abortus* in the GenBank database.

#### Keywords

BCSP-31,  
*Brucella abortus*,  
Polymerase chain  
reaction assays  
(PCR).

#### Article Info

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### Introduction

Brucellosis is a widespread, economically devastating and highly infectious zoonotic disease of animals prevalent throughout the

world (Dawood, 2008). The causative agents of brucellosis are facultative intracellular gram-negative, non-spore forming, minute

coccobacillary bacteria belonging to the genus *Brucella*. Brucellosis continues to be of great health significance and economic importance in many countries. The infection can result from direct contact with infected animals and can be transmitted to humans through the consumption of raw milk and milk products.

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; Radostits *et al.*, 2007). The disease causes serious economic losses to the livestock industry by reducing the productive and reproductive potential of the affected animals, loss of calves, sterility, infertility, as well as reduction or complete loss of milk yield after the abortion (Chahotal *et al.*, 2003).

One of the major abortion-causing agents in case of cattle population is *B. abortus* (Priyantha, 2011). The host specificity in different *Brucella* species is not very strict (Mustafa and Corbel, 1988; Erdogan *et al.*, 1993), they have been found to infect other host species such as *B. abortus* in sheep and goats (Elzer *et al.*, 2002; Ocholi *et al.*, 2005) or *B. melitensis* in cattle (Alvarez *et al.*, 2011).

To control a disease, accurate early detection of the pathogen is very important. Diagnosis of brucellosis is based on cultural isolation, serology and nucleic acid amplification. Cultural isolation of organism from the host tissues, the exudates, secretions followed by bacteriological characterization is considered as the “gold standard” (Surucuoglu *et al.*, 2009). However these methods require lot of skill to isolate the organism, are time consuming as the organism takes 2-3 days to grow and also involves risk of laboratory acquired infection (Alton *et al.*, 1998; Perez-Sancho *et al.*, 2013).

Serological diagnosis of brucellosis is relatively simple and inexpensive; however the specificity of serological tests is low (Ariza *et al.*, 1992). Further, the serological tests are prone for false positive reactions and exhibits cross-reactivity of *Brucella* with a number of other Gram-negative organisms (Corbel *et al.*, 1984; Fernandez-Lago *et al.*, 1982). 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 BCSP 31. Considering all these aspects the present study was planned for isolation, identification and molecular detection of *B. abortus* in cattle and buffalo.

## **Materials and Methods**

### **Collection of sample**

For the present investigation 610 serum samples, abomasal contents and tissue samples from 5 aborted fetuses were collected from dairy farms of cattle and buffalo in and around Mumbai and Pune region.

Out of 610 sera samples, 135 were detected positive by RBPT. These seropositive animals (n=135) were then subjected to blood collection and DNA isolation. Similarly vaginal swabs were collected from the seropositive females (n=92).

### **Isolation**

The vaginal swabs, abomasal content and foetal tissues were inoculated in brucella broth. These inoculums were separately streaked on Brucella Agar Medium with selective antibiotic supplements and incubated at 37°C anaerobically in an atmosphere of 5 per cent CO<sub>2</sub> for minimum of

15 days. The plates were observed at every 24 hours interval for the growth.

### Identification

The isolates were subjected to Gram staining and Modified Ziehl-Neelsen (MZN) staining for confirming the purity of cultures and morphological characters of *Brucella*. Identification of *Brucella* organism was done by biochemical tests like Oxidase test, Catalase test, Nitrate reduction test, Urease test, H<sub>2</sub>S Production test and Growth in the presence of dyes (OIE, 2009) etc. (Figs. 3-6).

### Molecular detection of *Brucella abortus*

#### DNA extraction

The extraction of genomic DNA of *B. abortus* from the blood samples of seropositive animals was carried out as per the protocol described by Leal-Klevezas *et al.*, (1995) with slight modifications and extraction of DNA from bacterial cultures and *Brucella* reference strains (*B. abortus*S19, *B. abortus*544 and *B. melitensis* Rev1) was done as per the protocol described by Romero *et al.*, (1995a) with slight modifications. Foetal tissue samples and abomasal contents were processed for DNA isolation as per the methods given by O'Leary *et al.*, (2006) and Leal-Klevezas *et al.*, (1995) with slight modifications, respectively.

#### Detection of *Brucella* using genus-specific BCSP 31 B4/B5 primer

A PCR was standardized in a total reaction volume of 25 µl, containing 1X PCR buffer, 100mM of each dNTP's, 2 mM of MgCl<sub>2</sub>, 10 pM of forward (B4 5'TGG CTC GGT TGC CAA TAT CAA3') and reverse (B5 5'CGC GCT TGC CTT TCA GGT CTG3') primers each, 1 U of Taq DNA Polymerase and 50-100 ng Template DNA. The reaction was standardized in a thermal cycler (Master Cycler, Eppendorf) with initial denaturation at

94°C for 3min, followed by 35 cycles at 94°C for 60 s, 62°C for 45 s and 72°C for 60s. Final extension was carried out at 72°C for 5 min. The amplified product was electrophoresed on 2% agarose gel stained with ethidium bromide (EtBr) and the products were visualized and documented using Automatic Computerized Gel Documentation and Analysis System (Uvitec).

#### Detection of *B. abortus* using species-specific IS711/AB primer

A PCR was standardized in a total reaction volume of 25 µl, containing 1X PCR buffer, 100mM of each dNTP's, 2 mM of MgCl<sub>2</sub>, 10 pM of forward (IS711/AB F 5' TGCCGATCACTTAAGGGCCTTCAT 3') and reverse (IS711/AB R 5' GACGAACGGAATTTTTCCAATCCC 3') primers each, 1 U of Taq DNA Polymerase and 50-100 ng Template DNA. The reaction was standardized in a thermal cycler (Master Cycler, Eppendorf) with initial denaturation at 94°C for 3min, followed by 35 cycles at 94°C for 3 min, 60°C for 45 s and 72°C for 1 min. Final extension was carried out at 72°C for 6 min. The amplified product was electrophoresed on 2% agarose gel stained with EtBr and the products were visualized as above.

#### PCR- RFLP analysis of *Brucella* isolates

For characterization of *Brucella* isolates, PCR-RFLP analysis was carried out on BCSP 31 B4/B5 PCR product using restriction enzyme HaeII. 30µl reaction mixtures including 16µl sterile distilled water, 3µl of 10X reaction buffer, 1µl restriction enzyme and 10µl PCR product were prepared. The mixture was incubated at 37°C for 2.5 hrs and the digested PCR products were electrophoresed on 2.5% agarose gel after staining with EtBr and visualized under UV light.

## Results and Discussion

### Isolation

In this study successfully recovered 14 *Brucella* isolates from the processed clinical field samples (vaginal swabs, abomasal content and foetal tissues; n=102). These isolates exhibited round, glistening and smooth colonies on *Brucella* agar medium (Fig. 1, Table 1). Further all the 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. While in MZN staining they appeared to be red coccobacillary organisms (Fig. 2). Similar morphology of organism was observed by Alton (1988), Ghodasara (2008) and Kanani (2007). The rate of isolation of *B. abortus* from vaginal swabs in our study was 13.72 per cent.

Several workers in India have made an attempt towards isolation of *B. abortus* with varying rates of isolation. Jeyaprakash *et al.*, (1999) recorded isolation of *B. abortus* in 15 per cent cases from vaginal swabs employing tryptose agar while Das *et al.*, (1990) using *Brucella* selective medium recorded an isolation rate of 38.18 per cent in cows and 14.28 per cent in buffaloes.

Isolation rate varies depending on the stage of disease, previous use of antibiotics, clinical specimen, the culture methods and technical approach. Isolation rates are much higher during first two weeks of symptoms (80 - 90% in acute form and 30-70% in chronic form) (Al Dahouk *et al.*, 2003).

### Molecular detection of *Brucella abortus*

*Brucella* genus specific PCR is designed to target an amplicon of 223 bp, within the region of the sequence encoding 31 kDa immunogenic bcs31 using primer pairs B4/B5 (Baily *et al.*, 1992). The targeted sequence is conserved across the *Brucella*

species. The *Brucella* genome also contains an insertion sequence (IS) element called IS711, specific to the genus. However the copy number of IS711 varies in the genome of the different *Brucella* species. *B. abortus* carries six complete and one truncated copy of IS711 element (Halling *et al.*, 2005) whereas, *B. melitensis* and *B. suis* contain seven complete copies of this element (Ocampo-Sosa and Garcia-Lobo, 2008). Other *Brucella* spp. viz., *B. ceti* and *B. pinnipedialis* has more than 20 copies (Bricker, 2000 and Zygmunt *et al.*, 2010) and *B. ovis* contains 38 copies of IS711 element (Tsolis *et al.*, 2009). The IS711/AB primer pair is designed to amplify 498 bp fragment, specific to the *B. abortus* spp. (Bricker and Halling, 1994).

In the present study, out of 135 DNA samples isolated from seropositive blood samples, 83 samples showed genus (*Brucella*) specific amplicon of 223 bp and 29 samples showed species (*B. abortus*) specific amplicon of 498 bp, upon PCR using BCSP31 B4/B5 and IS711/AB primer pairs, respectively (Fig.9 and 10).

Similar results were seen by Mukherjee *et al.*, (2007), Karthik *et al.*, (2014) and Mahmood *et al.*, (2016). Mukherjee *et al.*, (2007) compared efficacy of *bcs* and *omp2* PCR assays and ELISA for detection of *Brucella* spp. On bovine blood samples and observed that, *bcs* PCR assay was most sensitive (92.72 %) followed by *omp2* PCR (61.81 %) and ELISA (55.55 %). Karthik *et al.*, (2014) reported 15% samples positive for *B. abortus* out of 200 blood samples of cattle by BCSP 31 and IS711 PCR. Rashid *et al.*, (2016) processed 167 blood samples of cattle, buffalo and goat by culture, RBPT, cELISA and IS711 PCR. They found 1% (2/167) samples positive for infection by culture, 4% (7/167) by RBPT, 6% (10/167) by cELISA and 21% (35/167) by IS711 PCR.

In the present investigation the DNA isolated from abomasal content and tissues of aborted foetuses were also PCR tested using B4/B5 primers. Only 1 abomasal content and 2 foetal

tissue samples showed amplicon of 223bp confirming the infection of *Brucella*. Patel *et al.*, (2017) observed similar 223 bp amplicon in 3 foetal tissues and 1 stomach content.

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

Type of sample	No. of tested samples	No. of PCR positive clinical samples	No. of isolates recovered from clinical samples
Vaginal swab	92	10	10
Abomasal content	5	1	2
Foetal tissues	5	2	2
Blood samples	135	83 by BCSP 3129 by IS711	--

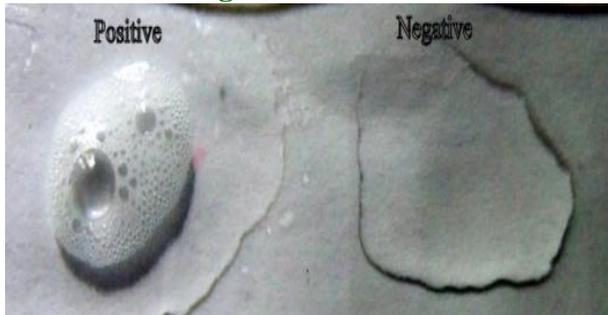
**Fig.1** Colonies of *Brucella* spp. on BAM



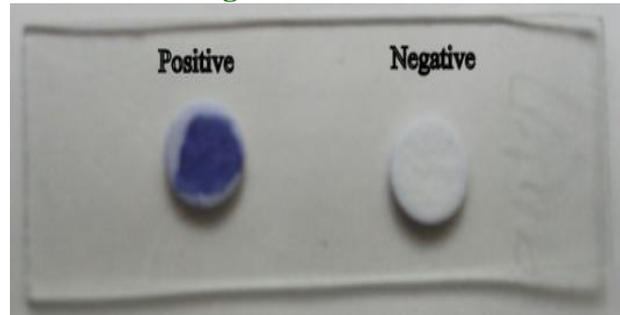
**Fig.2** Modified Ziel- Neelson staining of *Brucella* spp



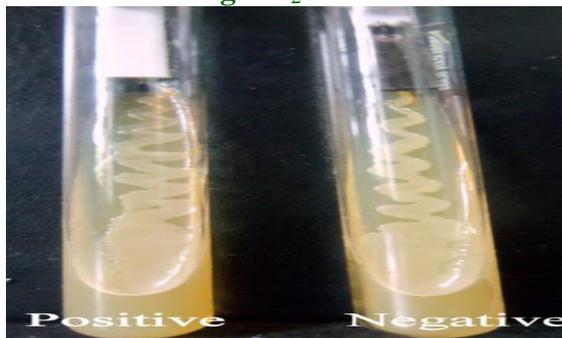
**Fig.3** Catalase test



**Fig.4** Oxidase test



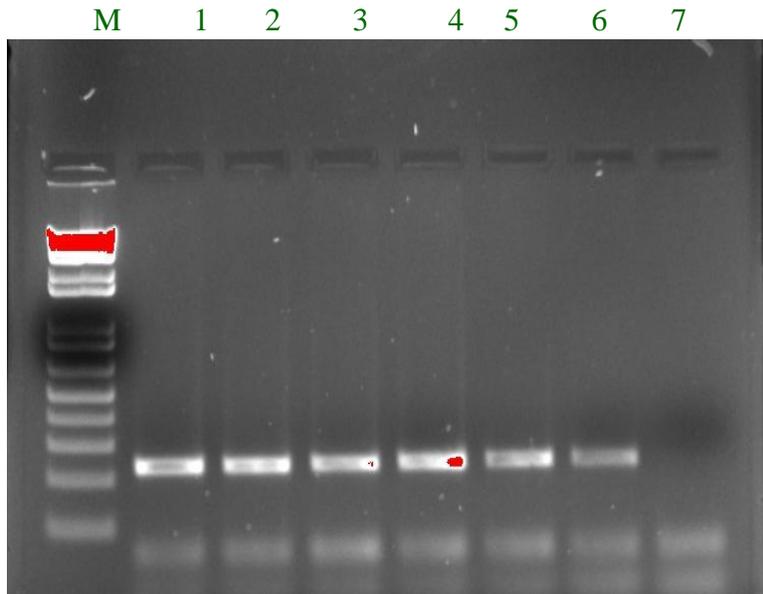
**Fig.5** H<sub>2</sub>S Test



**Fig.6** Urease Test

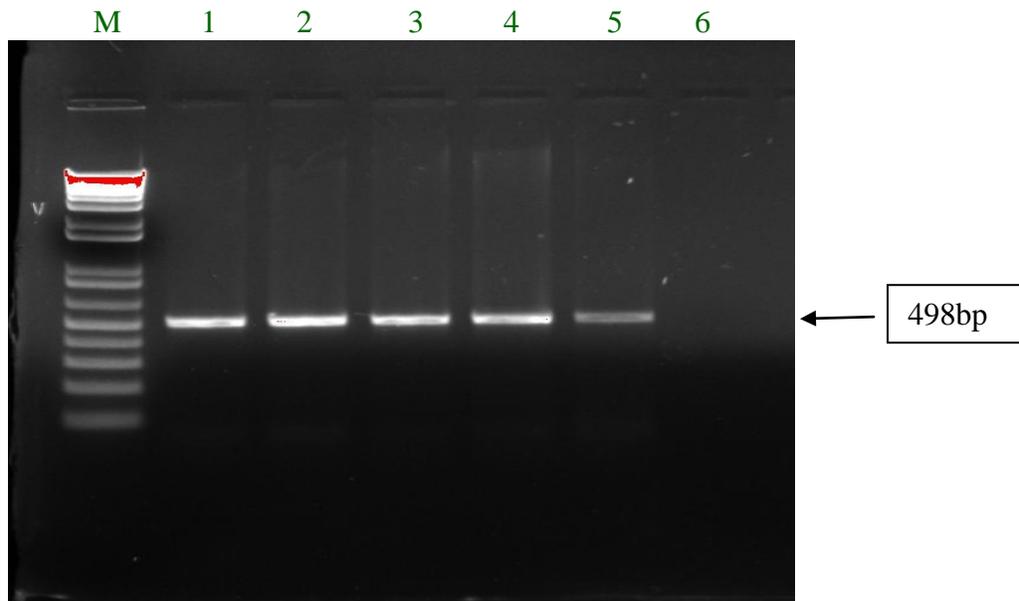


Fig.7 PCR amplification of *Brucella spp.* using the primer set B4 and B5



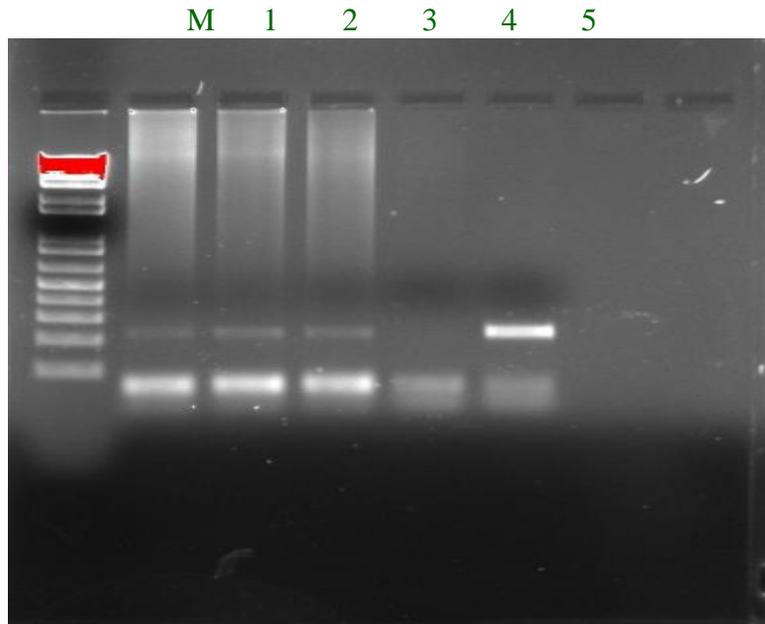
- Lane M - Molecular size marker (1kb plus)
- Lane 1 - *B. abortus*S19
- Lane 2 - *B. abortus*544
- Lane 3 - *B. melitensis* Rev1
- Lane 4, 5&6 - PCR amplified 223bp product of *brucella* isolates
- Lane 7 - Negative control

Fig.8 PCR amplification of *Brucella abortus* using the primer set IS711 and AB



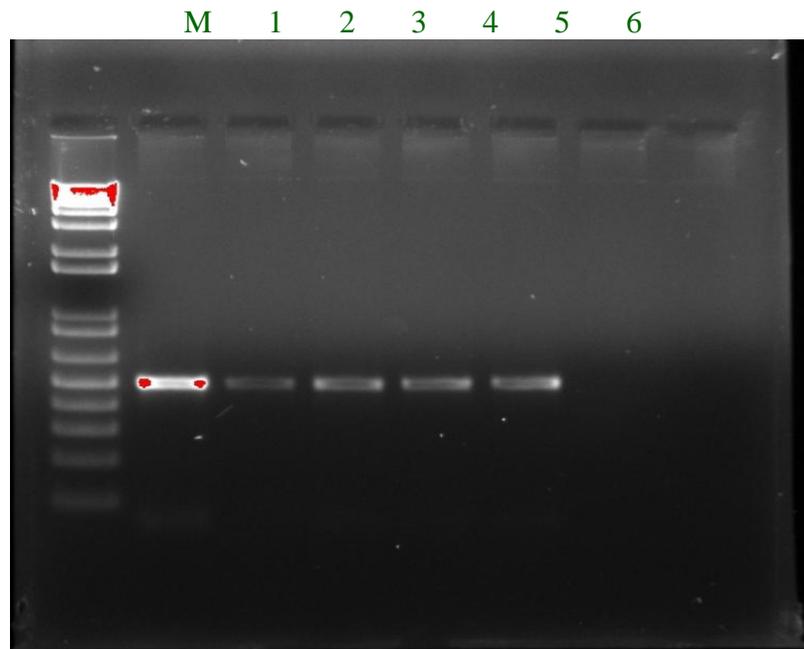
- Lane M - Molecular size marker (1kb plus)
- Lane 1 - *B. abortus*S19
- Lane 2 - *B. abortus*544
- Lane 3, 4&5 - PCR amplified 498bp product of *brucella* isolates
- Lane 6 - Negative control

**Fig.9** PCR amplification of *Brucella* spp from blood samples using the primer set B4 and B5



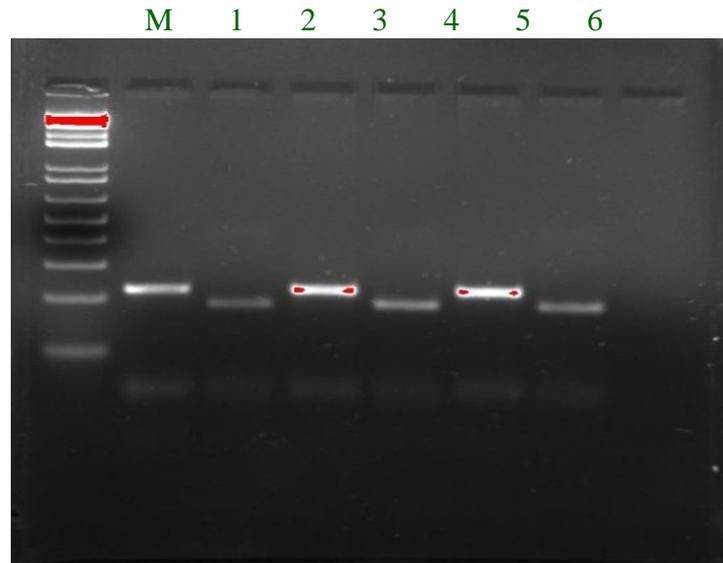
Lane M - Molecular size marker (1kb plus)  
Lane 1, 2&3 - P52, P54, P70  
Lane 4 - Negative control  
Lane 5 - *B. abortus*S19

**Fig.10** PCR amplification of *Brucella abortus* from blood samples using the primer set IS711 /AB



Lane M - Molecular size marker (1kb plus)  
Lane 1 - *B. abortus*S19  
Lane 2, 3, 4 &5 - Blood samples  
Lane 6 - Negative control

**Fig.11** HaeII digestion of PCR amplicon of BCSP 31 gene of *Brucella*spp



Lane M - Molecular size marker  
Lane 1 - PCR product of S19  
Lane 2 - HaeII digest S19  
Lane 3 - PCR product of Isolate 1  
Lane 4 - HaeII digest of Isolate 1  
Lane 5 - PCR product of Isolate 2  
Lane 6 - Hae II digest of Isolate 2

The bacterial isolates (n=14) recovered from clinical specimens in our study were found to be of genus *Brucella*, on BCSP31 PCR (Fig. 7). These results are in agreement with the findings of Casanas *et al.*, (2001) who applied BCSP31 PCR on various reference and field strains and observed similar amplification product of 223 bp. Similarly, Londhe *et al.*, (2014) processed 104 clinical specimens and 173 blood samples of farm animals for diagnosis of brucellosis by cultural isolation and BCSP 31 PCR. They recovered only 4 isolates from the clinical specimens, which were confirmed as *Brucella* spp, while 76.30% blood samples revealed presence of *Brucella* on BCSP 31 B4/B5 PCR.

The samples which were confirmed to be of genus *Brucella* were further processed for detection of species by IS711 PCR. In our study 10 out of 14 *Brucella* genus specific isolates yielded an amplicon of 498 bp,

confirming their identity as *B. abortus* (Fig. 8). Our results are in agreement with Patel *et al.*, (2017), who processed 192 different clinical samples by cultural isolation, BCSP31 B4/B5 and IS711A/B PCR. They recovered 7 isolates, which yielded an amplicon of 498bp, specific to *B. abortus* on IS711A/B PCR.

#### **PCR- RFLP analysis of *Brucella* isolates**

Polymerase chain amplification of selected sequences followed by restriction fragment length polymorphism (PCR-RFLP) analysis has provided evidence of polymorphism in a number of *Brucella* genes (Cloeckeaert *et al.*, 1995). PCR-RFLP is useful for characterization and differentiation of *Brucella* strains within a species.

In the present investigation the samples positive for *Brucella* genus (n=14) were

processed for PCR-RFLP using HaeII restriction enzyme. The restriction digestion of these 223 bp amplicons of B4/B5 primer pair, generated two DNA fragments (189 and 34 bp) (Fig. 11), 34 bp fragment was however not visible due to its small size. Our results indicated presence of a single restriction site in the *Brucella* genome within the region of the sequence encoding 31 k Da immunogenic bcsp31. The B4/B5 PCR products of reference strains of *Brucella* were also processed for PCR-RFLP using HaeII. All the PCR products of bacterial isolates and that of reference strains yielded similar restriction pattern, indicating prevalence of monomorphic *Brucella* organism in and around Mumbai and Pune region. Similarly, Hemade *et al.*, (2016) detected lack of polymorphism in the *Brucella* genome.

In conclusion we observed that the molecular approach to identify *Brucella* at species level is advantageous over traditional bacteriological techniques. The bacteriological methods required long duration for isolation of the organism, it took at least two weeks for the final identification at the species level. The traditional procedure needs to be performed by highly skilled personnel in a sophisticated laboratory. However, molecular techniques identified *Brucella* spp. In a very short duration. The BCSP31 B4/B5 and IS711 A/BPCR is very specific, rapid, safe and confirmatory diagnostic tool for detection of brucellosis where whole blood can be used as a sample of choice. Further use of PCR-RFLP will be a valuable tool for epidemiological and evolutionary studies of *Brucella*.

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