



Detection of animal brucellosis by cultural method

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ABSTRACT

Brucellosis, an infectious disease of many vertebrate species, is an important re-emerging zoonosis with a worldwide distribution. The present research was aimed for detection of brucellosis in animals by isolation and identification of *Brucella* species isolates by conventional method and PCR. Clinical specimens (61) which included vaginal swabs (48), foetal membranes (08), semen samples (03) and abomasal contents of aborted fetuses (02) were subjected for isolation on Brucella Agar Medium (BAM), wherein 15 *Brucella* spp. isolates were recovered with isolation rate of 24.59%. All 15 conventionally identified *Brucella abortus* species isolates were further confirmed by 2 different PCR assays using genus specific BCSP31 and species specific IS711 (AB and BM) primers. The BCSP31 (223 bp) PCR assay was simple, effective and sensitive in detection of *Brucella* isolates at genus level, whereas, IS711/AB PCR was useful to detect isolates at species level. In IS711/AB PCR, abortus specific amplification product of 498 bp was produced in all 15 isolates confirming their identity as *B. abortus*, whereas none of isolate showed *B. melitensis* specific amplification in IS711/BM PCR.

Key words: Animal brucellosis, *Brucella abortus*, *Brucella melitensis*, Cultural identification, Cultural isolation, PCR

Brucellosis, is an important re-emerging zoonosis with a worldwide distribution; although it has been eradicated in many developed countries (Geering *et al.* 1995), it continues to be endemic in most parts of the world especially the developing countries (Trujillo *et al.* 1994). The incidences of brucellosis cases are on the increasing side in recent times due to increased trade and all-over rapid movement of livestock (Renukaradhya *et al.* 2002), but often a neglected disease. Brucellosis attributes to serious economic losses through its adverse effects on the reproductive and productive potential of the affected animals (Chahotal *et al.* 2003) along with loss of calves, wool, meat and milk production (Corbel and Brinley-Morgan 1984). Brucellosis is a major impediment for trade and export. Singh *et al.* (2002) reported annual economic losses to the tune of ₹ 350 million due to this disease. Kollannur *et al.* (2007) estimated that, in India there is loss of US\$ 58.8 million / year due to brucellosis.

Bovine brucellosis is usually caused by *Brucella abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. Historically, the diagnosis of brucellosis has been based on conventional culture, serology and isolation of *Brucella* spp. from clinical specimens. Isolation and identification of a biovar of *Brucella* strains is an epidemiological indication that can help to investigate the origin of a given brucellosis

outbreak. *Brucella* species and their different biovars are distinguished by differential tests based on serotyping, phage typing, dye sensitivity, CO₂ requirement, H₂S production and metabolic properties (Alton *et al.* 1988).

PCR assays targeting different regions of *Brucella* DNA have been explored for their efficacy in precise identification of *Brucella* at genus and species level. The PCR techniques have proved to be useful due to specificity, sensitivity and the rapidness (Leal-Klevezaset *al.* 2000, Amin *et al.* 2001). A number of PCR based assays have been developed for the rapid identification of *Brucella* spp. Some of the targets that have evaluated extensively include 16S rRNA (Herman and Herman 1992), insertion sequence IS711 (Bricker and Halling 1994) and *bcs*p31 (Guarino *et al.* 2000, Baily *et al.* 1992).

Taking into consideration endemic nature of brucellosis in animal and importance of isolation technique in epidemiology; the present research was aimed for isolation, identification of field isolate of *Brucella* species from animals by conventional method and PCR.

MATERIALS AND METHODS

Reference bacterial strains: A total of 3 references ATCC *Brucella* spp. strains *Brucella abortus* 544, *Brucella abortus* S-19 and *Brucella melitensis* Rev-1 were used in the present study as standard controls.

Clinical specimens: A total of 61 clinical specimens which included abomasum contents (02), semen samples (03), foetal membranes (08) and 48 vaginal swabs from animals suspected for brucellosis formed the material for

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investigation of the present study. The samples of animals belonging to different districts of Maharashtra were collected.

Collection of specimens: The vaginal swabs were collected from cattle and buffaloes within 28 days of post abortion using readymade guarded sterile swabs with or without transport medium. For abomasum content, the carcass of freshly aborted foetus was opened and using sterile syringe or a Pasteur pipette, the abomasum content was aspirated and collected in sterile tube. The least healthy-looking (showing pathological changes/ lost normal appearance) cotyledons or portions were collected from the aborted foetal membranes. The semen samples from the breeding bulls of private farms used for natural service were collected aseptically by using artificial vagina. All specimens collected were transported on ice pack to the laboratory as early as possible and processed immediately.

Isolation and identification of *Brucella* spp. by conventional methods: All the work involving handling of zoonotic *Brucella* organism and infected aborted material was performed by taking appropriate bio-safety precautions including use of personal protective equipments (PPE). Isolation of *Brucella* spp. was carried out from the clinical specimens of animals using *Brucella* agar media (BAM) supplemented with *Brucella* selective supplement and 5% horse serum. Inoculated BAM plates were incubated at 37°C in CO₂ incubator in presence of 5% CO₂ till growth became visible on medium or maximum up to one week. Round, glistening and smooth or mucoid colonies on BAM plate were suspected to be of genus *Brucella*.

The colonies suggestive of *Brucella* spp. were examined for presence of gram negative, modified acid-fast organisms and such isolates were further processed for identification as *Brucella* spp. based on biochemical properties, growth in presence of dyes, requirement of CO₂, oxidase test, catalase test, urease test and H₂S production and rapid slide agglutination test using standard procedures (Quin *et al.* 1994, Alton *et al.* 1975).

Molecular identification of *Brucella* spp. by PCR: Extraction of DNA from cultures of *Brucella* spp. and other reference bacterial strains, viz. *B. abortus* 544, *B. abortus* S19 and *B. melitensis* Rev-I was done as per Romero *et al.* (1995). Quantification of DNA extracted from bacterial cultures was done spectrophotometrically at 260 nm and 280 nm using Nanodropspectro-photometer for determination of sample concentration and purity. The isolated genomic DNA samples were subjected to agarose gel electrophoresis in ethidium bromide stained agarose gel for assessing their integrity (Brown 2007). The integrity of the DNA was checked by visualization of the DNA using gel documentation system.

***Brucella* genus specific BCSP31 PCR assay:** The amplification of 223 bp region of BCSP 31 genetic element of *Brucella* was carried out using published oligonucleotide primer sequences (Table 1), as per Baily *et al.* (1992).

The PCR was set in a final volume of 25 µl, spinned briefly and set into thermal cycler. The cycling conditions

Table 1. Oligonucleotide primers for *Brucella* genus-specific PCR(BCSP 31)

Primer	Oligonucleotide sequence	Reference
B4	5'-TGG-CTC-GGT-TGC-CAA-TAT-CAA '3	Baily <i>et al.</i> (1992)
B5	5'- CGC-GCT-TGC-CTT-TCA-GGT-CTG-'3	

Table 2. Oligonucleotide primers for *B. abortus* and *B. melitensis* specific PCR

Primer	Oligonucleotide sequence	Reference
IS711AB	5' -TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT- 3' 5' -GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC -3'	Bricker and Halling (1994)
IS711BM	5' -TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT- 3' 5'- AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA-3'	

used were initial denaturation at 95°C for 3 min, 5 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min.

Brucella species specific IS711 PCR assays: The field isolates of *Brucella* spp. and reference strains were subjected to IS711 AB (*B. abortus*-specific) and IS711 BM (*B. melitensis*-specific) PCR assays. The amplification of 498 bp region of IS711 genetic element of *Brucella* spp. was carried out using published oligonucleotide primer sequences (Table 2) as per Bricker and Halling (1994).

Evaluation of PCR products: The amplification products of both BCSP31 and IS711 PCR assays were evaluated by agarose gel (1.5%) electrophoresis stained with ethidium bromide (Brown 2007). The products were visualized and documented using automatic computerized gel documentation and analysis system using UV light source. The size of PCR product was estimated with the help of Image Lab (Version 4.1) software available with the gel documentation system.

RESULTS AND DISCUSSION

Many developed countries have successfully controlled and eradicated brucellosis in livestock species by adopting a strict test and slaughter policy, however, the disease continues to be the major cause of economic loss and human suffering in developing countries (Al-Shamahy 1999, Singh *et al.* 2002). Since, test and slaughter policy cannot be adopted in India due to social, economic and religious reasons, the disease has assumed an endemic proportion. Moreover, due to the limitations associated with the available vaccines, immuno-prophylaxis is not widely practiced, thereby leading to perpetuation of the pathogen. Therefore, precise and timely diagnosis of such endemic disease (brucellosis) with molecular epidemiology is of

utmost importance in developing countries to initiate appropriate control measures and facilitate tracing the origins of outbreaks (Mehra *et al.* 2000, Renukaradhya *et al.* 2001, Sarumathi *et al.* 2003). With this view, the present research was conducted for detection of brucellosis by isolation and identification of *Brucella* spp. isolates using conventional methods and by PCR.

Isolation and identification of *Brucella* spp. by conventional methods: Stomach contents of aborted fetuses are good source for isolation of *Brucella* organism since they are less contaminated and contain high concentration of organisms. In cases of retention of placenta, the foetal membrane is considered as choice of sample. Under the conditions of non-availability of aborted specimens, vaginal secretion swabs are preferred since recovery of *Brucella* organisms can be achieved up to 6-week period following parturition or abortion (Corner and Alton 1982). Whereas, semen sample used forms a specimen for screening of suspected breeding bulls for brucellosis. Therefore, in the present investigation all above listed clinical specimens suspected for brucellosis were used for isolation of *Brucella* spp., since they are found to be most valuable for isolation of *Brucella* organisms (OIE 2009).

Isolation of organisms from clinical specimens followed by bacteriological characterization has been considered as the “gold standard” for diagnosis of brucellosis. The procedure is reliable and definitive since it provides direct evidence of presence of organism in the herd (Corner and Alton 1982, Bricker 2002, OIE 2009). Therefore, present research was performed based on the above technique.

The isolates recovered on BAM were tentatively identified as *Brucella* spp. on the basis of morphology and staining reaction in Gram's and modified Ziehl - Neelsen method (Fig. 1), colony characters (Fig. 2), bio-chemical and agglutination tests (Fig. 3) with known anti- *B. Abortus* serum. All isolates were found to be CO₂ dependant, oxidase producing, catalase, H₂S and urease. Moreover, all the isolates showed agglutination with known anti-*B. Abortus* serum confirming their identity as *Brucella abortus* species.

In the present study brucella agar medium (BAM) was used for isolation of *Brucella* spp., which was also employed

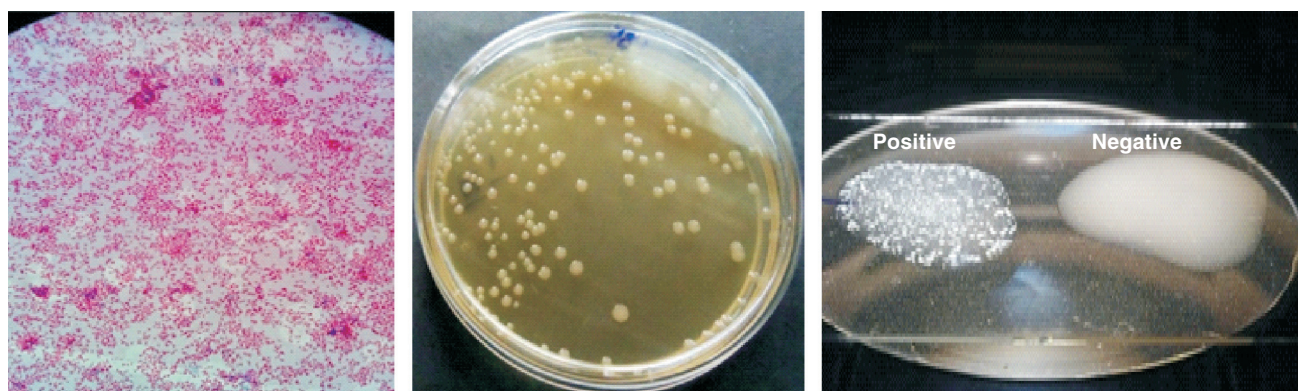
Table 3. Isolation rate of *Brucella* spp. from clinical specimens

Clinical specimen	Number of samples processed	Number (%) of <i>Brucella</i> spp. isolates recovered
Vaginal swab	48	07 (14.58%)
Foetal membrane	08	07 (87.50%)
Semen sample	03	00 (0%)
Abomasal content	02	01 (50%)
Total	61	15 (24.59%)

by many workers for isolation and evaluation of its suitability for growth of *Brucella* organisms (Farrell and Robertson 1972, Shin *et al.* 1978, OIE 2004). Isolation of *Brucella* was successful only from 15 specimens (24.59%) while remaining 41 specimens (75.41%) proved negative for cultural isolation. Out of the 15 isolates of *Brucella* spp. recovered, 07 (46.67%) were from vaginal swab, 07 (46.67%) were from foetal membrane and 01 (6.66%) was from abomasum contents of foetus while semen samples proved to be negative in cultural isolation.

In present study, isolation of *Brucella* was successful only from 15 specimens (24.59%) with highest recovery of *Brucella* spp. isolates was achieved from foetal membrane (87.5%) followed by abomasal content (50%) and vaginal swab (14.58%). However, isolation from semen sample could not yield recovery of *Brucella* organism (Table 3).

Several researchers in India have reported isolation of *Brucella* spp. from different clinical specimens with varying degrees of success. Das *et al.* (1990) found isolation rate of 38.18% in cows and 14.28% in buffaloes, whereas Jeyaprakash *et al.* (1999) investigated the cases of abortions and retained placenta in cows and could recover 10 *Brucella* isolates with an isolation rate of 15.62%. Rathore *et al.* (2002) achieved 39.02% isolation rate of *Brucella* spp. from clinical specimen and Chahota *et al.* (2003) reported 100% isolation rate of *Brucella* spp. using aborted foetal stomach contents. Farwachi *et al.* (2010) isolated *Brucella* spp. from 4 (33.3%) of the 12 samples (aborted foetal stomach content of sheep). Londhe (2009) recorded *Brucella* spp. with an isolation rate of 9.68% from various clinical specimens.



Figs 1–3. 1. Microscopic appearance of *Brucella* spp. (MZN stain, 1000×). 2. Colonies of *Brucella* spp. on BAM. 3. Agglutination with known anti *Brucella* serum.

Aher (2010) achieved isolation rate of 4.08% from vaginal swabs of aborted animals, whereas Madale (2011) reported isolation rate of 7.86% from various clinical specimens collected from animals.

The isolation rate of *Brucella* spp. reported by above authors lies between wide range of 4.08 to 100%, since number of factors are likely to influence the successful isolation of *Brucella* spp. from clinical specimens, especially the availability of appropriate material at the appropriate stage of the disease (Radostits *et al.* 2008) and growth requirements. The isolation rate (24.59%) of the present study laid in the range reported by above authors.

In the present study, all 15 *Brucella* spp. isolates recovered from animals were identified as *B. abortus* based on criteria of conventional methods (OIE 2009). The similar criteria were reported to be useful for conventional identification of *Brucella* spp. by Alton *et al.* (1988), Musa *et al.* (2000), Rathore *et al.* (2002), Kanani (2007), Bayatti and Thwani (2009) and Tuba *et al.* (2012).

Molecular identification of *Brucella* spp. by PCR: A number of PCR assays have been developed by targeting 31 kDa (*bcs*p31) outer membrane proteins that was useful in genus-level identification of *Brucella* (Baily *et al.* 1992, Guarino *et al.* 2000), whereas IS711 PCR assays developed by Bricker and Halling (1994) targeting insertion sequence IS711 had a potential for species-specific identification of *B. abortus* and *B. melitensis*. Considering the advantages offered by the molecular techniques in identification of *Brucella* organisms as reported by above authors, two PCR assays i.e. *Brucella* genus specific BSCP31 and *Brucella* species specific IS711 (IS711/AB- *abortus* specific, IS711/BM *melitensis* specific) PCR assays were carried out for identification and confirmation of *Brucella* animal isolates recovered in present study.

Genus specific BCSP 31 PCR assay: A total of 15 field isolates recovered and identified as *Brucella* spp. by conventional methods were further subjected to BCSP31 gene PCR assay for confirmation *Brucella* genus. Using genus-specific primer pair BCSP31 B4/B5 an amplification

product of 223 bp (Fig. 4) was found in all the 15 field isolates including 3 reference strains thereby confirming their identity as the members of genus *Brucella*. The results of BCSP31 PCR and those of conventional procedures thus were concordant with each other.

Casanas *et al.* (2001) evaluated the efficacy of 31 kDa immunogenic protein (*bcs*p31) PCR assay on *Brucella* strains, generating 223 bp amplification product concluding this the technique as specific and useful in identification of *Brucella* organisms. Kanani (2007) recovered 8 isolates from semen samples and identified them on the basis of cultural, morphological and biochemical characteristics and further confirmation was carried out by BCSP31 PCR assay. He suggested that B4/B5 primer pair is more suitable and sensitive for detection of *Brucella* at genus level. Similarly, Mukherjee *et al.* (2007) evaluated efficacy of 3 PCR assays, viz. BSCP31 kDa protein, *omp*2 and the 16S rRNA genes in the diagnosis of brucellosis. Result of the 3 PCR assays on 6 reference strains of *Brucella* were observed in complete relation. Moreover, Aher (2010) and Suryawanshi (2011) employed BCSP31 PCR assay on *Brucella* spp. isolates and all isolates tested showed specific amplification product of 223 bp confirming their identity as members of genus *Brucella*.

Findings of our study are in accordance with the above authors who employed BCSP31 PCR assay and found it to be specific and efficacious in detecting *Brucella* spp. without giving any false positive or negative amplification product.

Species specific IS 711 PCR assay: In IS 711/AB PCR, all 15 *Brucella* spp. isolates and reference strains *B. abortus* 544 as well as *B. abortus* S19 exhibited *B. abortus* specific amplification product of 498 bp, except *B. melitensis* Rev1 (Fig. 5). Whereas, in IS711/BM PCR, none of the field isolates of *Brucella* spp. generated *B. melitensis*-specific amplification product of 731 bp. Reference strain *B. abortus* 544 as well as *B. abortus* S19 were also found negative for

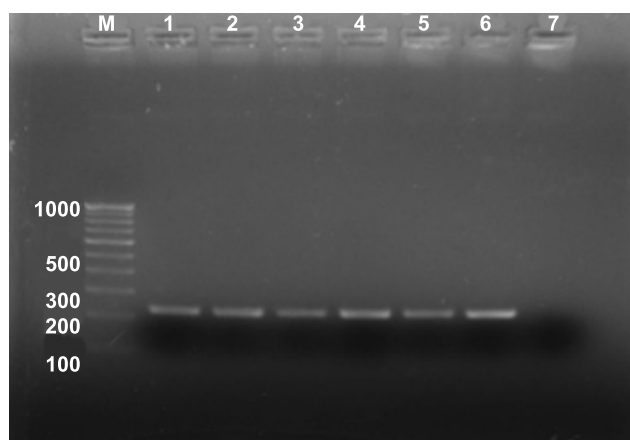


Fig. 4. BCSP31 *Brucella* genus specific PCR assay. Lane M: 100 bp DNA ladder; 1, *B. abortus* 544; lane 2–6, positive samples; 7, negative control.

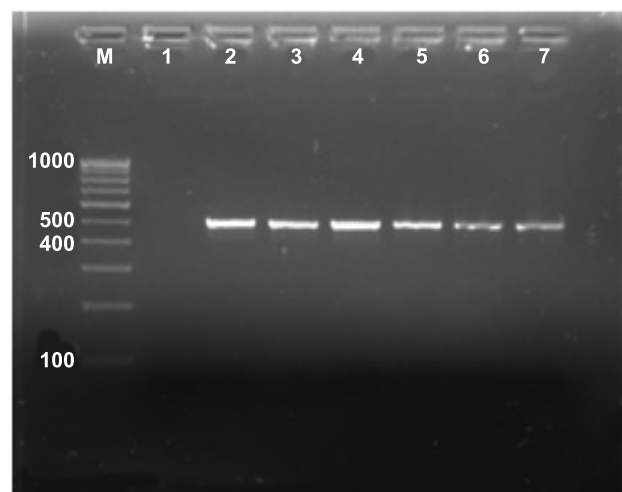


Fig. 5. IS711/AB PCR assay for detection of *B. abortus*. Lane M: 100 bp DNA ladder; 1, negative control; 2, *B. abortus* 544; 3–7, positive samples.

B. melitensis-specific IS 711 PCR, however *B. melitensis* Rev1 yielded *B. melitensis*-specific PCR amplification product of 731 bp. Thus, above 2 species specific primers discriminated the isolates of *B. abortus* and *B. melitensis* spp.

Bricker and Halling (1994) while evaluating the efficacy of *B. abortus*-specific IS711 PCR assay observed that the amplification product of 498 bp was generated in *B. abortus* spp. while no amplification was seen with *B. melitensis* and 731 bp amplification product was generated in *B. melitensis* specific IS711 PCR assay only with *B. melitensis* and no amplification with *B. abortus*. Londhe (2009), Aher (2010), Suryawanshi (2011) and Bhoigade (2012) employed *B. abortus* specific IS711 PCR for confirming the identity of *Brucella* isolates recovered from different clinical specimens using *B. abortus* specific primer pair IS711/AB. All *Brucella* field isolates showed an amplification product of 498 bp confirming their identity as *B. abortus*. The above workers found that the IS711 PCR assay was sensitive and specific in confirming the identity of *Brucella* isolates up to their species level.

The result of molecular detection by PCR in the present study were found in concordance with those of Londhe (2009), Aher (2010), Suryawanshi (2011) and Bhoigade (2012), who employed IS711/AB and IS711/BM species specific PCR assays for identification of species of Genus *Brucella* and found to be efficacious.

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