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Molecular characterization of *Brucella* species detected from clinical samples of cattle and buffaloes

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ABSTRACT

The present study was undertaken for molecular characterization of *Brucella* species of cattle and buffaloes. Clinical samples (1145) of unvaccinated cattle and buffaloes (200 blood samples, 710 sera, 190 vaginal swabs, 20 abomasal contents of foetus, 25 foetal tissues) and 146 blood samples of vaccinated animals were collected from dairy farms in and around Mumbai and Pune region. These samples were processed for isolation of *Brucella* organisms and further characterized by PCR and sequencing. A total of 26 (11.06%) *Brucella* isolates were recovered from 235 samples. Also, 5 isolates received from human cases were included in the study. BCSP 31 PCR showed an amplicon of 223 bp in all 31 isolates, 123 (61.5%) blood samples, 123 (64.73%) vaginal swabs and 27 (60%) aborted foetal material. IS711/AB and BM PCR showed an amplicon of 498 bp and 731 bp in 17 and 14 isolates, 42 (21%) and 38 (19%) blood samples, 43 (22.63%) and 34 (17.89%) vaginal swabs, while 7(15.55%) and 6 (13.33%) aborted foetal material, respectively. The phylogenetic analysis detected the ancestral origin of the organism. Rapid and correct diagnosis of brucellosis and vaccination is important to eradicate the disease. The molecular methods used in the present study speed up the diagnosis of the disease.

Keywords: *Brucella abortus*, *B. abortus* 544, BCSP 31, IS711 PCR assays

Brucellosis is a vital disease of cattle and buffaloes that causes infertility in male and female animals. The disease causes severe economic losses to the livestock industry by reducing the productive and reproductive potential of the affected animals. The disease remains neglected and results in major health, economic and livelihood burden due to inefficient control methods (Deka *et al.* 2018). It is a zoonotic infection, so humans may get the disease condition by contacting infected animals. Abortions are generally seen in cattle and buffaloes in the last trimester of pregnancy due to brucellosis. Infections in female animals may also cause stillborn or weak calves, retained placentas and reduced milk yield. In male animals, seminal vesicles, ampullae, testicles, and epididymides may be infected, and occasionally, the bacteria localizes in the joints causing arthritis (Shakuntala *et al*. 2021).

Brucellosis is often diagnosed by serology and culture. The detection of microbes from the tissue samples, milk or vaginal exudates followed by bacteriological characterization is considered as the 'gold standard' (Surucuoglu *et al*. 2009). But these approaches demand a high level of expertise to isolate the organism; they are time-consuming and involve the risk of laboratory-acquired infection (Shome *et al*. 2019). The serological diagnosis of brucellosis is relatively simple, inexpensive and widely used to diagnose in animals and human. However, the major limitation of this procedure is its doubtful specificity.

The nucleic acid-based detection methods developed in recent times are promising tools for diagnosing brucellosis. Amplification of DNA by PCR is currently used to diagnose several infectious diseases caused by fastidious or slowly growing bacteria. These techniques are sensitive, specific, quick and inexpensive. Additionally, the methods do not require the handling of living organisms, thereby reducing the safety concerns. Several targets have been explored to find out their suitability in identification and typing. Some of the targets that have been evaluated extensively include 16S rRNA, insertion sequence IS*711* and *Brucella* Cell Surface Protein (Mittal *et al*. 2018). Considering all these aspects, the present study was planned for molecular characterization of *Brucella* spp. from clinical samples of cattle and buffaloes.

MATERIALS AND METHODS

Permission was taken from Institutional Biosafety Committee for conducting research as per letter ref No.BVC/Dean/VPH/IBSC/221/2016 dated 02/07/2016.

Reference strains: Reference strains of *Brucella*, i.e. *Brucella abortus* 544, *Brucella melitensis* Rev 1 and *Brucella abortus* S-19 were purchased from Division of

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Biological Standardization, IVRI, Izatnagar, Uttar Pradesh Along with that, two isolates received from J.J. Hospital Mumbai and three isolates received from Bijapur Lingayat, District Educational Association (BLDE) University, Bijapur are also included in the current study.

Clinical specimens: A total of 1145 clinical samples derived from unvaccinated cattle and buffaloes including (200) blood samples, (710) sera, (190) vaginal swabs and (20) abomasal contents of foetus and (25) foetal tissues were collected in Tryptose broth from dairy farms in and around Mumbai and Pune. Apart from the clinical samples from unvaccinated animals indicated above, a total of 146 blood samples were collected from vaccinated animals from Cattle Breeding Farm, Bombay Gowrakshak Mandali, Betegaon Farm Bhoisar (95), Rahimatpur Cattle Farm, Satara (28) and Bhagyalakshmi Farm, Manchar, Pune (23).

Processing of specimens by conventional and Molecular methods: The sera samples were screened by Rose Bengal Plate Test. The vaginal swabs, abomasal content and foetal tissues were processed for isolation of *Brucella* by conventional methods. The isolates suspected of *Brucella* were subjected to biochemical tests for identification (OIE 2009). The isolates recovered were further subjected to PCR. The blood samples, vaginal swabs, abomasal content and foetal tissue samples were also used for direct DNA extraction and further characterized by PCR assays. The extraction of genomic DNA of *B. abortus* from the blood samples, bacterial cultures, *Brucella* reference strains and foetal tissue, abomasal contents was carried out by Leal-Klevezas *et al*. (1995), Romero *et al.* (1995) and O'Leary *et al.* (2006) and Leal-Klevezas *et al.* (1995) with slight modifications, respectively.

BCSP 31 and IS711 PCR assays: The BCSP 31, IS711 PCR was carried out on isolates and clinical samples to detect *Brucella* at genus and species level respectively. The oligos were manufactured and supplied by M/s Bangalore Genei, Bengaluru (India). The details of primers are given in Table 1.

Table 1. Oligonucleotide primers for Molecular Characterization

PCR Primer sequence	Reference
B4- (5' –TGG-CTC-GGT-TGC-CAA-TATCAA'3)	Baily *et al.* 1992
B5- (5'- CGC-GCT-TGC-CTT-TCA-GGT-CTG-'3)	
IS711 (5' -TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT- 3')	Bricker and Halling (1994)
AB (5' -GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC -3')	
IS711 (5' -TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT- 3')	Bricker and Halling (1994)
BM (5'– AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA-3')	

The PCR was set in a final volume of 25 µl consisting of nuclease free water (NFW)16.8 µl, 10× PCR buffer 2.5 µl, $MgCl_2$ (25 mM) 2 µl, dNTPs mix (10 mM) 0.5 µl, Primers B4-F/IS711-F (10 pM/ µl) 1 µl and Primer B5-R/ Primer AB/ BM-R (10 pM/ µl) 1 µl, Template DNA1 µl (200 ng) and *Taq* DNA polymerase (5 U /µl) 0.2 µl.

The PCR reaction was conducted in a thermal cycler (Master Cycler, Eppendorf) with initial denaturation at 94°C for 3 min, followed by 35 cycles [denaturation at 94°C for 1 min, annealing at 62°C (BCSP31 primers)/60°C (IS711/AB/BM primers) for 45 sec and 72ºC for 1 min]. The final extension was carried out at 72ºC for 5 min.

The amplified BCSP31 and IS711/AB and BM PCR products were visualized in ethidium bromide-stained 1.5% agarose gel under Automatic Computerized Gel Documentation and Analysis System (Gel Doc EZ Imager, Bio-Rad).

PCR products of BCSP 31 region of five *Brucella* Isolates including reference strain S19 and PCR amplicons of IS711/AB and BM region each of two *Brucella* isolates were subjected to sequencing. Sequences retrieved from ABI files were subjected for correct annotation using chromas lite software. The nucleotide sequences of BCSP31 were compared with that of other species of *Brucella* available in the GenBank. The multiple sequence alignment of field isolates and reference strain sequences was done using BioEdit (version 7.1.9) software. Phylogenetic tree was constructed using MEGA (7.0) software by using Neighbor-Joining method.

RESULTS AND DISCUSSION

Serological detection of Brucellosis: A total of 710 bovine sera samples were processed for the detection of *Brucella* antibodies using RBPT. 200 samples were found positive by RBPT with an overall serological prevalence of 28.16%. The data generated suggests that the serological prevalence of brucellosis was higher in the Pune (37.5%) region than in Mumbai, i.e. 26.42%. The present study's findings of seroprevalence of brucellosis corroborate with those of Aher (2010), who recorded a higher prevalence of brucellosis in Pune than Mumbai region.

Isolation: A total of 26 (11.06%) *Brucella* isolates were recovered from 235 clinical samples. All the isolates exhibited morphology and staining characteristics typical of *Brucella* spp., i.e. they were Gram- negative coccobacilli,

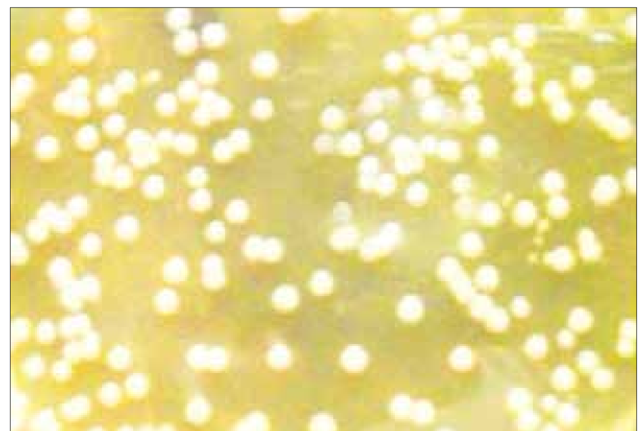

Fig. 1. Characteristic colonies of *Brucella* spp. on brucella agar media.

showed acid-fastness in MZN staining and appeared red coloured. The colonies of organisms isolated on BAM seemed to be round, glistening and smooth (Fig. 1); those on MacConkey agar were non-lactose fermenting, and the colonies were non-haemolytic on blood agar. The isolates recovered were further confirmed as members of *Brucella* spp. employing different biochemical tests. All isolates produced oxidase, catalase, urease, reduced nitrates, while none produced indole.

From 235 clinical samples processed, 26 isolates were recovered from clinical samples. The highest proportions of (30%) isolates were recovered from abomasal contents of aborted foetuses, followed by 16% isolates from foetal tissues. The isolation rate was relatively low (8.42%) from vaginal swabs (Table 2).

Table 2. Isolation of *Brucella* from clinical specimens

Clinical sample	Number of samples processed	Number of isolates recovered	Per cent positivity
Vaginal swab	190	16	8.42
Abomasal content	20	6	30.0
Foetal tissues	25	4	16
Total	235	26	11.06

Several workers in India have made attempts towards isolation of *Brucella* spp. with varying rates of isolation. Jeyaprakash *et al.* (1999) recorded isolation of *B. abortus* in 15% cases from vaginal swabs employing tryptose agar while Das *et al.* (1990) using *Brucella* selective medium recorded an isolation rate of 38.18% in cows and 14.28% in buffaloes. Isolation rates are much higher during the first two weeks of symptoms (80-90% in acute form and 30-70% in chronic form) (Al Dahouk *et al.* 2003). A relatively low isolation rate (11.06%) recorded in the present study agrees with the previous report of 6.4% (Kala *et al.* 2018). Slow growing and fastidious nature of the pathogen could be another explanation for a relatively lower isolation rate (Seleem *et al*. 2010).

Molecular characterization of Brucella *spp. by PCR assays BCSP 31 PCR assay*: All the 31 isolates and the reference strains *B. abortus* 544, *B. abortus* S19 and *B. melitensis* showed BCSP31 gene-specific amplicon of 223 bp, confirming their identity as members of genus *Brucella* (Fig. 2).

An amplicon of 223 bp was observed in 123 (61.5%) blood samples. Out of 146 samples from the Mumbai region, 94 were positive (64.38%), whereas of the 54 samples examined from the Pune region, 29 were positive (53.70%). An amplicon of 223 bp was observed in 123 (64.73%) vaginal swabs. Of 157 vaginal swabs from the Mumbai region, 100 were found positive, while 23 of 33 from the Pune region were positive. The per cent positivity of Brucella infection was found to be 63.69% and 69.69% in Mumbai and Pune regions, respectively. Abomasal contents of aborted fetuses and aborted foetal tissues (45) were processed for direct detection of *Brucella abortus* by

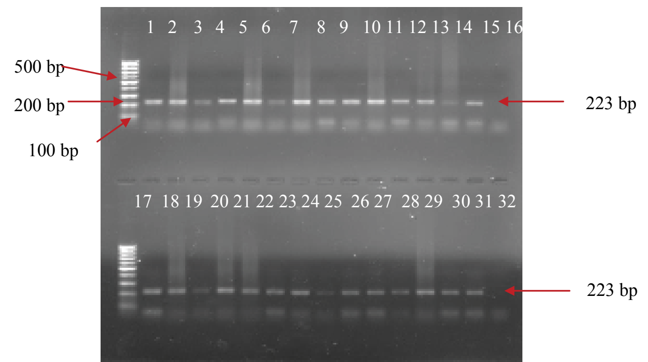

Fig. 2. Identification of field isolates of *Brucella* spp. by BCSP 31 (B4/B5) PCR. Lane 1 and 17: 100bp Ladder; 2 to 15: Isolates 1-14, 16 and 32: Negative Control; 18 to 31: Isolates 15- 28.

BCSP 31 PCR. 27(60%) were found positive, including 21 from Mumbai and six from the Pune region

The results of the efficacy of BCSP31 PCR and size of amplicons generated in detecting genus *Brucella* are in agreement with the findings of Mukherjee *et al.* (2007) and Tyasningsih *et al*. (2015). Londhe (2013), Suryawanshi (2017) and Kaur *et al.* (2018) found similar efficacy of the BCSP31 B4/B5 primers in detecting *Brucella* organism at the genus level.

IS 711 PCR assays: In IS711/AB PCR, *B. abortus* 544, *B. abortus* S19 and 17 clinical isolates generated a product of 498 bp, confirming the isolates as *B. abortus*. While in IS711/BM PCR, *B. melitensis* Rev1 and 14 clinical isolates produced amplicons of 731 bp showing isolates as *B. melitensis.*

B. abortus-specific and *B. melitensis*-specific amplicons of 498 bp and 731 bp were observed in 42 (21%) and 38 (19%) blood respectively (Fig. 3). Out of 146 samples from the Mumbai region, 35 (23.97%) and 25 (17.12%) were positive while 7 (12.96%) and 13 (24.07%) out of 54

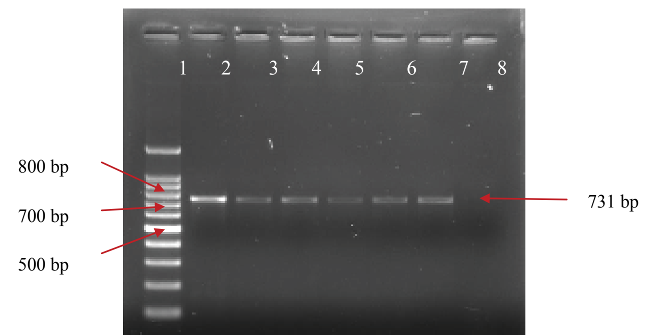

Fig. 3. Identification of *Brucella melitensis* in blood samples by IS 711 /BM PCR assay. Lane 1: 100bp Ladder; 2: Rev1; 3 to 5: Blood samples; 6 and 7: Vaginal swabs; 8: Negative control.

samples from the Pune region turned out to be positive for IS711/AB and IS711/BM PCR respectively. The per cent positivity of *Brucella melitensis* infection was found to be higher in the Pune region than Mumbai region

Amplicons of 498 bp and 731 bp were observed in 43 (22.63%) and 34 (17.89%) vaginal swabs respectively. Out of 157 vaginal swabs examined from the Mumbai region, 34 and 29 were found positive while, 9 and 5 out of 33 from

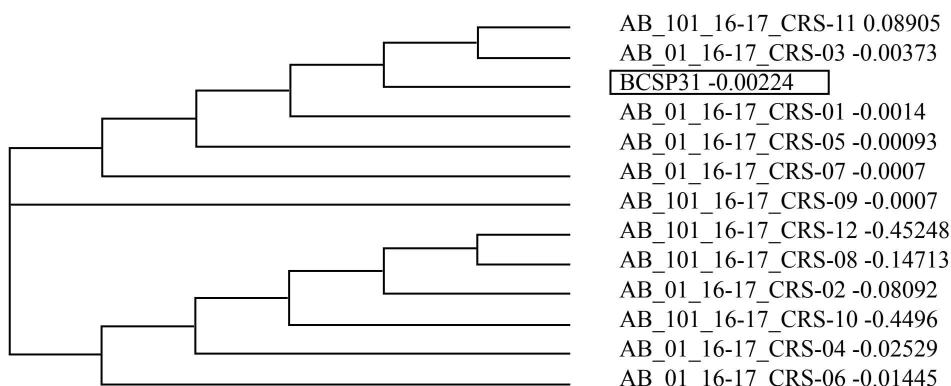

Fig. 4. Phylogenetic tree of *Brucella* isolates and reference strains based on BCSP 31 sequences.

the Pune region turned out to be positive by IS711 PCR. Out of the 45 abomasal contents and aborted foetal tissues examined 7(15.55% , i.e five from Mumbai and two from the Pune region) and 6 (13.33% i.e. 5 from Mumbai and one from the Pune region) were found positive by by IS711/AB (Supplementary Fig. 1) and BM PCR, respectively.

The results of IS711 AB and IS711BM PCR assays recorded in the present investigation agree with the findings of Awwad *et al. (*2015), Ilhan et al. (2007) and Aher (2010). They employed IS711/AB and IS711/BM PCR assays to identify Brucella abortus and melitensis and found it to be effective.

Molecular characterization of Brucella *species by nucleotide sequencing*

BCSP31 (223 bp): Five isolates and *B. abortus* reference strain S-19 were subjected to sequencing of 223 bp BCSP31 amplicon of BCSP31 gene. It was observed that amplicons of the BCSP 31 gene of the five isolates of *B. abortus* and reference strain *B. abortus* S19 had a homology ranging between 96 to 100% with the sequences of *Brucella* spp. available in NCBI data. The results further showed that the BCSP 31 gene nucleotide sequence of *Brucella* isolates recovered during the present study, and isolates from foreign countries had a high level of homology. The nucleotide sequences of BCSP 31 gene of five field isolates and one vaccine strain were deposited in GenBank under the accession numbers MH045842, MH045843, MH045844, MH045845, MH045846 and MH045841, respectively.

Phylogram constructed based on the nucleotide sequences of the isolates and BCSP31 nucleotide sequence of all the *Brucella* strains available in the GenBank showed that CRS_11, CRS_03 and BCSP31 formed a separate clad (Fig. 4). These sequences are closely related to each other. BCSP 31 nucleotide sequences are highly conserved in genus *Brucella* and can be used to identify the suspected *Brucella* isolates as *Brucella* or not, without any ambiguity.

IS711/AB (498 bp) and IS711/BM (731 bp): The PCR amplicons of IS711/AB and BM region of *Brucella* isolates

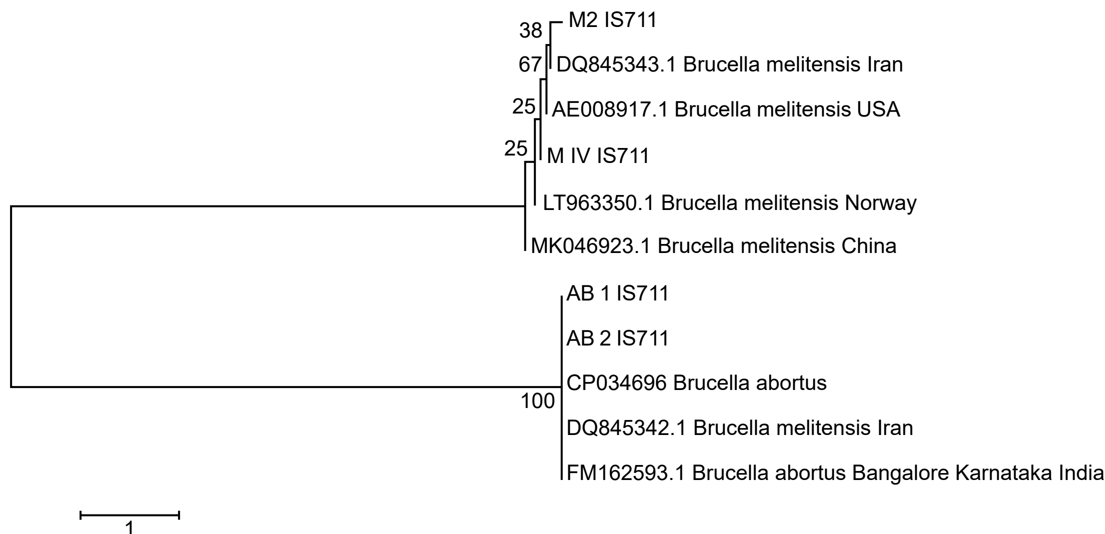

Fig. 5. Phylogenetic tree of *Brucella* isolates and reference strains based on IS711/AB and IS711/BM sequences.

were sequenced. BLAST results showed that isolate 1 of the present study was found to be 99% identical with *Brucella abortus*, Isolate 2 revealed 100% identity with *Brucella abortus*, Isolate 3 showed 99% homology with *Brucella melitensis*, whereas Isolate 4 showed 100% homology with *Brucella melitensis* (Supplementary Table 1).

Phylogenetic analysis was done and results indicated that *Brucella abortus* and *B. melitensis* showed clear differentiation at 100 bootstrap values. Both isolates belonging to *B. abortus*, i.e. AB1 IS711 and AB2 IS711, showed 100% similarity with isolates from Iran and India. *B. melitensis* isolate M2 IS711, isolate from Iran and M IV IS711 was associated with an isolate from the USA (Fig. 5). Similar phylogenetic analysis findings have been reported by Barua *et al.* 2016, Ahmed *et al.* 2017 and Thenamutha *et al.* 2017.

The overall serological prevalence of brucellosis in animals at studied locations was found to be 28.16%. Twenty six isolates of *Brucella* spp. were recovered from 235 clinical specimens with an isolation rate of 11.06%. Highest proportions of isolates were recovered from abomasal contents of aborted fetuses followed by foetal tissues. The isolation rate was relatively low from vaginal swabs. PCR assays proved useful in genus and species level identification of *Brucella* organisms and also found effective in direct detection of *Brucella* spp. from whole blood samples, vaginal swabs and aborted foetal tissues of animals. Sequence analysis of targeted genes followed in this study could be a practical approach for molecular characterization of *Brucella* isolates from animals.

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