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Research Article

Molecular detection of *Brucella abortus* detected from bovine clinical samples

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Abstract

The present investigation was undertaken to detect brucellosis in cattle using serological, bacteriological and molecular techniques. A total of 50 samples including milk (24), placenta (2), blood (22) and vaginal discharge (2) from cattle with a history of abortion were collected from farms located in and around Vasai region. The specimens were processed for detection of brucellosis by different methods: RBPT, MRT, bacteriology (cultural isolation and identification) and molecular techniques (BCSP 31 PCR, IS711/AB PCR assays). Out of 24 milk samples tested by MRT, 2 samples were found positive for the presence of *Brucella* antibodies with an overall prevalence of 8.33%. Whereas out of 22 sera samples of animals examined by RBPT, 5 (22.72%) were positive for the presence of *Brucella* antibodies. Three *Brucella* spp. isolates were recovered from 28 samples with a 10.71% isolation rate. Antibiotic sensitivity testing showed that all *B. abortus* isolates (100%) are sensitive to cefotaxime, doxycycline and gentamicin, and 66.66% of *B. abortus* isolates were resistant to erythromycin and streptomycin. The BCSP31 and IS711/AB PCR assay were carried out to confirm the identity of isolates recovered from clinical cases. The BCSP31 PCR showed an amplicon of 223 bp in all 03 isolates and reference strain *B. abortus* 544. The IS711/AB PCR analysis of the reference strain and all the isolates revealed a species amplicon of 498 bp.

Keywords: BCSP 31, *Brucella abortus*, IS711 PCR assays, MRT

Highlights

- The overall prevalence of brucellosis in animals at studied locations was found to be 8.33% and 22.72% by MRT and RBPT, respectively.
- Three isolates of *Brucella abortus* were recovered from 28 clinical specimens with an isolation rate of 10.71%.
- BCSP 31 PCR assay proved useful in genus level identification of clinical isolates of *Brucell*a spp. generating an amplification product of 223 bp in all the clinical isolates and reference strain.
- IS711/AB PCR assay was found to be effective in identifying *Brucella* isolates upto species level, i.e. *Brucella abortus*.
- Antibiotic sensitivity testing of *brucella abortus* showed that cefotaxime, doxycycline and gentamicin were the most effective antibiotics, while erythromycin and streptomycin were the least effective antibiotics.

INTRODUCTION

Brucellosis is an endemic infectious disease that is seen in animals and humans all over the world. It not only severely harms human health but also greatly influences the development of animal husbandry. It is an extremely widespread distributed zoonosis, which is also accountable for substantial losses (Probert *et al.*, 2004).

The mode of transmission of *Brucella* can be direct or indirect via horizontal or vertical routes in bovines. Potential sources of infection are aborted fetuses, placental membranes and uterine discharges. A high concentration of organisms is seen in the uterus of pregnant animals. Milk, urine, and semen of infected bulls are other sources of infection. Veterinarians, laboratory personnel, animal handlers, slaughterhouse staff, farmers etc. are under high-risk groups.

Samples for *Brucella* spp. isolation from cattle include fetal membranes, particularly the placental cotyledons, where the number of organisms tends to be very high. In addition, fetal organs such as the lungs, bronchial lymph nodes, spleen and liver, fetal gastric contents, milk, vaginal secretions and semen are samples of choice for isolation (Poester *et al.*, 2006). Milk samples should be a pool from all four mammary glands. Non-pasteurized dairy products can also be sampled for isolation (Lage *et al.*, 2008; Poester *et al.*, 2010).

The most reliable method for diagnosing brucellosis is the isolation of *Brucella* spp. (Alton *et al.*, 1988). Bacterial culture is the most preferable and gold standard

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method (Lucero *et al*., 2008; Selim *et al.,* 2015). However, culturing bacteria is complex and tedious, and poses a potential hazard for laboratories. The serological diagnosis of brucellosis is relatively simple and inexpensive, and has been widely employed to diagnose brucellosis in animals and humans. The nucleic acid-based detection methods developed in recent times are up-and-coming tools for diagnosing brucellosis. The techniques do not require handling living organisms, reducing safety concerns. Several targets have been explored to determine their suitability in identification and typing. Some of the targets that have been evaluated extensively include BCSP 31, omp-outer membrane proteins (Baily *et al*., 1992; Leal Klevezas *et al*., 1995; Rossetti *et al*., 1996), DnaJ (Da Costa *et al*., 1996), insertion sequence IS711 (Londhe *et al*., 2010) etc. Considering the above facts, the present study was undertaken for the detection of brucellosis by isolation of *Brucella abortus*, antibiotic sensitivity testing of isolates, serological diagnosis by MRT and RBPT, molecular diagnosis by BCSP 31 PCR and IS711/AB PCR assays.

MATERIALS AND METHODS

Ethical permission: Permission was taken from the Institutional Biosafety Committee for conducting research as per letter ref no. MVC/IAEC/11/2022 dated 07/06/2022

Sample collection: A total of 50 samples including milk (24), placenta (02), blood (22) and vaginal discharge (02) from cattle with a history of abortion collected from farms located in and around Vasai region, Mumbai, Maharashtra were included in the present study.

Procedure of Rose Bengal Plate Test (RBPT): The colored antigen required for the RBPT test was purchased from the Indian Veterinary Research Institute, Division of Biological Products, Izatnagar. The serum samples were processed for the detection of *brucella* antibodies by RBPT as per OIE, 2009.

Milk Ring Test (MRT): The antigen used for Milk Ring Test is *Brucella abortus* antigen (MRT Antigen) which was provided by IVRI Bareilly. The milk samples were processed for detection of *Brucella* antibodies by MRT as per OIE, 2018.

Isolation of *Brucella* spp.: Placenta and vaginal swabs were processed for isolation of *Brucella* spp. as per Poester *et al*. (2006) and Lage *et al*. (2008). Milk samples were processed for isolation of *Brucella* spp. as per standard bacteriological procedures (OIE, 2018). Brucella agar medium (BAM) plates were aseptically inoculated by smearing pellet and fat over the surface of agar with an inoculating loop. BAM plates were incubated at 37±2°C in air supplemented with 5% CO_2. The cultures were observed regularly for the appearance of growth. The colonies suggestive of *Brucella* spp. were examined for the morphology and staining characters employing Gram's stain and modified Ziehl Neelsen's staining method. The strains showing colony characters suggestive of *Brucella* spp. were processed further for identification of the organism using standard bacteriological procedures (Quinn *et al*., 1994).

Identification: The isolates suspected to be of *Brucella* spp. were subjected further to different identification tests like oxidase test, catalase test, nitrate reduction test, urease test, H_2S production test, growth in presence of dyes and indole test etc. recommended by OIE (2009).

***In-vitro* antimicrobial sensitivity test**: *In-vitro* antimicrobial sensitivity test of *Brucella i*solates was carried out according to Kirby and Bauer (1966) disc diffusion method on *Brucella* agar medium. Antibiotic discs of oxytetracycline, erythromycin, doxycycline, gentamicin, streptomycin, penicillin G and cefotaxime from Hi Media, Mumbai, were used. The antibiotics were graded as susceptible (S), intermediate (I) or resistant (R) based on diameter of zone of inhibition (millimetre) developed around the discs as per Clinical Laboratory Standards Institute (CLSI, 2018).

Identification of *Brucella* spp. by molecular methods

DNA extraction: For extraction of DNA from cultures of *Brucella* spp. and other reference bacterial strains, the protocol described by Romero *et al.* (1995) with modifications was followed.

BCSP 31 PCR assay: A PCR assay targeting BCSP-31 gene was employed for identification of *Brucella* spp. during the present investigation as described by Baily *et al*. (1992). The 223 bp region of BCSP 31 genetic element of *Brucella* spp. was amplified using published primer sequences B4 (5'-TGG-CTC-GGT-TGC-CAA-TAT-CAA- 3') and B5 (5'-CGC-GCT-TGC-CTT-TCA-GGT-CTG-3') manufactured and supplied by M/s Bangalore Genei, Bangalore (India). The PCR was set in a final volume of 25 µL consisting of 16.8 µL sterile water, 2.50 µL 10X PCR buffer, 0.50 µL dNTPs mix (10 mM), 2.0 µL $MgCl_2$ (25 mM), 1 µL Primer B4- F (10 pM/µL), 1 µL Primer B5- R (10 pM/µL), 1.00 µL (200 ng) Template DNA, 0.2 µL, Taq DNA polymerase (5 U/µL). The reaction mixtures prepared as above were subjected to cyclic conditions of initial denaturation at 94°C for 3

min; 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min.

IS711/AB PCR assay: The amplification of 498 bp region of IS711 genetic element of *Brucella abortus* was carried out using published oligonucleotide primer sequences IS711 (5'-TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-3') and AB (5'-GAC-GAACGG-AAT-TTT-TCC-AAT-CCC-3') as per Bricker and Halling (1994). The oligos were manufactured and supplied by M/s Bangalore Genei, Bangalore (India). The PCR was set in a final volume of 25 µL consisting of 16.8 µL sterile water, 2.50 µL 10X PCR buffer, 0.50 µL dNTPs mix (10 mM), 2.0 µL $MgCl_2$ (25 mM), 1 µL Primer IS711- F (10 pM/µL), 1 µL Primer AB-R (10 pM/µL), 1.00 µL (200 ng) Template DNA and 0.2 µL Taq DNA polymerase (5 U/µL). The reaction mixtures prepared as above were subjected to cyclic conditions of initial denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 3 min, annealing at 60°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 6 min. The amplification products of both BCSP31 PCR and IS711 PCR assays were evaluated by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The products were visualized and documented using Automatic Computerized Gel Documentation and Analysis System (Gel Doc EZ Imager, BioRad).

RESULTS

MRT: A total of 24 milk samples were subjected to Milk Ring Test (MRT) for detection of *Brucella* antibodies. Out of 24 milk samples tested, 2 samples were found positive for the presence of *Brucella* antibodies with an overall serological prevalence of 8.33% (Plate 1).

RBPT: A total of 22 bovine sera samples were processed for detection of *Brucella* antibodies using RBPT. Out of 22 samples, 5 samples were found positive for the presence of *Brucella* antibodies with an overall serological prevalence of 22.72%.

Isolation and Identification: Out of 24 raw milk samples processed, only one *Brucella* spp. isolate was recovered. The milk sample positive for culture, was positive for serological test, i.e., MRT. Out of 2 placenta samples and 2 vaginal swabs, only two *Brucella* spp. isolates could be recovered. Three *Brucella* spp. isolates were recovered with 10.71% (3/28) isolation rate.

All the isolates exhibited morphology and staining characteristics typical of *Brucella* spp. i.e., they were Gram negative coccobacilli, showed acid-fastness in MZN staining (Plate 2) and appeared red coloured. The colonies of organisms isolated on BAM appeared round, glistening and smooth (Plate 3); those on MacConkey agar were non lactose fermenting, and on blood agar, the colonies were non haemolytic. The isolates recovered were further confirmed as members of *Brucella* spp. employing different biochemical tests. All the isolates produced oxidase, catalase, urease, and reduced nitrate, while none produced indole. For the species-level identification of *Brucella* spp., all the isolates were subjected to tests viz. H_2S production, CO_2 requirement, growth on the media containing thionine and basic fuchsin. The results of the tests confirmed that the isolate required CO_2 for growth could produce H_2S and also showed growth on BAM containing basic fuchsin at 1: 50,000 (20 µg/mL) concentration for each. Based on the above results, the *Brucella* isolate was confirmed as *Brucella abortus*.

***In-vitro* antimicrobial sensitivity testing of *Brucella* spp.**
In-vitro antimicrobial sensitivity testing showed that all *B. abortus* isolates (100%) are sensitive to cefotaxime, doxycycline and gentamicin, followed by 66.66% to oxytetracycline and penicillin, 33.33% to erythromycin and streptomycin. Overall, 66.66% of *B. abortus* isolates were resistant to erythromycin and streptomycin, followed by 33.33% to oxytetracycline and penicillin.

Cefotaxime, doxycycline, and gentamicin were found to be the most effective antibiotics against *B. abortus* isolates, whereas erythromycin and streptomycin were the least effective (Plate 4 and Fig. 1).

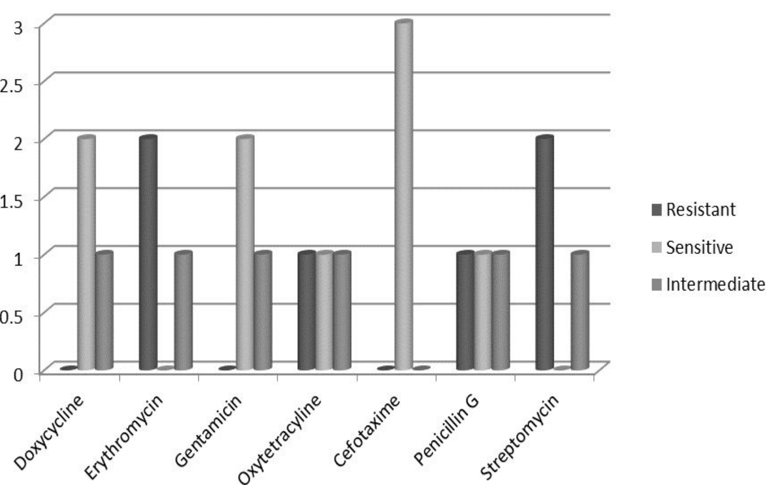


Fig. 1. *In-vitro* antimicrobial sensitivity testing of *Brucella* spp.

Molecular characterization of *Brucella abortus* in Mumbai region

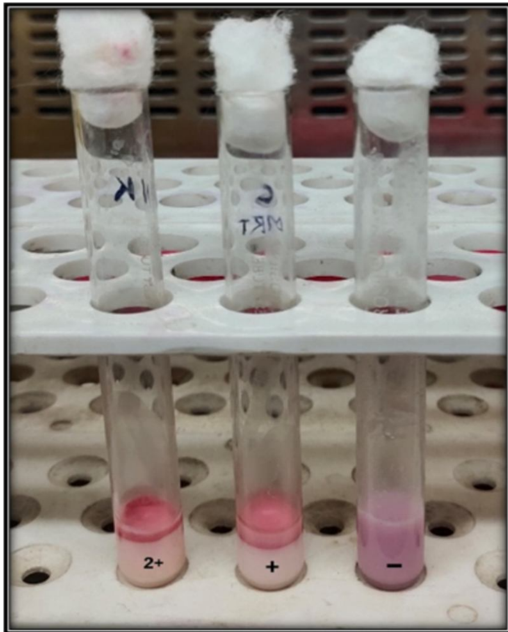


Plate 1. Detection *Brucella* antibodies by MRT

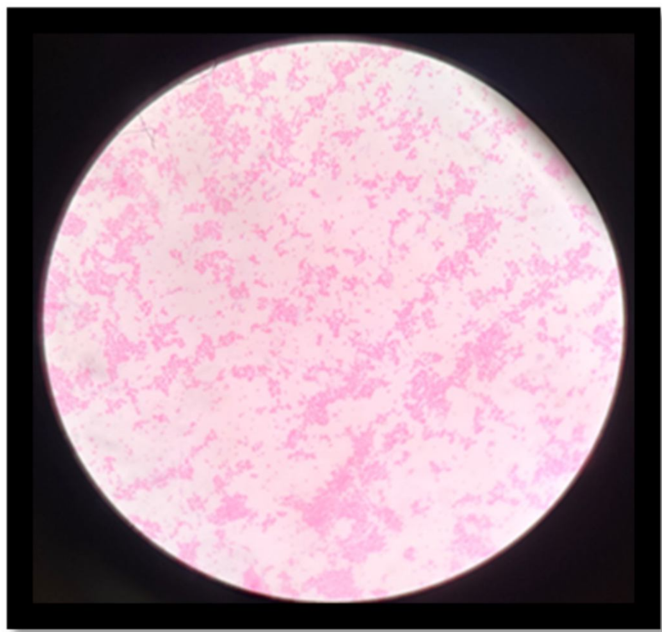


Plate 2. Modified Ziehl Neelsen staining of *Brucella* spp.

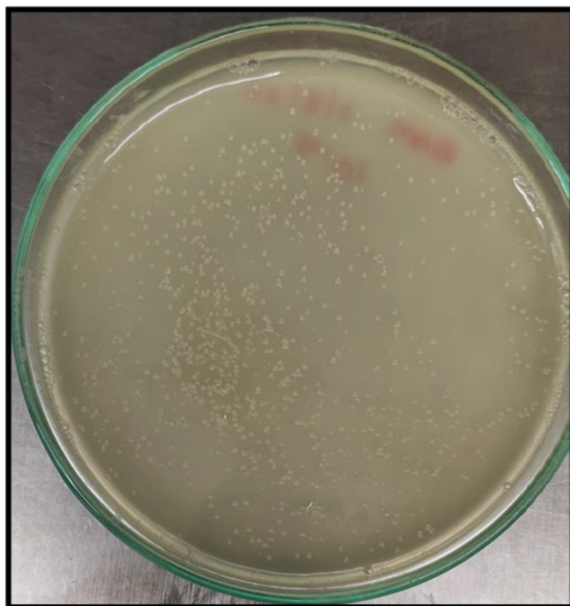


Plate 3. Characteristic colonies of *Brucella* spp. on Brucella agar medium

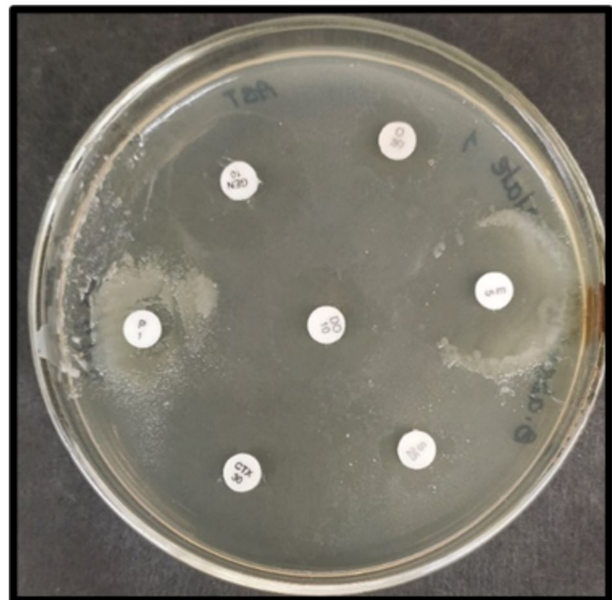
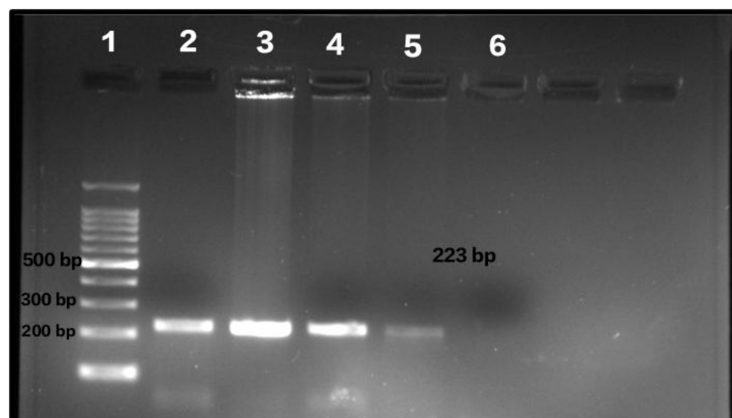


Plate 4. *In vitro* antimicrobial susceptibility test of *Brucella* spp.

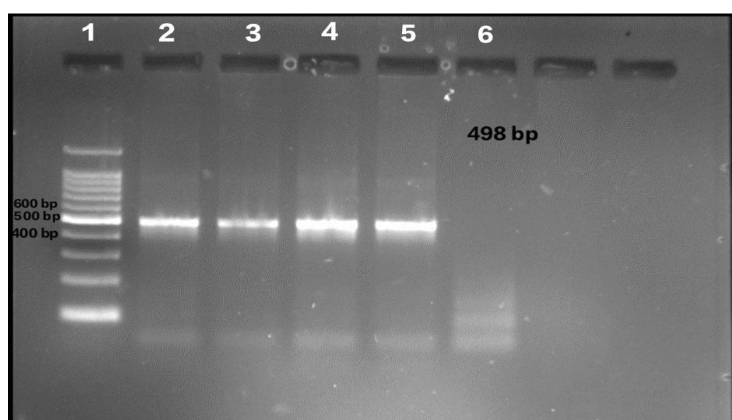


[Lane 1 - Ladder 100 bp, Lane 2 - *Brucella abortus* isolate 1, Lane 3 - *Brucella abortus* 544, Lane 4 - *Brucella abortus* isolate 2, Lane 5 - *Brucella abortus* isolate 3, Lane 6 - Negative control]

Plate 5. Identification of *Brucella* spp. isolates by BCSP31 PCR

BCSP 31 PCR assay: The genus-specific BCSP31 PCR assay using B4/B5 primers was initially carried out for confirming the identity of isolates recovered from bovine cases during the present study. A total of 3 isolates which were identified as *Brucella* spp. by conventional methods were included in PCR assay along with the reference strain *B. abortus* 544 as control. All the 3 isolates and the reference strain *B. abortus* 544 showed BCSP31 gene specific amplification product of 223 bp confirming their identity as members of genus *Brucella* (Plate 5).

IS 711 /AB PCR assay: The PCR assay targeting insertion sequence IS711 was applied on all the isolates in order to confirm their identity at the species level. The reference strain *B. abortus* 544 was included as a control. In IS711/AB PCR, *B. abortus* 544 and 3 clinical isolates generated a product of 498 bp, confirming the identity of clinical isolates as *B. abortus* (Plate 6).



[Lane 1 - Ladder 100 bp, Lane 2 - *Brucella abortus* isolate 1, Lane 3 - *Brucella abortus* isolate 2, Lane 4 - *Brucella abortus* 544, Lane 5 - *Brucella abortus* isolate 3, Lane 6 - Negative control]

Plate 6. Identification of *Brucella abortus* isolates by IS711/AB PCR

DISCUSSION

MRT is used as a screening test for the detection of brucellosis in dairy cows. Several workers in India have studied the prevalence of brucellosis in dairy cows by MRT. In the present study, MRT results showed overall serological prevalence of 8.33% of brucellosis in dairy cows. The findings of the present study concurred with the report of Al-Mashhadany (2019), who collected 210 samples of milk for the detection of brucellosis by MRT and found 8.6% prevalence of brucellosis. Saleha *et al.* (2014) collected 142 milk samples of bovines from different private farms in Pakistan. These samples were processed for detection of brucellosis by MRT and found 8.4% prevalence. The presence of *Brucella abortus* in milk poses a risk to human beings as many rural people consume raw milk. So, rural people need to be aware of the importance of boiling milk before consumption in the household.

RBPT is a cheaper, sensitive, readily available field screening test used for the diagnosis of brucellosis. Several workers studied the seroprevalence of brucellosis by RBPT in bovines. The findings of current studies of percent positive of 22.72% concurred with the analysis of Khan *et al.* (2021), who screened 220 samples, out of which 49 samples were found positive with 22.7% prevalence. Chand and Chhabra (2013) screened 2967 samples, out of which 663 samples were positive with 22.34% prevalence. Tasiame *et al.* (2016) screened 315 samples, out of which 72 samples were found positive with 22.9% prevalence.

Isolation rate of *Brucella abortus* from clinical samples in our study was 10.71%. The highest proportion of (50%) isolates were recovered from vaginal swab and placenta, followed by 4.16% isolates from milk samples. Several workers in India have made an attempt towards isolation of *B. abortus* with varying rates of isolation. The present study agreed with the findings of Jain *et al.* (2013), who processed 84 samples of vaginal mucus (35), foetal stomach content (31), foetal membranes (11) and uterine discharges (7) from aborted cattle (29) and buffaloes (55). Nine (10.7%) were positive through isolation. Whereas Mugizi *et al.* (2015) processed 110 cow milk samples, 11 isolates recovered from milk samples with

percent positive of 10%. Geresu *et al.* (2016) processed 46 seropositive samples, 9 placental cotyledons samples and 23 vaginal swab samples. 3 samples out of 46, one sample out of 9 and 2 samples out of 23 samples were culture positive with positive percentages of 6.52%, 11.1% and 8.69%, respectively.

The present study showed that cefotaxime, doxycycline and gentamicin were found to be the most effective antibiotics, whereas erythromycin and streptomycin were the least effective antibiotics against *B. abortus* isolates. Results of *in vitro* antimicrobial sensitivity testing are in agreement with Schurig *et al.* (1991), who reported the sensitivity of all four *B. abortus* strains towards gentamicin, oxytetracycline, carbenicillin and cephalothin. Abro *et al.* (2017) found that *B. abortus* was found highly sensitive to gentamicin, tobramicin and penicillin, with sensitivity percentages of 75%, 100% and 100%, respectively. Jain *et al.* (2013) revealed that *B. abortus* was sensitive to doxycycline, oxytetracycline, and gentamicin but resistant to penicillin, cephalothin, cotrimoxazole and erythromycin. Dadar *et al.* (2023) found that *B. abortus* was susceptible to doxycycline, gentamicin and ceftriaxone.

BCSP 31 PCR assay proved useful in genus level identification of clinical isolates of *Brucella* spp. generating an amplification product of 223 bp in all the clinical isolates and reference strain. The present study showed all three isolates as *Brucella* spp. by generating 223bp amplicon. Several workers attempted detection of *Brucella* spp. by BCSP 31 PCR assay. Ali *et al.* (2014) detected 30 *Brucella* spp. by using BCSP 31(B4/B5) PCR. Khan *et al.* (2017) detected *Brucella* spp. using BCSP 31 (B4/B5) PCR. Bounaadja *et al.* (2009) evaluated standard assay for the diagnosis of *Brucella* spp. in humans and animals, and found that BCSP 31 PCR was useful for the detection of isolates at the genus level. Kaur *et al.* (2018) obtained a total of four isolates of *Brucella abortus* from 100 clinical samples of foetal stomach contents, vaginal mucus and uterine discharges. These isolates were confirmed as *Brucella* spp. by PCR using B4/B5 primer pair by generating 223 bp amplicon.

IS711/AB PCR assay was found to be effective in the identification of *Brucella* isolates upto species level, i.e., *Brucella abortus.* The present study showed all three isolates as *Brucella abortus* by generating 498 bp amplicon. Several workers attempted detection of *Brucella abortus* isolates by IS711/AB PCR assay. Bounaadja *et al.* (2009) evaluated IS711/AB PCR for the diagnosis of *Brucella abortus* isolates in humans and animals. Suryawanshi *et al.* (2017) evaluated twelve *Brucella* species isolated from animals, which were characterized up to the species level, and 8 isolates were identified as *B. abortus*.

The overall prevalence of brucellosis in animals at studied locations was found to be 8.33% and 22.72% by MRT and RBPT, respectively. Three isolates of *Brucella abortus* were recovered from 28 clinical specimens with an isolation rate of 10.71%. Cefotaxime, doxycycline and gentamicin were found to be effective antibiotics against *B. abortus* isolates recovered from clinical samples collected from farms in and around Vasai region. BCSP 31 PCR assay proved useful in genus level identification of clinical isolates of *Brucella* spp. IS711/AB PCR assay was found to be effective in the identification of *Brucella* isolates upto species level, i.e., *Brucella abortus*. Detection of *Brucella abortus* in milk samples causes a risk to the human population as many rural people consume raw milk. So, it may be suggested that control programs may be implemented to prevent further spread of the disease.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: DDI: Writing, original draft preparation; VDT: Writing, review and editing, conceptualization, data curation; RSG: Project administration, supervision; RRP: Visualization, investigation; PDG: Technical support in research.

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