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REGULAR ARTICLES

Brucellosis in migratory sheep flock from Maharashtra, India

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Abstract Brucellosis is a zoonotic disease worldwide distributed and having the economic as well as public health importance. The prevalence of brucellosis among sheep flock having history of abortions was studied. A total of 229 samples comprising of 157 blood and 72 clinical samples (vaginal swabs) were collected from 157 animals. Clinical samples were processed for the isolation of *Brucella melitensis*. Serum samples (n = 157) were tested by Rose Bengal plate test (RBPT) and i-ELISA. A total of 68 (43.31%) and 104 (66.24%) samples were positive by RBPT and ELISA, respectively. *Brucella* isolates (n = 2) were recovered from clinical samples. Both isolates demonstrated amplification for *bcsp 31* and *IS711* genes. On AMOS PCR, both the isolates amplified at 731 bp, i.e., belongs to *B. melitensis* species. The incidence of *B. melitensis* in a migratory flock warns the thorough testing and culling of *Brucella*-infected sheep from the flock on a continuous basis; otherwise, such incidence will be routine and poor farmers will be at a loss.

Keywords Sheep · *Brucella melitensis* · Seroprevalence · Isolation

Introduction

Brucellosis is a zoonotic disease worldwide distributed and having the economic as well as public health importance. Brucellosis due to *Brucella melitensis* is recorded more in the Mediterranean but also distributed in the Middle East, Central Asia, around the Arabian Gulf, and some countries of Central America. The disease has also been reported from India and African countries. In small ruminants, brucellosis is caused by *B. melitensis* which is further categorized into three biovars (biovars 1, 2, and 3), and all these three biovars are responsible for causing the disease in small ruminants, but varies in their geographical distribution (CDC 2005). Along with the *B. melitensis*, *B. abortus* and *B. suis* are also responsible for causing the disease in small ruminants, but clinical disease seems to be rare (Mantur and Amarnath 2008). Goats and sheep are the natural host for *B. melitensis*. The infection with *B. melitensis* in small ruminants is similar to *B. abortus* in cattle with similar clinical manifestation of abortion, stillbirth, and usually manifested once in the animal's lifetime (Blasco and Molina-Flores 2011; Elzer et al. 2002).

Vertical transmission of *B. melitensis* is documented in lamb and kids, but latent infection occurs mostly by ingestion of infected colostrum or milk (Grillo et al. 1997). Many of the non-pregnant sheep and goats remain asymptomatic. Humans may acquire infection when they come in contact with the infected aborted materials like the placenta, dead fetus through abraded skin; however, the most common route of infection is the consumption of contaminated raw, unpasteurized milk and milk products (Georgiou's et al. 2005; Rahman et al. 2011).

There are several laboratory techniques to diagnose brucellosis which include serology, isolation of bacteria from clinical samples, and its identification by molecular techniques including PCR and qPCR. Among all, the isolation of a *Brucella* organism is the most reliable and undeniable method for diagnosing animal brucellosis (Alton et al. 1988). There are various serological tests for detection of the brucellosis and includes Rose Bengal plate test (RBPT), serum agglutination test (SAT), plate agglutination test, enzyme-linked immunosorbent assay (ELISA), complement fixation test (CFT),

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hemolysis in gel test (HIGT), milk ring test, and modified SAT. But to detect all the stages of infection in live animals, there is no single detection technique which is specific and sensitive (McGiven et al. 2003; Poester et al. 2010). In serological testing, there is a lacuna as sometimes these tests show false positive results because of the cross reactivity of antibodies against *Yersinia enterocolitica* (See et al. 2012).

Though isolation confirms the disease, but it is not always successful as the isolation of *Brucella* is very time consuming and involves the hazard to the person involved in the isolation procedures and handling (Refai 2003). Among other diagnostic tools, PCR has proven to be the potential and better diagnostic technique for several infectious diseases caused by the fastidious or slow growing bacteria (Romero et al. 1995; Bricker 2002). The molecular diagnosis of brucellosis can be performed using genus-specific polymerase chain reaction (PCR) assays (Pathak et al. 2014). Molecular assays targeting the *IS711* insertion element and the *bcsp31* gene, coding for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* spp. are the most common molecular targets in clinical applications (Baily et al. 1992). To identify the species and biotypes in the genus *Brucella*, AMOS PCR is frequently used as it is very reliable to detect various species in single reaction. In the present paper, we report the prevalence of *B. melitensis* in migratory sheep flock with history of abortions. Disease was confirmed by isolation of *B. melitensis* and further characterization by PCR assay.

Materials and methods

Samples

A flock of about 430 sheep (63 M and 367 F) was selected for study which was having history of abortion to the tune of 51.12% (188 abortions out of 367 female) in the flock. Another parallel flock of 277 sheep (25 M and 252 F) with different owners without history of abortion was also investigated. The animals were not vaccinated for brucellosis. A total of 157 animals were randomly selected for the collection of samples. A total of 229 samples comprising of blood (157) and vaginal swabs (72) were collected from migratory sheep flock of Akola district, Maharashtra province of India. From the affected flock blood samples of 10 males, 125 females, and 72 vaginal swabs of affected females were collected. From another healthy flock, blood samples of 1 male and 21 females were collected. The blood samples were collected in clot activator vacutainers. All the samples were transported to laboratory under chilling condition. The clinical samples (vaginal swabs) were processed for isolation of *Brucella*. Serum was separated and stored at − 20 °C till further processing.

Isolation of *Brucella* organisms

The clinical samples, i.e., vaginal swabs, were processed for isolation of *Brucella* as per Alton et al. (1988). Vaginal swabs were inoculated in the *Brucella* broth (211,088, BD, BBL) with modified *Brucella* selective supplement (SR0209E, OXOID) containing polymyxin B as sulfate (2500 IU), bacitracin (12,500 IU), natamycin (25 mg), nalidixic acid (2.5 mg), nystatin (50,000 IU), and vancomycin as HCl (10 mg). The samples were incubated at 37 °C with 5% of CO_2 for 3–5 days for enrichment till the turbidity was observed in the broth. Then the inoculum streaked on *Brucella* agar (211,086, BD, BBL) was supplemented with 5% horse serum (Himedia) and modified *Brucella* selective supplement (Oxoid). The plates were incubated at 37 °C with 5% of CO_2 till colonies appeared on agar plate; approximately, the plates incubated for 30 days. The colonies of *Brucella* were translucent, honey tinged, smooth, round, and about 1–2 mm in diameter.

Identification

The isolates were confirmed by PCR technique. For molecular identification, the genomic DNA of isolates was extracted by QIAamp DNA Mini Kit (QIAGEN) as per manufacturer's instructions. Briefly, cultured bacterial cells (approx. 5×10^6 cells) were centrifuged for 5 min at 300×*g* in a 1.5-ml microcentrifuge tube. The supernatant was removed and discarded, taking care not to disturb the cell pellet. Cell pellet was resuspended in PBS to a final volume of 200 μl. To this, 20 μl QIAGEN Protease was added and incubated at 56 °C for 10 min. Added 200 μl ethanol (96–100%) to the sample, and mixed again by pulse vortexing for 15 s. After mixing, the 1.5-ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid. The mixture was passed through a QIAamp spin column (in a 2-ml collection tube) without wetting the rim, the cap closed, and centrifuged at 6000×*g* (8000 rpm) for 1 min. Then the QIAamp spin column was placed in a clean 2-ml collection tube, and the tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500 μl buffer AW1 was added without wetting the rim. Cap is closed and centrifuged at 6000×*g* (8000 rpm) for 1 min. The QIAamp spin column was placed in a clean 2-ml collection tube, and the collection tube containing the filtrate was discarded. The QIAamp spin column as carefully opened and 500 μl buffer AW2 was added without wetting the rim and centrifuged at full speed (20,000×*g*; 14,000 rpm) for 3 min. Later, the QIAamp spin column was placed in a clean 1.5-ml microcentrifuge tube. A total of 200 μl buffer AE was added to the QIAamp spin column and incubated at room temperature (15–25 °C) for 1 min, and then centrifuged at 6000×*g* (8000 rpm) for 1 min. Purity and concentrations of DNA were evaluated by Nanodrop

Biospectrometer (Eppendorf), and extracted DNA of isolates was stored at – 20 °C till further processing.

The extracted DNA samples were subjected for *Brucella* genus-specific, i.e., *bcsp 31* and *IS711* gene (Baily et al. 1992, Henault et al. 2000), and species-specific AMOS PCR (Bricker and Halling 1994). Details of primers are given in Table 2. For confirmation of genus-specific PCR, duplex PCR for *IS711* and *bcsp 31* genes were standardized and PCR was performed. PCR reaction for genus-specific PCR was carried out in 25 μl volume mixture including 0.5 μl of 10 pmol/μl of each forward and reverse primer, 12.5 μl of 2X GoTaq® Green master mix (Promega), 1.5 μl of DNA template, and 9 μl of nuclease free water. To detect the *bcsp 31* and *IS711* gene, the cycling conditions were initially denatured at 95 °C for 3 mins followed by 35 cycles of denaturation at 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis using 1.5% agarose gel, and the gel was visualized under a UV transilluminator and photographed by Bio-rad GelDoc system. The isolates showing the amplification at 223 and 350 bp were considered as a positive for *bcsp 31* and *IS711* gene, respectively, conforming belonging to genus *Brucella* spp.

After confirmation of genus of *Brucella* spp., the isolates were subjected to AMOS PCR for confirmation of species. The AMOS PCR is a multiplex PCR performed using five different primers having the one common reverse primer, i.e., IS711 with four forward primers for *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis* (Table 1). Four of the species of *Brucella*, i.e., *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis* were amplified at 498, 731, 976, and 285 bp, respectively. The reaction mixture of 25 μl consist of 12.5 μl of 2X GoTaq® Green master mix (Promega), 0.5 μl of each five primers (10 pmole/μl), 1.5 ul of DNA template, and 8.5 μl of nuclease free water. The cycling conditions were initial denaturation at 95 °C for 2 min followed by 30 cycles of denaturation 95 °C for 1.15 min, annealing at 55.5 °C for 2 min, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. The PCR products were separated and analyzed by gel electrophoresis using 1.5% agarose gel, and the gel was visualized under a UV transilluminator and photographed by Bio-rad Gel Doc system.

Seroprevalence of brucellosis

The antibodies against brucellosis in serum were tested by RBPT and indirect ELISA. RBPT was performed by mixing a drop of RBPT reagent with an equal volume of serum, and agglutination was read after 2 to 3 min. Indirect ELISA (to detect IgG antibodies) was performed by using (smooth Lipopolysaccharide) sLPS antigen.

Results

In the present study, two flocks of small ruminants were studied, i.e., with history of abortions and without history of abortion. In the flock with history of abortion, the prevalence was observed of 40.00% (4/10) by both RBPT and i-ELISA in males and 48.00% (60/125), 71.20% (89/125), and 47.20% (59/125) were found positive by RBPT, i-ELISA, and by both RBPT and i-ELISA in females, respectively. Among healthy flock, in males there was no positivity observed while in females 19.04% (4/21), 52.00% (11/21), and 19.04% (4/21) were positive by RBPT, i-ELISA, and by both RBPT and i-ELISA, respectively. Overall out of the 157 serum samples of sheep, overall 68 (43.31%) were found to be positive by RBPT and 104 (66.34%) by indirect ELISA and 67 (42.67%) were positive by both RBPT and indirect ELISA. The details of the result are given in Table 2. The prominent clinical signs recorded in affected flock were abortion in females. In males, no clinical symptoms were observed.

For the confirmation of brucellosis in animals, the isolation of pathogen from aborted materials, fetal tissues, and vaginal swabs of infected animals is the gold standard. In the present investigation, on bacteriological analysis of 72 clinical samples (vaginal swabs), two *Brucella* isolates were obtained. The

Table 1 Details of primers used for genus and species detection of *Brucella* organisms

	Primer sequence	Product size
Primers		
Bcsp-F	TGGCTCGGTTGCCAATATCAA	223 bp
Bcsp-R	CGCGCTTGCCTTTCAGGTCTG	
IS711-F	CTGGCTGATACGCCGGACTTTGAA	350 bp
IS711-R	GGAACGTGTTGGATTGACCTTGAT	
AMOS		
IS711 (R)	TGCCGATCACTTAAGGGCCTTCAT	
B. abortus (F)	GACGAACGGAATTTTTCCAATCCC	498 bp
B. melitensis (F)	AAATCGCGTCCTTGCTGGTCTGA	731 bp
B. ovis (F)	CGGGTTCTGGCACAATCGTCG	976 bp
B. suis (F)	GCGCGGTTTTCTGAAGGTTCAGG	285 bp

Table 2 Prevalence of antibodies against *Brucella* in serum samples of sheep

Species	Health status of animal		RBPT positive (%)	i-ELISA positive (%)	RBPT + ELISA positive (%)
Sheep	Healthy M	1	0	0	0
	Healthy F	21	4 (19.04)	11 (52)	4 (19.04)
	Total	22	4 (18.18)	11 (50)	4 (18.18)
	Suspected M	10	4 (40)	4 (40)	4 (40)
	Suspected F	125	60 (48)	89 (71.2)	59 (47.2)
	Total	135	64 (47.40)	93 (68.88)	63 (46.66)
Grand total		157	68 (43.31)	104 (66.24)	67 (42.67)

isolates were confirmed by using PCR, the isolates amplified at 223 bp for *bcsp* gene and at 350 bp for *IS711* gene (Fig. 1) which confirms the isolates belong to the genus *Brucella*. On performing AMOS PCR, the isolates amplified the PCR product of 731 bp (Fig. 2) which confirms the isolates belong to *B. melitensis* spp.

Discussion

Brucellosis in small ruminants is a major hurdle in the growth of sheep and goat rearing industry worldwide. Also, the situation is not different in a tropical country like India. Brucellosis is a notifiable disease distributed worldwide and responsible for causing the economic losses due to abortions and reduced milk production. Serology is used worldwide for the screening of the brucellosis in flocks. But due to the cross reaction of *B. melitensis* to the other bacteria particularly *Yersinia enterocolitica* O:9, serology may give a false positive result which leads to misdiagnosis of the disease (See et al. 2012).Various researchers carried out the study on seroprevalence of brucellosis worldwide and showed varied seroprevalence rates. The agglutination test for brucellosis like RBPT is known to have high analytical sensitivity (OIE 2000).

In the present investigation, we report abortions by *B. melitensis* in migratory sheep flock on the basis of seropositivity and isolations. The seroprevalence observed in the present study in sheep flock by RBPT, i-ELISA, and both by

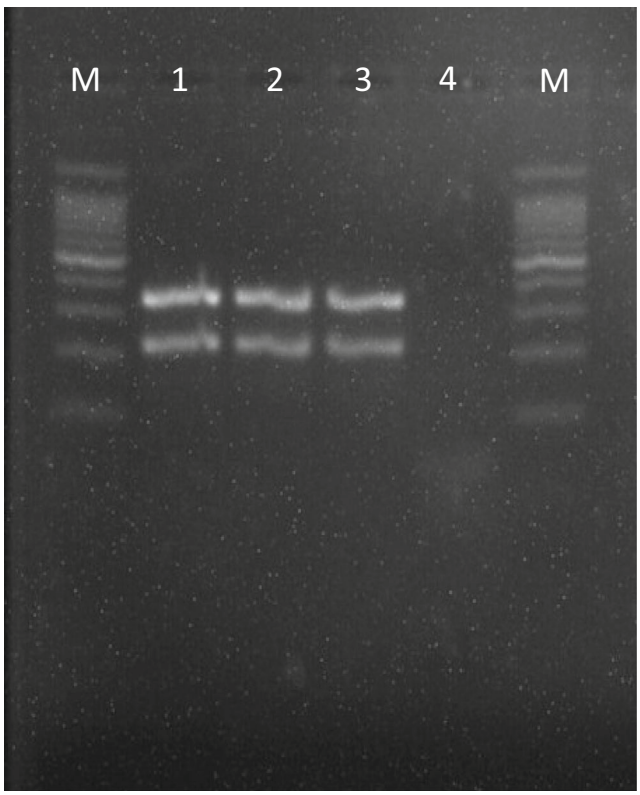

Fig. 1 Genus-specific duplex PCR for *Brucella* genus, *lane M* 100-bp DNA ladder; *lanes 1–2 Brucella* genus DNA samples for bcsp 31 (223 bp) and IS711 (350 bp); *lane 3* positive control for *Brucella* genus; *lane 4* negative control

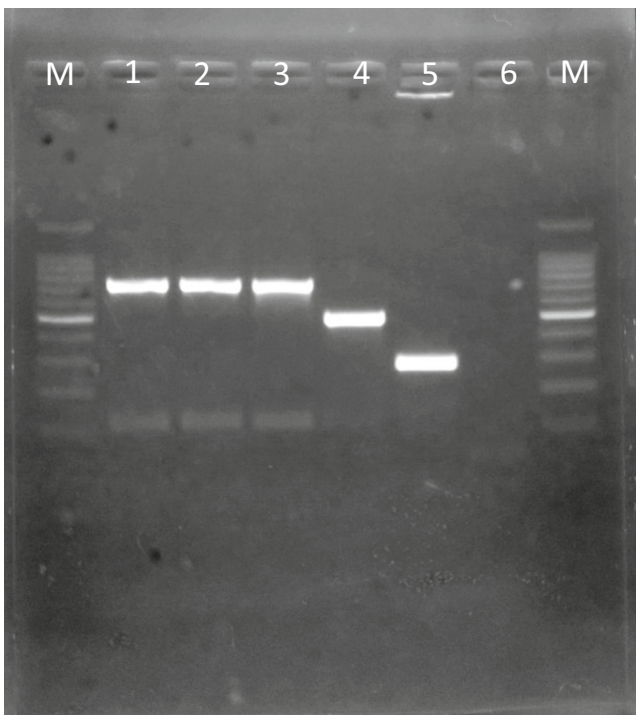

Fig. 2 AMOS PCR for *Brucella* species, *Lane M* 100-bp DNA ladder; *lanes 1–2 B. melitensis* DNA samples (731 bp); *lane 3* positive control for *B. melitensis*; *lane 4* positive control for *B. abortus* (498 bp), *lane* 5 positive control for *B. suis* (285 bp), *lane 6* negative control

RBPT and ELISA were 43.31, 66.24, and 42.67%, respectively.

Worldwide varied range of seroprevalence is reported among small ruminants. A total of 0.5% of sheep was found positive for brucellosis in Ethiopia when tested by RBPT (Lemu et al. 2014). In nearby country Bangladesh, Rahman et al. (2011) observed overall seroprevalence in sheep to the tune of 2.31 and 3.15% in goats. In Egypt, 18.09% positivity for *B. melitensis* in sheep population was recorded (Mahboub et al. 2013); which is also a tropical country and prevalence recorded is too less as compared to our observation. On comparison of the results of the present study with the studies carried out in India, the results vary with the different researchers. The seroprevalence observed in the different studies was at the lower side while comparing with the present study. Suryawanshi et al. (2014) recorded seroprevalence to the tune of 17.68% on RBPT test of serum samples of sheep from Maharashtra. In the same study, seroprevalence was recorded up to 18.33% in sheep from Akola district. However, we reported very high seroprevalence in the present investigation of flock, having history of abortions. In another state of India, Gujrat, the seropositivity for *B. melitensis* was recorded only up to 4.41% (Sutariya et al. 2014). Sadhu et al. (2015) reported overall seroprevalence in small ruminants as 11.30% by RBPT and 8.80% by i-Elisa in Northern Gujarat where sheep population is more. There may be a difference in the sheep husbandry practices in these states. In all these reports, the prevalence was recorded in apparently healthy animals, while, we report the seroprevalence in a flock with a history of abortion.

The prevalence and chances of spread of disease increases with increase in flock/herd size (Abbas and Agab 2002). During abortion, the contamination of environment with the *Brucella* organism takes place (Gameel et al. 1993) and helps to rapid transmission of causative agent. The poor management practices of animal husbandry are directly related to infection rate (Abou-Eisha 2000; Wernery and Kaaden 2002). So the flock size appears to be the major risk factor for contributing the disease. We also investigated the healthy flock from the same locality and few females were positive for brucellosis, while till the stage of investigation none of the males were found positive. But these positive females can be the source of infection to males and other females, and disease may spread in the flock. The seroprevalence recorded in the present study in apparently healthy sheep flock was 50%. In the healthy sheep flock, investigation showed seroprevalence to the tune of 28% among sheep in Tajikistan. The rate of isolation of the *Brucella* species depends on the number of viable organisms and stages of the infection at which samples were collected from growth of other fast growing organisms. Organisms like *B. ovis*, *Chlamydophila abortus*, *or Coxiella burnetii* are morphologically similar so staining could cause misleading diagnosis; hence, further isolation and characterization of the organism by PCR is mandatory (Garin-Bastuji et al. 2006). As per Alton (1990), vaginal excretion of *B. melitensis* is abundant and persists for several weeks even after abortion followed by udder infection (Marin et al. 1996). Hence, we thought vaginal swabs could be the best sample taken for the isolation of the *B. melitensis* from sheep. In the present case, we isolated *B. melitensis* from the outbreak and confirmed by PCR from the suspected flock. The incidence of *B. melitensis* in migratory flock warns the thorough testing and culling of *Brucella*-infected sheep from the flock on a continuous basis; otherwise, such incidence will be routine and farmers will be at a loss. Though there is no clinical sign among the animals at a farm, the farm should be tested for brucellosis regularly irrespective of abortions in animals as it will help to control the further spread of the disease among flocks.

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Compliance with ethical standards

Conflict of interest statement The authors declare that they have no conflict of interest.

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