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Detection of *Brucella melitensis* in milk and serum samples of goats by serological and molecular techniques

SWAPNIL LONKAR[1], VARSHA THORAT[1]✉, RAJASHREE GANDGE[1], RAJESH PHARANDE[1] and SANDEEP GAIKWAD[1]

Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra 440 006 India

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ABSTRACT

The present investigation was planned for the detection of brucellosis in goats using serological and molecular techniques. A total of 500 clinical samples (250 milk and 250 serum) of goats were collected. All the milk samples were screened by Milk Ring Test and Enzyme-Linked Immunosorbent Assay and serum samples with i-ELISA. Further, the positive milk and serum samples were subjected to 16SrRNA and OMP-31 PCR assays. Results showed the seropositivity of brucellosis was 34.8% and 16% from milk and serum samples of goats by ELISA, respectively and 2.8% by MRT. The results of 16SrRNA PCR detected *B. abortus*S19, in 32 (36.78%) milk samples and 10 (25%) serum samples by showing an amplicon of 1412 bp. Similarly, the OMP-31 PCR assay tested positive for *B. melitensis Rev*1, in 22 (25.28%) milk and 05 (12.5%) serum samples by showing an amplicon of 720 bp. Hence, this study is helpful for the detection of brucellosis in goats by using milk and serum samples. The study has generated evidence on the distribution of *Brucella melitensis* in milk and demonstrated the need to aware people about the importance of boiling milk before consumption in household and thus reduce the risk of infection in human beings.

Keywords: Brucellosis, Indirect ELISA, Lateral flow assay, Milk ring test, Polymerase chain reaction

Brucellosis in goats is mainly caused by *Brucella melitensis*, gram-negative coccobacillus of the genus *Brucella* and is most common zoonotic disease worldwide, causing serious public health problems. Goats acquire the infection by contact with organisms excreted in secretions like vaginal discharges, foetal fluids, placenta, etc. Infected goats can shed *B. melitensis* in secretions like milk, urine, semen and vaginal discharges, etc. The disease is characterized by infertility, delayed heat, interrupted lactation, loss of kids, breeding failure, and decreased wool, meat, and milk production. Abortions, stillbirths, and the delivery of immature offspring are the most common signs in naturally infected goats. Males may develop epididymitis and acute orchitis, which can lead to sterility (OIE 2018). The disease can be transmitted through direct contact with sick animals, aborted fetuses or placenta, consumption of raw milk and milk products in the human population. It is also an occupational hazard that affects shepherds, slaughterhouse employees, veterinarians, dairy sector specialists and laboratory personnel (Van den Brom *et al*. 2020).

The "Gold standard" for diagnosing brucellosis is by culture and isolation of the *B. melitensis* from the vaginal exudates, placenta and milk, followed by bacteriological characterization. The isolation needs expertise, more time for isolation as it takes 2-3 days to grow. It also possesses the danger of laboratory-acquired infection. Serological tests like Rose Bengal Plate Test (RBPT), Milk Ring Test (MRT) and Enzyme-Linked Immunosorbent Assay (ELISA) are used for herd testing for brucellosis. Serological tests are still considered the gold standard for herd screening (Ren and Peng 2020).

PCR-amplification is used to detect *Brucella* DNA from serum and milk samples of the goats. This technique improves safety as it does not need to deal with live organisms, and also rapid and affordable confirmatory diagnosis is achieved. Furthermore, DNA-based approaches are valuable for identifying organisms and typing organisms and investigating their epidemiology. Some of the targets that have been widely studied include 16SrRNA and OMP-outer membrane proteins (Herman and De Ridder 1992, Baily *et al.* 1992).

Amplification of the sequence exclusively available in the *B. melitensis* is used for its specific detection. Amplifying the same gene from milk samples offers a good target for amplification. A PCR assay from goat milk, with primers derived from the OMP 31 gene sequence of the *B. melitensis*, can be used. This approach will help build a sensitive PCR method to detect *B. melitensis*. It is crucial to create a sensitive and reliable PCR assay to detect

Present address: [1]Mumbai Veterinary College, Maharashtra Animal and Fishery Sciences University, Nagpur, Maharashtra.
✉Corresponding author email: drvarshamicro@gmail.com

B. melitensis in clinical samples of goats and humans. To reduce the risk of infection to humans, the present study was planned to detect *B. melitensis* in milk and serum samples of goats by serological and molecular techniques.

MATERIALS AND METHODS

Sample collection: A total of 500 clinical samples (250 serum and 250 milk samples) were collected from goats having history of abortions in and around Sangli district, Maharashtra. Milk samples were collected aseptically in a clean 15 ml centrifuge tube after cleaning the teats with the spirit. Blood samples were collected in plain vacutainers and allowed to clot at room temperature for around 2 h to obtain sera. The serum was then separated and aliquoted in sterile 2 ml microcentrifuge tubes. All samples were transported to the laboratory on ice and kept in refrigerator for overnight and thereafter at -20°C till further use.

Serological diagnosis: Milk Ring Test (MRT) was carried out using MRT antigen. Milk samples collected were kept at 4°C overnight and then brought to room temperature along with antigen. Samples were shaken gently to disperse the cream evenly and 1ml of sample was transferred to narrow test tube. MRT antigen (30µl) was added to 1 ml of milk sample. The milk samples were mixed with antigen and were incubated at 37°C for 1 h and results were recorded. A strong positive reaction was indicated by formation of dark pink ring above the skim milk column.

Enzyme-Linked Immunosorbent Assay (ELISA): AsurDx™*Brucella* Antibodies ELISA Test Kit (Bovine/ Ovine/Caprine) manufactured by Biostone Animal Health LLC, Dallas, Texas (USA) was used for detection of antibodies against *Brucella* spp. The test was performed as per the manufacturer's instructions.

Molecular diagnosis

Extraction of Brucella DNA from milk and serum samples: The genomic DNA of *Brucella* spp. was extracted from milk samples as per Romeo and Lapez-Gani (1995) with slight modifications. The boiling method was chosen for DNA isolation from serum samples.

Quantification of DNA: The A_{260}:A_{280} ratio was determined after DNA was collected from blood samples and was spectrophotometrically quantified at 260nm and 280nm using a nanodrop spectrophotometer.

Identification of Brucella spp. by PCR assay: Identification of *Brucella* isolates using genus specific 16S-rRNA primer sequences were performed (Singh *et al*. 2013). Primers used for 16SrRNA PCR were Forward-(5'–AGA-GTT-TGA-TCC-TGG-CTC-AG-3') and Reverse-(5'-ACG-GCT-ACC-TTG-TTA-CGA-CTT-3'). PCR Master Mix 12.5 µl, forward primer (20 pM/ µl) 1 µl, reverse primer (20 pM/ µl) 1 µl; template (300 ng) 1 µl and sterile distilled water 9.5 µl were mixed to make total reaction mixture of 25 µl. The cycling conditions used for amplification were as initial denaturation at 95°C for 5 min 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 1 min 30 s, final extension at 72°C for 10 min.

The amplification of OMP-31 gene of *B. melitensis* was carried out using oligonucleotide primer sequences: Forward - (5'-TGA-CAG-ACT-TTT-TCG-CCG-AA- 3') and Reverse - (5'-TAT-GGA-TTG-CAG-CAC-CGC-3'). PCR Master Mix 12.5 µl; forward primer (20 pM/ µl) 1 µl; reverse primer (20 pM/ µl) 1 µl; template (300 ng) 2 µl and sterile distilled water 8.5 µl were mixed to make total reaction mixture of 25 µl. The cycling conditions used for amplification were as initial denaturation at 95°C for 5 min. 35 Cycles of denaturation at 95°C for 1 min, annealing at 51°C for 30 s, extension at 72°C for 1 min, final extension at 72°C for 10 min.

Sequencing of PCR product: The sequencing of the one PCR product of 16SrRNA PCR was carried out at Eurofins India Pvt. Ltd., Bengaluru. The sequences obtained from the ABI files were analyzed and curated using Chromas light software.

Annotation of sequences using bioinformatics tools: The curated sequences were submitted to search for their similarity in the NCBI database, using BLAST tool. All the sequences were aligned and phylogenetic analysis was carried out using Neighbor-Joining method.

RESULTS AND DISCUSSION

Milk ring test: Two samples of Sheep and Goat Farm, Ranjani, Dist. Sangli and 5 samples of goat farm, Miraj were found positive by MRT (Table 1). In the present study, very less prevalence of brucellosis, i.e 2.8% was found by MRT.

Table 1. Detection of *Brucella* antibodies in milk by MRT

Place/Farm	Number of samples tested	Number of positive samples	Per cent positivity
Sheep and Goat Farm, Ranjani, District Sangli	121	2	1.65
Goat farm, Miraj	129	5	3.87
Total	250	7	2.8

The seropositivity of brucellosis in goats has been studied in different regions and showed a considerable range of variation in infection. Milk samples (120) of goats were screened for brucellosis by MRT and 11 (9.16%) samples were found positive (Ali 2014). Among 100 milk samples of goats screened for Brucellosis by MRT in two districts of Punjab, Pakistan, 6% samples were found positive for brucellosis (Khan *et al.* 2018). Prevalence of 4.97% was reported in goats of Pakistan by MRT (Nawaz *et al.* 2021) while 14/354 (3.96%) samples positive by MRT (Ghani and Nada 1983).

MRT is valuable for screening dairy cows but shows limitations in the diagnosis of brucellosis in goats. The smaller fat globuli of goat cream absorb agglutinated stained *Brucella* less efficiently in positive milk samples and do not rise to form the typical colored ring. Another problem of MRT is low content of antibodies in goat

Table 2. Detection of *Brucella* antibodies by i-ELISA in goats

Place/farm	Number of samples tested			Number of positive samples (%)		
	Milk	Serum	Total	Milk	Serum	Total
Sheep and Goat Farm, Sangali	121	121	242	46 (52.8)	20(16.5%)	66
Goat farm, Miraj	129	129	258	41(47.1)	20(15.5%)	61
Total	250	250	500	87(34.8)	40(16%)	127

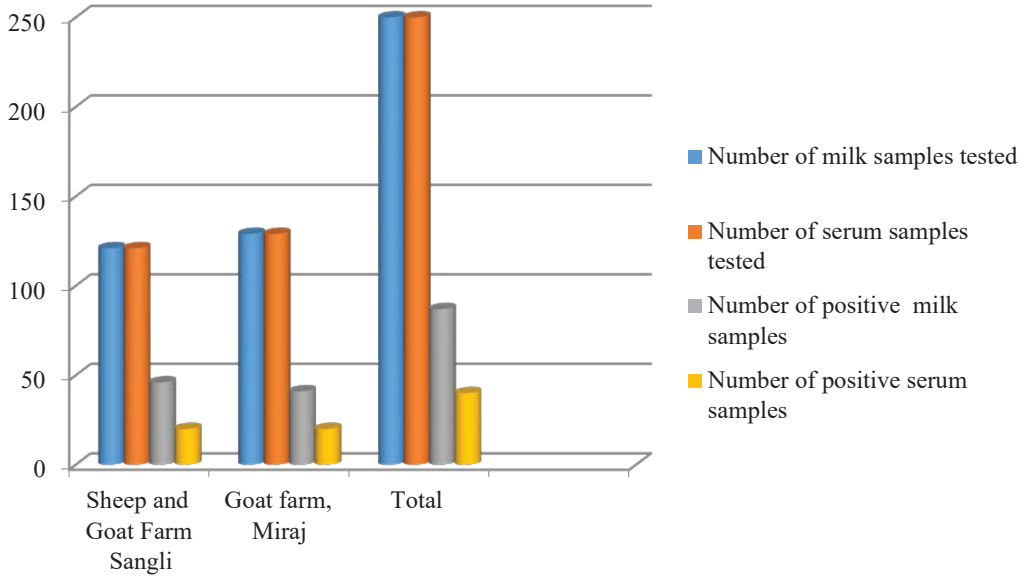


Fig. 1. Results of milk and serum i-ELISA.

milk which makes this test less efficient for diagnosis of brucellosis (Kolar J 1984).

Enzyme-Linked Immunosorbent Assay (ELISA): Total of 87 (34.8%) samples out of 250 milk samples were positive, and 40 (16%) samples out of 250 serum samples tested positive in i-ELISA (Table 2 and Fig.1).

The findings of ELISA corroborate with Sankhe *et al.* (2022) who reported a seropositivity of 20.54% by serum i-ELISA in Mumbai and Pune region. Further, Bait *et al.* (2019) reported 10.66% seroprevalence of brucellosis in small ruminants of western Maharashtra. Sonekar *et al.* (2017) collected 229 samples comprising of 157 blood and 72 clinical samples (vaginal swabs) from 157 animals. These serum samples (n = 157) were tested by i-ELISA and 104 (66.24%) samples were found positive by ELISA. Chaudhari *et al.* (2021) collected 9855 serum samples from animals for detection of brucellosis and reported 11.69% seropositivity of brucellosis by ELISA in Maharashtra region.

The findings of ELISA of the present study are similar to those of Kotadiya (2012), who reported a seropositivity of 18.20% in Gujarat, and Manasa *et al.* (2019) who found a seropositivity of 22.7% in sheep and goats in Andhra Pradesh and Telangana regions. While Kanani *et al.* (2018) found a seropositivity of 17.24%, Khan *et al.* (2018) found 22.6% and 28.6% prevalence in Jhang and Okara regions of Punjab.

The *Brucella* antibodies found in milk samples of goats, pose a zoonotic risk to the human population that drinks raw milk. Hence, milk ELISA is a useful method and should be used as a regular screening test of animals for brucellosis at farm level. Further detection of *Brucella* organism in milk samples by molecular techniques is needed to assure the risk to the human population (Kamwine *et al.* 2017). It was found in the present study that ELISA was a better serological test than other tests like MRT and it could be advocated for screening of goats for brucellosis. Information on the prevalence of brucellosis in goats is necessary to define control measures for zoonotic brucellosis (Godfroid *et al.* 2005). The results of this study are helpful in controlling brucellosis in goats.

Detection of Brucella spp. in milk and serum samples by 16Sr RNA PCR assay: An amplification product of 1412 bp was observed in 32 (36.78%) samples out of 87 milk samples confirming the presence of *Brucella* species. Out of 40 serum samples, an amplification product 1412 bp was found in 10 (25 %) serum samples (Table 3 and Fig.2).

Detection of Brucella melitensis in milk and serum samples by OMP 31 PCR assay: An amplification product

Table 3. Detection of *Brucella melitensis* by PCR

Sample	PCR	Number of samples tested	Number of positive samples	Per cent Positive
Milk	16SrRNA	87	32	36.78
	OMP31	87	22	25.28
Serum	16SrRNA	40	10	25
	OMP31	40	05	12.5

of 720 bp was observed in 22 (25.28%) samples out of 87 milk samples confirming the presence of *Brucella melitensis.* While 5 (12.5%) serum samples were found positive by showing amplicon of 720 bp (Table 3 and Fig.3.).

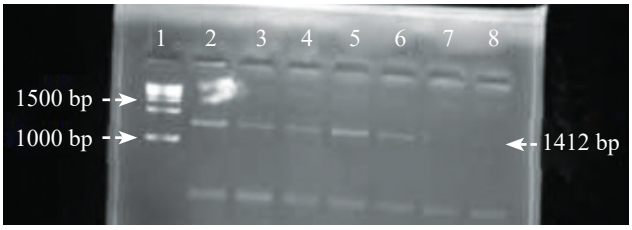


Fig. 2. Identification of milk and serum samples by 16SrRNA PCR assay. Lane 1- 1000 bp ladder, Lane 2- S 19, Lane 3,4 and 5- Milk samples, Lane 6 and 8-Serum samples, Lane 7- Negative control.

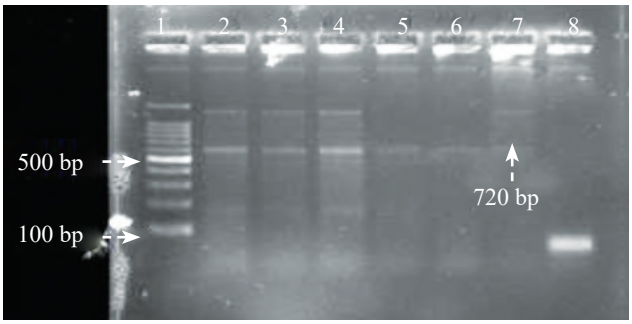


Fig. 3. Identification of milk and serum samples by OMP 31 PCR assay. Lane 1- 100 bp ladder, Lane 2 & 3- Milk samples, Lane 4- Rev 1, Lane 5 to 7- Serum samples, Lane 8- Negative control.

The genus-specific 16SrRNA PCR is used for molecular diagnosis of brucellosis. Ntirandekura *et al.* (2020) characterized *Brucella* species infecting humans, cattle and goats in the Kagera region using PCR amplification of the 16SrRNA gene. Out of the 12 milk samples, 2 (16.7%) samples were found positive by 16SrRNA PCR. Gupta *et al.* (2014) detected *Brucella* species in serum samples of goat by different PCR assays. They found highest sensitivity, i.e 95%, of 16SrRNA PCR assay for detection of brucellosis in goats. Ntirandekura *et al.* (2020) detected *Brucella* spp. in 2/12 (16.7%) serum samples by 16SrRNA PCR.

Amplification of the sequence exclusively available in the *B. melitensis* is to be detected for specific *Brucella melitensis* by OMP 31 PCR assay. Gupta *et al.* (2005) collected 54 milk samples from Barbari and Jamnapari of nearby villages of CIRG, Makhdoom and found 48 (89%) samples positive by milk OMP 31 PCR assay. Leal Klevezas *et al.* (2000) reported 11 (64%) samples positive by OMP31 PCR. Rahimi *et al.* (2020) detected 8 samples positive by TD-OMP 31 PCR assay. Wolde (2017) found 3 (9.37%) milk samples and 7 (23.33%) blood samples positive by OMP PCR assay.

Similar studies were carried out by Gupta *et al.* (2017) who collected 76 serum samples from goats of local abattoir at Agra and Mathura, UP and found 41 (54%) serum samples positive by OMP 31 PCR assay. Saini (2017) also found 52 (49.05%) samples positive by OMP31 real time PCR and Beena *et al.* (2017) reported 12 (15.78%) samples positive by OMP 31 PCR.

Detection of *Brucella melitensis* in milk samples becomes public health hazard as many rural people drink raw milk. Apart from that, detection of *Brucella* in goat milk poses a risk to other animals that are reared jointly, like cattle and buffaloes. Hence, the correct diagnosis of brucellosis using PCR assay is beneficial and speedy method and should be used as a regular screening test to diagnose brucellosis in goats.

Molecular characterization of Brucella spp by sequencing of 1412 bp region of 16SrRNA: One PCR amplicon of 16SrRNA region of *Brucella* spp. derived from milk sample was sequenced. Chromas lite software was used for annotation of the retrieved sequences from ABI files. The sequences were compared with the available sequences at GenBank. BLAST search helped to confirm the identity and species of *Brucella* organisms. Sequence analysis of the PCR products revealed that the PCR amplicon under study exhibited identity with different strains of *Brucella melitensis* (Table 4).

Table 4. Homology of the *Brucella* 16SrRNA gene sequences with sequences available in NCBI

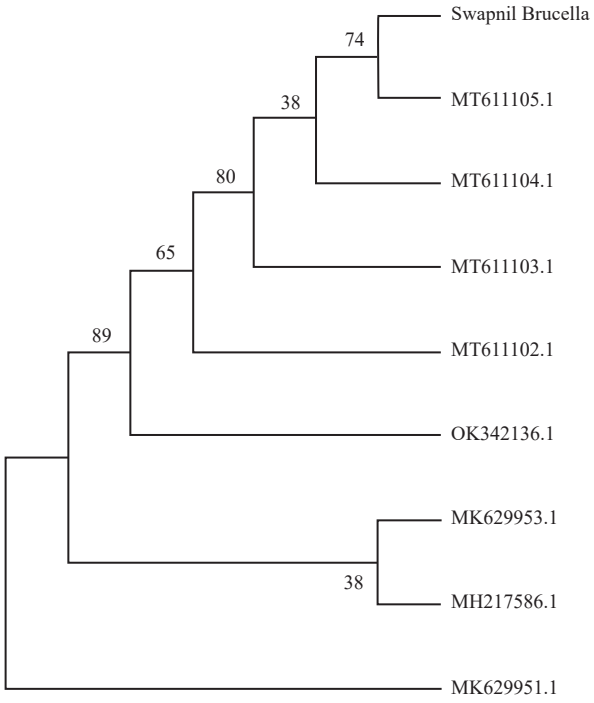
Sample	Genus and Species	Per cent Identity	Origin	Accession Number
Sample 1	*Brucella melitensis*	94.22%	Greece	MK142739.1
		96.39%	China	MT611104.1
			Norway	LT962939.1
			India	CP029757.1
		94.7%	China	CP035795.1
			India	CP025680.1

Sample of the present study was found to be 94.22%, 94.7% and 96.39% identical with *Brucella melitensis*. The *Brucella* isolate sequence in this study was identified using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and related sequences were downloaded for phylogenetic analysis. All sequences were aligned using MAFFT online software. Results of phylogenetic analysis showed that the *Brucella* sequences were divided into three lineages and *Brucella* sequences under study were grouped under lineage II (Table 5). Lineage I and III contained *Brucella* sequences from Philippines, China, Greece and Croatia. The *Brucella* sequences of lineage II discovered a link between sequences from China.

Phylogenetic analysis of 16SrRNA gene sequence of the *Brucella* spp. is useful method to detect the ancestral origin of the organism. Similar findings have been reported by O' Leary *et al.* (2005), Barua *et al*. (2016) and Mukherjee *et al*. (2007).

The high seropositivity of brucellosis in and around Sangli district of Maharashtra was observed. ELISA is a better serological test than MRT for screening of goats for brucellosis. 16SrRNA and OMP-31 PCR assays proved useful in identification of *Brucella* organisms and also

Table 5. Phylogenetic tree of *Brucella melitensis* with *Brucella* sequences from NCBI



in direct detection of *Brucella* spp. from milk and serum samples of animals. Detection of *Brucella melitensis* in milk samples poses a risk to the human population as many rural people drink raw milk. So it is suggested that control programs may be implemented to prevent further spread of the disease.

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