Detection of \textit{Brucella melitensis} in milk and serum samples of goats by serological and molecular techniques

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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 \textit{B. abortus} 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 \textit{B. melitensis Rev} 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 \textit{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 \textit{Brucella melitensis}, gram-negative coccobacillus of the genus \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{B. melitensis}, can be used. This approach will help build a sensitive PCR method to detect \textit{B. melitensis}. It is crucial to create a sensitive and reliable PCR assay to detect...
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\text{260} / A\text{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'-TAT-GGA-TGT-GCA-CAT-GGC-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 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-CTT-TGG-CGG-AA- 3') and Reverse - (5'-TAT-GGA-TGT-GCA-CAT-GGC-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>
<thead>
<tr>
<th>Place/Farm</th>
<th>Number of samples tested</th>
<th>Number of positive samples</th>
<th>Per cent positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep and Goat Farm,</td>
<td>121</td>
<td>2</td>
<td>1.65</td>
</tr>
<tr>
<td>Ranjani, District Sangli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat farm, Miraj</td>
<td>129</td>
<td>5</td>
<td>3.87</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>7</td>
<td>2.8</td>
</tr>
</tbody>
</table>

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 serum and do not rise to form the typical colored ring. Another problem of MRT is low content of antibodies in goat
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. Sonetkar 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 16S rRNA 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>
<thead>
<tr>
<th>Place/farm</th>
<th>Number of samples tested</th>
<th>Number of positive samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>Serum</td>
</tr>
<tr>
<td>Sheep and Goat Farm, Sangli</td>
<td>121</td>
<td>121</td>
</tr>
<tr>
<td>Goat farm, Miraj</td>
<td>129</td>
<td>129</td>
</tr>
<tr>
<td>Total</td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

**Table 3. Detection of *Brucella melitensis* by PCR**

<table>
<thead>
<tr>
<th>Sample</th>
<th>PCR</th>
<th>Number of samples tested</th>
<th>Number of positive samples</th>
<th>Per cent Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>16SrRNA</td>
<td>87</td>
<td>32</td>
<td>36.78</td>
</tr>
<tr>
<td></td>
<td>OMP31</td>
<td>87</td>
<td>22</td>
<td>25.28</td>
</tr>
<tr>
<td>Serum</td>
<td>16SrRNA</td>
<td>40</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>OMP31</td>
<td>40</td>
<td>05</td>
<td>12.5</td>
</tr>
</tbody>
</table>
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.).

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, Mahkdoom 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).
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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