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Molecular Detection of *Mycoplasma* in Milk Samples of Goat Herds in Borujerd County

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Abstract

Mycoplasmas are among the smallest bacteria which are responsible for economically significant infections in small ruminants. This study aimed to detect *Mycoplasma* in goat milk samples from Borujerd County, Iran. Ninety-six milk samples were collected from seven regions and screened for subclinical mastitis using the California Mastitis Test (CMT) and somatic cell count (SCC). Thereafter, total DNA was extracted, and *Mycoplasma* detection was performed by a PCR assay using genus-specific primers. High SCC and CMT scores were observed in 7.3% and 31.25% of samples, respectively. PCR analysis confirmed *Mycoplasma* infection in 5.2% (n=5) of the milk samples. The presence of *Mycoplasma* infection in goat herds in Borujerd county highlights the need for breeders and veterinary supervisors to take effective measures to control and prevent these infections. Besides, these findings suggest PCR assay as a rapid and reliable method for identifying *Mycoplasma* in milk samples.

1. Introduction

Goats are among the most important domestic ruminants, widely raised across the world, including many regions of Iran, for meat and milk production. Small size, adaptability, low nutritional demands, and resistance to adverse environmental conditions are those attributes that make goats especially valuable for rural and small-scale farmers. However, bacterial infectious diseases, particularly mycoplasma infections, have posed a significant threat to goat production (Zamiri and Heidari, 2006).

Mycoplasmas are small, pleomorphic bacteria that typically cause chronic infections (McGowin and Totten, 2017; Hajizadeh *et al.*, 2018). Contagious agalactia, a notable mycoplasma disease of small ruminants, presents with inflammation of the udder, joints, and conjunctiva, and is endemic in many Mediterranean countries (Jay and Tardy, 2019). In this case, *Mycoplasma agalactiae* is the primary causative agent, though other species including *M. mycoides*, *M. capricolum*, *M. putrefaciens* and *M. arginini* have also been implicated (Quinn *et al.*, 2011). Infected mammary glands may secrete purulent material, progressing to fibrosis, and permanent loss of milk production. Economic impacts include abortions, weak offspring, udder deformities, joint swelling, and blindness (Madanat *et al.*, 2001). These outcomes underscore the importance of accurate detection and control.

Traditional diagnosis of mycoplasma infections relies on clinical signs, bacteriological culture, and serology; however, the fastidious growth requirements of mycoplasmas limit these methods. Molecular techniques such as PCR offer rapid, sensitive, and specific detection (Templeton *et al.*, 2003).

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Table 1
Primer sequences used in *Mycoplasma* genus-specific PCR

Primer	Sequence 5'-3'	Amplicon size (bp)	Reference
GPF- Forward	GCTGGCTGTGTGCCTAATACA	1013	Lierz *et al.*, 2007
MGSO- Reverse	TGCACCATCTGTCACTCTGTTAACCTC		

Considering the importance of mycoplasma infections and the economic losses caused by these bacteria in goat herds, the present study was carried out to molecularly investigate mycoplasma infection in milk samples from goat herds in Borujerd county.

2. Materials and Methods

2.1. Sampling

Milk samples were collected from 96 clinically healthy goats in seven regions of Borujerd county (Shisheh, Shahviran, Jahanabad, Karvaneh, Asadkhani, Darehgorg, and Kenarvand) between 2018 and 2019. Sampling was performed seasonally: Shisheh and Shahviran (summer 2018), Jahanabad and Karvaneh (autumn 2018), Asadkhani and Darehgorg (winter 2018), and Kenarvand (spring 2019). Twenty-four samples were collected from Kenarvand, and 12 from each of the remaining regions.

To do sampling, teats were washed, disinfected with 70% ethanol for 30 s, and the first streams of milk were discarded. Approximately 10 mL of milk was collected from the third stream onward into sterile containers, labeled with animal data, and transported on ice to the Bacteriology Laboratory, Faculty of Veterinary Medicine, Bu-Ali Sina University. None of the animals had received antimicrobial treatment at the time of sampling.

2.2. California Mastitis Test (CMT)

Somatic cell levels were estimated using the California Mastitis Test (Ruegg, 2017). Equal volumes of milk and CMT reagent were mixed in test wells and reactions were scored within 10 s as negative, +1, +2, or +3 based on clot formation.

2.3. Somatic Cell Count (SCC)

SCC was determined using Giemsa staining. Ten microliters of milk samples were smeared over 1 cm^2 on a glass slide, air-dried, fixed in xylene (5 min) and ethanol (3 min), stained with Giemsa (1 min), and decolorized in absolute ethanol (5–10 s). Slides were then examined under a 40× light microscope, and counts from 10 fields were multiplied by 5×10^4 to yield SCC/mL. Samples with $>3\times10^5$ cells/mL were classified as high SCC (Dohoo *et al.*, 1984).

2.4. DNA Extraction

Total DNA was extracted from milk samples using a commercial kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. DNA quality was assessed by agarose gel electrophoresis and suitable samples were stored at −20°C until analysis.

2.5. PCR Detection of Mycoplasma

Genus-specific PCR targeting a conserved region of the *Mycoplasma* genome was performed using primers GPF and MGSO (Lierz *et al.*, 2007). Each 25 μL PCR reaction contained 12.5 μL master mix, 1 μL of each primer (10 pmol), 5 μL DNA template, and 5.5 μL sterile distilled water. Thermal conditions were as follow: 94 °C for 5 min; 40 cycles of 94 °C, 55 °C, and 72 °C for 1 min each; and a final extension at 72 °C for 10 min. DNA from a commercial contagious agalactia vaccine (Razi Vaccine and Serum Research Institute, Karaj, Iran) served as a positive control, and distilled water as a negative control. Amplicons were visualized on 1% agarose gels.

3. Results

3.1. California Mastitis Test

All 96 milk samples were evaluated by CMT and classified into four categories: negative, +1, +2, and +3. As shown in Table 2, 20% (n=19) of samples were negative, while 80% (n=77) were positive distributed across the +1 to +3 categories.

Table 2
Percentage frequency of milk samples in terms of score in CMT

CMT Scores	No. of samples (%)
Negative	17 (17.7)
+1	22 (22.91)
+2	27 (28.12)
+3	30 (31.25)

3.2. Somatic Cell Count

SCC was determined according to the Giemsa staining method (Table 3). Samples with SCC >300,000 cells/mL were classified as high-SCC. An example of Giemsa-stained somatic cells from a high-SCC sample is shown in the Fig. 1.

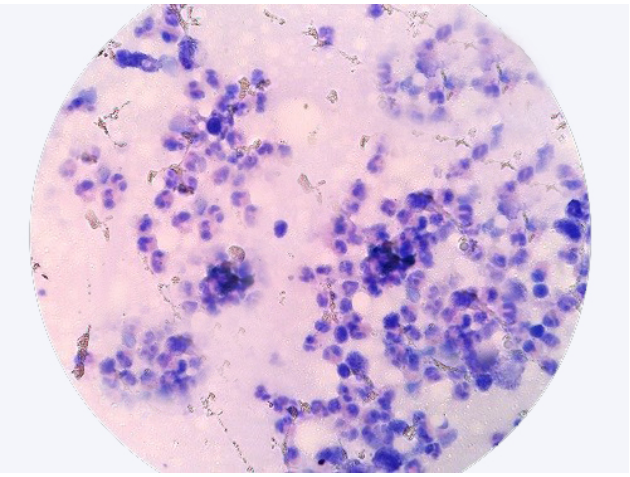

Fig. 1. Microscopic view of a Giemsa-stained smear for somatic cell counting (×40 magnification).

Table 3
Frequency distribution of milk samples in terms of somatic cell count.

Group	SCC	No. of samples (%)
A	$< 10^5$	50 (52.08)
B	10^5–2×10^5	29 (30.21)
C	2×10^5–3×10^5	10 (10.42)
D*	$3\times10^5 <$	7 (7.29)

D: Milk samples with high SCC

3.3. PCR Detection of Mycoplasma

PCR assay, as a molecular method with high sensitivity and specificity, was performed for molecular detection of *Mycoplasma* contamination in DNA samples extracted from goat milk samples. Positive samples yielded a 1,013 bp amplicon on agarose gel electrophoresis (Fig. 2). Totally, PCR results indicated that *Mycoplasma* DNA was present in 5 of 96 goat milk samples (5.2%). The characteristics of animals infected with *Mycoplasma* are presented in Table 4. As shown, *Mycoplasma* infection occurred in animals across different age groups.

4. Discussion

Mastitis, a common complication in cattle, has received less attention in small ruminants. However, given the large goat population in Iran and their considerable milk production, mastitis in goats warrants serious attention, as it can cause significant economic losses. Among the major causes of mastitis in goats is *Mycoplasma*, particularly *Mycoplasma agalactiae*, the causative agent of contagious agalactia which is a disease known for nearly two centuries. This disease is characterized by mastitis, arthritis, and keratoconjunctivitis (Kumar *et al.*, 2014; Quinn *et al.*, 2015). Although goats are generally less susceptible to mastitis than cattle, chronic, acute, and gangrenous forms can occur. Infections with *M. agalactiae* and *M. mycoides subsp. mycoides* often present with mastitis as a prominent symptom. Since mastitis is often subclinical, SCC and CMT tests are valuable for early detection. The CMT is a rapid, field-friendly test for estimating somatic cell counts, though in goats, high SCC values may occur in the absence of infection, limiting the test's diagnostic specificity. Nevertheless, increased SCC typically indicates mastitis (Contreras *et al.*, 2007).

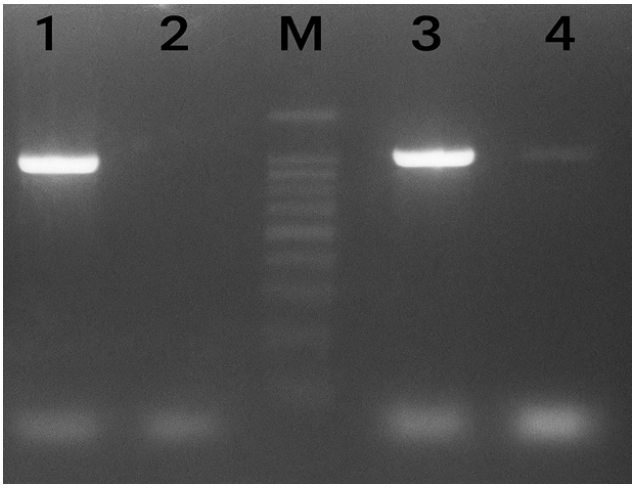

Fig. 2. Electrophoresis results of PCR products to identify genus *Mycoplasma*. Lanes 1 and 4: examined milk samples, lane 2: negative control, distilled water, lane 3: positive control, DNA extracted from the agalactia vaccine, lane M: a 100 bp DNA ladder.

In the present study, 96 goat milk samples from seven regions of Borujerd county were tested. In the CMT test, only 17 samples (17.7%) were negative; the remaining samples scored +1 to +3, with 30% scoring +3, indicating subclinical mastitis. In the SCC test, 7.3% of samples were in group D (>300,000 cells/mL). All high-SCC samples were also in the CMT 3+ group, confirming agreement between the two methods. Similar correlations between CMT and SCC have been reported by Persson and Olofsson (2011), who found both tests suitable for identifying mastitis in goats. In the evaluation of subclinical mastitis using SCC and CMT tests, all regions except Asadkhani had relatively similar conditions. No cases suspected of subclinical mastitis were found in Asadkhani (Table 4). Whereas, goats infected with *Mycoplasma* were detected only in the Shisheh and Jahanabad regions. All samples with high SCC values (except one) were classified in group D and corresponded to the CMT 3+ category. Furthermore, our regional analysis showed no subclinical mastitis in Asadkhani, maybe due to better hygiene and management practices.

Table 4
Number of milk samples with high SCC and CMT scores, and *Mycoplasma*-infected samples by region.

Sample collection area	No. of collected samples	No. of samples with high SCC	No. of samples with high CMT score	No. of *Mycoplasmainfected* samples
Shisheh	12	2	5	2
Shahviran	12	0	3	0
Jahanabad	12	1	5	2
Karvaneh	12	2	7	0
Asadkhani	12	0	0	0
Darehgorg	12	0	4	1
Kenarvand	24	2	6	0

Differences in SCC and CMT results between studies may be attributed to factors such as stress, breed, season, milking frequency, and infection type. Seasonal variation also influences SCC, with the highest counts from October to mid-February. Additionally, SCC accuracy can vary depending on whether automated or manual counting methods are used (Contreras *et al.*, 2007).

Sporadic reports of *Mycoplasma*-associated mastitis in Iran have emerged in recent years (Moradi *et al.*, 2011; Pourbakhsh *et al.*, 2013). Limited data exist on *Mycoplasma* infections in goat herds in Iran, as most studies focus on sheep due to their larger population. However, given that *M. agalactiae* causes severe milk yield losses and goats are a key dairy species, investigating its prevalence in goats is critical.

In this study, total DNA was extracted from milk samples using a commercial kit, and the *Mycoplasma* genus was detected by a PCR assay using specific primers. Five samples (5.2%) were positive. All of these *Mycoplasma*-positive goats were found only in Shisheh and Jahanabad.

Similar studies have reported varying prevalence rates across Iran, influenced by region-specific factors and diagnostic methods. However, PCR is generally more sensitive than culture (Moradi *et al.*, 2011; Mohammadpour *et al.*, 2015; Rahimabadi *et al.*, 2017).

Moradi *et al.* (2011) collected 367 milk samples from sheep and goats in Sanandaj and screened them for *Mycoplasma* contamination by culture on PPLO medium and by a PCR method using a specific primer pair for *Mycoplasma agalactiae*. Twelve samples (5.5%) were culture-positive, and five were PCR-positive for *M. agalactiae*, including one goat milk sample. Pourbakhsh *et al.* (2013) analyzed 142 sheep and 85 goat samples (joint fluid, eye secretions, and milk) using PCR. *Mycoplasma* was detected in 59 sheep (41%) and 46 goats (54%), of which 17 sheep (29%) and 28 goats (61%) were *M. agalactiae*. The authors reported a higher prevalence in goats and identified Kerman as a high-risk area due to large goat populations. In Ardabil, Hajizadeh *et al.* (2018) examined 116 sheep and 16 goats. Of the 132 animals tested, 33 (25%) were culture-positive in milk samples. PCR identified *M. agalactiae* in 47% of these, *Mycoplasma putrefaciens* in 43%, *Mycoplasma capricolum* in 7.5%, and *Mycoplasma mycoides* in 2%. The findings indicated that non-*agalactiae Mycoplasma* species are common in Iranian flocks and may be transmitted between sheep and goats. Mohammadpour *et al.* (2015) investigated 25 milk samples from five sheep flocks in Guilan. Culture identified nine positive samples (36%), while PCR detected 17 positives (68%), including five *M. agalactiae*. The study supported PCR as a reliable detection method as well. Shamsaddini *et al.* (2017) tested milk, joint fluid, and eye secretions from sheep and goats. Culture yielded 15% positivity, while PCR targeting the 16S rRNA gene detected *Mycoplasma* in 36% of samples. Rahimabadi *et al.* (2017) examined 71 samples (vaginal swabs, ear swabs, and milk) in Guilan. *Mycoplasma* colonies were observed in 50 samples (70.4%) by culture, while PCR confirmed *Mycoplasma* identity in 40 (80%) of these samples including 11 *M. agalactiae* (27.5%). Overall, prevalence rates vary widely by location, likely due to regional differences in management and environmental conditions. Most studies report PCR to be more sensitive and accurate than culture, though Tatay-Dualde *et al.* (2015) found both methods highly reliable for detecting *M. agalactiae* in goat milk and recommended culture of bulk-tank milk over PCR for routine surveillance. Another important aspect of *Mycoplasma* epidemiology is vertical transmission, which facilitates herd-level spread (De la Fe *et al.*, 2009). While semen samples were not tested in this study, future work should include semen and vaginal secretions to provide a more complete epidemiological picture. Previous research has found high *Mycoplasma* prevalence in goat semen using both culture and PCR, underscoring the importance of reproductive materials in disease transmission as well (Pourbakhsh *et al.*, 2017).

5. Conclusion

This study confirms the presence of *Mycoplasma* contamination in goat herds in Borujerd, albeit at a low prevalence. Nevertheless, the risk of transmission to other livestock, including sheep, warrants attention due to the potential for insidious reductions in herd productivity. The results also suggest the PCR assay as a rapid, sensitive, and reliable molecular tool for detection of *Mycoplasma*.

Acknowledgments

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