Original Article https://doi.org/10.47748/tjvr.1624707

TJVR 2025; 9 (1): 49-55

Turkish Journal of Veterinary Research

https://dergipark.org.tr/tr/pub/tjvr

e-ISSN: 2602-3695

Detection of *Mycoplasma agalactiae* in small ruminants in eastern of Türkiye

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Received: 22.01.2025 Accepted: 26.02.2025

ABSTRACT

Objective: In this study, it was aimed to investigate the presence of *Mycoplasma* (*M.*) *agalactiae* in small ruminants with clinical symptoms such as milk withdrawal, milk reduction, and discoloration by molecular techniques in the Bitlis region.

Materials and Methods: For this purpose, 120 samples were collected from milk, blood, eye fluid, ear fluid, and joint fluid from animals, and the clinical symptoms were shown in the Bitlis Centre and Districts. DNA isolation of the collected samples was performed according to commercial kit protocols. Then, a Polymerase Chain Reaction (PCR) step was performed with primer pairs (*uvrC*) synthesized specifically for *M. agalactia*.

Results: For the *uvrC* gene region, 36.55% (34/93) of the milk samples and 28.30% of the whole samples were positive. It was determined that 33 (%32.4) of the positive samples originated from goat milk and 1 (%5,6) from sheep milk sample. As a result, the positivity rate determined by the molecular method in this study was higher than the classical methods (isolation, identification). This study is the first study investigating the presence of *M. agalactia* in the Bitlis region.

Conclusion: Further studies are needed to determine the prevalence of the agent in the region. In addition, further studies are required to control and eradicate the circulating agent in the area.

Keywords: *Small Ruminant, Mycoplasma, PCR, Mastitis*

INTRODUCTION

Infectious agalactia in sheep and goats is a disease that has been known for about two centuries (WOAH, 2018). The infection is seen in many world regions, especially in the Mediterranean basin (Tardy et al., 2012; WOAH, 2018). Morbidity and mortality rates can reach up to 100% in infectious agalactia infections (Yatoo et al., 2018). Infectious agalactosis occurs seasonally in an enzootic fashion and primarily affects sheep and goats. It usually causes mastitis in lactating sheep and goats, while it usually manifests itself with arthritis, keratoconjunctivitis, and respiratory system problems in male animals and offspring. Severe respiratory system infections may cause the death of the offspring. Diagnosis of infectious agalactia agents is based on isolation and identification by conventional culture method. The disease is combated with antibiotic treatment and vaccination in the regions affected by the disease (Leonovich, 2024). Infectious agalactia primarily affects sheep and goats. The causative agents of the disease include *M. agalactiae*, *M. capricolum* subsp. *capricolum* (Mcc) and *M. mycoides* subsp. *capri* (Mmc) (CFSPH, 2018; Dawood et al., 2022; Heller et al., 2015). In addition, due to the similar clinical picture, especially in goats, Mmc, Mcc, and Mp infections have been recognised as disease agents for about 30 years. However, due to the acceptance of *M.*

agalactiae as the causative agent of the disease in national and international animal disease regulations, it was proposed to remove the other 3 agents from the OIE list in 2021, except for *M. agalactiae* (Migliore et al., 2021).

Considering the rugged geographical structure, varying climatic conditions, and socio-economic structure in different regions of our country, ovine breeding has come to the forefront. Although small ruminant breeding is more labor intensive than other types of animal breeding, its financial income is higher. Among the livestock, the fact that goats can better use areas unsuitable for agriculture and rocky areas compared to other animals reveals the importance of small ruminant breeding (Karatekeli, 2020). The goat population is much higher than the sheep population in the Bitlis region (Ertaş, 2019). There is no research on the presence of *M. agalactiae*, one of the infectious agalactia agents seen in sheep and goats in the Bitlis region.

The most frequently used gene regions in the molecular diagnosis of mycoplasmas are *adk*, *gpsA*, *polC*, and *uvrC*. However, it has been reported that these genes can be used as clinical detection markers to distinguish between *M. bovis* and *M. agalactiae*. These genes can be important markers for clinical diagnosis and reveal genetic differences between species. *uvrC* gene region confirms the study results and distinguishes between *M. agalactiae* and *M. bovis* agents. In recent studies, it was reported that point mutations were detected in the *M. bovis uvrC* gene, which may cause false negative PCR results to identify *M. bovis* strains (Sun et al., 2020; Yücetepe et al., 2022). Research has demonstrated the successful utilisation of the *uvrC* gene region for detecting and characterising *M. agalactiae* in small ruminants (Göçmen et al., 2016). In this study, it was aimed to investigate the presence and prevalence of *M. agalactiae*, one of the causative agents of infectious agalactiae in sheep and goats raised in Bitlis and its region, by using the molecular method of PCR and *uvrC* gene region. In addition, the study draws attention to the first study in which the disease agent was investigated using the molecular method in the region.

MATERIALS and METHODS

Material

A total of 120 samples (1 joint fluid, two ear swabs, five eye swabs, 19 blood, 93 milk) were collected from 113 different animals (18 sheep and 102 goats) showing clinically infectious agalactia symptoms (milk reduction, milk withdrawal, milk discoloration, keratoconjunctivitis, arthritis) in 46 different enterprises in 29 villages of 6 districts of Bitlis in 2024 (Table 1). Among the 93 milk samples collected, 89 were obtained from goats and 4 from sheep. The collected samples were brought to the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University, Van Yuzuncu Yil University, as soon as possible under the cold chain.

Table 1. Distribution of samples according to species and location of collection

Sample regions	Samples					Total
	Milk	Blood	Eye Swab	Ear Swab	Joint Fluid	
Centre	27	10	1	0	0	38
Mutki	19	5	3	1	0	28
Güroymak	17	2	1	1	0	21
Tatvan	12	2	0	0	1	15
Hizan	9	0	0	0	0	9
Ahlat	9	0	0	0	0	9
Toplam	93	19	5	2	1	120

Reference strains

The quality control analyses performed in the study used the *Mycoplasma agalactiae* AIK, NCTC 10123 strain obtained from Pendik Veterinary Control Institute, Mycoplasma Laboratory.

Methods

After centrifugation at 16,000 rpm for 20 minutes for 93 milk samples taken in sterile tubes under aseptic conditions from animals with mastitis findings, the fat layer accumulated on the upper part and the clear liquid phase just below it was discarded with the help of an automatic pipette, and 1 ml of the

precipitate was transferred to sterile eppendorf tubes and stored at -20°C until used in the analyses. Swap samples were placed in vortexed tubes containing 2 ml of sterile PBS, and the liquid phase content was transferred to the liquid phase. Then, the tube with PBS was centrifuged at 2,500 rpm for 10 minutes, 1.5 ml of the supernatant was discarded, and the precipitate was transferred to sterile Eppendorf tubes and stored at -20°C until used in the analyses. Blood samples were collected in anticoagulated (EDTA) vacuum tubes (Greiner, Bio-One, Germany) and stored at +4°C until analyses.

DNA isolation and amplification

Genomic commercial kits were used for DNA extraction from the study's samples. Two different DNA kits were used for tissue (HY-DDNA-100, Hydra Biotechnology R&D, Van, Türkiye) and milk (Norgen Biotek Corporation, Ontario, Canada). Both DNA extraction procedures were performed according to the manufacturer's specifications.

The *uvrC* gene was selected as the specific primer for *M. agalactiae,* and the amplification length of the product to be obtained was synthesised as 1624 bases. Accordingly, the forward and reverse amino acid sequences of the *uvrC* gene are given in Table 2.

Tablo 2. Oligonucleotide sequences of primer pairs used in the PCR step

Gen	Primers	Oligonucleotide sequences (5′-3′)	Size	Annealing T.	References
uvrC	MAGAUVRC1-L	CTCAAAAATACATCAACAAGC	1624bp	60°C	(Subramaniam et al., 1998)
	MAGAUVRC1-R	CTTCAACTGATGCATCATAA			

Table 3. PCR mixture and heat cycling protocol were used to detect the *uvrC* gene region of *M. agalactiae* in the analysed samples.

Component	Amount used	Heat cycling protocol
2x Taq PCR Master mix	12.5 µl	95°C - 5 min 94°C - 30 sec 60°C - 30 sec 72°C - 90 sec 72°C - 7 min 33 Cycle
Primer -F (10 pmol/µl)	0.5 µl	
Primer -R (10 pmol/µl)	0.5 µl	
Deionised water	8.5 µl	
DNA	3.0 µl	
Total	**25 µl**	

PCR was performed using a 2x Taq PCR Master mix (K0171, Thermo, Lithuania) according to the procedures specified by the manufacturer. The samples' DNAs were analysed for the PCR step to amplify the *uvrC* gene region using the methods described by Subramani et al. (1998) (Subramaniam et al., 1998). The reaction components, mixtures, and heat cycling protocol for PCR of the synthesised primers were carried out in a Thermal Cycler (Corbett Research, Qiagen GmbH) (Table 3).

DNA obtained from *Mycoplasma agalactiae* AIK (NCTC 10123) reference strain was used as a positive control, and deionised water was used as a negative control. The PCR amplicons obtained were run on an agarose gel electrophoresis setup (Thermo Scientific, OwlR Easy Cast TM B1) using 2% agarose gel and then analysed under UV light in a Gel Imaging device (Genesis®).

Statistical Analysis

Descriptive statistical data were given as numbers and percentages. The evaluation of the disease according to districts and animal species was performed by chi-square test. Statistical analyses were performed with the SPSS Ver 26 package programme. The statistical significance level was accepted as $p<0.05$.

Ethical consideration

Van Yuzuncu Yil University Animal Research Local Ethics Committee (Decision no: 2023/ 09-06), Van, Türkiye, granted ethical permission for the study.

RESULTS

In this study, in order to investigate the presence of the *uvrC* gene region of *M. agalactiae*, genomic DNA extraction was performed from a total of 120 specimens collected from the Bitlis region and its

districts and stored at -20˚C in the laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Van Yuzuncu Yil University. After DNA extraction, qPCR was performed with a commercial Master mix kit using appropriate (forward/reverse) primers. As a result of qPCR, the presence of nucleic acid for the *M. agalactiae uvrC* gene was detected in 34 samples out of 120 sheep and goat samples. No positivity was obtained in the study samples except milk. A 36.55% (34/93) positivity rate was detected in milk samples. The positivity rate was determined as 28.30% in all study samples. The agarose gel image of the amplicons of the analysed samples is shown in Figure 1.

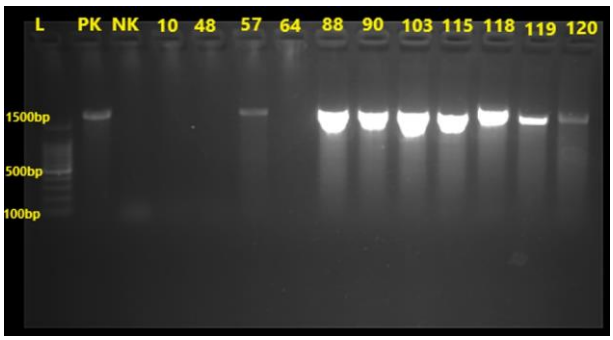

Figure 1. Gel Image of *uvrC* gene region (1-L=Ladder, 2-PK= Positive Control, 3-NK= Negative Control, 4-5-7= Negative Study Samples numbered 10, 48, 64, 6-8-9-10-11-12-13-14= Positive Study Samples numbered 57, 88, 90, 103, 115, 118, 119, 120)

Table 4. Distribution of *uvrC* gene detected in samples from districts

Sample regions	Positive (%)	Negative (%)	Total	p
Centre	15 (39.5)	23 (60.5)	38	
Mutki	6 (21.4)	22 (78.6)	28	
Güroymak	5 (23.8)	16 (76.2)	21	
Tatvan	2 (13.3)	13 (86.7)	15	0.407
Hizan	3 (33.3)	6 (66.7)	9	
Ahlat	3 (33.3)	6 (66.7)	9	
Total	34 (28.3)	86 (71.7)	120	

Table 5. Distribution of the presence of the *uvrC* gene region according to animal species

Animal species	Positive (%)	Negative (%)	Total	p
Sheep	1 (5.6)	17 (94.4)	18	
Goat	33 (32.4)	69 (67.6)	102	0.022*
Total	34 (28.3)	86 (71.7)	120	

*: $p<0.05$

Statistical Analysis Results

The chi-square method analyses showed no statistically significant difference in *uvrC* gene regions between the districts (Table 4).

For the *uvrC* gene region, the highest positivity rate (39.5%) was observed in Bitlis-Centre, while the highest negativity rate (86.7%) was determined in the Tatvan district. The reason statistically significant differences could not be determined in the evaluation of the *uvrC* gene was the small number of samples collected in the Tatvan, Hizan, and Ahlat districts, which limited the study.

In addition, a chi-square test was performed separately for *uvrC* gene regions to determine whether there are differences between species. The results are presented in Table 5.

For the *uvrC* gene region, the positivity rate in goats was 32.4%, while the negativity rate in sheep was 94.4%. Although the low number of sheep in the study may limit the chi-square analyses, no statistically significant difference was detected between sheep and goats (*uvrC*= $p<0.022$).

DISCUSSION

M. agalactiae is an important bacterial pathogen that primarily affects small ruminants, mainly sheep and goats, and causes contagious agalactiae syndrome. *M. agalactiae* is spread primarily through direct contact and contaminated milk, and infected animals often become chronic carriers that can shed the pathogen intermittently (Migliore et al., 2024). It is emphasised that live attenuated vaccination is successfully applied in Türkiye, where the disease

is endemic (Dudek et al., 2022). The disease is particularly prevalent in the East and Southeast of Türkiye, where small ruminant farms are dense and considered an important part of the agricultural economy (Ocak et al., 2023). According to the literature, the disease agent is endemic in many provinces of Türkiye, affecting small ruminant herds, and its seroprevalence varies between 5% and 50%. In addition, studies show that infection rates are higher in traditional farming than in modern farming. This is due to factors such as management practices and biosecurity measures applied in the enterprises (Jaye et al., 2021). This study used molecular methods to investigate the presence of *M. agalactiae* in small ruminants with clinical symptoms in Bitlis and its region. No data on the disease agent in this region existed before. Therefore, the data obtained from this study are considered important epidemiological data about the circulating disease in the region.

There are similar studies in Türkiye. In a study conducted in Mersin to determine *M. agalactiae* from goats with mastitis complaints by molecular and bacteriological methods, the identification of 10 (8%) isolates as molecular methods confirmed *Mycoplasma* spp. and all isolates (100%) were confirmed to be *Mycoplasma* spp. by 16S rRNA PCR. Of the 10 isolates identified based on the genus, 7 (70%) were identified as *M. agalactiae* as a result of *M. agalactiae* 16S rRNA-specific PCR (Uluganligil, 2019). In another study conducted in Elazig, haematological and biochemical parameters were determined in goats with clinical signs of contagious agalactiae caused by *M. agalactiae*. Although the concentrations of biochemical parameters were emphasised in the study, it was reported that *M. agalactiae* was detected as positive by PCR (Kizil & Ozdemir, 2006). In a study investigating the presence of infectious agalactiae in the Isparta and Afyonkarahisar regions in 2018, *Mycoplasma* spp. was bacteriologically isolated from only 3 nasal swabs (1.03%). PCR was performed with the *polC* gene region for *M. agalactiae* for 3 isolates isolated as *Mycoplasma* spp. but the agent could not be detected (Karatekeli, 2020). A study conducted on sheep and goats from Bursa, Balıkesir, Çanakkale, and Edirne provinces aimed to determine the presence of contagious agalactia disease using bacteriological and molecular methods. It was determined that 29 (8,55%) isolates were positive for *Mycoplasma* spp. of these, 25 (7.37%) were identified as *M. agalactiae*. In molecular diagnosis, it was reported that 9.14% were positive for *M. agalactiae* as a result of *polC*-PCR. When PCR findings were compared with bacteriological findings, it was reported that five milk samples and one lung sample were positive for *M. agalactiae* by *polC*-PCR but negative by culture. According to *polC*-PCR results, 14.19% of milk samples, 13.33% of joint fluid samples, 2.72% of eye swab samples, and 50% of lung samples were positive. As a result of the study, it was emphasised that *M. agalactiae* was the primary causative agent of infectious agalactiae (Göçmen et al., 2015). A previous study conducted in the same provinces detected the uvrC gene region in 22 (9.4%) of 234 samples. This research confirmed the presence of both *M. agalactiae* and other *Mycoplasma* species in goats, highlighting the necessity for implementing effective control strategies against infectious agalactia and various *Mycoplasma* species throughout Türkiye (Göçmen et al., 2016). In another study in the Ağrı-Diyadin region, the seroprevalence of ewes during the lambing period was investigated. As the results of the study, it was reported that 1 (0.207%) of the 482 blood samples taken was seropositive and belonged to a 6-year-old ewe showing mastitis symptoms (Yaşar, 2008).

Our study is not similar to those of Kizil and Ozdemir (2006) and Yaşar (2008). The reason for this difference seems to be using serological methods in other studies. Because it is known that molecular methods, such as PCR, are more sensitive than serological methods (Balachandra et al., 2021). In addition, classical bacteriological isolation and identification methods were not used in our study. The studies of Göçmen et al. (2015), Uluganlıgil (2019), and Karatekeli (2020) are relatively similar to the method used in our study. However, there are differences in the positive rates detected in these studies. For example, in Uluganlıgil (2019), the fact that only five different herds were collected in Uluganlıgil (2019) explains the low positive rate and suggests that this and the other two studies may be due to the fact that the disease agent circulates at a lower rate in the west (Ocak et al., 2023). It is also challenging to culture *Mycoplasma* bacteria. For example, the use of antibiotics in the diseased animal or the inability to bring the suspicious sample to the laboratory under suitable conditions are seen as disadvantages in isolation. In addition, our study's absence of positive results from swabs and blood samples may be attributed to the limited sample size. The exclusive detection of positive results in milk samples clearly demonstrates that the pathogen is present in milk from clinically

symptomatic animals and is actively shed through this medium.

CONCLUSION

These findings have important implications for animal health and regional livestock management, as *M. agalactiae* is the primary causative agent of infectious agalactia, which can cause significant economic losses in small ruminant farming. Further studies are needed to determine the prevalence of the agent in the region. The relatively high prevalence rate detected in this initial survey highlights the need for comprehensive surveillance programmes and the development of targeted control strategies. Further epidemiological studies are needed to better understand the distribution patterns, risk factors, and economic impacts of *M. agalactiae* infections in the Bitlis region, which will be crucial for implementing effective disease prevention and control measures. Future studies aim to expand the epidemiological data and provide more detailed information about the pathogen using whole genome sequence (WGS) analysis.

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ACKNOWLEDGMENTS

Author contributions: AK and KA designed the study. AK and KA performed laboratory studies. KA performed statistical analysis. KA and AK participated in drafting and revising the manuscript. AK: Atanur Koçyiğit, KA: Kadir Akar

Financial Disclosure: This study was supported by Van Yuzuncu Yil University BAP Coordination Unit with project number TYL-2023-10814.

Conflict of Interests: The authors declared that there is no conflict of interests.

Additional information: All authors have read and agreed to the published version of the manuscript Correspondence and requests for materials should be addressed to KA.

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