Detection of *Mycoplasma* spp. and *Mycoplasma bovis* DNA in Mastitic Cow Milk Samples by PCR

Ocak, F.,[1,a] Avsever, L.,[2,b] Turkyilmaz, M. K.[3,c] and Turkyilmaz, S.[4,d,*]

[1]Department of Biology, Basic and Industrial Microbiology, Manisa Celal Bayar University, Manisa, TÜRKİYE.
[2] Department of Plant and Animal Production, Akhisar Vocational High School, Manisa Celal Bayar University, Manisa, TÜRKİYE.
[3] Department of Animal Science, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, TÜRKİYE.
[4] Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Aydin, TÜRKİYE.
Orcid: [a]: 0000-0002-6301-4480, [b]: 0000-0003-2959-663X [c]: 0000-0002-7600-2390, [d]: 0000-0002-1363-4534.

* **Corresponding author:** Dr. S. Turkyilmaz, Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Isikli, Aydin Türkiye. E-mail: sturkyilmaz@adu.edu.tr

ABSTRACT

Mycoplasma mastitis is a highly contagious disease of dairy cattle that generally does not respond to treatment, adversely affect milk yield as well as animal health, causing significant economic losses. Therefore, rapid and reliable identification of this pathogen is required to develop control strategies on farms. One of the most important mycoplasma agents causing mastitis is *Mycoplasma bovis*, an invasive agent. In this study, it was aimed to identify *Mycoplasma* spp. and M. *bovis* DNA by PCR, which are important mastitis pathogens but often neglected, in cow milk with mastitis. For this purpose, 312 milk samples with mastitis, 84 with clinical and 228 with subclinical mastitis, were investigated from 17 farms. DNA extraction from milk samples was carried out using the phenol chloroform method. Identification at the species and genus level were performed by polymerase chain reaction (PCR). In PCR, primers targeting 16S rDNA for *Mycoplasma* spp. and *uvr*C gene target regions for *M. bovis* were used. *Mycoplasma* spp. and *M. bovis* DNA were detected in milk samples at a rate of 19.6% (61/312) and 13.8% (43/312) respectively from 11 farms. The rate of *M. bovis* among all mycoplasmas was determined as 70.5% (43/61). Isolation of mycoplasmas, which are the causative agents of mycoplasma mastitis, by classical conventional methods tends to be long and laborious. Where mycoplasmal mastitis is suspected, bacterial DNA detection by PCR may be an ideal way to make a diagnosis in a short time. However, in order to develop accurate treatment strategies, it would be beneficial to examine all mycoplasma agents along with not overlooking other pathogens that could lead to mastitis.

Keywords: Cow Mastitis; *Mycoplasma bovis*; PCR; Milk.

INTRODUCTION

Mycoplasma species have recently attracted attention as an important mastitis pathogen, in addition to being a respiratory pathogen and polyarthritis agent in cows (1). Among the *Mycoplasma* species, *Mycoplasma bovis* is the species that is the most prominent cause of mycoplasma mastitis in cows. The first report of *M. bovis* in mastitis was made in the USA in 1961 (2). It was named *Mycoplasma agalactiae* var. *bovis* due to its similarity to *Mycoplasma agalactiae* in small ruminants and was renamed *M. bovis* in 1976 (3). *M. bovis* has caused significant economic losses by causing mastitis in dairy cows (4). It has been reported that the annual economic loss due to *M. bovis* in the USA is around 108 million dollars (5). The morbidity of *M. bovis* mastitis is high (70%), and animal sales between countries has placed an important role in the spread of the disease worldwide (6,7). In Türkiye, 15 mil-

lion cattle were imported into the country in 2015 resulting in a rapidly increasing rate of infection (8). Mastitis due to mycoplasma is an important problem frequently encountered in dairy farms in Türkiye. In recent years, there has been an increase in the incidence of mastitis due to mycoplasmas along with an increase in herd size in different regions of our country. The lack of an effective treatment option against mycoplasmas increases the importance of prevention and control programs (9).

Staphylococcus spp., *Streptococcus* spp. and *Escherichia coli*, which are easy to isolate and identify, are detected as the main mastitis agents in many routine diagnostic laboratories. For this, general media such as blood agar are used and incubation period is limited to 1-2 days at most and incubators with aerobic conditions are used in order to produce the agent in a short period of time and then to perform an antibiogram test. Under these conditions, mycoplasma species that cannot be produced before 7-10 days in a specific medium and in a CO_2 environment would be overlooked (5,10). Prolonged culture of mycoplasma may result in late or sometimes false negative reports. Ideally, in cases of mastitis, mycoplasma species should be taken into account and isolate the causative agent (1,5). However, in general, it may take upto two weeks to identify the pathogens that cause mycoplasmal mastitis by culture of bacteria. The presence of mixed infections or the inability to perform biochemical tests for related species can always cause problems. For these reasons, although the culture method is accepted as the gold standard in the identification of mammary gland pathogens, the technique is regarded as tedious, time-consuming and sometimes inefficient (5). To overcome identification problems such as growth failure of bacteria, the use of polymerase chain reaction (PCR) in conjunction with direct bacterial DNA isolation from milk samples may be the most practical way ahead (11). Where, mycoplasma DNA can be detected in a short time by PCR from milk, an effective treatment can be applied immediately by using appropriate and effective antibiotics (12). The disadvantage of this may be the use of unnecessary antibiotics (13), as finding DNA of mycoplasma in milk does not nessarily prove that it is a main factor in a case of mastitis (11).

In recent studies, it was determined that *M. bovis* mastitis prevalence has increased. For example, in a study conducted in Türkiye, 172 isolates were obtained from dairy farms in seven geographical regions and it was determined that 87.6% (149 isolates) of them were *M. bovis* (14). In a study conducted in the USA, mycoplasma species were investigated in 214518 samples taken from 2757 dairy cattle and it was determined that the most common species (75.1%) was *M. bovis* (15).

Since there is no study in recent years on the prevalence of mastitis caused by *Mycoplasma* spp. in bovine farms in western Türkiye, our knowledge on this subject is somewhat limited. In this study, we aimed to detect *Mycoplasma* spp. and *M. bovis* DNA by PCR in cow milk with mastitis.

MATERIAL AND METHODS

Ethical Statement

In the application made to the Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University, it was stated that according to Article 8 of the Animal Experiments Local Ethics Committee Directive, ethics committee approval is not required for clinical applications for milking, diagnosis and treatment.

Sampling

In this study, 312 milk samples from Holstein cows (84 with clinical mastitis (CM) and 228 with subclinical mastitis (SCM)) from 17 farms in western Türkiye were collected over a period of approximately one year (January 2021 to January 2022). Milk samples were taken from animals that had not been treated with antibiotics for at least two weeks prior to sampling. The age of the cows from which the material was taken varied between 3-11 years of age and the number of cows in each farm varied between 22-41. Automatic milking was used in all farms.

For subclinical mastitis screening, California Mastitis Test (CMT; Bavivet CMT Liquid, Kruuse®) was applied after udder cleaning in dairy farms. Transactions and interpretations were made as previously described (10). Clinical mastitis was defined by the presence of inflammatory symptoms (swelling, warmth, redness, and pain) of the udder and/or changes in color, fluidity and odor of the milk during routine examination of the mammary lobes just prior to milking by veterinarians.

While collecting milk, the first few drops of milk were discarded after cleaning the nipples with 70% alcohol. Approximately 5-10 ml of milk sample taken from the single udder lobe was sent to the laboratory under aseptic conditions under cold chain conditions on the same day.

Table 1. All primers used in this study.

Primer	Target Gene	Sequence (5'-3')	T_m (°C)	Amplicon (bp)	Reference
16S20 F	16S rRNA	AGAGTTTGATCCTGGCTCAG	58.4	1371	(18, 19)
16S1391 R		GACGGGCGGTGTGTACAA	58.4		
Myco16SF772	*Mycoplasma* spp.	GGGAGCAAACAGGATTAGATACCC	57.6	269	(20)
Myco16SR1041		TGCACCATCTGTCACTCTGTAACCT	57.9		
MycouvrCF364	*M. bovis*	TTACGCAAGAGAATGCTTCA	47.6	171	(20)
MycouvrCR545		TCATCCAAAAGCAAAATGTTAAA	46.3		

Determination of Milk Samples Containing Aerobic Bacterial Agents

Milk samples were centrifuged at 3500 rpm for 5 minutes and the supernatant was discarded. The sediment was vortexed and a loopful was inoculated onto blood agar supplemented with 7% defibrinated sheep blood (Merck 1.10886, Germany) and MacConkey agar (Merck 1.05465, Germany). Plates were incubated aerobically for 48 hours at 37°C. DNA extraction was performed on 312 milk samples, 168 with bacterial growth and 144 without bacterial growth, which were examined by classical conventional methods.

DNA Extraction from Milk Samples

Phenol-chloroform-isoamyl-alcohol extraction method was used for DNA extraction. For this purpose, 400 µl of post-centrifugation milk samples was mixed with 400µl of denaturation solution containing Na guanidine thiosionate, trisodium citrate dihydrate, N-lauryl sarcosine and mercaptoethanol and vortexed for 15 seconds. 300µl of acid phenol and 300µl of chloroform/isoamylalcohol (49/1) were vortexed for 15 seconds. One-hundered microliters of 3M Na acetate was added to the mixture. The mixture was centrifuged at 12,000 rpm for 10 minutes. Seven hundred micrliters of the supernatant was taken and transferred to clean eppendorf tubes. Seven hundered microliters of isopropyl alcohol cooled at -20^{0}C was added and the samples were vortexed for 15 seconds. Thereafter the mixture was maintained at -80^{o}C for 1 hour for the precipitation of the nucleic acids. After the samples were thawed at room temperature, they were centrifuged at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was discarded and 300 µl of 70% ethanol was added to the pellet, which had settled at the bottom of the tube, and centrifuged at 12,000 rpm for 3 minutes. After centrifugation, the supernatant was discarded and the samples were left to dry at 37^{0}C. Fifty microliters of sterile distilled water was added to the dried samples (16). Three microliters of supernatant were used as template DNA in each PCR reaction.

Molecular Detection

DNA purity and quantity control: Three microliters of supernatant were used as template DNA in each PCR reaction. DNA purity and quantity controls were also performed. The ratio of OD260/OD280 was found to be between 1.6-2.0, indicating that the DNA was pure (17). With the performed electrophoresis of raw DNA as a measure of quality and quantity on 1% agarose gel, the presence of DNA bands in the UV transilluminator was investigated.

Primers: Firstly, the bacterial presence and DNA extraction by amplification of the 16S rRNA gene (18,19) was confirmed. Later, species-level identifications were confirmed by PCR. In the PCR assay, primers targeting 16S rDNA for *Mycoplasma* spp. and *uvr*C gene target regions for *M. bovis* were used (20) (Table.1.).

To amplify the genes, 30 µL of reaction mixture was prepared containing 2 mM $MgCl_2$, 0.4 mM of each of the four dNTPs, 0.1 mM oligonucleotide primers, 1.5 U Taq polymerase (Fermentas, Massachusetts, USA) and 20 ŋg template DNA. The prepared tubes were loaded in the thermal cycler (Boeco, Hamburg, Germany). The DNA was amplified using the following protocol: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation (95°C for 30 seconds), annealing for 30 seconds 54°C and extension (72°C for 1 minute), with a single final extension for 7 minutes at 72°C. On electrophoresis, a 2% agarose gel stained with Safe View (100 ml/6 µl) (ABM, Richmond, Canada) was used and the gel was exposed to 100 volts for 45 minutes. After electrophoresis, the gel was placed in the chamber of the transilluminator device, which was connected

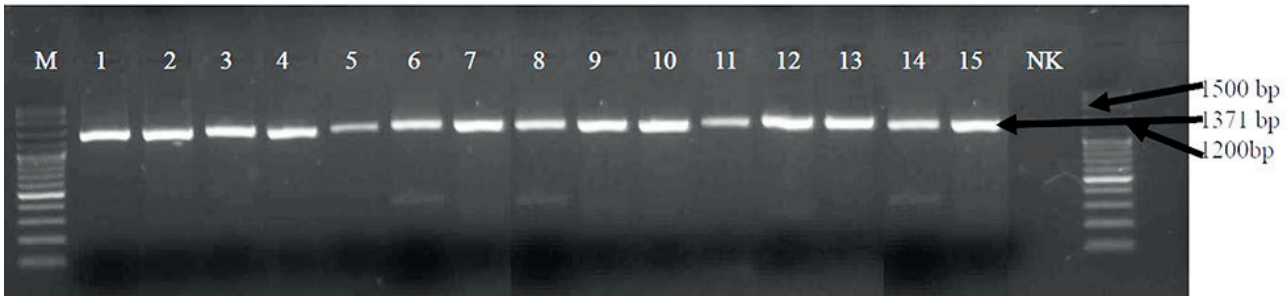

Figure 1. PCR performed by using 16S rRNA universal primers. **1-14:** 16S PCR performed with the DNA extracted directly from milk samples by phenol-chloroform method **15:** Positive Control (*M. bovis* NCTC 10131) **NK:** Negative Control (without DNA mastermix), M: Marker (100 bp DNA Ladder, Fermentas).

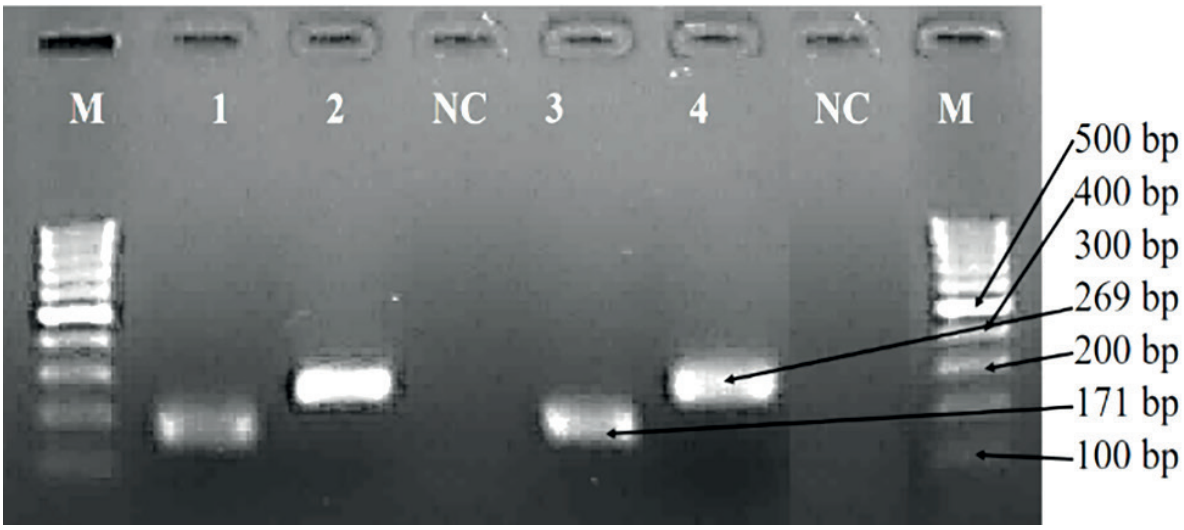

Figure 2. Gel electrophoresis image of *M. bovis* and *Mycoplasma* spp. positive DNA **1:** *M. bovis* (171 bp) DNA positive field isolate **2:** *Mycoplasma* spp. (269 bp) positive field isolate **3:** Positive Control *Mycoplasma* spp. (*M. bovis* NCTC 10131) (171 bp) **4:** Positive Control *Mycoplasma* spp. (*M. bovis* NCTC 10131) (269 bp) **NC**: mastermix without DNA 3. **M**: 100 bp DNA Ladder, (Fermentas, USA).

to the computer and photographed under UV light (Vilbert Lourmat, Collegien, France). When the amplified product formed a band of the expected size (Table 1.), it was assumed to carry the gene examined. In PCR, DNA of *M. bovis* NCTC 10131 strain was used as positive control and without DNA mastermix as negative control.

RESULTS

DNA Extraction

After PCR, using 16S universal primers, 1371 base bp long bands were obtained from 94.2% (294/312) milk samples (all 168 milk samples with bacterial growth and 126 of 144 samples without bacterial growth) (Figure 1.). Thus, it was apparent that DNA extraction from milk with the phenol-chloroform isoamylalcohol method was performed correctly.

Molecular Detection

DNAs obtained by DNA isolation from milk samples by phenol-chloroform isoamylalcohol method were subjected to PCR coupling with the species-specific primer pairs of *Mycoplasma* spp. and *M. bovis*. Amplification products were detected with molecular sizes of approximately 171 bp and 269 bp, which are considered indicative for *M. bovis* and *Mycoplasma* spp., respectively (Figure 2).

Mycoplasma spp. and *M. bovis* DNA were detected in 19.6% (61/312) and 13.8% (43/312) of all milk samples where 5.8% (18/312) were mycoplasma species DNAs other than *M. bovis*. The positivity rate of *M. bovis* DNAs among

Table 2. Detection of *Mycoplasma* spp. and *M. bovis* in clinical and subclinical mastitis cases.

	CM (n=84) (%)	SCM (n=228) (%)				Total number of milk samples with mastitis (n=312) (%)
		CMT Score 1	CMT Score 2	CMT Score 3	Total SCM samples	
Mycoplasma spp.	6 (7.1)	2	1	9	12 (5.3)	18 (5.8)
M. bovis	13 (15.5)	3	4	23	30 (13.1)	43 (13.8)
Total	19 (22.6)	5	5	32	42 (18.4)	61 (19.6)

Mycoplasma spp. DNAs was 70.5% (43/61). However, mycoplasma DNA was found in 22.6% (19/84) of clinical mastitis and 18.4% (42/228) of milk samples with subclinical mastitis.

Mycoplasma spp. DNA was detected from 38 (26 *M. bovis*, 12 *Mycoplasma* spp.) of 168 milk samples with bacterial growth detected and from 23 of 144 milk samples without bacterial growth (17 *M. bovis*, 6 *Mycoplasma* spp.).

DISCUSSION

Bovine mastitis is known to be caused by more than 100 pathogens, including viruses, bacteria, fungi and mycoplasma (20). The most commonly isolated bacterial species mastitis in cows are *Streptococcus* spp., staphylococci and Gram-negative bacteria such as *Escherichia coli*. Depending on the cause, mastitis can be classified into two categories: contagious mastitis and environmental mastitis. Contagious mastitis is caused by *Staphylococcus aureus*, *Streptococcus agalactia*e and *Mycoplasma* species. The likelihood of infectious mastitis can be reduced by following appropriate hygienic measures (20).

Mycoplasma spp. are considered important infectious pathogens causing bovine mastitis in Türkiye (9) as well as world wide (2,3). The transmission of these pathogens primarily occurs during the milking period (7). In recent years, with the increase in herd sizes in different regions of Türkiye, there has been an increase in the incidence of mastitis due to mycoplasmas (9). Studies have shown that more than 10 species of mycoplasma microorganisms cause mastitis in cows. The main mycoplasma species that cause mastitis are; *M. bovis*, *M. alkalescens*, *M. bovigenitalium*, *M. arginini*, *M. canadense*, *M. californicum* and *M. dispar* (2). Among *Mycoplasma* species, *M. bovis* is responsible for the majority of mastitis outbreaks in dairy herds (5). Cows of all ages, including non-lactating cows, are susceptible to an intramammary infection caused by *Mycoplasma* spp. (1). Despite this, mycoplasmas are often ignored in the diagnosis and treatment of mastitis. The antibiotics used are generally among the drug groups that affect the cell wall structure of bacteria, however, they are are ineffective against mycoplasmas which do not possess a cell wall. This causes both the inability to treat the mammary gland and results in the progression of the disease, as well as the development of antibiotic resistance to bacteria (1,5).

On the other hand, isolation of mycoplasma species from milk is quite difficult because mycoplasma species have very complex reproductive requirements and working with mycoplasma requires experienced personnel. Therefore, perhaps the ideal way would be to demonstrate mycoplasma DNA in milk and then immediately begin specific and beneficial treatment (7). In previous studies, it was reported that PCR can provide higher sensitivity (96.2%) and specificity (99.1%) compared to microbiological culture (21). However, it should be kept in mind that isolation is the gold standard method in bacterial diagnosis and it should be noted that dead agents could also be detected by PCR (11). This is often regarded as a herd problem. On the other hand, mycoplasma strains isolated from milk are less likely to be environmental pathogens. *M. bovis* is considered a contagious pathogen for cattle. This disadvantage can be eliminated by discarding the first milk when taking the sample. Normal udder cleansers are alcohol-based and do not destroy DNA.

It has been reported that the treatment of mycoplasma mastitis is extremely difficult, especially because of the multi-antibiotic resistance that mycoplasmas develop (7,22). Therefore, environment and milking hygiene are very important (22,23). Because the agent is highly contagious, animals with PCR positive should also be quarantined, and separate caregivers and separate equipment should be used (24). PCR should also be preferred as a rapid and reliable test method for detecting and removing mycoplasma-positive animals from the herd.

Mastitis caused by mycoplasmas are generally highly contagious and unresponsive to antibiotic therapy (25,26).

In this study, the cows from which the material was taken were not receiving antibiotics at least two weeks before. However, cows on farms from which milk samples were taken frequently developed reinfection. In this case, it was estimated that fungal mastitis or antibiotic resistance was common in cows from which material is taken.

Mycoplasma spp. isolation is quite difficult even under ideal conditions. Collection and transport method, nutritional requirements of the pathogen, number of live mycoplasmas in clinical materials (1,5,6). Gonzalez and Wilson reported that an incidence of 0.5-35.0% of *M. bovis* as the causative agent of bovine mastitis in USA (24). In a recent study in Southern Türkiye, the isolation rate from clinical mastitis was reported to be 8.2% (27). In our study, *Mycoplasma* spp. was detected in 61 (19.6%) and *M. bovis* was detected in 43 (13.8%) of 312 milk samples with mastitis. Among the isolated mycoplasmas, the *M. bovis* rate was 70.5% (43/61). Our study is compatible with other studies in Türkiye in terms of both the density of mycoplasma species in mastitis and the predominance of *M. bovis* among mycoplasma species (11,27,28).

Mycoplasma isolation rates vary from region to region in Türkiye. In a study conducted in 2016, it was reported that mycoplasma isolation from milk in the Aegean region of Türkiye was lower than in many other regions. The highest isolation rate was reported in the Eastern and Southeastern Anatolia regions (29). The reason for this may be that the dairy enterprises in the Aegean region were more modern and hygienic conditions were better than the Eastern and Southeastern Anatolia regions. However, in this study conducted in the Aegean region, we found the detection rate of mycoplasma in milk with mastitis was 19.6%, which indicates that mycoplasma species are also spreading in herds in the Aegean Region with the decline in hygiene standards.

Mycoplasmal mastits may be seen as clinical mastitis or subclinical mastitis (1). In this study, mycoplasma DNA was detected from 22.6% (19/84) of cows with clinical mastitis and 18.4% (42/228) of cows with subclinical mastitis. Although the difference between clinical and subclinical mastitis in this study is quite small, it is consistent with the finding that mycoplasmas are mostly seen in clinical mastitis cases (1,10,12).

In conclusion, this study determined that mycoplasma-induced mastitis is a significant problem in Holstein dairy farms in western Türkiye, with *M. bovis* being the main cause of these infections. The PCR method appears to be a suitable option for rapid detection of mycoplasma DNA; however, further research on more practical and user-friendly DNA extraction methods would be beneficial. Developing and implementing faster and more effective DNA extraction protocols in such studies could facilitate early diagnosis of mycoplasma infections and expedite treatment processes. Additionally, strict adherence to hygiene and preventive measures on dairy farms is crucial in preventing the spread of mycoplasma infections.

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CONFLICT OF INTERESTS STATEMENT

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript. Original work and is not under review at any other publication.

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