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A phylogenetic analysis of *Mycoplasma* strains circulating in sheep pneumonia in the Kars region of Turkey

Olcay ÖZTÜRKLER[1], **Salih OTLU**[2,*]

[1]Institute of Health Sciences, Kafkas University, Kars, Turkey

[2]Department of Microbiology, Faculty of Veterinary Medicine, Kafkas University, Kars, Turkey

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Abstract: This study aimed to investigate *Mycoplasma* species by cultural and molecular methods in 250 pneumonics and 30 healthy appearance sheep lungs brought from slaughterhouse and butcheries in the Kars region of Turkey. The phylogenetic positioning of the bacterial strains to be obtained in the present study was another remarkable outcome of the study. *Mycoplasma* isolation was achieved solely in 26 (10.4%) of 250 sheep lungs with pneumonia. *Mycoplasma ovipneumoniae* was found predominant species in 12 (46.15%) of samples and that was followed by *Mycoplasma arginini* in 4 (15.38%) of strains. Moreover, two samples were found to be co-infected with the aforementioned *Mycoplasma* species and 8 were found negative. The partial sequence analysis of the 16S rRNA gene of 22 (84.61%) isolates emerged that 11 of the isolates were *M. ovipneumoniae* and 4 were *M. arginini*. Two mixed samples were further identified as *M. arginini* by the sequence analysis. Five isolates that were not identified by cultural and PCR procedures were identified as *M. bovigenitalium* by sequence analysis. The evolutionary analysis of the 16S rRNA sequence yielded three main clusters as *M. ovipneumoniae*, *M. arginini,* and *M. bovigenitalium* which took part in the Hominis group that was previously described in Mycoplasma phylum. The inter se proximity of the main clusters was thus described polyphyletic for *M. ovipneumoniae* and *M. arginini* and monophyletic for *M. bovigenitalium*. However, in the general view, *M. arginini* and *M. bovigenitalium* isolates had two distinct phylogenetic positions, but their proximity to each other was higher than those obtained for *M. ovipneumoniae* isolates.

Key words: Mycoplasma, sheep, pneumonia, 16S rRNA, sequence analysis, phylogeny

1. Introduction

Respiratory system diseases in small ruminants are common in many countries of the world and cause major economic losses in enterprises. Sudden animal deaths take place on the top among these losses. In the United States, 4.8% of sheep deaths and 12.6% of lamb deaths were reported to be caused by respiratory system infections such as pneumonia and shipping fever. In addition to sudden deaths in respiratory system diseases, productivity losses due to persistent infections, discarding and subsequent slaughtering of animals, increased labor and operational management costs, and protection and control expenses are regarded as among the other important economic losses [1].

The etiology of respiratory system diseases in small ruminants is multifactorial and highly complex. *Mannheimia haemolytica* infections, which are called pneumonic pasteurellosis, are particularly important in neonates and sheep. *Pasteurella* spp., *Actinomyces* spp., *Haemophilus* spp., *Staphylococcus* spp., *Streptococcus* spp. and *Mycoplasma* spp. are other bacterial agents that cause infection in sheep [2,3]. Although the role of *Mycoplasma ovipneumoniae* in Mycoplasmas is often overlooked, this bacterium may be the primary agent in infections or predisposes to other bacterial and viral infections. *M. ovipneumoniae* infections, called atypical pneumonia or enzootic pneumonia, progress slowly and form a chronic disease. It is known that other species such as *Mycoplasma arginini, Mycoplasma mycoides* subsp. *capri* and *Mycoplasma agalactiae* cause similar diseases [4,5].

In Mycoplasma infections, clinical manifestations may range from mild to severe respiratory infections due to the accompanying environmental and biological factors. The common clinical manifestations in affected sheep are chronic, soft and persistent cough, and eye and nasal discharges. After a few weeks of infection, the entire herd may be affected, and the disease is rarely fatal and ends without diagnosis [5]. Nonetheless, by taking appropriate samples, diseases in animals can be diagnosed by various laboratory methods [3].

* Correspondence: salihotlu@gmail.com

Mycoplasma-related respiratory system infections have been reported in sheep which are more resistant to Mycoplasma infections than goats in terms of animal species sensitivity. Re-evaluation of Mycoplasma infection emerges a particular necessity in countries as Turkey and some Middle East countries where mixed animal farming is engaged. Data on the etiology of atypical pneumonia in sheep in the Kars region and the prevalence of Mycoplasma infections are limited. Therefore, in this study, it was aimed to learn more about the roles of Mycoplasma agents in pneumonia cases in sheep, to determine the phylogenetic relationship and global position of the bacterial species to be isolated and to give special emphasis to Mycoplasma pneumonia.

2. Material and methods

2.1. Study material

The legal permission of this study was obtained from Kafkas University Animal Experiments Local Ethics Committee with a code of 2018/004. The material of this study, which was carried out as a modified cross-sectional study, consisted of 250 pneumonic sheep lungs obtained from slaughterhouse and butcheries between 2017 and 2018 from sheep farms in Kars province and its districts. Thirty healthy sheep lungs were evaluated as a control group. Pneumonic lung samples were taken as 10×10 cm sections containing macroscopic lesions during sheep slaughtering and the samples were examined by cultural methods in a short time.

2.2. Isolation and identification of Mycoplasma species from lung samples

The surface of the lung was burned with a superheated spatula to remove superficial contamination. Samples were taken from the living tissue in deep and transferred to Falcon tubes containing 5 mL Mycoplasma Broth which kept in an ice mold. The tissue fragment was homogenized with a homogenizer (Daihan, HG-15D) for 30 s and the supernatant obtained with brief centrifugation (at 3000 rpm for 5 min) was used for cultural purposes. 0.1 mL homogenate was plated on Mycoplasma agar base and Mycoplasma broth base media. The plated media were incubated in microaerobic conditions at 37 °C for 5–7 days. Following the incubation, the growth of the solid medium was examined by a stereomicroscope (Nikon SMZ-10A, Nikon Inc., Tokyo, Japan). On the other hand, turbidity was evaluated as a possible bacterial growth in broth culture. The growths of both media were purified by subculturing on a Mycoplasma agar base medium.

2.3. Molecular identification

The identification of *Mycoplasma* species isolated from lung samples was performed by PCR and sequence analysis. DNA extraction of the isolates was performed by the heat treatment method by using a single colony lysis buffer (SCLB) [6]. For this purpose, a single bacterial colony was transferred to PCR tubes containing 40 µL of SCLB. The samples were heat-treated in a thermal cycler cycle at the following temperatures (10 min at 80 °C, 10 min at 55 °C, and 10 min at +4 °C). Then, 80 µL nuclease-free water was added to the tubes, and approximately 50 µL supernatant formed after the centrifugation at 7000 rpm for 2.5 min was collected as template DNA.

2.4. PCR analysis

Identification of Mycoplasmas at the genus and species level was performed by PCR analysis which provides amplification of the 16S rRNA gene regions. For this purpose, GPO3 (5'-TGGGGAGCAAACAGGATTAGATACC-3') and MGSO (5'-TGCACCATCTGTCACTCTGTTAACCT C-3') [7] primers for genus-specific identification; LMF1-F (5′-TGAACGGAATATGTTAGCTT-3′) and LMF1-R (5′-GACTTCATCCTGCACTCTGT-3′) primers for identification of *M. ovipneumoniae* and MAG (5′-GCATGGAATCGCATGATTCCT-3′) and GP4 (5′-GGTGTTCTTCCTTATATCTACGC-3′) [8] primer for identification of *M. arginini* were used. PCR reactions were adjusted in a volume of 25 µL for each sample as follows; 2.5 µL 10xPCR buffer, 0.5 µL dNTP mix (10 mM), 3 µL $MgCl_2$ (25mM), 1 µL primer F (10 pmol), 1 µL primer R (10 pmol), 0.4 µL Taq polymerase (250 U), 3 µL template DNA and 13.6 nuclease-free water. The thermal cycle was carried out as follows; 5 min initial denaturation at 94 °C followed by 35 cycles consisting of 30 s denaturation at 94 °C, 30 s primer binding at 55 °C, 60 s elongation at 72 °C, and 5 min final elongation at 72 °C. Amplification of the 16S rRNA gene region was performed with 2 primers (27F- 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R-5'-GGTTACCTTGTTACGACTT-3') to be used in the sequence of this region of *Mycoplasma* strains obtained from cultural surveys, genus and species-specific PCR analyzes. The reaction components were adjusted as in genus-specific PCR. The thermal cycle was carried out as follows; 5 min initial denaturation at 95 °C followed by 30 cycles consisting of 15 s denaturation at 94 °C, 30 s primer binding at 59 °C, 45 s elongation at 72 °C and 5 min final elongation at 72 °C. Analysis of the amplified products was performed on horizontal gel electrophoresis containing 1.5% agarose gel. As a result of the electrophoretic analysis, the presence of 270 bp products was evaluated as *Mycoplasma* spp., 361 bp products as *M. ovipneumoniae,* and 545 bp products as *M. arginini.* After PCR analysis of the 16S rRNA gene region to be used for sequence purposes, the presence of 1465 bp of amplified products was considered.

2.5. Sequence analysis

Sequence analysis of *Mycoplasma* isolates was performed in Erciyes Technopark of Erciyes University (Kayseri,

Turkey). The 16S rRNA gene region was sequenced on the ABI 3500 Genetic Analyzer using the BigDye Terminator v3.1 Cycle Sequencing Kit in the presence of primers (27F and 1492R) used for PCR amplification of this region. Analysis of the obtained sequences was performed with the CLC Main Workbench 7.7.3 (Qiagen) program and the data were compared with *Mycoplasma* species in NCBI GenBank. *Mycoplasma* species names were given according to the similarity scores obtained from NCBI GenBank by BLAST analysis [9].

2.6. Statistical analysis

The results of agent isolation, molecular identification, and sequence analysis of pneumonic sheep lungs were presented and interpretation of the values was recorded as a percentage.

3. Results

3.1. Isolation and identification results

Mycoplasma suspected agent was isolated from 26 (10.4%) of 250 pneumonic lung samples examined by cultural analysis. Mycoplasma isolation could not be performed from 30 sheep lung samples of the control group. The colonies formed on solid media were examined under a stereomicroscope at 40× magnification. The first and simple identification of *Mycoplasma* species was conducted with the presence of centered or decentralized, regular, or irregularly terminated granular colonies called "fried eggs."

3.2. PCR results

Genus-specific PCR identified 26 isolates (100%) as *Mycoplasma* spp. (Figure 1, Table 1). Of the 26 isolates identified as *Mycoplasma* spp. after cultural analysis and genus-specific PCR, 12 (46.15%) were identified as *M. ovipneumoniae* and 4 (15.38%) were identified as *M. arginini* by species-specific PCR (Figure 2). *M. ovipneumoniae* and *M. arginini* were determined simultaneously in two (7.69%) of the samples. Eight isolates (30.76%) identified as *Mycoplasma* spp. were found negative for the presence of *Mycoplasma* species (*M. ovipneumoniae* and *M. arginini*) (Table 1). The 16S rRNA gene region was amplified in 22 (84.61%) of 26 isolates identified as *Mycoplasma* spp. by cultural analysis and PCR (Figure 3). The 16S rRNA gene region could not be amplified in 3 *Mycoplasma* spp. and 1 *M. ovipneumoniae* strain (Table 1).

3.3. Sequence results

The 16S rRNA gene region of 22 *Mycoplasma* isolates was amplified and subsequently sequenced. Following the sequencing, *Mycoplasma* species names were given according to a ≥ 99% similarity score obtained from NCBI GenBank by BLAST analysis. Eleven *M. ovipneumoniae* and 4 *M. arginini* isolates identified by PCR were confirmed with sequence analysis. The two samples in which *M. ovipneumoniae* and *M. arginini* were determined simultaneously were identified as *M. arginini* by sequencing. Five isolates identified as *Mycoplasma* spp. were identified as *M. bovigenitalium* after the sequence analysis. In summary, after sequence analysis of the 16S rRNA gene region, 11 (50%) of 22 isolates were identified as *M. ovipneumoniae*, 6 (27.27%) were identified as *M. arginini* and 5 (22.72%) were identified as *M. bovigenitalium* (Table 1).

3.4. Phylogenetic analysis results

In the dendrogram generated by neighboring joining method using sequence data of the 16S rRNA gene region of 22 *Mycoplasma* strains, it was seen that the strains were divided into three main clusters as *M. ovipneumoniae* (n = 11), *M. arginini* (n = 6) and *M. bovigenitalium* (n = 5). Out of the 11 *M. ovipneumoniae* strains, 10 (MYCO1,

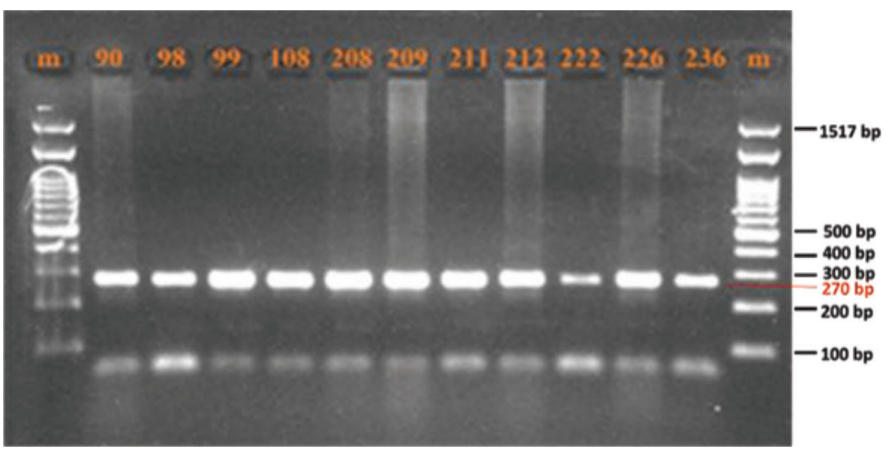

Figure 1. Gel electrophoresis image of amplified products of genus-specific PCR. *Mycoplasma* spp. yielded 270 bp amplified product. Marker: 100 bp DNA Ladder (NEB, N3231S).

Table 1. PCR and sequence-based *Mycoplasma* identification results of culture-positive samples.

Strain code	Genus-specific PCR	Species-specific PCR	Sequence Result	Strain name	NCBI accession number
73	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO1	MK789475
75	*Mycoplasma* spp.	*M. arginini*	*M. arginini*	MYCO2	MK789476
77	*Mycoplasma* spp.	Negative	*M. bovigenitalium*	MYCO3	MK789477
78	*Mycoplasma* spp.	Negative	*M. bovigenitalium*	MYCO4	MK789478
79	*Mycoplasma* spp.	Negative	*M. bovigenitalium*	MYCO5	MK789479
80	*Mycoplasma* spp.	Negative	*M. bovigenitalium*	MYCO6	MK789480
81	*Mycoplasma* spp.	*M. arginini*	*M. arginini*	MYCO7	MK789481
85	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO8	MK789482
87	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO9	MK789483
88	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO10	MK789484
90	*Mycoplasma* spp.	*M. arginini* and *M. ovipneumoniae*	*M. arginini*	MYCO11	MK789485
98	*Mycoplasma* spp.	*M. arginini* and *M. ovipneumoniae*	*M. arginini*	MYCO12	MK789486
99	*Mycoplasma* spp.	*M. arginini*	*M. arginini*	MYCO13	MK789487
108	*Mycoplasma* spp.	Negative	*M. bovigenitalium*	MYCO14	MK789488
208	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO15	MK789489
209	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO16	MK789490
210	*Mycoplasma* spp.	Negative	Absent	Absent	Absent
211	*Mycoplasma* spp.	*M. arginini*	*M. arginini*	MYCO17	MK789491
212	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO18	MK789492
222	*Mycoplasma* spp.	Negative	Absent	Absent	Absent
224	*Mycoplasma* spp.	Negative	Absent	Absent	Absent
226	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO19	MK789493
234	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO20	MK789494
236	*Mycoplasma* spp.	*M. ovipneumoniae*	Absent	Absent	Absent
249	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO21	MK789495
250	*Mycoplasma* spp.	*M. ovipneumoniae*	*M. ovipneumoniae*	MYCO22	MK789496

MYCO8, MYCO9, MYCO10, MYCO15, MYCO16, MYCO18, MYCO20, MYCO21, MYCO22) showed great homogeneity within their cluster, however, they took place in different branches with the sequences obtained from GenBank. *M. arginini* strains, which constitute the other cluster of the dendrogram, were divided into 2 groups in terms of their proximities. MYCO13 and MYCO17 strains representing the first group were closest to each other, whereas these 2 strains were in the same branch with MYCO7 strain. The second group consisted of MYCO2 and MYCO12 strains close to each other and these were found in the same branch with MYCO11 strain. The last phylogenetic group consisted of *M. bovigenitalium* strains. While 4 of these strains (MYCO3, MYCO4, MYCO5, MYCO14) showed a great homogeneity within the cluster, these 4 strains were found in the same branch with MYCO6 strain (Figure 4).

4. Discussion

Sheep breeding is a promising livestock branch that provides important contributions to the national economy with its rapid reproduction and growth potential. Increasing the quality and quantity of the products to be obtained from this sector makes the sheep industry sustainable. Many infectious diseases can lead to loss of product quality and quantity in sheep breeding and thus adversely affect this sector. Among these diseases, diarrhea, perinatal lamb deaths and respiratory system diseases cause the most significant economic losses [10–12]. Respiratory system infections account for 5.6% of all infections in small ruminants and are characterized by diseases such as rhinitis, laryngitis, pneumonia, and pleurisy. However, pneumonia is the most important of the respiratory disorders which play a critical role in terms of prevalence, prognosis, and economic losses. Pneumonia,

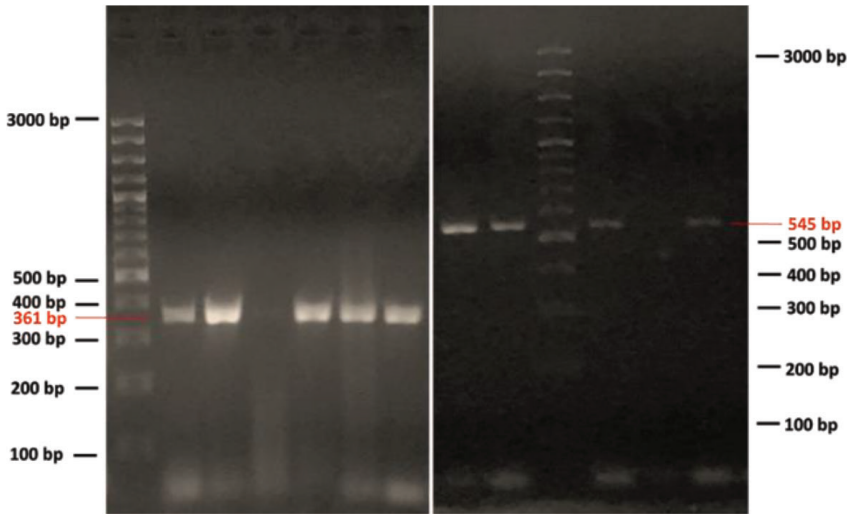

Figure 2. Gel electrophoresis image of amplified products of species-specific PCR. *M. ovipneumoniae* with 361 bp amplified product and *M. arginini* with 545 bp amplified product. Marker: GeneRuler 100 bp plus DNA Ladder (ThermoFisher Sci., SM0322).

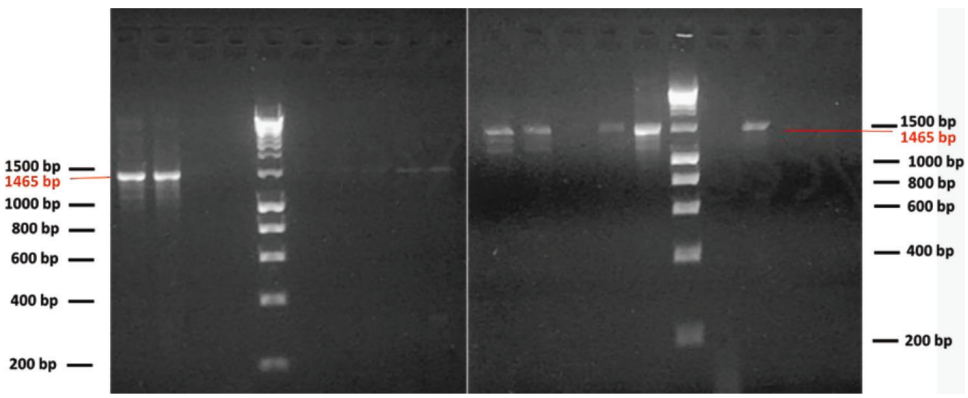

Figure 3. Gel electrophoresis image of the 16S rRNA gene region which yielded 1465 bp products. Marker: HyperLadder 1kb (Bioline, BIO- 33053).

a disease complex caused by an interaction between susceptible host, pathogen, and environmental factors, is caused by a virus, parasite, and bacterial pathogens and may exhibit a multifactorial character [13–14]. *M. haemolytica*, *P. multocida,* and *M. ovipneumoniae* are the most common bacterial agents causing pneumonia in sheep and lambs [15]. *Mycoplasma* species can take part as a primary [16,17] or secondary agent [2,18] in pneumonia cases. *Mycoplasma* infections have been reported in 56%–79% of sheep pneumonia depending on the region, animal breed, and age [2,19,20]. On the other hand, although it has been reported that the pathogenic *Mycoplasma* species should not exist in the upper respiratory tract flora, but they have been also reported in healthy animals [2,18]. The prevalence of Mycoplasma pneumonia in sheep in Turkey is relatively less compared to other studies [3,21,22]. In this study, *Mycoplasma* was isolated from 26 (10.4%) of 250 sheep lungs with pneumonia taken from slaughterhouse and butcheries in the Kars region. The isolation rate has been quite low when compared to the isolation rates from pneumonic sheep lungs around the world [2,19,20]. This may be due to the different circumstances in the geographies studied and the current country-specific prevalence of Mycoplasma pneumonia including our country which moves about 5.5% to 24.69% [3,21,22]. In this context, the most recent study of Mycoplasma-induced sheep pneumonia in Kars province was conducted by Otlu [3] and reported an isolation rate of 24.69%. Although the low isolation rate in this study is similar to those obtained by Otlu [3] in the Kars region, it should be considered that the periods studied are different and may be due to the decreasing incidence of Mycoplasma pneumonia in parallel with increased protection and control measures in that period.

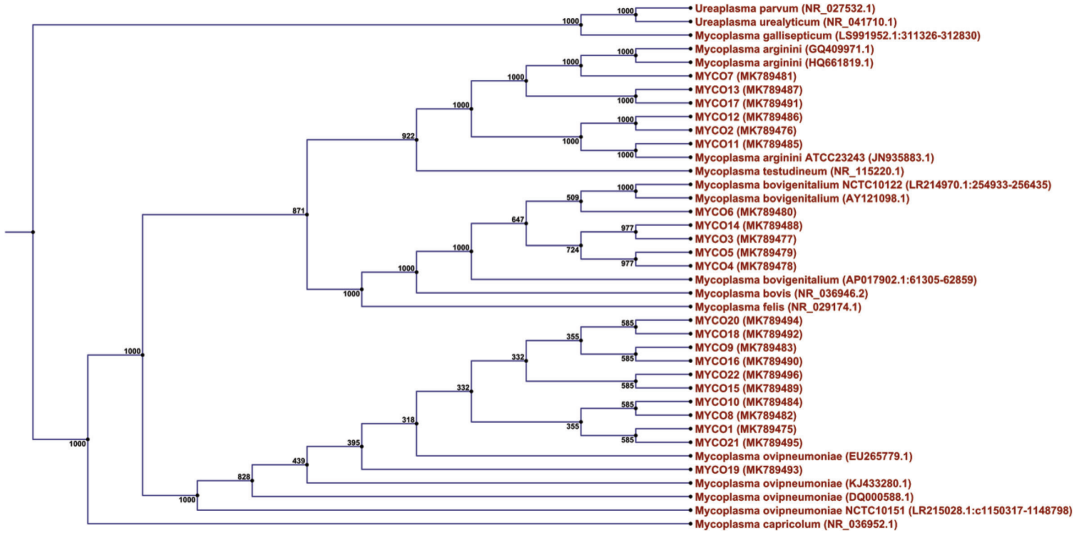

Figure 4. The dendrogram and evolutionary relationships generated by the neighboring joining method for 22 *Mycoplasma* strains analyzed according to the sequences of the 16S rRNA gene region. GenBank access numbers of the strains are indicated in parentheses.

As known, among the *Mycoplasma* species, the primary responsible agent for sheep pneumonia is *M. ovipneumoniae*. In a study in southern Norway, Bakke [18] examined the samples of 126 pneumonics and 83 healthy lungs and he isolated *M. ovipneumoniae* as the primary agent in 87% of the lungs with pneumonia and 6% of the healthy lungs. The aforementioned species predominancy was reported by Pasic and Popovic [20] (49.29%) and Sheehan et al. [23] (73.33%). Whether *M. arginini* is a pathogenic agent in sheep pneumonia is controversial. This agent is known to exacerbate pneumonia caused by the other microorganisms [24,25]. Nevertheless, many pneumonia studies have been reported in which *M. arginini* is detected as pathogen [3,26,27]. While Güler [26] determined 72.52% *M. arginini* from lamb, sheep, and goat lungs, Otlu [3] reported that *M. arginini* was the predominant species with a rate of 70.5% in sheep pneumonia and *M. ovipneumoniae* was the second most common species with 29.5%. In this study, *M. ovipneumoniae* was identified as the dominant species in a total of 14 (53.84%) isolates, followed by *M. arginini* with 6 (23.07%) isolates. Although the different isolation rates of these two species, which constitute the main *Mycoplasma* species found in the lungs of the sheep with pneumonia in Kars region, they are similar in general to the Mycoplasmas detected in sheep pneumonia. Anyway, additional tests may be needed for their role and the controversial pathogenicity [24,25] of *M. arginini*, all have already isolated from pneumonic sheep lungs, as an etiological factor in the cases. Two samples that were found to be coinfected with *M. ovipneumoniae* and *M. arginini* were reidentified as *M. arginini* after 16S rRNA sequencing. While this indicates a coinfection in the pneumonic lung [23,28], it was interpreted as DNA is not selected purely to represent a single bacterium and relatively higher quality and concentrated DNA, that probably of *M. arginini*, was used in sequencing. Besides, *M. bovigenitalium* species were identified from 5 pneumonic sheep lungs. These species, which were excluded from the evaluation in PCR analysis since it was not predictable at the beginning of the study, could only be identified after 16S rRNA sequence analysis. This is in line with recent reports [29,30] of increased isolation of *M. bovigenitalium* from both experimental and natural genital tract infections and mastitis in sheep and goats. Moreover, it can be emphasized that this is the first international report on sheep pneumonia caused by *M. bovigenitalium*. In Kars, where cold and long winter conditions prevail, the habit of raising different animal species together in the same farms can be speculated as a predisposing factor in the transmission of *M. bovigenitalium* to sheep.

PCR and DNA sequencing techniques are used as an alternative to cultural methods in the diagnosis of *Mycoplasma* agents. The availability of bacterial 16S rRNA gene sequences in databases and the ability to amplify these protected regions with various universal primers has made this gene region a popular target in many molecular and sequence applications [31]. There are many studies on diagnosis and phylogenetic analysis of *Mycoplasma* agents using the 16S rRNA gene region in sheep and goats. Azizi et al. [32] have succeeded in identifying *M. ovipneumoniae* and *M. arginini* from pneumonic sheep lungs with the 16S rRNA-based PCR technique. Kalshingi et al. [30] isolated 34 *Mycoplasma* spp. from healthy and sick sheep (vulvitis and balanitis) and identified the *Mycoplasma* species by

PCR, cloning, and gene sequence analysis. Abdel Halium et al. [27] performed a partial sequence analysis of the 16S rRNA gene region of *M. arginini* and *M. ovipneumoniae* strains isolated from sheep. In this study, sequence analysis of the 16S rRNA gene region was achieved in 22 (84.61%) out of 26 isolates which were identified as *Mycoplasma* spp. by cultural and PCR analysis. *Mycoplasma* species identified by the PCR method and the species identified after the sequence analysis of the 16S rRNA gene region showed great agreement. Also, it was determined that the 16S rRNA sequence analysis has allowed the detection of *Mycoplasma* species (*M. bovigenitalium*) whose sheep are not the primary host. The 16S rRNA sequence analysis showed that the isolates had high (≥ 99.9) sequence similarity with the reported strains from different species and took part in the same clusters with foreign strains around the world. It is not surprising that the strains obtained in this study coincided with the phylogenetic groups and showed similarities with the strains from different countries that are not geographically close to Turkey. This is due to the lack of sequence analysis and country-specific phylogenetic clusters of the *Mycoplasma* strains isolated in Turkey. That is, with the identification of such new bacterial genome sequences, country-specific phylogenetic clusters will be created, bacterial genealogies can be revised and it will be possible to reinterpret these similarities.

The first phylogenetic classification of Mycoplasmas was made in 1989 based on the 16S rRNA gene region and 5 groups named Anaeroplasma, Asteroplasma, Hominis, Pneumonia, and Spiroplasma were defined [33]. Two new groups, Entomoplasma and Mesoplasma, were added to these later. Hominis phylogenetic group is the largest group with more than 20 species and hosts the main *Mycoplasma* species responsible for the infections in the field of Veterinary Medicine. Of these, *M. ovipneumoniae* takes part in the *M. neurolyticum* cluster of the Hominis group; *M. arginini* is in the subcluster of *M. alcalescens* and *M. bovigenitalium* is in the subcluster of *M. bovis*. Although *M. bovigenitalium* and *M. ovipneumoniae* belong to the same group, these are phylogenetically partially distant bacteria. Maniloff [34] reported a different phylogenetic classification for Mycoplasmas consisting of α, β, γ, δ branches. Among these branches, α and β- Mycoplasmas have the closest homology, whereas these are in the same cluster with γ- Mycoplasmas. δ- Mycoplasmas constitute the most distant group. All branches include important human and animal pathogens such as *M. bovis*, *M. bovigenitalium*, *M. agalactiae* and *M. meleagridis* in α- Mycoplasmas; *M. hominis* and *M. arginini* in β- Mycoplasmas; *M. ovipneumoniae* and *M. conjunctivae* in γ- Mycoplasmas and *M. pneumoniae*, *M. gallisepticum* and *M. iowae* in δ- Mycoplasmas. Ten *M. ovipneumoniae* strains identified in this study had a great homogeneity among themselves, while the sequence obtained from GenBank showed a polyphyletic branching (Figure 4). These 10 strains are similar to *M. ovipneumoniae* strain (EU265779.1) obtained from the pneumonic lamb of American wild sheep raised in Hells Canyon of USA. One *M. ovipneumoniae* strain (MYCO19) with the paraphyletic feature was in the same branch as *M. ovipneumoniae* (KJ433280.1) isolated from Musk ox in Norway. The other phylogenetic group of the study consisted of *M. arginini* strains and was divided into 2 groups with a polyphyletic distribution. MYCO13 and MYCO17 were the closest members of the first group, while these 2 strains were found in the same branch with MYCO7 strain, which is close to *M. arginini* strain (HQ661819) isolated from vaginal swab samples of Dorper sheep in South Africa and *M. arginini* strain (GQ409971) of unknown origin from England. The second group consists of MYCO2 and MYCO12 strains which are close to each other and MYCO11 strain in the same branch. The latter was similar to the *M. arginini* strain (ATCC23243) isolated from pneumonic sheep lungs (Figure 4). The other phylogenetic group included *M. bovigenitalium* strains. Four of these strains (MYCO3, MYCO4, MYCO5, MYCO14) showed a monophyletic distribution. MYCO6 strain, which shows a paraphyletic distribution, was in the same branch as *M. bovigenitalium* (AY121098) and *M. bovigenitalium* (NCTC10122) strain isolated from bovine genital system in 1947 (Figure 4). In summary, 22 *Mycoplasma* strains identified in this study were included in Hominis phylogenetic group by dendrogram formed according to 16S rRNA sequence analysis and it was found that they were collected in three main clusters: *M. ovipneumoniae*, *M. arginini,* and *M. bovigenitalium*. When these clusters were examined, *M. arginini* and *M. bovigenitalium* strains were found to have 2 different phylogenetic positions, but their proximity to each other was higher than those obtained for *M. ovipneumoniae* isolates. This is similar to the homology between *M. bovigenitalium* in α- Mycoplasmas and *M. arginini* in β- Mycoplasmas reported by Maniloff [34].

In conclusion, as a result of isolation and identification studies from 250 pneumonic sheep lung samples obtained from slaughterhouse and butcheries in the Kars region, *Mycoplasma* agents were identified in 26 of them and the prevalence of Mycoplasma was determined as 10.4%. Considering the negative effects of Mycoplasma-induced respiratory tract infections on sheep breeding, the isolation rate obtained from this study is significant. The other remarkable aspects of the study are the use of agent isolation method, which is still used as the gold standard in the diagnosis of many bacterial infections, the smooth confirmation of the strains by PCR and the findings obtained by these methods which are in great agreement with the results of modern diagnostic and typing method, sequence analysis. It is thought that increasing such phylogenetic studies, which could be

predicted to be integrated with conventional methods, will be beneficial in effective diagnosis and taxonomic studies on Mycoplasmas.

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Conflict of interest
The authors declare no competing or financial interests.

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