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Original Article

Isolation of Mycoplasma spp. from Geese with Pneumonia and Identification of Microbial Isolates via Molecular Methods

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■Keywords

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ABSTRACT

This study aimed to investigate *Mycoplasma* species in the lungs of 500 geese with pneumonia from the Kars region (Turkey) via cultural and molecular methods. The samples were cultured on Frey's Broth and Agar media. To identify *Mycoplasma* species a Growth Inhibition Test was used. The identification was continued with species-specific PCR and sequence analysis which provide amplification of the genes *dnaX*, *pcrA*, *rpoB,* and the sequence of the 16S rRNA gene, respectively. In addition, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from pneumonic lung samples were directly analyzed via Multiplex Real-time PCR. As a result, 51 *Mycoplasma* strains were isolated and 32 were identified as *Mycoplasma anatis*, 9 as *Mycoplasma anseris*, 5 as *Mycoplasma cloacale* and 3 as *Mycoplasma anserisalpingitis*. Two *Mycoplasma* isolates that could not be identified were grouped in the same branch as a result of 16S RNA sequencing and their nearest neighbour was found to be *Mycoplasma* sp. 2045 (GenBankNo.MK615061.1). *M. gallisepticum* DNA was detected in 3 pneumonic lung samples and *M. gallisepticum*/*M. synoviae* DNAs were found simultaneously in 1 sample. While some *Mycoplasma* species identified in this study consolidated their place as pneumonic agents, some increased their potential to become a pneumonic agent when compared with cases caused by well-recognized *Mycoplasma* strains. Two isolates were identified as -*Mycoplasma* spp. as their 16S rRNA gene sequence identity levels scored below the threshold of 98.7% for species demarcation and still need to be defined whether they are possible representatives of a novel *Mycoplasma* species.

INTRODUCTION

Mycoplasma species have been defined as the microorganisms that lead to several clinical infections, primarily reproductive system infections, peritonitis, airsacculitis, pneumonia and sudden death in poultry. *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are responsible for respiratory system infections, arthritis and embryonal deaths in chicken and turkeys, the latter of which results from the widespread *in ovo* transmission property of the bacteria. In waterfowls such as geese and ducks, *Mycoplasma anatis*, *Mycoplasma anseris*, *Mycoplasma cloacale* and *Mycoplasma anserisalpingitis* (formerly called *Mycoplasma* sp. 1220) are common pathogenic *Mycoplasma* species (Stipkovits & Szathmary, 2012; Otlu, 2016; Grózner *et al.,* 2019b; Gyuranecz *et al.,* 2020). These *Mycoplasma* species, together with other infectious microorganisms and environmental factors, play an important role in the immune system of geese. They cause certain pathological lesions and clinical symptoms due to differences in their pathogenesis (FAO, 2002). Respiratory system infections caused by *Mycoplasma* species progress with nasal discharge, wheezing, coughing and have a moderate mortality rate (5-9%) in geese

of 1-2 weeks of age, while arthritis is added to these symptoms at 3-4 weeks of age and mortality increases partially. In adult geese, a regression of respiratory system infections is observed with access to fresh air. Infertility, embryonal deaths, cloaca and phallus infections join to respiratory system infections that re-emerged during the laying period. In geese of almost all ages, these clinical symptoms are accompanied by pathological lesions such as pneumonia, tracheitis, air sac inflammation, peritonitis and salpingitis (Otlu, 2016).

While *M. anseris* causes classic respiratory tract infections observed in geese, *M. anatis* also causes neurological disorders. *M. cloacale* leads to infertility and causes a decrease in egg production. *M. anserisalpingitis* was first detected in the early 1980s from the inflammation of the cloaca and phallus, and its prevalence gradually increased in the following years (Stipkovits *et al.,* 1986; 1987). Such reproductive diseases caused by *M. anserisalpingitis* leads to significant economic losses in geese breeding (Stipkovits & Szathmary, 2012). Reports on *Mycoplasma* sp. 2045 are very limited (Spergser, 2008), and the clinical significance of the microorganism in poultry infections is not yet fully known. *Mycoplasma* species may cause co-infections in poultry as well as coexist with different viruses and bacteria and negatively affect the course and prognosis of the disease (Tiong, 1990; Stipkovits & Szathmary, 2012; Samy & Naguib, 2018). *Mycoplasma* species can also be found in the normal microbiota of healthy waterfowl such as ducks and geese. Diseases caused by these microorganisms may occur due to stress factors such as insufficient housing conditions and feding, crowded breeding, or an intense laying period (Stipkovits & Kempf, 1996; de Sá *et al.,* 2015).

The definitive diagnosis of Mycoplasmosis and the identification of the constitutive microorganism at the species or variant level provide valuable data into illuminating the epidemiology of the disease, taking appropriate protection and control measures and assist vaccine development studies. In addition, if the microorganism can be cultured, many analyses permitted by living microorganisms can be performed and antimicrobial activities that will guide therapeutic applications can be tested (Grózner *et al.,* 2016). Cultural and serological methods are mostly used for the diagnosis and identification of *Mycoplasma* species. However, the use of these methods is limited by the fact that they are fragile microorganisms when it comes to reproduction in artificial environments, their cultures are time-consuming, their species cannot be distinguished easily due to their close microbiological characteristics, in addition, there is a lack of qualified antisera to be used in serological tests in routine experiments and they show low diagnostic sensitivity. Moreover, these methods are inadequate in the differentiation of the aforementioned co-infection and mixed infections (Kılıç *et al.,* 2010; Umar *et al.,* 2016).Nucleic acid-based molecular methods are widely used for more precise and practical identification at the species level and can even reveal new species and variants and their taxonomic positioning (Grózner *et al.,* 2019a, b). In addition, molecular methods can scan asymptomatic or porter animals by directly detecting the microorganism from clinical samples. In this context, conventional and real-time PCR techniques and probes that provide *in vitro* enzymatic amplification of bacterium-specific conserved gene regions such as *mgc2*, *vlhA*, *dnaX*, *rpoB*, *pcrA*, *16S rRNA*, and sequencing methods that perform the sequence analysis of nucleic acid belonging to certain microorganisms have been widely used in recent years (Ferguson-Noel *et al.,* 2012; Fraga *et al.,* 2013; Grózner *et al.,* 2019a, b).

The study aimed to investigate *Mycoplasma* species in geese with pneumonia via lung samples taken during their slaughtering in Kars (Turkey), where traditional geese breeding is a wide-held practice, via cultural and molecular methods.

MATERIALS AND METHODS

Study material

Permission from the ethics committee for this study was obtained from the Kafkas University Local Ethical Committee of Animal Experiments (KAÜ-HADYEK) via Decision No. "2018-20".

As part of the study, a total of 500 pneumonic lung samples from geese living on 12 different farms in the Kars region and slaughtered in a slaughterhouse between 2019 and 2020 were examined for the presence of *Mycoplasma* species. All pneumonic lung samples were taken per asepsis and antisepsis rules and delivered to the laboratory without disrupting the cold chain. To be used in all analyses, reference strains such as *M. anatis* (NCTC 10156), *M. anseris* (ATCC 49234), *M. cloacale* (NCTC 10199) and *M. anserisalpingitis* (ATCC BAA-2147) were obtained from Dr. Miklos Gyuranecz (the Institute for Veterinary Medical Research, Center for Agricultural Research, Hungarian Academy of Sciences) and *M. gallisepticum* S6 and *M. synovia* 1853 strains were obtained from the culture collection of the Pendik Veterinary Control Institute (Turkey).

Cultural analysis

Tissue homogenate was prepared from samples of pneumonic lungs from geese in accordance with the protocol (Öztürkler & Otlu, 2020).The homogenate was inoculated (10% rate) on; a) Frey's broth (Fluka-F6797) supplemented with 20 ml horse serum (Sigma-H1138), 1 ml 1% NAD, 1 ml 1% cysteine, 1 ml penicillin (20000 IU), 1 ml 50% glucose, 1 ml 50% pyruvate, 5 ml 4% cresol red and b) Frey's agar (Fluka-F6797) plates supplemented with 20 ml horse serum (Sigma-H1138), 1 ml 1% NAD, 1 ml 1% cysteine, 1 ml penicillin (20000 IU) and 1.5 g agar bacteriological. The plated media were incubated at 37 °C for 4-7 days in a humid environment with 5% CO_2. During the incubation period, a colour change due to the pH indicator was observed in Frey's broth and in positive cases, 10 µl of the medium was inoculated onto Frey's agar plates and left to incubate under the same conditions. At the end of this period, pure cultures of the colonies with suspected mycoplasma were subcultured 3 times onto the Frey's agar media, which were examined with 35x magnification under a stereomicroscope. A digitonin test was performed to distinguish *Mycoplasma* spp. isolates from *Acholeplasma* and a urease test to distinguish them from *Ureaplasma* species. The isolates were distinguished from L-forms by passaging them into non-inhibitor media (Tully, 1983). Species identification of the isolates confirmed as *Mycoplasma* spp. was performed via the Growth Inhibition Test (GIT) in the presence of the reference hyperimmune serums (*M. anatis*, *M. anseris*, *M. cloacale* hyperimmune sera obtained from the Pendik Veterinary Control Institute, Turkey) (OIE, 2008).

Molecular analysis

DNA extraction

DNA extraction from pure Mycoplasma cultures and reference strains was carried out via the boiling method (Barbosa *et al.,* 2016). For this purpose, 1 ml of fresh liquid culture of the bacterium was taken and centrifuged at 14.000 rpm for 30 minutes. 25 µl of Sterile PCR grade water was added to the sediment obtained after centrifugation. The tubes were boiled in a hot water bath for 10 minutes, and then kept on the ice mould for 10 minutes. Samples kept in the oven at 37 °C until they were completely dissolved were then centrifuged at 14.000 rpm for 5 minutes, and the supernatant containing DNA was collected and stored at -20 °C until it was used in the PCR analysis. DNA needed for the Real-Time PCR analysis was extracted from the lung samples using a commercial kit (DNeasy Blood & Tissue Kits, Qiagen) and as per the manufacturer's instructions.

Species-specific PCR

Species-specific PCR methods and primers that provide amplification of *dnaX*, *pcrA*, *rpoB* gene regions were used for identifying the species of the *Mycoplasma* isolates (Grózner *et al.,* 2019b).A PCR reaction was prepared uniformly for each *Mycoplasma* species and the reaction volume was set as 25 µl consisting of 2 µl template DNA, 2.5 µl 10XPCR buffer (Qiagen, HotStarTaq DNA Polymerase, Germany), 2 µl $MgCl_2$ (25 mM), 0.5 µl dNTP mix (10 mM), 2 µl primer F, 2 µl primer R (10 pmol / µl), 0.125 µl HotStarTaq DNA polymerase (5 U/µl), and the remaining volume was made up of nuclease free water. The PCR was carried out with a common thermal cycle consisting of initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, binding for 1 min at 61 °C and an extension for 1 min at 72 °C. The reaction was finalized with a final elongation stage at 72 °C for 5 minutes. Amplified products were stained with Xpert Green DNA dye (Grisp, Portugal) and run in a 1% agarose gel electrophoresis system and viewed under UV. Ultrapure water was used as a negative control matrix to monitor cross contamination. The amplification quality of the extracted Mycoplasma DNA was tested using species-specific PCR primers and certain amplicons were evaluated.

Real-Time PCR

The direct analysis of *M. gallisepticum* and *M. synoviae* on pneumonic lung samples obtained from geese was performed via the IDEXX RealPCR MG/MS Multiplex Real-time PCR. The Real-time PCR mix for each sample in 35 µl volume was set with a 20 µl reaction mix (10 µl *M. gallisepticum*/*M. synoviae* DNA mix and 10 µl DNA master mix), 5 µl negative control, 5 µl positive control and 5 µl DNA. The Real-time PCR was performed on the LightCycler 96 (Roche, Switzerland) device. The Real-time PCR protocol was carried out with pre-denaturation at 95 °C for 1 minute, 45 cycles consisting of denaturation at 95 °C for 15 seconds and amplification at 60 °C for 30 seconds. FAM for *M. gallisepticum* and CY-5 for *M. synoviae* were selected as reporter dyes. HEX-labeled internal control (IC) was used, which provides sample-borne inhibition control and kit reagent control. Samples that produced an amplification curve and had a threshold (Cq) value of > 38 were considered positive.

Genus-specific PCR and Sequencing

The sequence analysis of the *Mycoplasma* strains was performed at the Pendik Veterinary Control Institute of the Ministry of Agriculture and Forestry of the Republic of Turkey. The 16S rRNA gene region amplified by *Mycoplasma* genus-specific PCR was used for the sequence analysis. The PCR analysis of this region was performed in the presence of primers GPO-3 (5'-GGGAGCAAACAGGATTAGATACCCT-3') and MGSO (5'-TGCACCATCTGTCACTCTGTTAACCTC-3'), which provides 280 bp amplicon (Van Kuppeveld *et al.,*1992). A PCR reaction with a total volume of 25 µl for each sample was consisted of 2.5 µL 10xPCR buffer, 0.5 µL dNTP mix (10 mM), 3 µL MgCl2 (25 mM), 1 µL primer F (10 pmol), 1 µL primer R (10 pmol), 0.125 µl Taq DNA polymerase (5U/µl), 3 µl template DNA and 13.875 µl nuclease-free water. The thermal cycle was created from initial denaturation at 94 °C for 5 min, 35 cycles consisting of denaturation at 94 °C for 30 sec, primer binding at 58 °C for 30 sec, elongation at 72 °C for 30 sec. The reaction was finalized with the last elongation step at 72 °C 5 sec. The analysis of PCR products was performed via electrophoresis with 2% agar gel. The final PCR products obtained were purified with an agarose gel DNA extraction kit (Roche). The sequence analysis of the purified PCR products was performed at the Genetics Laboratory of the Istanbul Pendik Veterinary Control Institute using the Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystem) and ABI 3130XL DNA Analyzer. The obtained chromatogram and nucleotide sequence were analyzed by the BioEdit and Chromas programs. After the comparative analysis of the sequences via the web-based BLAST program, species identification was performed.

Phylogenetic analysis

The 16S rRNA gene sequences of the avian *Mycoplasma* type strains were downloaded from the Genbank in NCBI and aligned using CLUSTAL_W in MEGA version X (Hall, 2013). Also, *Mycoplasma* sp. strain 2045, which is the closest strain according to Genbank, was added to the phylogenetic analysis. Phylogenetic trees were constructed using neighbour-joining (NJ) (Saitou & Nei, 1987). The evolutionary distance model of Kimura's two-parameter (Nishimaki & Sato, 2019) was used to generate evolutionary distance matrices for NJ algorithms with 95% partial deletion. For NJ, the rate variation among sites was modelled using the gamma distribution. The confidence values of the nodes were evaluated by bootstrap analysis based on 1000 re-samplings (Felsenstein, 1985; Saticioglu *et al.,* 2021a, b).

Statistical analysis

The results of the agent isolation, molecular identification, and sequence analysis of the lungs of geese with pneumonia were presented, and the interpretation of these values was recorded as a percentage.

RESULTS

Phenotypic analysis findings

In this study, Mycoplasma growth was detected in 51 (10.2%) of the 500 pneumonic lung samples collected from geese on 12 different farms in the Kars region of Turkey as a result of the cultural analysis on Frey's media. All isolates were differentiated from Acheloplasmas, Ureaplasma and L-forms, thus defined as *Mycoplasma* spp.

As a result of the GIT, 32 (62.75%) of 51 *Mycoplasma* spp. were identified as *M. anatis*, 9 (17.65%) as *M. anseris* and 5 (9.8%) as *M. cloacale*. Five (9.8%) isolates could not be identified at the species level using the aforementioned test method (Table 1).

Molecular analysis findings

Species-specific PCR findings

Molecular confirmation of the phenotypically identified *Mycoplasma* species was performed by species-specific PCR that amplified the *dnaX*, *pcrA*, *rpoB* gene regions, and the identification of 32 *M. anatis*, 9 *M. anseris* and 5 *M. cloacale* species was confirmed by the PCR (Figure 1). Three (5.88%) of the 5 isolates that were defined as *Mycoplasma* spp. via phenotypic methods but could not be identified at the species level were later identified as *M. anserisalpingitis* by species-specific PCR. The two remaining isolates could not be identified by species-specific PCR (Table 1).

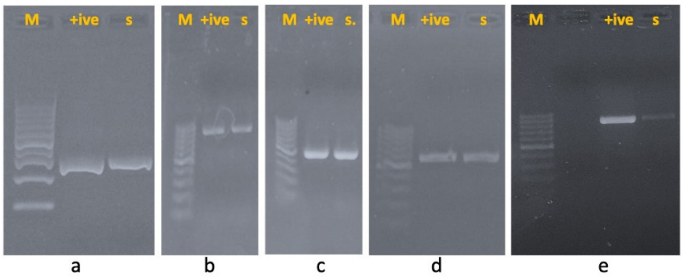

Figure 1 – Gel electrophoresis image of the PCR products representing the certain *Mycoplasma* species. PCR products of genus-specific PCR (a), *M. anatis* (b), *M. anseris* (c), *M. cloacale* (d), *M. anserisalpingitis* (e) species-specific PCR with 280 bp, 895 bp, 504 bp, 591 bp, 857 bp, respectively. M: marker, +ive: positive control (*M. anatis* NCTC10156 for 'a' and 'b', *M. anseris* ATCC 49234 for 'c', *M. cloacale* NCTC10199 for 'd', *M. anserisalpingitis* ATCC BAA-2147 for 'e'), s: field isolate.

Table 1 – *Mycoplasma* species and their identification properties.

Mycoplasma Species	Growth Inhibition Test	Species-Specific PCR	Sequence Analysis	NCBI Data				Direct Analysis of the Tissue with Real-Time PCR
				Strain Name	Accession Number	Identity Score (%)	ACNO of Bacterium Genealogized	
M. anatis	32	32	N/A	N/A	N/A	N/A	N/A	N/A
M. anseris	9	9	N/A	N/A	N/A	N/A	N/A	N/A
M. cloacale	5	5	N/A	N/A	N/A	N/A	N/A	N/A
M. anserisalpingitis	-	3	N/A	N/A	N/A	N/A	N/A	N/A
Mycoplasma sp. 2045	-	N/A	2	M4 M48	MW858371 MW858373	98.17 97.80	MK615061.1	N/A
M. gallisepticum	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3
M. gallisepticum and *M. synoviae*	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1

N/A: not applicable ACNO: accession number

Real-Time PCR findings

The direct analysis of other *Mycoplasma* species, *M. gallisepticum* and *M. synoviae*, from pneumonic lung tissue samples was performed via the Real-time PCR. *M. galliseptium* in 3 samples and *M. gallisepticum*/*M. synoviae* in 1 sample came up positive in the samples (Table 1).

Genus specific PCR/Sequence/Phylogenetic analysis findings

Genus-specific PCR followed by sequencing was applied to the 2 isolates that could not be identified via GIT and species-specific PCR. The 16S rRNA gene sequences of the isolates M4 and M48 (275 bp and 276 bp in length, respectively) were obtained and subjected to comparative analysis. The 16S rRNA gene sequence similarity of the strain M4 was found 95.60% with the *Mycoplasma cynos* strain H831. The 16S rRNA gene sequence identity level between the strains M4 and M48 was 99.64%. Two isolates were grouped in the same branch and the nearest neighbour was *Mycoplasma* sp. 2045 (MK615061.1) (Spergser, 2008) (Figure 2).

DISCUSSION

The tempting properties of food products in terms of consumption and their preference as a filler in textiles carry the geese farming sector to the forefront of poultry breeding in many countries. In Turkey, geese farming is generally grown to meet the food needs of the local population and has just begun to gain importance as a luxury food for a wide scope of consumers (Arslan, 2013). While the *Mycoplasma* species cause significant economic losses by causing pneumonia, arthritis, osteodystrophy, genital system infections and immunosuppression in geese with their horizontal and privileged vertical transmissions, the most important of these are their destructive effects on the respiratory system (Nascimento *et al.,* 2005; Dobos-Kovács *et al.,* 2009; Stipkovits & Szathmary, 2012; Otlu, 2016). In this study, *Mycoplasma* species were detected in 11% (55/500) of pneumonic lung samples obtained from geese. A direct proportional comparison could not be made since there are no similar studies on the prevalence of *M. anatis*, *M. anseris*, *M. cloacale* and *M. anserisalpingitis* in cases of geese with pneumonia. Most of these studies are case reports or sequence and taxonomic analyses of a collection of strains obtained from different periods or locations (Grózner *et al.,* 2019a, b). Nevertheless, although the prevalence of *M. gallisepticum* and *M. synoviae* is low, other *Mycoplasma* species and their prevalence are similar to those described in the aetiology of pneumonia in geese (Benčina *et al.,* 1987; Stipkovits *et al.,* 1987; Ivanics *et al.,* 1988; Stipkovits & Kempf, 1996; Ferguson-Noel *et al.,* 2012; Stipkovits & Szathmary, 2012; Grózner *et al.,* 2016).

M. gallisepticum and *M. synoviae* are the causative agents of Chronic Respiratory Disease (CRD) and Infectious Synovitis (IS), respectively, and cause infections mainly in chickens and turkeys. Lower egg yield and quality are more prominent in *M. synoviae* infections. The frequency of asymptomatic infections can reach 90%. Other bird species may also be affected by these microorganisms, but there are no clear clinical findings (Mohammed *et al.,* 1985; Lutrell *et al.,* 1996). In studies conducted in our country, *M. gallisepticum* and *M. synoviae* positivity was detected in 0.7% - 20.9% of poultry (Ülgen, 1991; Güler, 1992). In this study, frequency of *M. gallisepticum* and *M. synoviae* was lower than in other studies (Ülgen, 1991; Güler, 1992) with 0.008% and 0.002%, respectively, and even they could not be cultured in a similar way (Dakman *et al.,* 2009). However, even if they were detected via direct PCR, the presence of a *M. gallisepticum* and *M. synoviae*

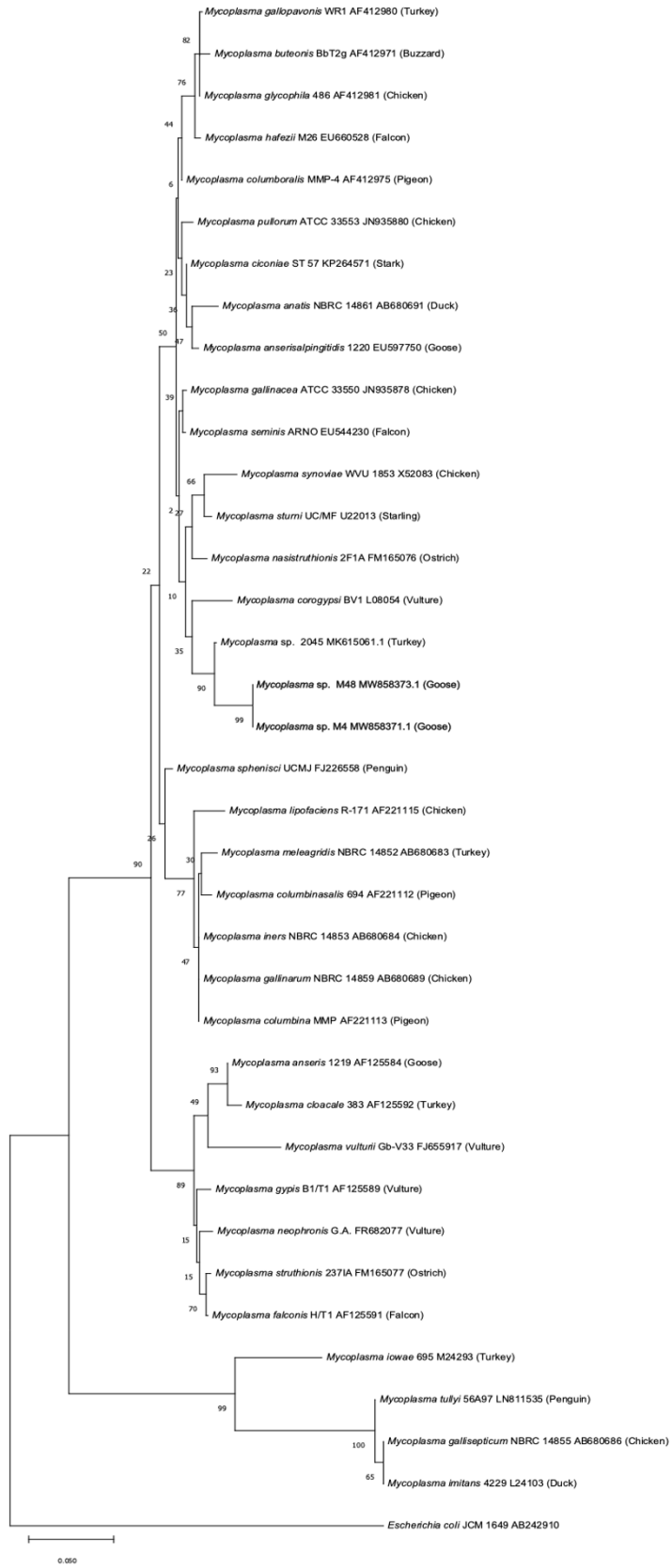

Figure 2 – The neighbour-joining phylogenetic tree constructed from 16S rRNA gene sequences showing the phylogenetic position of the strains M4 and M48 among avian *Mycoplasma* species. The numbers on the branch nodes represent the percentages of 1000 bootstrap replicates. GenBank accession numbers for the 16S rRNA gene sequences are shown after the strain name. *Escherichia coli* JCM 1649^{T}was used as an outgroup.

mixed infection was similar to what was previously reported (Bagal *et al.,* 2019). The positivity rates of *M. gallisepticum* and *M. synoviae*, which are known as the predominant species of respiratory system infections in poultry species such as chickens and turkeys, were found to be lower in geese in this study as expected, contrary to those previously reported (Ülgen, 1991; Güler, 1992). This low positivity rate may be influenced by the host specificity of *Mycoplasma* species, as well as the lower prevalence of CRD and IS infections, which are more infectious in restricted chicken and turkey populations and observed as local epidemics. This low positivity rate is an expected situation in such collection materials which were randomly generated from goose lung samples obtained from different farms during slaughtering.

M. anatis together with the other *Mycoplasma* species (*M. anseris*, *M. cloacale*, *M. anserisalpingitis*) was identified in the respiratory system, genital system and nervous system infections, and usually as co-infections in ducks. The pathology and roles of *M. anatis* in respiratory system infections (lymphohistiocytic infiltration in the lungs), of which colonization is more limited in geese, is little known (Ivanics *et al.,* 1988). In this study, the prevalence of *M. anatis* in the lungs of pneumonic geese was determined to be 6.4% (32/500) and it was the most common species among the *Mycoplasma* species with a rate of 58.18%. Considering the presence of *M. anatis* in tissues such as the oropharynx, trachea, cloaca, and oviduct in healthy geese (Benčina *et al.,* 1987; Grózner *et al.,* 2019b), it encourages us to persue further study to elucidate the role of this highly prevalent bacterium in the pathology of pneumonia. In addition, the prevalence of this *Mycoplasma* species, which is usually isolated from ducks, in cases of pneumonia in geese can be considered a result of the mixed breeding of these geese with ducks in the area of the study.

M. anseris, which was first isolated from the phallus lymph node of a male goose in Hungary (Bradbury *et al.,* 1988), gradually increased the prevalence of similar infections in geese in the following years, while cases of cloaca inflammation and airsacculitis, peritonitis and embryonal deaths have also been reported (Stipkovits & Kempf, 1996; Stipkovits & Szathmary, 2012). Unlike other pneumonic bacteria, *M. anseris* has a much less significant role in respiratory system infections. It is known that the disease incidence varies depending on herd structure and density, as well as farm hygiene. Obvious clinical symptoms (nasal discharge, coughing, dyspnea and low mortality) and pathological lesions

(pneumonia, airsacculitis and peritonitis) are limited in respiratory system infections that occur following practices such as keeping geese in a closed environment for a long time and forced feeding (Stipkovits & Kempf, 1996). In this study, the prevalence of *M. anseris* in the sampled lungs was found to be 1.8% (9/500) and ranked second among the *Mycoplasma* species defined with a rate of 16.36%. The high incidence of *M. anseris*, whose presence in clinical samples is interpreted as absolute infection (Stipkovits & Kempf, 1996; Stipkovits & Szathmary, 2012), as was the case in this study, and especially its culturability from inflamed lungs, further strengthen its place as a causative agent of pneumonia.

M. cloacale, which is not as host specific as other *Mycoplasma* species (Braburry *et al.,* 1988), has been reported in geese both in infertility cases (Stipkovits & Kempf, 1996) and in healthy animals (Benčina *et al.,* 1987; Hinz *et al.,* 1994). The presence of *M. cloacale* in geese is generally seen along with *M. anseris* and *M. anserisalpingitis* as a co-occurrence or co-infection (Hinz *et al.,* 1994). However, *M. cloacale* has not been associated with lung infections. In this study, the prevalence of *M. cloacale* in pneumonic geese was found to be 1% (5/500) and 9.09% among the *Mycoplasma* species identified. Additional studies are needed to explain the role of *M. cloacale* in the pathology of pneumonia, as within other *Mycoplasma* species.

M. anserisalpingitis leads to serious economic loss by causing inflammation in the cloaca and phallus, salpingitis, a decrease in egg production and embryonal death in geese (Stipkovits *et al.,* 1986; Dobos-Kovács *et al.,* 2009). The bacterium has also been reported in the liver, ovaries, testes and peritoneum (Volokhov *et al.,* 2020). Apart from this, *M. anserisalpingitis* has also been detected in respiratory system infections such as airsacculitis, and in the trachea and as lung inflammation in waterfowl such as geese and ducks (Stipkovits *et al.,* 1987; Grózner *et al.,* 2016). In this study, the prevalence of *M. anserisalpingitis* in pneumonic lungs was determined to be 0.6% (3/500) and 5.45% among the *Mycoplasma* species identified. A proportional comparison could not be made due to limited reporting on cases of pneumonia caused by *M. anserisalpingitis* in geese.

Literary data on *Mycoplasma* sp. 2045 is currently only one. It consisted of the information obtained from a turkey's choana sample in Austria in 2008 and contained the partial sequence analysis of the 16S rRNA gene in the NCBI database (accession number MK615061.1) (Spergser, 2008). In this study, two *Mycoplasma* strains, which could not be identified by GIT and PCR but were found identical to the *Mycoplasma* sp. 2045 strain (MK615061.1) as having 98.17% and 97.8% nucleotide similarity after the partial sequence analysis of the 16S rRNA gene region. Hence, as shown by their 16S rRNA gene sequence identity levels below the threshold of 98.7% for species demarcation with all other *Mycoplasma* species, both isolates were identified as *Mycoplasma* spp., a fact also confirmed by their phylogenetic trees (Figure 2). However, differences observed in the GPO-3/MGSO amplicons should not be overestimated since the genus-specific primers yield a small fraction of the 16S rRNA gene when compared to the whole 16S rRNA gene. For now, these isolates have been genealogized to the relevant strain in line with their similarity scores obtained from the BLAST analysis, thus polyphasic studies consisting of genotypic (especially a full-length 16S rRNA sequence of type strain), chemotaxonomic, and phenotypic methods are advised (Chun *et al.,* 2018) to introduce them into the taxonomy and give them a taxonomic position as a novel *Mycoplasma* strain. In addition, due to a lack of reports, the prevalence and pathogenicity of these *Mycoplasma* species concerned are questionable. However, the absence of other dominant or recessive *Mycoplasma* species in the lung samples where this bacterium was isolated suggests the possibility of this bacterium being a pathogenic species. The possible predisposition of other bacteria (*Pasteurella multocida*, *Avibacterium paragallinarum* and *Ornithobacterium rhinotracheale*) and viruses (Infectious Bronchitis Virus and Newcastle Disease Virus) in the respiratory tract (Van *et al.,* 2020) also strengthens this possibility. Nevertheless, conducting host or host-independent pathogenicity tests would provide valuable data to clarify these concerns.

In conclusion, the prevalence of *Mycoplasma* species identified in cases of pneumonia in geese was found to be significantly high (11%). It is seen that the species dominance in the aetiology of pneumonia of *Mycoplasma* species, which share similar chances in terms of their *in vitro* culturing, may vary from time to time. Considering the existence of these *Mycoplasma* species in classical respiratory system infections and their economic damages as well as their prevalence in embryonal deaths, phallus and cloaca infections and their vertical transmission characteristics, these will also improve the importance of studies on these species in geese with low reproductive efficiency and fertility. It shows that species-specific PCR and the 16S rRNA gene

sequencing have sufficient specificity to identify these agents in the differentiation of *Mycoplasma* species that are morphologically and biochemically very close.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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