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Mycoplasma infections in dairy cattle farms in Turkey

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Abstract: Mycoplasmas cause the most severe and economically costly diseases of cattle throughout the world. In this study, *Mycoplasma* species were isolated from calves and cows with suspected mycoplasmosis in Holstein dairy cattle farms within 7 geographical regions of Turkey between May 2010 and December 2015. Mycoplasma infections were positive in 17 (80.9%) of 21 dairy cattle farms and the overall percentage was calculated as 32.1%. The highest isolation rate occurred in the Southeastern Anatolia Region (42.8%), and the lowest was observed in the Mediterranean Region (19.6%). In total, 172 *Mycoplasma* spp. were isolated from samples. Using PCR analysis, 149 (87.6%) isolates were identified as *Mycoplasma bovis* (*M. bovis*). Eleven (6.3%) isolates were identified as *M. alkalescens*, 2 (1.1%) were *M. canis*, and 10 (5.8%) were *M. bovigenitalium*. The isolation rate was found to be increasing annually. In conclusion, mycoplasmosis is a common problem in Holstein dairy cattle farms in Turkey, and *M. bovis* is the most frequently encountered cause of mycoplasma infections. The isolation rate seems to have increased in correlation with increased live cattle imports. Additionally, *M. alkalescens* and *M. canis* were isolated and identified in respiratory tract infections in cattle from Turkey for the first time.

Key words: Cattle, *Mycoplasma bovis*, *Mycoplasma bovigenitalium*, *Mycoplasma alkalescens*, *Mycoplasma canis*, Turkey

1. Introduction

Mycoplasmas are wall-less bacteria that belong to the class Mollicutes (1). Numerous *Mycoplasma* species cause respiratory tract infections, mastitis, arthritis, genital tract infections, otitis, keratoconjunctivitis, abortus, and deaths in cattle. This leads to millions of dollars in losses in the cattle industry worldwide (1–3). Species include *M. bovis*, *M. alkalescens*, *M. bovihirnis*, *M. dispar*, *M. bovigenitalium*, *M. canadense*, and *M. mycoides* subsp. *mycoides* small colony (*M. mycoides* SC). Recently, *M. canis* was also isolated from the lungs of cattle with pneumonia in North Europe, Great Britain, and Canada. This species is known as one of the primary agents of urogenital tract infections in dogs (2,4–6).

Clinical and pathological signs are not characteristic for *Mycoplasma* infections and are often overlooked by laboratories (1). Because of the lack of cell wall in *Mycoplasma* strains, certain groups of antibiotics are ineffective. To date, there is no effective vaccine to prevent *Mycoplasma* infections (7). Control is possible through determination and exclusion of infected animals from the herd and through supply of animals from healthy herds (1). Due to cross reactions with apathogenic mycoplasmas, the credibility of serological diagnosis of *Mycoplasma* infections is low. Although culture is time-consuming and requires special media, it is the gold standard. PCRs have been used to detect mycoplasmas directly in clinical samples and to identify cultured isolates (8).

The incidence of *Mycoplasma* infections is reported to be increasing in cattle in European countries, Canada, and the USA (9). The agent is transmitted to countries that are free from infection by cattle imports (9,10). According to data from the Turkish Statistical Institute for 2015, there are approximately 14 million cattle in Turkey, and figures for cattle imports have been increasing year by year (https://biruni.tuik.gov.tr/hayvancilikapp/hayvancilik.zul). Despite this dramatic increase, there is no up to date study regarding the occurrence of *Mycoplasma* infections in dairy cattle in this country.

In the present study, *Mycoplasma* species were isolated from calves and cows with suspected mycoplasmosis in Holstein dairy cattle farms located in 7 geographical regions of Turkey between 2010 and 2015. Isolates were identified by PCR. The aim was to provide preliminary findings concerning mycoplasma infections in dairy cattle.

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2. Materials and methods

2.1. Sampling and culture

A total of 535 samples were collected from 21 different Holstein dairy cattle farms located within 7 different geographical regions of Turkey (Central Anatolia, Aegean, Marmara, Mediterranean, Southeastern Anatolia, Black Sea, and Eastern Anatolia). Samples were collected between May 2010 and December 2015 and included 67 bronchoalveolar lavage fluid (BALF) samples, 95 lung samples, and 26 synovial fluids from arthritis, which were taken from 2-week to 10-month-old calves with pneumonia. In addition, 310 milk samples and 37 uterus lavage samples were obtained from cows with mastitis and metritis, respectively (Table 1). Samples were inoculated onto Mycoplasma agar base (Oxoid, CM0401, UK), supplemented with Mycoplasma-selective supplement-G (Oxoid, SR0059C, UK), and processed according to the method described by Nicholas and Ayling (2).

2.2. DNA isolation

DNA isolation from *Mycoplasma* positive cultures was performed in accordance with the method indicated by Karahan et al. (11).

2.3. PCR analysis

Initially, PCR was performed with *Mycoplasma* spp. genus specific primers (GPF, MGSO) (12). PCR analyses were performed using 2X PCR master mix according to the manufacturer's instructions (Thermo, K0171). The 50-µL PCR mixture included 25 µL of master mix, 20 pmol of each primer, 25 ng of target DNA, and nuclease-free water. The PCR cycling parameters were as follows: initial denaturation at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 53 °C for 15 s, 72 °C for 72 s, and 72 °C for 5 min. Next 10 µL of the PCR products was separated by ethidium bromide (5 µg/mL, Sigma) agarose gel (1.5%, w/v) electrophoresis with a 100 bp DNA ladder (Solis Biodyne, Estonia). An amplification product of 1013 bp was indicative of the *Mycoplasma* spp. For positive samples, PCRs were performed with *M. bovis* (13), *M. alkalescens*, *M. bovihirnis*, *M. bovigenitalium* (14), *M. dispar* (15), *M. canadense* (16), *M. mycoides* SC (17), and *M. canis* (2) specific primers. A list of the primers used in the study is given in Table 2.

2.4. Statistical analysis

The statistical analyses were performed using SPSS version 12. The *Mycoplasma* isolation rate comparisons between BALF and lung samples of calves were performed using the chi-Squared test at $P < 0.05$.

3. Results

In total, 172 (32.1%) *Mycoplasma* spp. were isolated from 535 samples including 26 BALF, 40 lung, 17 synovial fluid, 73 milk, and 16 uterus lavage. *Mycoplasma* infections were positive in 17 (80.9%) of 21 dairy farms, and isolation rates ranged from 4.3% to 60%. The overall percentage was calculated as 32.1% (172 of 535). The highest isolation rate occurred in the Southeastern Anatolia Region (42.8%), and the lowest was seen in the Mediterranean Region (19.6%). The highest frequency for isolation was obtained from the synovial fluids of calves with arthritis (65.3%).

All culture positive isolates were confirmed as *Mycoplasma* spp. by genus specific PCR analysis. In species specific PCR analysis, 149 (87.6%) of 170 isolates were identified as *M. bovis*, which were obtained from 23 BALF, 30 lung, 17 synovial fluid, 73 milk, and 6 uterus samples. Of 170 isolates, 11 (6.4%) were identified as *M. alkalescens*, 2 (1.1%) were *M. canis*, and 10 (5.8%) were *M. bovigenitalium*, which were obtained from respiratory samples of calves and uterus samples (Table 3). *M. bovis* was isolated from both synovial fluids and BALF samples from 15 calves. In a few cattle (n = 4), *M. bovigenitalium* and *M. bovis* were simultaneously isolated from uterus and milk samples, respectively. *Mycoplasma* isolation was

Table 1. Regions for sampling.

Farm location (region) (n = farm number)	Samples					
	Milk	Lung	BALF	Synovial fluid	Uterus lavage	Total
Central Anatolia (n = 5)	55	30	32	12	12	141
Aegean (n = 3)	32	19	17	7	0	75
Marmara (n = 3)	35	12	2	2	6	57
Mediterranean (n = 3)	48	2	4	0	2	56
Southeast Anatolia (n = 3)	50	12	5	4	6	77
Black Sea (n = 2)	52	9	5	0	8	74
Eastern Anatolia (n = 2)	38	11	2	1	3	55
Total	310	95	67	26	37	535

Table 2. Primer sequences for the detection of *Mycoplasma* species used in PCR.

Mycoplasma strains	Sequence (5'→3')	Annealing temperature	Fragment size (bp)	References
Mycoplasma spp.	**GPF** - F - GCTGGCTGTGTGCCTAATACA **MGSO** - R - TGCACCATCTGTCACTCTGTTAACCTC	56 °C	1013	(12)
M. bovis	F - TATTGGATCAACTGCTGGAT R - AGATGCTCCACTTATCTTAG	59 °C	348	(13)
M. alkalescens	F - GCTGTTATAGGGAAAGAAAACT R - AGAGTCCTCGACATGACTCG	60 °C	704	(14)
M. bovihirnis	F - GCTGATAGAGAGGTCTATCG R - ATTACTCGGGCAGTCTCC	60 °C	316	
M. bovigenitalium	F - CGTAGATGCCGCATGGCATTTACGG R - CATTCAATATAGTGGCATTTCCTAC	60 °C	312	
M. dispar	F - TTAAAGCTCCACCAAAAA R - GTATCTAAAGCGGACTAAA	53.6 °C	433	(15)
M. canadense	F - ACACCATGGGAGCTGGTAAT R - CTTCATCGACTTTCAGACCCAAGGCAT	55 °C	150	(16)
	NESTED - F - GTTCTTTGAAACTGAAT **NESTED** - R - GCATCCACCAAAAACTCT			
M. mycoides SC	F - CTAAAGAGCTTGGAGTTCAGTG R - CCAGCTCAACCAGCTCCAG	62 °C	1100	(17)
M. canis	F - TGATGATTAGCTGATAGTAGAACT R - GATTTGCTTGACGTCGCCGTT	60 °C	400	(2)

determined to be increasing annually, and the highest isolation rate was 30% (51 of 190) in 2015 (Figure).

4. Discussion

Previous studies showed that mycoplasmas have important roles in different infections in cattle (18–20), especially in calf pneumonia and mastitis outbreaks. Nicholas and Ayling (1) reported that mycoplasmas are responsible for at least one quarter to one third of pneumonia cases in calves. In different studies, the corresponding rate was between 40% and 100% (20,21).

In the present study, mycoplasmas were detected at a rate of 32.1% (n = 172) in different samples. In a previous study, Karahan et al. (11) investigated lungs, eye swabs, nasal swabs, and milk samples (n = 148) in Eastern Turkey. They isolated *M. bovis* from 23% of samples in three different farms and concluded that *M. bovis* is relatively common in the eastern region of the country. Similarly, the *Mycoplasma* isolation rate in the present study was 23.6% (n = 13) in two different farms in the Eastern Anatolia Region. Similar results have also been observed by other researchers (22), who collected a total of 127 tracheal swab samples from 6- to 12-month-old beef cattle with respiratory problems from seven different geographically distinct farms in Turkey. In that study, 12.6% (16/127) of the samples were positive for *M. bovis* in 4 different farms according to PCR. They stated that *M. bovis* infection is a common respiratory problem for cattle in Turkey. In another study, Özen et al. (23) reported a *Mycoplasma* isolation rate of 19% in 100 lung samples collected from a slaughterhouse in Kars city located in the Eastern Anatolia Region. They concluded that the low isolation rate was due to sampling performed in beef cattle that are more resistant to diseases. In our study, the lowest isolation rate occurred in the Eastern Anatolia Region (23.6%), followed by the Mediterranean Region (19.6%). However, the cattle were Holstein dairy cows, in contrast to those studied by Özen et al. (23). Additionally, the highest isolation rate occurred in the Southeast Anatolia Region (40.2%, n = 31). The reason for the high isolation rate in this region could be the large numbers of animals in farms included in the study and that animals of different ages were housed together (data not shown). All the relevant studies conducted in Turkey thus far were performed using different materials and diagnostic methods, which makes comparison among data virtually impossible.

Mastitis, arthritis, cattle pneumonia, and reproductive problems usually coexist in farms with a history of

Table 3. Distribution of *Mycoplasma* species detected using PCR by regions and types of samples.

Farm location (region)	Mb					Ma		Mbvg	Mc	Total (%)
	Milk	Lung	BALF	Synovial fluid	Uterus lavage	Lung	BALF	Uterus lavage	Lung	
Central Anatolia	10†/55††	9/30	10/32	8/12	2/12	4/30	3/32	4/12	-/30	50/141 (35.4)
Aegean	5/32	2/19	4/17	4/7	-	3/19	-/17	-	-/19	18/75 (24)
Marmara	11/35	6/12	-/2	-/2	1/6	1/12	-/2	2/6	-/12	21/57 (36.8)
Mediterranean	9/48	-/2	2/4	-	-/2	-/2	-/4	-/2	-/2	11/56 (19.6)
Southeast Anatolia	17/50	5/12	2/5	4/4	1/6	-/12	-/5	2/6	2/12	33/77 (42.8)
Black Sea	16/52	3/9	3/5	-	2/8	-/9	-/5	2/8	-/9	26/74 (35.1)
Eastern Anatolia	5/38	5/11	2/2	1/1	-/3	-/11	-/2	-/3	-/11	13/55 (23.6)
Total (%)	73/310 (23.5)	30/95 (31.5)	23/67 (34.3)	17/26 (65.3)	6/37 (16.2)	8/95 (8.4)	3/67 (4.4)	10/37 (27)	2/95 (2.1)	172/535 (32.1)

Mb: *M. bovis*, Ma: *M. alkalescens*, Mbvg: *M. bovigenitalium*, Mc: *M. canis*
† Number of positive samples / †† Number of samples

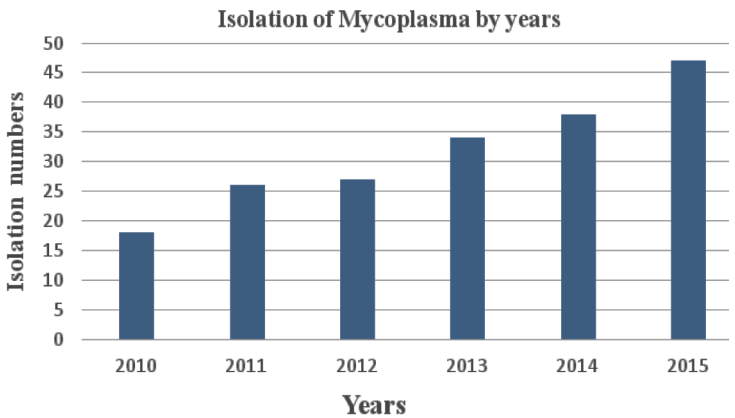

Figure. *Mycoplasma* isolation rates between 2010 and 2015.

mycoplasmosis. Consistent with previous studies (7,13,19), it was determined in the present study that mycoplasmas were common in cattle pneumonia (40.7%, 66 of 162), arthritis (65.3%, 17 of 26), and mastitis (23.5%, 73 of 310) in dairy cattle farms, suggesting that the agent causes different infections in the same farm. *M. bovis* was isolated from both synovial fluid and BALF samples from 15 calves. These findings are consistent with the results of other researchers (17,19), who reported a significant relationship between pneumonia and arthritis both caused by mycoplasmas. Other researchers reported that different species of *Mycoplasma* can be isolated simultaneously from infections of the same or different systems (17,24). Similarly, in our study, *M. bovigenitalium* was isolated from the metritic uterus, and *M. bovis* was isolated from a mastitic milk sample from the same animal (n = 4).

The most significant losses among *Mycoplasma* infections occur due to mastitis, for which the annual loss is reported to be 576 million euros in Europe (1), 108 million dollars in the USA, and 54 million pounds in the UK (2). Moreover, mycoplasma mastitis is important as an agent transmitted to calves (13). *Mycoplasma* colonization has been determined to occur to a high degree in the respiratory tract mucosa of calves fed with infected milk, and a significant relationship has been found between *Mycoplasma* mastitis and calf arthritis (17). In our study, a significantly positive correlation (r = 0.8) was found between the *M. bovis* isolation rate from milk samples (n = 73), from respiratory samples (n = 53), and from synovial fluid samples (n = 17) from calves in the same farm.

M. bovis is the primary agent, but more than 20 different *Mycoplasma* species have been isolated from cattle with different clinical symptoms of a disease (2). Similarly, a majority of mycoplasmas isolated in this study (86.6%, n = 149) were identified as *M. bovis* by PCR. Like other mycoplasmas, *M. alkalescens* is also found in the respiratory tract mucosa of cattle. This species has commonly been reported in cases of mastitis and arthritis but rarely in respiratory tract infections (25). We isolated *M. alkalescens* from only respiratory samples (n = 11). To the best of our knowledge, there are no previous reports of *M. alkalescens* in bovine respiratory samples in Turkey.

Numerous studies have demonstrated the presence of *Mycoplasma* species in the genital tracts of diseased and healthy cows (26–28). Genital infections caused by mycoplasmas are generally asymptomatic but have been reported to be the most important cause of infertility (26,28). In a previous study, *M. bovis* and *M. bovigenitalium* were isolated from genital samples of 76 infertile and 86 healthy cows at rates of 18.7% and 15.1%, respectively (26), while only *M. bovigenitalium* (7.4%) was detected in metritic uterus samples in another study, which aimed to determine 7 different species of *Mycoplasma* (28). In the present study, 16.2% and 27% of mycoplasmas isolated from uterus samples were *M. bovis* and *M. bovigenitalium,* respectively. In any case, it is difficult to compare the figures with those from previous reports since sampling sites and diagnostic methods differ.

M. canis has been isolated from cattle with respiratory disease by some researchers, but it is known primarily as a cause of reproductive disease in dogs (2,4–6). In our study, *M. canis* was isolated and identified from two lung samples taken from calves in the same farm. To the best of our knowledge, this is the first isolation from cattle in Turkey. It was firstly isolated from pneumonic calves in Holland in 1993 (4). Isolations from calf pneumonia (5,6) and mastitis (29) were later reported. The most important means of *M. canis* transmission to cattle has been thought to be close contact with dogs, which are the primary hosts. However, the agent is beginning to be considered a part of the normal flora of the respiratory tract in cattle since the discovery that the agent is commonly encountered in cattle, and typical *Mycoplasma* pneumonias have developed in cattle by experimental infection (29).

Byrne et al. (10) reported that mycoplasmas were introduced to Ireland via importing cattle. Filioussis et al. (9) published a similar report after isolating *M. bovis* in 8.2% of dairy cows with mastitis. All of the infected cows had been imported to Greece from European countries. However, the origins of cattle used in our study differed, making it difficult to interpret the higher ratio of bovine mycoplasmosis based on cow origins.

BALF samples are considered more suitable materials compared to nasal swab samples in the diagnosis of respiratory tract infections (6,22). In our study, 41% (n = 67) of respiratory samples were BALF samples and 58% (n = 95) were lung samples. The *Mycoplasma* isolation rate from lung samples (40%) was higher than that from BALF samples (38.8%), but this difference was not statistically significant ($P > 0.05$). These results support the current idea that BALF samples can be used reliably for the antemortem diagnosis of respiratory infections in cattle.

In conclusion, *Mycoplasma* infections are a common problem in Holstein dairy cattle in Turkey. The *Mycoplasma* isolation rate is becoming more serious than before and seems to correlate with the popular practice of live cattle imports. The most common species causing the infection is *M. bovis*. *M. alkalescens* and *M. canis* were also isolated from respiratory tract infections from cattle in Turkey for the first time. Problems with the ineffectiveness of chemotherapeutics are common in the control of infection, and implementing measures on animal movement is difficult. Together, these suggest a requirement for more effective primary and secondary prevention methods.

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