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HÜBAN GÖÇMEN

RUBEN SEBASTIAN ROSALES

ROGER DAVID AYLING

MİHRİBAN ÜLGEN

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Comparison of PCR tests for the detection of *Mycoplasma agalactiae* in sheep and goats

Hüban GÖÇMEN[1,*], Ruben Sebastian ROSALES[2], Roger David AYLING[2], Mihriban ÜLGEN[3]
[1]Department of Microbiology, Faculty of Veterinary Medicine, Near East University, Nicosia, North Cyprus
[2]Mycoplasma Group, Animal and Plant Health Agency, Addlestone, Surrey, United Kingdom
[3]Department of Microbiology, Faculty of Veterinary Medicine, Uludağ University, Bursa, Turkey

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Abstract: The aim of this study was to compare PCR tests for the detection of *Mycoplasma agalactiae* in sheep and goats. Samples obtained from sheep and goats in Turkey were tested to determine the presence of *M. agalactiae*. Mycoplasma culture and two direct PCR methods based on the *uvr*C gene and *pol*C gene were compared with PCR of the 16S rRNA gene followed by denaturing gradient gel electrophoresis (PCR/DGGE) on DNA extracted from clinical samples (following culture enrichment). A total of 234 samples were examined: 9.4% and 13.2% were positive for *M. agalactiae* based on the *uvr*C gene and *pol*C gene PCRs, respectively, while the culture method revealed that 12.8% of the samples were mycoplasma-positive. The PCR/DGGE method identified *M. agalactiae* in 15.0% of the samples; it also detected and identified other *Mycoplasma* species in the samples. *M. agalactiae* was only detected in goats in this study. This is the first time 16S rRNA PCR/DGGE was performed on various samples from sheep and goats in Turkey. This study confirmed that *M. agalactiae* and other *Mycoplasma* species are present in goats, indicating a requirement for effective control strategies for contagious agalactia and *Mycoplasma* species in Turkey.

Key words: *Mycoplasma agalactiae*, PCR/DGGE, *uvr*C, *pol*C, goat, sheep

1. Introduction

Mycoplasma species cause several diseases in small ruminants, which often manifest with clinical signs including mastitis, arthritis, keratitis, pneumonia, and septicaemia (1). Contagious agalactia (CA) is caused by *Mycoplasma agalactiae*, while *Mycoplasma mycoides* subsp. *capri*, *Mycoplasma capricolum* subsp. *capricolum*, and *Mycoplasma putrefaciens* cause diseases similar to CA, all of which have been highlighted by the World Organisation for Animal Health (1) due to the significant economic impact associated with reduced milk production and quality (2). CA is widespread and occurs in many countries bordering the Mediterranean in Europe, Asia, and Africa (2,3). Additionally, CA has been endemic in several regions of Turkey for many years. The presence and distribution of mycoplasma diseases in sheep and goats in Turkey has been explored previously (4–6).

Mycoplasma species are fastidious organisms, which makes traditional diagnosis and identification by culture, biochemical, and serological methods difficult and time consuming. Therefore, molecular methods such as the polymerase chain reaction (PCR) are quicker and generally more sensitive than culture. However, with more than 120 different known mycoplasma species, a single molecular technique that can detect and identify the different species in one test differentiate has negated the need to develop 120 different PCR tests. PCR of the 16S rRNA gene followed by denaturing gradient gel electrophoresis (PCR/DGGE) determines the genetic diversity of microorganisms, as it can theoretically detect single base mutation in DNA and also identify the majority of small ruminant mycoplasmas (7,8). This method uses G-C rich clamped primers based on the 16S rDNA gene and results in amplicons that are separated in a urea and formamide gradient polyacrylamide gel heated at 60 °C (9). The PCR/DGGE method can detect and differentiate 67 mycoplasma species of veterinary importance including causative bacteria of CA (1,7,10). PCR/DGGE can be applied directly to clinical samples or cultured isolates. It can also detect new mollicute species and detect and identify mixed cultures (11). In this study, we compared two *M. agalactiae*-specific PCR methods (12,13) with PCR of the 16S rRNA gene followed by denaturing gradient gel electrophoresis (PCR/DGGE) (7) using samples collected from sheep and goats. These experiments also indicated the presence of *Mycoplasma* species and their association with clinical and subclinical signs of CA in sheep and goats.

* Correspondence: huban.gocmen@neu.edu.tr

2. Materials and methods

2.1. Sampling

The 234 samples were collected from 220 small ruminants in different districts and provinces of Turkey and included animals with and without clinical signs of CA (Table 1). The 234 samples were 127 milk samples, 83 eye swabs, 15 joint fluids, 5 nasal swabs, and 4 lung tissue samples. These samples originated from 164 adult female goats, 23 ewes, 12 rams, 8 lambs, 7 young female goats, and 6 male goats (Table 1). The samples were obtained aseptically and then transported to the laboratory in chilled containers and processed within 24 h.

2.2. Bacteriology, isolation, and culture conditions

All the samples were inoculated directly—or, in the case of tissue samples, homogenised and inoculated—into modified Hayflick broth (Oxoid, UK), and three ten-fold serial dilutions were made. Following the serial dilutions, a few drops of the broth media containing the sample were plated onto Hayflick Mycoplasma agar (Oxoid, UK). All media were incubated at 37 °C in a 5%–10% CO_2 atmosphere (Oxoid CO_2 Gen, UK) and observed for 3 to 7 days (1,14). The broth media were examined daily for any signs of mycoplasma growth, such as slight turbidity, swirls of growth, a change in pH, or opalescence. The agar media were examined for mycoplasma-like colonies and typical 'fried-egg' colonies using a stereomicroscope (Olympus SZ2-ILST Zoom stereomicroscope, Tokyo, Japan). If any of the broth cultures showed signs of bacterial contamination, the broths were filtered using a 0.45-µm Minisart filter (Sartorius AG, Goettingen, Germany) (15,16). Mycoplasma-like growth was tested for digitonin sensitivity and urease activity using standard methods (15).

2.3. DNA extraction

Genomic DNA was extracted directly from 1 mL of joint fluid and milk samples and 1 mL of enrichment mycoplasma broth from lung tissue samples and eye and nasal swabs. The samples were centrifuged at 14,000 × *g* for 5 min in 1.5-mL microtubes. The pellets were resuspended in 200 µL of phosphate-buffered saline (PBS) (pH 7.4). The DNA was extracted using the High Pure PCR Template Preparation kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

2.4. PCR assays

Two *M. agalactiae* PCR methods were used with all the samples. These PCR methods target two different genes—*pol*C (12) and *uvr*C (13)—and were optimised for use in our laboratory. Briefly, the *pol*C PCR was carried out as described using 1 µL of template DNA in 24 µL of reaction mix containing 0.4 µM MAPol-1F and MAPol-5R primers, 2.5 µL of 10X Taq buffer, 5 U/µL Taq polymerase, dNTPs (300 µM dATP and dTTP; 150 µM dGTP and dCTP), and 2 mM $MgCl_2$ (all from the Fast Start Taq DNA polymerase kit and dNTPack; Roche Diagnostics, Mannheim, Germany). The *pol*C-based PCR was performed using a gradient thermal cycler (Techne TC-3000G; Bibby Scientific, UK) as described previously (12). For the *uvr*C PCR, the volumes were adjusted for a 25-µL reaction. In this case, 25 pmol of each primer was used with 5 µL of 5X Go Taq green buffer (Promega, UK), 5 U/µL *Taq*Gold DNA polymerase (Applied Biosystems), 10 mM each dNTP (Promega, UK), and 1.5 mM $MgCl_2$ (Applied Biosystems). The *uvr*C-based PCR was performed with a GeneAmp 9700 (Applied Biosystems) thermal cycler under the following cycle conditions: an initial denaturation for 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C (annealing temperature), and 30 s at 72 °C, with a final elongation step for 5 min at 72 °C. The amplicons were analysed using 2% agarose gel electrophoresis, stained with ethidium bromide, and visualised using UV light.

2.5. PCR/DGGE for mycoplasma detection and identification

The 16S rRNA gene PCR and denaturing gradient gel electrophoresis was performed according to the method of McAuliffe et al. (7) using GC341-F (universal) and R543 (mycoplasma specific) primers. The profiles were compared with *M. agalactiae* PG2-type strain (NCTC 10123) and AIK strain from Pendik Veterinary Control Institute and other *Mycoplasma* species that may affect small ruminants, including *M. mycoides* subsp. *capri* (Mmc) (F30), *M. capricolum* subsp. *capricolum* (Mcc) (NCTC 10137), *M. capricolum* subsp. *capripneumoniae* (Mccp) (NCTC 10192), *M. putrefaciens* (NCTC 10155), *M. ovipneumoniae* (NCTC 10151), and *M. arginini* (NCTC 10129).

Briefly, PCR/DGGE was performed using the Ingeny phorU 2 × 2 apparatus (GRI Molecular Biology, Essex, UK). Samples (20 µL) were loaded onto 10% polyacrylamide/bis (37.5:1) gels with denaturing gradients from 30% to 60% [where 100% is 7 M urea and 40% (v/v) deionised formamide] in 1X TAE electrophoresis buffer. Electrophoresis was carried out at 100 V at 60 °C for 18 h. Gels were stained with SYBR Gold (Cambridge BioScience, UK) in 1X TAE for 30 min at room temperature and visualised under UV illumination.

2.6. Sensitivity, specificity, and agreement value calculation

The diagnostic sensitivity (Se) and specificity (Sp) of the molecular techniques were calculated by a two-sided comparison of each individual test. Se was estimated as the ratio of true positive samples over the true positive plus (multiplied by) false negative samples [Se = TP/(TP × FN)]. Sp was estimated as the ratio of true negatives over the true negatives plus (multiplied by) false positives [Sp = TN/(TN × FP)] (17).

Table 1. Bacteriological findings obtained with samples collected from sheep and goats.

Province/Turkey	Flock/herd number host	Type and number of samples	Number of *Mycoplasma* spp.-positive samples[a] (%)[b]	Clinical signs
1. Bursa	1. Herd - goat	18 Milk samples 18 Eye swabs	7 (7.7%) -	Goats had no clinical signs* in milk samples and eye swabs. Herds 2, 3, and 4 had conjunctivitis, opacity of eyes, and respiratory symptoms.
	2. Herd - goat	5 Milk samples 27 Eye swabs 11 Joint fluids	- - -	
	3. Herd - goat	4 Milk samples 4 Eye swabs	- -	
	4. Herd - goat	5 Milk samples 6 Eye swabs	- -	
	5. Flock - sheep	34 Milk samples 2 Eye swabs	- 2 (3.5%)	Sheep had conjunctivitis and opacity of eyes.
2. Bursa	1. Herd - goat	16 Milk samples 4 Joint fluids	12 (41.4%) 2 (50.0%)	All goats had mastitis, conjunctivitis, and arthritis.
3. Bursa	1. Herd - goat	16 Milk samples 7 Eye swabs	- -	No clinical signs*
4. Bursa	1. Herd - goat	12 Milk samples	-	No clinical signs*
5. Bursa	1. Herd - goat	4 Lung tissue samples	2 (50.0%)	Lungs had marmoreal appearance.
	1. Flock - sheep	2 Milk samples 2 Nasal swabs 1 Eye swab	- 2 (100.0%) 1 (100.0%)	Sheep had nasal discharge, no mastitis, and mild conjunctivitis.
Balıkesir	1. Herd - goat	3 Eye swabs	1 (5.5%)	Goats had mastitis, conjunctivitis, and udder tissue lesions.
	2. Herd - goat	2 Eye swabs	-	
	3. Herd - goat	2 Eye swabs	-	
	4. Herd - goat	1 Eye swab	-	
	5. Herd - goat	3 Eye swabs	-	
	6. Flock - sheep	3 Nasal swabs 7 Eye swabs	1 (25.0%) -	Sheep had respiratory symptoms.
Çanakkale	1. Herd - goat	1 Milk sample	-	Goats were lame and coughing.
	2. Flock - sheep	1 Milk sample	-	
	3. Flock - sheep	1 Milk sample	-	Sheep had eye discharge and opacity, joint swelling, agalactia, and abortion.
	4. Flock - sheep	1 Milk sample	-	
	5. Flock - sheep	1 Milk sample	-	
Edirne	1. Flock - sheep	10 Milk samples	-	No clinical signs*
Total	22	234	30 (12.8%)	

[a] Samples identified as *Mycoplasma* species based on bacteriological examination.
[b] Percentage of culture-positive samples.
*These managements were observed as clinical signs (mastitis, conjunctivitis) in the past.

Cohen's kappa statistic was used to determine the strength of agreement between test results and was calculated using PASW Statistics 18.0 (IBM Inc.). The kappa (k) value was interpreted as described by Landis et al. (18).

3. Results

3.1. Bacteriology

Bacterial growth was observed in 30 (7 sheep and 23 goats) out of 234 samples (12.8%), as indicated by turbidity in the mycoplasma broths and mycoplasma-like colonies on the mycoplasma agar plates. Growth was observed in 19 of the 77 goat-milk samples (11.2%). The full results are provided in Table 1.

3.2. Comparison of *Mycoplasma agalactiae* conventional PCR assays and PCR/DGGE

The *pol*C *M. agalactiae* PCR produced amplicons of 265 bp in 31 of 234 (13.2%) samples, while the *uvr*C *M. agalactiae* PCR produced amplicons of 1624 bp in 22 of the 234 (9.4%) samples (Figures 1 and 2). The PCR/DGGE method identified 35 of the 234 (15.0%) samples as *M. agalactiae*. The PCR/DGGE method also identified 11 *M. arginini*, 3 *M. ovipneumoniae*, 1 *M. capricolum* subsp. *capricolum*, and 1 *M. putrefaciens*; multiple mycoplasma species were identified in three of the samples (Figure 3). Full details are provided in Table 2.

The Se and Sp values obtained after reciprocal analysis of each test are presented in Table 3. The highest Se values were obtained when comparing *pol*C and PCR/DGGE to *uvr*C and comparing PCR/DGGE to *pol*C (Se = 100%). The highest Sp values were found when comparing *pol*C and *uvr*C tests to PCR/DGGE and comparing *uvr*C to *pol*C (Sp = 100%). Additionally, high Sp values were found when comparing PCR/DGGE to *pol*C (Sp = 96.9%), *pol*C to *uvr*C (Sp = 96.0%), and PCR/DGGE to *uvr*C (Sp = 93.1%).

The k values showed an almost perfect agreement between PCR/DGGE and *pol*C (k = 0.90) and between

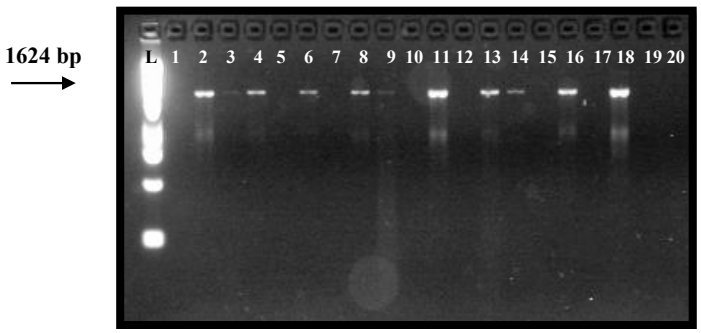

Figure 1. Agarose gel electrophoresis (2%) used for PCR-products–based *M. agalactiae- uvrC* gene. L: Ladder marker; lane 1: negative control; lane 2: *M. agalactiae* positive control (NCTC 10123); lanes 2–4, 6, 8, 9, 11, 13, 14, 16, and 18: positive results; lanes 5, 7, 10, 12, 15, 17, 19, and 20: negative results.

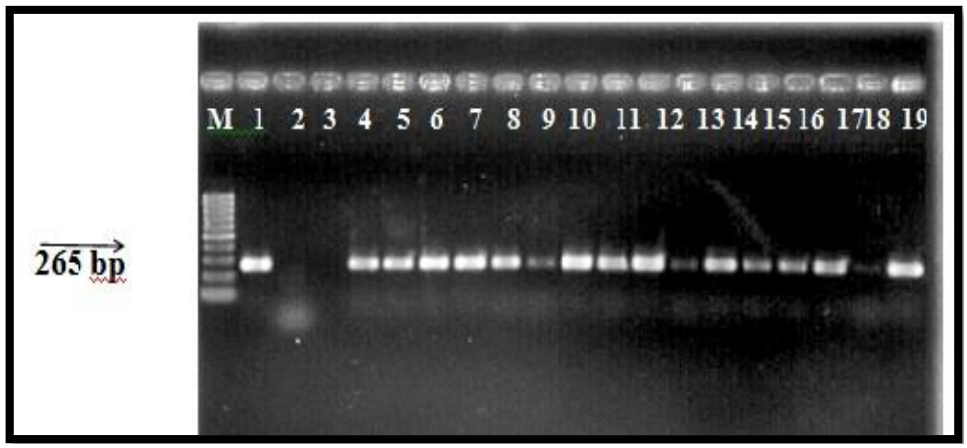

Figure 2. Agarose gel electrophoresis (2%) image of *M. agalactiae pol*C-PCR products. M: Marker (100 base pair) (Thermo Scientific, SM0242); lane 1: *M. agalactiae* positive control (AIK strain from Pendik Veterinary Control Institute); lane 2: negative control (*M. putrefaciens*-NCTC 10155 strain); lane 3: negative control (PCR grade); lanes 4–19: positive samples.

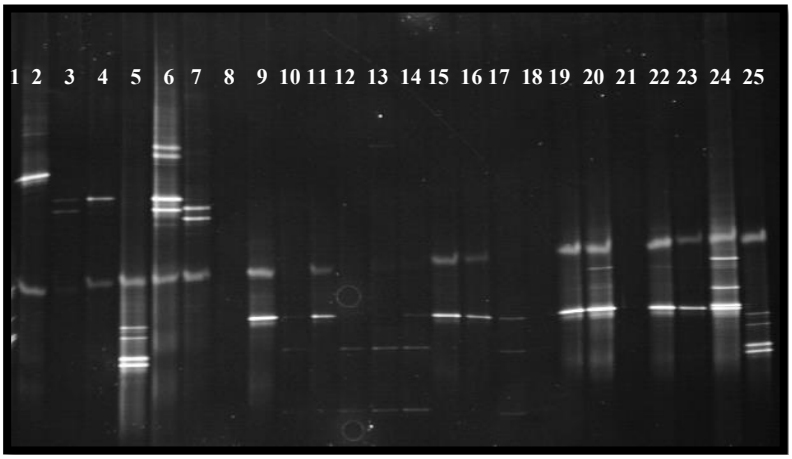

Figure 3. DGGE analysis of the amplified V3 region of 16 S rDNA gene from isolates of direct samples. Lanes 1, 24: *M. agalactiae* positive control (NCTC 10123); 2: *M. ovipneumoniae* positive control (NCTC 10151); 3: *Mccp* positive control (NCTC 10192); 4: *Mmc* positive control (NCTC F30); 5, 25: *M. arginini* positive control (NCTC 10129); 6: *Mcc* positive control (NCTC 10137); 7: *M. putrefaciens* positive control (NCTC 10155); 8: negative control; 9, 11, 14–16, 19, 20, 22, 23: *M. agalactiae*; 10, 14, 17: *M. agalactiae* and unknown profiles; 12, 13: unknown profiles; 18, 21: negative.

*pol*C and *uvr*C (k = 0.83). Substantial agreement was found between PCR/DGGE and *uvr*C PCR (k = 0.73).

4. Discussion

In this study we demonstrated that, under the conditions used, *pol*C PCR was able to detect more positive *M. agalactiae* samples than the *uvr*C method; however, a more stringent annealing temperature than originally described by Subramaniam et al. (13) was used. More positive *M. agalactiae* samples were observed when using PCR/DGGE compared to both conventional PCR tests. Overall, the PCR/DGGE method presented the highest combined sensitivity and specificity compared with the other PCR methods tested; it also detected and identified other *Mycoplasma* species, including some mixed infections. Here, we have demonstrated that the PCR/DGGE method is more sensitive than the standard PCRs we tested, and the PCR/DGGE method can also identify most *Mycoplasma* species. Unusual or new mycoplasma species can also be detected and subsequently identified via DNA sequencing of the PCR amplicon. One disadvantage of the PCR/DGGE method is the cost of the specialised equipment and the specialist technical skills required to perform the test and analyse the results. As of yet, no other test has delivered similar sensitivity and flexibility in identifying and detecting a range of *Mycoplasma* species, although a microarray described by Schnee et al. (19) may be the method of choice in the future.

Limited information is available regarding the occurrence of these economically important mycoplasma diseases in sheep and goats in Turkey. However, CA outbreaks have been reported by Özdemir et al. (5) and Cetinkaya et al. (6), who demonstrated the presence and distribution of mycoplasma species in Turkey. Here, we have shown that as many as 35/234 (15%) of the animals sampled in provinces were infected with *M. agalactiae*, and 51/234 (21%) were infected with *Mycoplasma* species according to PCR/DGGE method. The majority of CA-positive animals had clinical signs that included conjunctivitis, arthritis, or nasal discharge; however, positive detection was also achieved using milk samples, although no mastitis was observed. One unusual observation was the detection and identification of *M. capricolum* subsp. *capricolum* from a sheep that shared a pasture with goats. This organism is more often linked with CA-like disease in goats. This finding is significant for countries that import sheep, where this organism is reportable. Compared to a study from Iran by Khezri et al. (20), who reported 66.7% of 69 samples were positive using PCR and 32.6% were positive by culture alone, the detection of CA in 15% of the sampled animals is relatively low. In an endemic region of Spain, *M. agalactiae* was detected in 13 of 45 (28.8%) bulk-tank sheep milk samples by culture and PCR (16), and another study found that 339 of 922 (36.8%) samples were positive for *M. agalactiae* by real-time PCR, with 85 (9.2%) being positive by culture (21). However, other studies showed that *Mycoplasma* species were present in 75/692 (10.8%) of goat nasal discharge samples by culture and PCR (22), which supports the findings of this study.

Table 2. Comparison of *Mycoplasma agalactiae* molecular diagnostic tests by animal species and sample type.

Host	Sample type	No. of samples	No. of *M. agalactiae* *uvr*C - PCR positive (%)	No. of *M. agalactiae* *pol*C - PCR positive (%)	PCR/DGGE results (number of positive samples)
Goat	Milk sample	77	20 (26.0%)	28 (36.4%)	29 *M. agalactiae* 5 *M. arginini* 1 *M. ovipneumoniae* 1 *M. putrefaciens*
	Eye swab	73	-	1 (1.4%)	2 *M. agalactiae* 3 *M. arginini* 1 *M. ovipneumoniae*
	Joint fluid	15	1 (6.7%)	1 (6.7%)	2 *M. agalactiae*
	Lung tissue sample	4	1 (25.0%)	1 (25.0%)	2 *M. agalactiae* 1 *M. arginini* 1 *M. ovipneumoniae*
Sheep	Milk sample	50	-	-	1 *M. capricolum* subsp. *capricolum*
	Eye swab	10	-	-	1 *M. arginini*
	Nasal swab	5	-	-	1 *M. arginini*
Total		234	22 (9.4%)	31 (13.2%)	35 *M. agalactiae* 11 *M. arginini* 3 *M. ovipneumoniae* 1 *M. capricolum* subsp. *capricolum* 1 *M. putrefaciens*

Table 3. Sensitivity, specificity, and agreement analysis of the different molecular detection tests investigated.

	Gold standard											
	PCR/DGGE				*pol*C				*uvr*C			
	Se%	Sp%	k	k P-value	Se%	Sp%	k	k P-value	Se%	Sp%	k	k P-value
*pol*C	83.78%	100%	0.90	<0.005	-	-	-	-	100%	96.04%	0.83	<0.005
*uvr*C	62.16%	100%	0.73	<0.005	74.19%	100%	0.83	<0.005	-	-	-	-
PCR/DGGE	-	-	-	-	100%	96.91%	0.90	<0.005	100%	93.07%	0.73	<0.005

The economic impact of CA disease is such that suitable methods of control, such as vaccines or effective antimicrobials, are required. A live attenuated vaccine has been reported to provide better protection than inactivated vaccines (23), although the use of this type of vaccine for the control of CA is forbidden in Europe. Therefore, improved vaccines for *M. agalactiae* that can also target other mycoplasma species are required for the control of mycoplasma infections in sheep and goats in Turkey.

5. Conclusion

The sensitivities of the two PCRs tested here were inferior to the PCR/DGGE method; thus, improved tests that are cheap and can easily be performed are required. This

is the first study to perform 16S rRNA PCR/DGGE on various samples from sheep and goats in Turkey. We also demonstrated that *M. agalactiae* and other *Mycoplasma* species are often present in sheep and goats, with and without clinical signs, and we suggest that suitable vaccines should be developed as a control measure for the management of CA.

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