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The Investigation of the Infectious Agalactiae Infection in Sheep and Goat Milk Samples

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ABSTRACT

Many infections are known to be responsible for ruminants of *Mycoplasma*, especially in Europe and North America all over the world, mainly in cattle, goats and sheep cultivation causes great economic losses. Stress, immune system failure, incorrect antibiotic treatment, animal transport, breeding, artificial insemination with sperm infected with mycoplasmosis in the increase of cases of importance.

In this study, Elazig and Malatya lots of sheep and goats belonging to 300 milk collected from sample Agalaksi Contagious (Contagious agalactia) disease molecular methods to detect the presence of (specific PCR). Molecular diagnosis of 300 in the case of milk as a result of specific PCR rate of 45% found positive for *Mycoplasma* sp. 135 the Mycoplasma sp. positive milk sample was found *Mycoplasma agalactiae* at 99.

Keywords: Sheep, Goat, *Mycoplasma*, Milk, PCR

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Introduction

Infectious agalactia, caused by *Mycoplasma agalactiae*, commonly seen in small ruminants, causes mortality in sheep and goats, as well as diminishing milk yield and causing abortions in pregnancy, leading to significant economic loss. *Mycoplasma* is present all over the world and also leads to diseases in our country, which lead to severe clinical signs and deaths in cattle, sheep and goats. Although many clinical signs of infectious agalactia are present, the disease is characterized by inflammation of the breast, joints and eyes. This disease is the most serious disease of small milk ruminants. Initially, it was considered that the primary etiologic agent of the infectious Agalactic disease was *M. agalactiae* (Ma); the large colony, *M. capricolum subsp. capricolum* and *M. mycoides subsp capri*. It has been reported that Mycoplasma mycoides cluster group, including capri (Mmc), is a member of the cluster group (Arda et al., 1982, Kumar et al., 2014, Becker et al. 2012). The entry of the disease into the body occurs via the intake of contaminating water and feedstuffs, and sometimes via the conjunctival route (Keskin, 2013). Healthy animals can also be transmitted with the hands of the milkers. Milking, maintenance, feeding and hygiene conditions are not good business in the disease is more frequently encountered. In addition to sick animals, animals called carriers (clinically unspecified) are also effective in spreading the disease to healthy herds (Amores et al., 2010, Gómez-Martín et al., 2011). The main source of *M. agalactiae* infection is feed and water contaminated with the agent, milk, urine and stool of infected animals, and nasal discharge or tear flow. The incubation period of this disease is between 7 and 56 days. Most cases of infections occur during the summer months, during pregnancy, and throughout the peak level of lactation (Khezri et al., 2012).Contagious agalactia is a serious infection with three distinct clinical manifestations such as mastitis, arthritis and keratoconjunctivitis (Khezri et al., 2012; Bergonier et al., 1996). Causing infectious agalactia mycoplasma species the diagnosis and the identification, molecular diagnostic methods proved its effectiveness. PCR methods are useful in direct diagnosis of *M. agalactiae* in milk samples taken from sheep and reduce the duration of diagnosis in excess samples (Tola et al., 1996).

This study was carried out in Elazığ and Malatya sheep and goat flocks The presence of contagious agalactia (Contagious agalactia) a total of 300 milk samples identify with molecular methods .This study was carried out to identify the presence of contagious agalactia (Contagious agalactia) in 300 sheep and goat milk samples belonging to Elazig and Malatya by molecular methods.

Material and methods

This study material is consisted milk samples taken 150 sheep and 150 goat from 5 sheep and 5 goat herds (totaling 30 animals in each herd) and without clinical symptoms and showing mastitis findings, one of the symptoms of infectious agalactia in Elazığ and Malatya provinces. All milk samples were obtained from rapidly, It has been studied in terms of *Mycoplasma sp., Mycoplasma agalactiae* and *Mycoplasma mycoides subsp capri* by the direct PCR method in the lab. In this study, while the collected milk samples were examined, we tried to optimize the extraction method from direct milk in order to speed up the identification. For this purpose, milk samples brought to the laboratory were centrifuged for 15 minutes at 3,000 rpm. At the end of the centrifugation, 300 µl was taken from the clear and DNA extraction was performed according to the procedure of the commercial extraction kit (QIAamp DNA mini kit Qiagen, France). The purified DNA was then stored at -20^0 C for processing in the PCR.

Mycoplasma sp. Group Specific PCR

The PCR amplification 2 min at 94 ºC Following the pre-denaturation step, 94 ºC for 30 sec.

denaturation, 30 sec at 53 ºC. hybridization and 1 min at 72 ºC. synthesis a total of 35 PCR cycles were performed . The last cycle is 5 min at 72 ºC. extra synthesis was performed. The final products obtained from the Thermal Cycler were run on a 1.5% agarose gel in an electrophoresis apparatus for 90 minutes at 100 volts.

Final products from Thermal Cycler are obtained from the device Cycler 1.5% strength agarose gel electr ophoresis device was run at 100 volts for 90 minutes. Then The gel was transferred to the Carestream Gel Logic 212 PRO imaging system; *Mycoplasma* sp. bands for 280 bp length were searched for positivity. To determine the molecular weight of the resulting tape 100 bp DNA ladder was used . Reference vaccine strains were used as positive control for All PCR applications (Pendik Veterinary Control Institute, Turkey) and distilled water was used as a negative control.

Mycoplasma agalactiae Specific PCR

In order to investigate existence *M. agalactiae* from DNA obtained from milk isolates using a pair of primers specific to the MgA F and MgA R genes reported by Pankaj et al. (2011) (Table 1)PCR was performed. PCR amplification at 95 ºC for 5 min Following denaturation step at ,94 ºC for 1 min . denaturation , 1 min at 57 ºC . hybridization and 1 min at 68 ºC . synthesis . a total of 40 PCR cycles were performed.The last cycle is 10 min at70 ºC . extra synthesis was performed (Pankaj et al ., 2011). Thermal End products were obtained from the device Cycler 1.5% strength agarose gel electrophoresis device was run at 100 volts for 90 minutes. The gel were then transferred to the Carestream Gel Logic 212 PRO imaging system; bands in the length of 360 bp were searched for *Mycoplasma agalactiae* positivity,. in all PCR applications were used Reference Infectious agalactia vaccine strain as a positive control (Pendik Veterinary Control Institute, Turkey) and distilled water was used as a negative control.

Mycoplasma mycoides subsp. capri Specific PCR

M. mycoides subsp . *capri* PCR was performed using a pair of primers specific for P4 and P6 genes (Table 1) were reported by Pankaj et al. (2011).

At PCR Following at 94 ºC for 1 min the pre-denaturation step, at 94 ºC for 1 min. denaturation, 1 min at 46 ºC. hybridization and 2 min at 72 ºC. synthesis as a total of 30 PCR cycles were performed. The last cycle was extra synthesis 5 min at 72 ºC was applied (Pankaj et al ., 2011). The final products were obtained from the thermal Cycler at 1.5% strength agarose gel electrophoresis device was run at 100 volts for 90 minutes. Then, in the Carestream Gel Logic 212 PRO imaging system; *Mycoplasma mycoides subsp. capri* 194 bp bands were searched for positivity . The reference strain as a positive control in PCR applications (Pendik Veterinary Control Institute, Turkey) and the distilled water was used as a negative control.

Results

Mycoplasma sp. Group Specific PCR Findings From milk samples

DNA samples were collected from the milk obtained were subjected to PCR amplification using *Mycoplasma sp.* specific primers, Of the 300 PCR products, 135 (45%) were isolated from *Mycoplasma sp.* positivity was found (Table 2). 49 (16.33%) milk samples in sheep and 86 (28.66%) goat milk samples were found to be mycoplasma sp positivity (Table 2).According to PCR results, milk samples found positive 53 (35.33%) of the Elazığ and 82 (54.66%) were of Malatya was found to be *Mycoplasma sp.* positive reason (Table 2).

In addition, 43 (28.66%) of PCR products were obtained from 135 milk samples belonging to Elazığ and Malatya were belonged to healthy

flocks and 92 (61.33%) belonged to patient flocks (Table 3).

PCR Results of *Mycoplasma agalactiae* Specific Milk Samples

135 milk samples of *Mycoplasma sp.* Positive, 99 (73.33%) M. agalactiae were detected (Figure 1). 39 (28.88%) of the sheep and the 60 (44.44%) goat samples were found positive in which was determined to belong to *M. agalactiae* positive 99 milk samples (Table 4).According to the PCR results, 46 (86.79%) of the 53 positive milk samples of Elazığ province *Mycoplasma sp.* 53 (64.63%) of the 82 milk samples of Malatya province were found to be positive for *Mycoplasma agalactiae* (Table 4).

Table 1. Oligonucleotide primers specifically used in the genes investigated in the PCR analyzes

Primer	Specificity	Sequence (5'-3')	bp	Literature
GPO3 GMSO	*Mycoplasma* (f) *Mycoplasma* (r)	TGGGGAGCAAACAGGATTAGATACC TGCACCATCTGTCACTCTGTTAACCTC	280	(BOTES, 2005)
Mga F Mga R	*Mycoplasma agalactiae* (f) *Mycoplasma agalactiae* (r)	CCT TTT AGA TTG GGA TAG CGG ATG CCG TCA AGG TAG CGT CAT TTC CTA C	360	(PANKAJ, 2011)
P4 P6	*Mycoplasma mycoides* subsp.*capri* (f) *Mycoplasma mycoides* subsp.*capri* (r)	ACT GAG CAA TTC CTC TT TTA ATA AGT CTC TAT ATG AAT	194	(PANKAJ, 2011)

Table 2. Milk samples from sheep and goats were collected from *Mycoplasma sp.* Specific PCR findings

Province	Number of samples		*Mycoplasma sp.*-PCR Results				*Mycoplasma sp.*-PCR Results	
	Sheep	Goat	Sheep Number of samples	Rate%	Goat number of samples	Rate%	Total sheep goat sample number	Rate%
ELAZIĞ	90	60	20	22.22	33	55	53	35.33
MALATYA	60	90	29	48.33	53	58.88	82	54.66
Total	300		49	16.33	86	28.66	135	45

Table 3. In Patient and healthy sheep and goat milk samples *Mycoplasma sp.* Specific PCR findings

Province	Number of healthy flock samples		*Mycoplasma* sp.-PCR Findings				Patient Herd Sample Number		*Mycoplasma* sp.-PCR Findings			
	Sheep	Goat	Number of sheep sample	Rate%	Number of goat sample	Rate%	Sheep	Goat	Sheep sample number	Rate%	Goat sample number	Rate %
ELAZIĞ	60	30	13	21.66	7	23.33	30	30	7	23.33	26	86.66
MALATYA	30	30	12	40	11	36.66	30	60	17	56.66	42	70
Total	150		43			28.66	150		92			61.33

Table 4. *Mycoplasma sp.* specific PCR results of positive milk PCR products in terms of *M. agalactiae*

Province	Number of samples		*M. agalactiae* - PCR Findings				*M. agalactiae* - PCR Findings	
	Sheep	Goat	Sheep Number of samples	Rate %	goat sample number	Rate%	Total Sheep-Goat Number	Rate%
ELAZIĞ	20	33	19	95	27	81.81	46	86.79
MALATYA	29	53	20	68.96	33	62.26	53	64.63
Total	135		39	28.88	60	44.44	99	73.33

It was determined that 27 (62.79%) of the total of 99 milk samples positive for *M. agalactiae* belonging to Elazığ and Malatya were belong to healthy flocks and 72 (78.26%) belonged to the patient flocks (Table 5).

Table 5. The spesific PCR findings for Mycoplasma agalactiae from from milk samples Mycoplasma sp. positive which belong to sick and healthy looking sheep and goats

Province	Number of healthy flock samples		*M. agalactiae* - PCR findings				Patient Herd Sample Number		*M. agalactiae* - PCR findings			
	Sheep	Goat	Sheep Number of sample	Rate %	Goat Number of sample	Rate%	Sheep	Goat	Sheep Number of sample	Rate %	Goat number of sample	Rate %
ELAZIĞ	13	7	13	100	3	42.85	7	26	6	85.71	24	92.30
MALATYA	12	11	3	25	8	72.72	17	42	17	100	25	59.52
Total	43		27			62.79	92		72			78.26

Mycoplasma mycoides subsp. capri Specific PCR Results

135 *Mycoplasma sp.* positive milk, *Mycoplasma mycoides subsp. capri*-specific positive bands were not found.

Discussion

Today, The *Mycoplasma* identification is mostly done with cultural and serological methods. However, both methods are consuming and laborious. In addition, the application is also expensive methods (Corrales ve ark., 2007; Sımeckave ark., 1993). It has also been demonstrated in the study conducted by Çetinkaya et al. (2006-2008) that it is possible to obtain results in a very short time by direct PCR in cases where there are problems with mastitis in diagnosing such troublesome and time consuming mycoplasma infections. In our study, *Ma* was detected in the milk samples of sheep and goat cultures belonging to Elazığ and Malatya, *Mmc* not found. *Mycoplasma agalactiae* under natural conditions It is usually transmitted orally, sometimes through the respiratory or breast. The spread of disease agents with milk for at least 12 months and at most 8 years is a condition that should be taken into account in epidemiological terms (Bergonier et al ., 1997). It is not always considered that mycoplasmas are also found in healthy animals (Langford , 1975). It should not be overlooked that an animal that is not a symptom of disease and is a carrier is always a threatening reservoir for other healthy animals.

It is important to develop methods that accurately
and specifically identify mycoplasmas . In recent years molecular methods have been used to identify mycoplasmas. The PCR method is a technique that yields rapid results and replicates a single DNA sequence millions of times within a few hours (Çetinkaya ve ark., 2006-2008).

Tola et al., (1997) 357 samples from 21 new infected infections (Group 1) and 87 (Group 2) from the past 8 infections, sheep milk sample was examined by direct PCR and culture methods for *Mycoplasma agalactiae*.

Tola et al., (1997), in terms of *Mycoplasma agalactiae* the 357 sheep milk sample (Group 1) from 21 new infected herds and the 87 sheep milk sample (Group 2) from the 8 herds infected in the past , in terms of *Mycoplasma agalactiae* was examined with direct PCR and culture methods. In Group 1, 175 positive for PCR and 153 positive for culture, whereas in Group 2, milk samples were found negative by PCR and culture method. In this study, 135 (45%) *Mycoplasma sp.* were found positive; *Mycoplasma sp.* positive 99 of the PCR products (73.33%) were isolated as *M. agalactiae*. In this study, the PCR technique has been demonstrated that from culture is much faster and in a shorter time from culture results. It has also been shown that the PCR technique can routinely be used to diagnose infectious agalactia caused by *Mycoplasma agalactiae.*

In a study by Göçmen et al. (2015) for investigating the infectious agalactiae disease in sheep and goats with bacteriological and PCR methods, *Mycplasma sp.* were identified in 29 samples of from 339 samples (162 milk samples, 147 eye swabs, 15 joint fluids, 11 nasal swabs and 4 lung tissues) collected from sheep and goats belonging to Canakkale and Edirne province. When PCR and culture data were compared, 5 milk samples and 1 lung sample were found to be positive by polC-PCR and negative by culture. The major cause of the disease was found to be *Ma*, and none of the other mycoplasma agents causing the disease were found. In a study carried out by Bidhendi et al. (2011), *Mycoplasma sp*. were cultured positive 20 out of 367 milk samples taken from the sheep and goat flocks of the Iranian province of Kurdistan, 5 (belong to 1 goat and 4 of sheep) of these positive isolates were positive with *Mycoplasma agalactiae* primers. In addition, PCR was positive for *Mycoplasma agalactiae* in 11 of 367 milk samples. Rosetti et al. (2010) reported that real-time PCR is a specific, sensitive and rapid method for the diagnosis of *Mycoplasma bovis* from direct milk and tissue specimens without DNA extraction. Cai et al. (2005) concluded that direct real-time PCR of *Mycoplasma bovis* from lung tissue showed very high specificity (100%) and sensitivity (96.6%). In the present study, the direct PCR technique from milk was used as a rapid and economical method to detect infectious agalaxin compared to the culture method.

As a result of the study conducted, it was determined that *M. agalactiae* was the main cause of the disease according to the findings, and *M. mycoides subsp. capri* wasn't found. The results of this study proves that infectious agalasin is common in Elazığ and Malatya provinces. *M. agalactiae* origin mastitis infectio ns lead to major problems and economic losses in terms of flocks in terms of small ruminants. *Mycoplasma* induced mastitis considering the infectious nature of the infections , effective and radical control strategies should be implemented and vaccination programs should be established in the affected diseases.

Aknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

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