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Original Article

Detection and Isolation of *Mycoplasma capricolum* Subspecies *Capricolum* from East Azerbaijan Sheep Flocks

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ABSTRACT

Mycoplasma capricolum subspecies *capricolum* (*Mcc*) is one of the causative agents of contagious agalactia (CA), which is an important disease in sheep and goats in the Mediterranean and Middle East countries. *Mycoplasma agalactiae* is the classic agent of CA in sheep and goats. *Mycoplasma mycoides* subspecies *Capri* (*Mmc*), *Mycoplasma capricolum* subspecies *capricolum* (*Mcc*), and *Mycoplasma putrefaciens* (*Mp*) produce a clinically similar disease, more often in goats. The aim of the present study was to detect *Mcc* in sheep flocks in East Azerbaijan Province of Iran. Milk, ear canal, and eye swab samples were collected from 49 sheep flocks with clinical signs of CA or a history of a disease. All the samples were examined using both culture and molecular methods. In the molecular method, positive samples for the *Mycoplasma* genus were tested for *M. mycoides* cluster and *Mcc*. From 272 samples, 67, 87, and 62 samples were shown to be positive using the culture method, polymerase chain reaction (PCR) method, and both culture and PCR methods, respectively. *Mcc* was detected in all the four *M. mycoides* cluster positive samples, including milk, ear canal, and eye swab samples. This is the first report of *Mcc* detection from East Azerbaijan. Our results showed that eye, milk, and ear canal samples could be suitable sources for *Mcc* detection in sheep flocks.

Keywords: Mycoplasma *capricolum* subspecies *capricolum,* Sheep, Culture, PCR, East Azerbaijan Province

Isolement et identification de *Mycoplasma capricolum*subsp.*capricolum* provenant des brebis dela province d'Azerbaidjan oriental en Iran

Résumé: *Mycoplasma capricolum*subsp. *capricolum*est l'un des agents infectieux responsable de l'agalactie contagieuse chez les brebis et les chèvres dans les pays de la Méditerranée et du Moyen-Orient. *Mycoplasma agalactia*, *Mycoplasma mycoides*subsp. *capri* et *Mycoplasma capricolum subsp.capricolum* sont les agents pathogènes de l'agalactie contagieuse. L'objectif de cetteétude étaitl'isolement et l'identification de *Mycoplasma capricolum*subsp.*capricolum*chez lesbrebis soupçonnées d'être atteintsd'agalactie contagieuse dans la province de lAzerbaidjan oriental en Iran. Un total de 272 échantillons, comprenant74 échantillons de lait, 72 prélèvements oculaires et 126 prélèvements auriculaires ont été prélevés à partir de 49 brebis soupçonnées d'être contaminées par l'Agalactie contagieuse. Tous les échantillons ont été testés simultanément par des méthodes de culture et moléculaire. Dans la méthode moléculaire, tous les cas de mycoplasmes positifs ont été examinés en termes d'appartenance aux espèces *M. mycoides* et *M.capricolum* subsp. *capricolum*. Sur un total de 272 échantillons, 67 échantillons ont été identifiés positifs par la méthode de culture, 87 échantillonsse sont avérés positifs par la méthode de réaction en chaîne par polymérase et les deux méthodes ont montré que 62 échantillons étaient positifs aux mycoplasmes . *M. mycoides* a été identifié dans 2 prélèvements oculaires, 2

prélèvements auriculaires et 1 échantillon de lait. L'espèce *M ;capricolum*subsp. *capricolum* a été isolée et identifiée à partir de 4 échantillons positifs de *M. mycoides*. Cet article représentele premier rapport soulignant la présence de *Mycoplasma capricolum* subsp.*capricolum* chez les brebis dela province d'Azerbaidjan oriental. Les résultats de cette étude montrentque les prélèvements oculaires, les prélèvements auriculaires et les échantillons de lait sont appropriés pour l'isolement et l'identification de *M. capricolum* subsp.*capricolum.*

Mots-clés: Brebis, culture, réaction en chaîne par polymérase, province d'Azerbaidjan oriental en Iran

INTRODUCTION

Contagious agalactia (CA), one of the main diseases in sheep and goats, is characterized by mastitis, arthritis, keratoconjunctivitis, and occasionally abortion (OIE, 2015). CA has a devastating impact on the dairy industry and is an important concern for animal welfare due to its possible complications including blindness and lameness (Corrales et al., 2007). Antimicrobial therapy is an effective treatment for CA; however, the withholding period and the presence of antimicrobial residues in milk products limit this approach (Gómez-Martín et al., 2013). CA has been reported in more than 30 countries (OIE, 2015), and its incidence rate is expected to increase if surveillance networks are not set up in all countries (Corrales et al., 2007). Mycoplasma agalactiae is the classic agent of CA in sheep and goats. Mycoplasma mycoides subspecies Capri (Mmc), Mycoplasma capricolum subspecies capricolum (Mcc), and Mycoplasma putrefaciens (Mp) produce a clinically similar disease, more often in goats (OIE, 2015). Originally a goat pathogen, Mcc is receiving more attention nowadays as it has become a potential risk for endangered wild goats (Capra falconeri) population (Ostrowski et al., 2011). Mcc is not a human pathogen; however, recently for the first time, one Mcc case with severe septicemia and meningoencephalitis complications was reported in an old man in Germany. Consumption of dairy goat products during an excursion or accidental contact with goat herds were cited as the potential transmission routes (Heller et al., 2015). In Iran, the first report of CA was published in 1963 (Borry and Entessar, 1963)and since then, Iran has been considered as an endemic area for this infectious disease (OIE, 2015). Many studies have been carried out on Ma isolation and detection in different regions of Iran Contagious agalactia due to Mycoplasma spp. in small dairy ruminants: epidemiology and prospects for diagnosis and contro(Kheirkhah et al., 2011; A.R, 2013; Moslemi et al., 2013; Khezri and Pourbakhsh, 2014; Khezri et al., 2015; Pooladgar et al., 2015)However, there is only one Mcc report from Qom Province (Pourbakhsh et al., 2015). East Azerbaijan is an important hub of sheep dairy products. Therefore, the aim of the present study was to detect Mcc as one of the casual agents of CA in sheep in the East Azerbaijan Province through using both culture and molecular methods.

MATERIALS AND METHODS

Sampling. A total of 272 samples were collected from 49 sheep flocks in East Azerbaijan Province, Iran. The samples included milk secretions, ear canal swabs, and eye swabs from animals with clinical signs of CA or with a history of disease. The samples were transferred cold with a transport medium to the *Mycoplasma* Reference Laboratory of the Razi Vaccine and Serum Research Institute in Karaj, Iran.

Mycoplasma culture. Pleuropneumonia-like organisms (PPLO) broth and PPLO agar medium (BBL, USA) were used for the cultures. Inoculated cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2. *Mcc* reference strain (NCTC 10154) was used as the positive control and uncultured PPLO broth as the negative control. The cultures were examined daily to detect any sign of growth. In the positive PPLO agar cultures, typical fried-egg *Mycoplasma* colonies were observed under

the microscopic examination. Positive PPLO broth cultures were determined via color change and fine turbidity of the medium. Glycerol was added to the positive PPLO broth cultures and then frozen at -70°C for long-term storage (Nicholas et al., 2008; May et al., 2014).

DNA extraction and polymerase chain reaction (PCR). DNA was extracted from the samples using the conventional procedures (Pourbakhsh et al., 2015). Table 1 shows the primers applied for the *Mycoplasma* genus detection through using the PCR method. All the genus-positive samples were tested using *M. mycoides* cluster specific PCR. Finally, all the samples that were positive for the *M. mycoides* cluster were specifically tested with PCR for *Mcc* (Hotzel et al., 1996). Table 1 presents the primers employed in the PCR method. The PCR Master Mix was prepared in a total volume of 25 µl per sample that contained 2.5 µl of 10X PCR buffer, 2 µl of 50 mM $MgCl_2$, 5 mM of dNTPs, 10 ppm of each primer, and 0.5 U of Taq DNA polymerase (CinnaGen Co., Iran). Then, 15.3 µl of uncultured PPLO broth and 2 µl of the extracted DNA as the template were added to the mixture. *Mcc* reference strain (NCTC 10154) was used as the positive control and the uncultured PPLO broth as the negative control. The PCR assay was carried out in a Gradient Mastercycler (Eppendorf AG, Germany). The amplified products were visualized through using UV illumination after electrophoresis (1% agarose gel in 1 × Tris-acetic acid-EDTA [TAE] buffer) and ethidium bromide staining (Pourbakhsh et al., 2015).

RESULTS

Flocks with at least one positive culture or PCR result were considered positive for the *Mycoplasma* genus; therefore, 73.4% of the examined flocks were positive. Out of the 272 samples, 67, 87, and 62 samples were found to be positive using the culture method, PCR method, and both culture and PCR methods, respectively (Table 2).

Four samples were positive for the *M. mycoides* cluster (Figure 1). *Mcc* (Figure 2) was also detected in all the four *M. mycoides* cluster positive samples. Table 2 demonstrates the results in detail.

Table 1. Primers used in the polymerase chain reaction

	Primer	Reference
Mycoplasma genus	M1F: 5'-GCTGCGGTGAATACGTTCT-3' M3R: 5'-TCCCCACGTTCTCGTAGGG-3'	(Pourbakhsh et al., 2015)
Mycoplasma mycoides cluster	P1F: 5'-TATATGGACTAAAAAGAC-3' P2R: 5'-AATGCATCATAAATAATTG-3'	(Hotzel et al., 1996)
*Mcc**	P4F: 5'-ACTGAGCAATTCCTCTT-3' P8R: 5'-GTAAACCGTGTATATCAAAT-3'	(Hotzel et al., 1996)

* *Mycoplasma capricolum* subspecies *capricolum*

Table 2. Number of positive samples in the sheep flocks

Sample	Number	Culture	Polymerase chain reaction		
			Mycoplasma	*Mycoplasma mycoides* cluster	*Mcc**
Milk	74	12	22	1	1
Ear canal swabs	126	33	37	1	1
Eye swabs	72	22	28	2	2
Total	**272**	**67**	**87**	**4**	**4**

* *Mycoplasma capricolum* subspecies *capricolum*

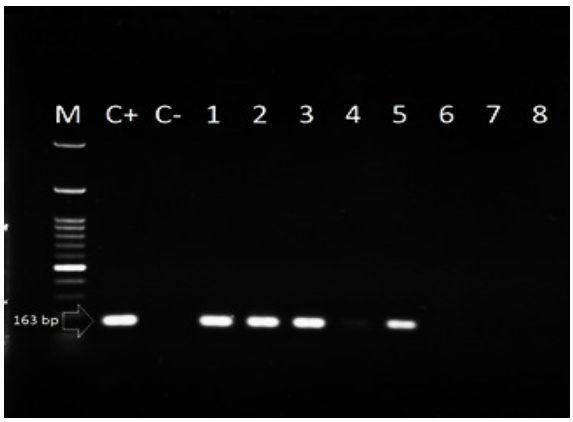

Figure 1. *Mycoplasma* genus polymerase chain reaction:M-marker (100bp DNA ladder). *Lane C+*: positive control (*Mycoplasma*genus, NCTC 10123) and *Lane C-*: negative control. *Lanes* 1 to 3 and 5 are *Mycoplasma* isolates in this study.

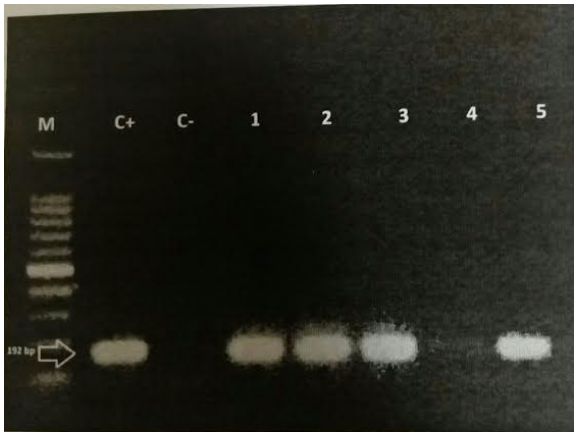

Figure 2. *Mycoplasma capricolum* subspecies *capricolum* (*Mcc*) polymerase chain reaction: *M*-marker (100bp DNA ladder). *Lane C*+: positive control (*Mcc*, NCTC 10154) and *Lane* C-: negative control. *Lanes* 1 to 3 and 5 are the *Mcc* isolates in this study.

DISCUSSION

In this study, *Mcc* was detected for the first time in sheep flocks of East Azerbaijan, Iran, by using culture and PCR methods. In a similar study, Pourbakhsh *et al.* (2015) detected *Mcc* in the lung and mammary lymph nodes of goats with clinical signs of CA in Qom Province, Iran. A total of 111 samples including 46 eye, 28 ear canal, 2 lung, 6 joint, 1 mammary lymph node, and 28 milk samples were collected for the experiment. In that study, 31 samples were found to be positive by using the culture method, while 51 of them were determined to be positive using the PCR method. Pourbakhsh also detected *Mcc* only in one lymph node and one lung sample. One of the lung samples was found positive by using the culture method, but it was not clear whether it was *Mcc* or not. In our study, however, three samples were tested positive for *Mcc* using PCR and one sample was positive using both culture and PCR methods. It is worth mentioning that, although culture method is the gold standard in microbiology, distinguishing between different *Mycoplasma* species is difficult unless molecular methods are applied (May et al., 2014). Our results indicated a higher degree of sensitivity for the PCR method in comparison with the culture method. The use of culture and PCR methods in this study could be compared with the report by Pourbakhsh *et al.* (2015). The number of eye, milk, and ear canal samples in the current study were two times higher than that of the above-mentioned report. Therefore, it could be argued that this study benefits from higher chances of *Mcc* detection from milk, eye, and ear canal samples. Awan *et al*(Awan et al., 2009)stated that nasal swabs and lung samples were the best materials for *Mcc* and *Mp* detection. During a CA outbreak in the Pishin district of Pakistan, lung, trachea, liver, kidney, intestine, fore-limb joints, eye, and nasal swab samples were obtained from euthanized goats with varying degrees of respiratory manifestations for *Mcc* isolation and detection. In that study, the samples were cultured and PCR tests were performed on the purified DNA from the cultures of *Mycoplasma* isolates. Awan *et al.* (2009) detected and isolated *Mcc* from 12 (40%) nasal swabs and 12 (40%) lung samples. Other sample types were found negative for *Mcc*. In addition, Benkirane *et al.* (Benkirane et al., 1993)detected *Mcc* in milk, lung, synovial fluid, nasal discharge, and ocular discharge samples obtained from sheep and goats in Morocco. These samples were collected from sheep and goats with septicemia, mastitis, polyarthritis, or pneumonia. In general, 16 Moroccan *Mcc* isolates were cultured from the samples. In the current study, *Mcc* was detected in ocular lesions, while in Moroccan isolates, ocular discharge samples were in association with respiratory symptoms. Following an unusually long period of bad weather, several CA outbreaks were reported in goat herds in the island of Lanzarotein, Spain. Clinical and subclinical mastitis in lactating goats and some cases of arthritis and pneumonia were detected in goat kids. Samples were collected from milk, ear canal swab, and synovial fluid of the affected animals. Seven *Mcc* isolates were confirmed as the casual agents of the CA outbreaks. Culture and PCR methods were used for *Mcc* isolation. This was the first report of *Mcc* isolation on the island of Lanzarote. Human and livestock migrations may be an important factor in *Mcc* outbreaks in both Morocco

and island of Lanzarote (De la Fe et al., 2007). The type strain California kid (NCTC 10154) was isolated for the first time from an outbreak of severe arthritis in baby goats with high mortality rates in California (Cordy et al., 1955). In addition, a study on *Mcc* isolation from sheep nasal swabs and milk samples was conducted in Jordan (Al-Momani et al., 2006). In all the studies mentioned above, *Mcc* was in relation with respiratory, joint, and mammary gland diseases. However, what the current study adds to the literature is the detection of *Mcc* in eye and ear canal swab samples, as well as milk samples. The detection of *Mcc* in the sheep eye swabs was a finding of the current research, which paves the way for the future studies.

In conclusion, the detection of *Mcc* in East Azerbaijan and Qom provinces presented many interesting findings. However, in order to broaden our knowledge about CA epidemiology, it is highly recommended to detect and isolate *Mcc* and other *Mycoplasma* species from other parts of Iran. Based on the findings of this report, *Mcc* is detected in 1.5% and 1.8% of samples in East Azerbaijan and Qom provinces, respectively. However, further studies in other geographical regions of the country are necessary to reach a more comprehensive conclusion. At the end, the authors highly recommend using eye and ear canal swab samples in addition to the respiratory system, lymph node, and milk samples for *Mcc* detection in sheep.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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