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

**Isolément et identification de Mycoplasma capricolumsubsp.capricolum provenant des brebis dela province d’Azerbaidjan oriental en Iran**

**Résumé:** Mycoplasma capricolum subsp. capricolumest 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 agalactiae, Mycoplasma mycoides subsp. capri et Mycoplasma capricolum subsp. capricolum sont les agents pathogènes de l’agalactie contagieuse. L’objectif de cette étude était l’isolement et l’identification de Mycoplasma capricolumsubsp.capricolum chez les brebis souffrantes d’être atteintes d’agalactie contagieuse dans la province d’Azerbaidjan oriental en Iran. Un total de 272 échantillons, comprenant 74 échantillons de lait, 72 prélèvements oculaires et 126 prélèvements auriculaires ont été prélevés à partir de 49 brebis souffrantes 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 échantillons qui 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
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% CO₂. 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 MgCl₂, 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYF: 5'-GCTGCCTTAACGTTCCTTCT-3'</td>
<td>Pourbakhsh et al., 2015</td>
</tr>
<tr>
<td>M3R, 5'-TCCTCACTTCTCGTACGGG-3'</td>
<td></td>
</tr>
<tr>
<td>P1F, 5'-TATATGGCTTTTAAAAACG-3'</td>
<td>Hotzel et al., 1996</td>
</tr>
<tr>
<td>P2R, 5'-AATGCATAAATATAATG-3'</td>
<td></td>
</tr>
<tr>
<td>Mcc*: P4F, 5'-ACTGAGCGATTCTCTT-3'</td>
<td>Hotzel et al., 1996</td>
</tr>
<tr>
<td>P8R, 5'-GTTAACCTGCTGTATCAAT-3'</td>
<td></td>
</tr>
<tr>
<td>*Mycoplasma capricolum subspecies capricolum</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Number of positive samples in the sheep flocks

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number</th>
<th>Culture</th>
<th><em>Mycoplasma</em></th>
<th><em>Mycoplasma mycoides</em> cluster</th>
<th>Mcc*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>74</td>
<td>12</td>
<td>22</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ear canal swabs</td>
<td>126</td>
<td>33</td>
<td>37</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Eye swabs</td>
<td>72</td>
<td>22</td>
<td>28</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>272</strong></td>
<td><strong>67</strong></td>
<td><strong>87</strong></td>
<td><strong>4</strong></td>
<td><strong>4</strong></td>
</tr>
</tbody>
</table>

* *Mycoplasma capricolum subspecies capricolum*
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 Lanzarote, 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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