Original Article

Isolation and Identification of Mycoplasma agalactiae by Culture and Polymerase Chain Reaction Methods in the Sheep Herds in Guilan Province, Iran

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ABSTRACT
Contagious agalactia is an infectious syndrome of sheep that is characterized by mastitis with reduction of milk production, arthritis, abortion, and keratoconjunctivitis. The disease is rapidly spread by the contact of the infected animals with the healthy ones. Domestic sheep and goats of both sexes can be infected at an equivalent frequency. Most of the researchers use culture and molecular methods for the isolation and identification of Mycoplasma. Mycoplasma agalactiae is the main cause of the disease in sheep. The aim of this study was to isolate and identify M. agalactiae by using culture and polymerase chain reaction (PCR) assay in the sheep herds in Guilan province, Iran. A total of 71 specimens were collected from seven sheep herds with clinical signs of agalactia disease. All of the seven sheep herds (100%) were positive either in PPLO agar or Mycoplasma PCR test. Out of the 71 specimens, 50 (70.4%) cases were positive; however, 21 (29.6%) samples were negative. Furthermore, 40 (80%) cases of the positive samples were detected for the presence of Mycoplasma by the PCR method; nonetheless, 34 (68%) samples were positive in culture. Additionally, out of the 40 positive samples in Mycoplasma PCR, 11 (27.5%) samples were positive in M. agalactiae-specific PCR. The samples that were positive for Mycoplasma were mostly detected in the ear/vaginal, milk, and ear swab samples, respectively, by culture and PCR methods. The most positive samples of Mycoplasma / M. agalactiae were obtained from the ear and vaginal samples. Our findings demonstrated that Mycoplasma was one of the main etiological agents of the contagious agalactia in Guilan province. In addition, PCR was found to be more successful than the culture method in the detection of Mycoplasma.

Keywords: Mycoplasma agalactiae, PCR, Sheep, Guilan

Isolement et identification de Mycoplasma agalactiae provenant des troupeaux de brebis de la province de Guilan en utilisant les méthodes de culture et de réaction en chaîne de la polymérase

Résumé: Les infections à Mycoplasmes chez les petits ruminants dans les pays de la Méditerranée et les pays africains ont été identifiées; ces infections engendrent de nombreux dégâts économiques. L’agalactie contagieuse est un syndrome infectieux du mouton qui est diagnostiqué par le gonflement du sein, l’interruption soudaine de la production de lait, l’enflure des articulations, l’avortement et l’inflammation de la cornée et de la conjonctive. Mycoplasma agalactiae est une cause majeure de la maladie chez les brebis. L’objectif de la présente étude est l’isolement et l’identification de Mycoplasma agalactiae provenant des troupeaux de brebis de la province de Guilan en utilisant les méthodes de culture et de réaction en chaîne de la polymérase. Pour ce faire, 71 échantillons provenant de 7 troupeaux de brebis avec des signes cliniques de la maladie d’Agalactie ont été recueillis. Tous les sept troupeaux (100%) échantillonnés étaient positifs à un des deux expériences de culture d’Agar ou la réaction en chaîne par polymérase. 50 échantillons sur 71 (70,4%) étaient positifs et 21
INTRODUCTION

Contagious agalactia is an important infectious disease in the dairy sheep and goats. This disease is identified by mastitis, arthritis, pneumonia, keratoconjunctivitis, and occasional abortion (Nicholas et al., 2008). The disease is rapidly spread by the contact of the infected animals with the healthy ones. Domestic sheep and goats of both sexes can be infected with this disease at the same frequency (Madanat et al., 2001) however, morbidity is most often associated with pregnant and lactating females (Ruffin, 2001). The economic impact of the disease lies in the reduction or loss of milk production and, less often abortions in pregnant dams. Contagious agalactia may appear in form of an acute, sub-acute, and chronic disease (Greco et al., 2001). The incubation period of infection with *Mycoplasma agalactiae* in goats and sheep ranges within 1-8 weeks. Initially, the infected animals are affected by depression, anorectics, and pyrexia, followed by sudden drop of milk production and occurrence of mastitis, agalactia, and polyarthritis mainly in the carpal and tarsal joints. Moreover, pneumonia and abortion can occur in the chronically infected animals. The majority of the researchers use culture and molecular methods for the isolation and identification of *M. agalactiae* (Azevedo et al., 2006; Al-Momani, 2006). Culturing can be costly and time-consuming, and also inconclusive (Kheirkhah et al., 2011). Molecular diagnostic tests have been used to identify *M. agalactiae* since the last decade. In this regard, polymerase chain reaction (PCR) assay have been considered as an efficient method for the detection of *M. agalactiae* in the milk samples (Tola et al., 1997). *M. agalactiae* has been isolated and identified by culture and PCR assays in the goats and sheep breeding in various provinces of Iran (Kheirabadi and Ebrahimi, 2007; Kheirkhah et al., 2011). Nonetheless, there is no investigation detecting the agent of this syndrome in Guilan province. Regarding this, the present study was conducted to isolate and identify *M. agalactiae* in the sheep with clinical signs of the disease in Guilan province using culture and PCR assays.

MATERIALS AND METHODS

Equal volume of the material in the tube was added to phenol and mixed well on a vortex mixer, and then centrifuged at 18,000 g for 15 min. Subsequently, all aqueous layer (top layer) was removed, and the solution was transferred to a new tube. Added phenol: chloroform (1:1) in tube equal volume of the tube containing. The tubes were centrifuged at 18,000 g for 15 min, and all aqueous layers (top layer) were transferred into a new tube. Added chloroform in the tube, equal volume of the tube containing mix them well by vortex and centrifuged at 18,000 g for 15 min. All aqueous layer was transferred to a new tube, and sodium acetate was added at the volume of 1:10 of the tube containing and mixed well. Added to them ethanol (ETOH) two fold of material in tube. This solution was
placed on -20 for 20 min and centrifuged for 15 min at 18000 g. Discarded liquid containing of tube softly and 200 μl of 70% ETOH, centrifuged for 5 min at 18000 g. After discarding ETOH and drying tubes, 50 μl distilled water was added.

**Amplification with specific primers.** In this study, the specific detection of *M. agalactiae* at genus and species levels was accomplished by using the primer sets published previously. The primers for genus *Mycoplasma* included M1F (5'-GCTGCGGTGAATA CGTTCT-3’) and M3R (5'-TCCCCACGTTCCTCGT AGGG-3’) (Van Kuppeveld et al., 1992). Additionally, the *M. galactiae*-specific amplification primers were FS1 (5’-AAAGGTGCTTGAGAAATGGC-3’) and FS2 (5’-GTTGCCAGAAAAGTCCAATCA-3’) as described by Tola et al. (1997). The PCR master mix was performed in a total volume of 25 μl per sample, containing 2.5 μl of 10 XPCR buffer (CinnaGen), 2 μl of 50 mM MgCl2, 5 μl MdNTPs, 10 pm of each primer and 0.5 U TaqDNA polymerase (CinnaGen). Consequently, 15.5 μl of deionized distilled water and 2 μl of extracted DNA as template were carried out. The PCR assay was conducted in a gradient Mastercycler (Eppendorff, Germany). The *Mycoplasma* PCR (MPCR) was performed for 7.5 minute at 94 °C, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 56 °C, and 1 minute at 72 °C, with a final extension cycle of 5 minute at 72 °C. Furthermore, the *M. agalactiae*-specific PCR (MAPCR) was performed for 5 minute at 95 °C, followed by 34 cycles of 1 minute at 94 °C, 1 minute at 50 °C, and 1 minute at 72 °C with a final extension cycle of 5 minute at 72 °C. The visualization of the amplified products was carried out by UV illumination after electrophoresis (1% agarose gel in 1×Tris-acetic acid-EDTA [TAE] buffer) and ethidium bromide staining.

**RESULTS**

According to the results, all of the seven sheep herds (100%) were positive in either PPLO agar or MPCR test. After clinical examinations, 71 samples were collected from the most significant lesions that were observed in each sheep. Out of the 71 samples, 50 (70.4%) cases were positive, and the rest (n=21, 29.6%) were negative. Furthermore, 40 (80%) and 34 (68%) cases of the positive samples were scored positive for the presence of Mycoplasma by the PCR and culture methods, respectively. In addition, 11 (27.5%) samples of the 40 positive samples in MPCR were positive in MAPCR (Table 1).

### Table 1. Results of culture, *Mycoplasma* polymerase chain reaction and *Mycoplasma agalactiae*-specific polymerase chain reaction

<table>
<thead>
<tr>
<th>Samples</th>
<th>Culture</th>
<th>MPCR</th>
<th>MAPCR</th>
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</thead>
<tbody>
<tr>
<td>50 (70.4%)</td>
<td>34 (68%)</td>
<td>40 (80%)</td>
<td>11 (27.5%)</td>
</tr>
<tr>
<td>21 (29.6%)</td>
<td>16 (31.3%)</td>
<td>10 (20%)</td>
<td>29 (72.5%)</td>
</tr>
<tr>
<td>71 (100%)</td>
<td>50 (100%)</td>
<td>50 (100%)</td>
<td>40 (100%)</td>
</tr>
</tbody>
</table>

**MPCR:** *Mycoplasma* polymerase chain reaction, **MAPCR:** *Mycoplasma agalactiae*-specific polymerase chain reaction

In this study, Mycoplasma was mostly detected in the ear/vaginal, milk, and ear swab samples, respectively, by culture and PCR methods (Table 2). The DNA fragment of Mycoplasma with approximately 163 bp was amplified in the PCR method (Figure 1). As illustrated in Figure 2, MAPCR product was 375 bp in length.

**DISCUSSION**

In spite of the great improvement in medium formulations, the isolation of Mycoplasma is considered as one of the most difficult tasks for the diagnostic laboratories due to the inability of this genus
to easily grow in laboratory media. The PCR can be used more reliably on Mycoplasma growing in culture.

### Figure 1. Mycoplasma polymerase chain reaction (PCR): PCR electrophoresis analysis in %1 gel agarose, M: marker (100 bp DNA ladder), lane PC: positive control (163 bp band, Mycoplasma genus, NCTC 10123), lane PC: negative control (uncultured PPLO broth), Lanes 1-8: Mycoplasma isolates.

### Figure 2. Mycoplasma agalactiae-specific polymerase chain reaction (PCR): PCR electrophoresis analysis in %1 gel agarose, M: marker (100 bp DNA ladder), lane PC: positive control (375 bp, Mycoplasma agalactiae, NCTC 10123), lane PC: negative control (uncultured PPLO broth), lanes 1-8: Mycoplasma genus samples.

In this regard, a 24-hour enrichment of Mycoplasma in the appropriate medium greatly facilitates PCR detection even in the presence of bacterial contamination (Nicholas et al., 2008)(Foddai, 2005). In this study, the isolation and identification of Mycoplasma was achieved for the first time in Guilan in the clinically affected small ruminants. The results of this study showed that all of the seven sheep herds (100%) were positive in either PPLO agar or MPCR test. Our findings demonstrated that Mycoplasma was one of the main etiological agents of the contagious agalactia in Guilan province. According to our results, 80% and 68.7% of the samples were positive in MPCR and culture methods, respectively. In line with the results of the recent studies, this finding revealed the higher efficiency of PCR in the detection of Mycoplasma, compared to that of the culture technique (Tola et al., 1997; Amores et al., 2010; Kheirkhah et al., 2011). In this research, 11 (27.5%) out of 40 positive isolated Mycoplasma were positive with MAPCR. AL-Momani et al. (2006) isolated 44 Mycoplasma that was identified by a newly developed PCR-based molecular technique. In the mentioned study, 1 out of 44 isolates was *M. agalactiae*. In a study conducted by Pirali and Ebrahimi (2007) in the west central of Iran, based on the PCR method, 8 out of 47 (17%) milk samples were positive with *M. agalactiae* primers. Several MAPCRs have been developed based on different gene sequences; nonetheless, they have shown similar levels of sensitivity (Bashiruddin, 2005)(Dedieu et al., 1995; Tola et al., 1997; Subramaniam et al., 1998). Zendulkova et al., 2007) claimed that the PCR was not an efficient method for the detection of *M. agalactiae* in the sheep and goats of Jordan. In the present study, 29.5% of the total samples were negative. Our findings were indicative of the presence of other species, such as *Mycoplasma mycoides*, *Mycoplasma putrefaciens*, or *Mycoplasma capricolum*, as important etiological agents in Guilan province. In a study performed by Hajizadeh et al. (Hajizadeh et al., 2012) 79.25% of the samples were negative.

This study showed the samples that were positive for Mycoplasma were mostly detected in the ear/vaginal, milk, and ear swab samples, respectively, by culture and PCR methods. The isolation of *M. agalactiae* from milk, joint exudates, and eye swab has been also reported in the studies conducted in Iran and other countries. Pirali et al. (2007) also detected *M. agalactiae* from the eye and milk samples of the sheep and goats. Zendulkova et al. (2007) claimed that the eye and ear samples were not suitable for the isolation and identification of *M. agalactiae*. In order to
complete this study, it is necessary to identify other species of Mycoplasma using MPCR.

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.

References


Nicholas, R., Ayling, R., McAuliffe, L., 2008. Mycoplasma diseases of ruminants, CABI.


