Molecular identification of *Mycoplasma agalactiae* in Iran, based on P30 gene

Babazadeh ¹, M., Pourbakhsh ² *, S.A., Noormohammadi ¹, Z., Esmaelizad ³, M., Goudarzi ², H.

¹. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran  
². Mycoplasma Reference Laboratory, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran  
³. Central Laboratory Department, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

*Author for correspondence. E-mail: a.pourbakhsh@rvsri.ac.ir*

ABSTRACT

*Mycoplasma agalactiae* (*M. agalactiae*) is known as the main etiological agent of contagious agalactia (CA) which is a disease affecting dairy sheep and goats and its main characteristics include keratoconjunctivitis, arthritis and mastitis. This pathogen results in milk production reduction and suppression and hence leads to serious economic loss. In the present research, 125 sheep and goat samples were collected from the 15 provinces of Iran. Cultural and molecular methods were used for sample characterization. After extracting genomic DNAs using phenol/chloroform method, PCR technique was employed to detect *Mycoplasma* genus in 163bp fragment of 16S rRNA gene (M-PCR) and *M. agalactiae* in 800bp fragment of conserve and specific P30 lipoprotein gene (P30-PCR) in Cultural and also clinical samples. Finally, to validate the experimental approach, a 375 bp amplicon of P80 lipoprotein was amplified (MA-PCR). Forty three cases of the 125 samples under investigation were positive, as *Mycoplasma* colonies were observed in the PPLO (pleuropneumonia-like organisms) agar culture. Based on
the results of M-PCR method, 61 specimens (out of 125 samples) were scored positive for *Mycoplasma* presence. Twenty samples were positive according to P30-PCR data. We also performed MA-PCR based on P80 gene on 125 total samples to further verify our results for *M. agalactiae* detection. Based on the obtained data, P30 and P80 genes were presented and amplified in all Iranian *M. agalactiae* isolates (Twenty samples). Our results indicated that P30 gene is conserve and specific in all Iranian *M. agalactiae* isolates and this new P30-PCR method (as an MA-PCR technique) may be useful in the detection of this pathogen.

**Key words:** *Mycoplasma agalactiae*, Contagious agalactia, Culture, PCR, P30 gene

**INTRODUCTION**

*Mycoplasma agalactiae* has been recognized as the main agent of contagious agalactia (CA) disease which affects dairy sheep and goats and is mainly identified by keratoconjunctivitis, arthritis and mastitis (OIE, 2008). This pathogen results in milk yield reduction and suppression and consequently, serious economic loss (Oravcova et al., 2008). This syndrome is produced by *Mycoplasma agalactiae* subsp. *agalactiae*, though other Mycoplasmas (such as *M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *capri*, *M. mycoides* subsp. *Mycoides*, and *M. putrefaciens*) have been also reported to cause this disease (Oravcova et al., 2008).

Contagious agalactia has been observed in several countries and is considered as an endemic disease in the majority of Mediterranean countries (Bergonier et al., 1997; Zendulkova et al., 2004). Surface lipoprotein phase variation is a major mechanism in Mycoplasmas, and is considered as an important adaptive strategy in this family of pathogens (Nouvel et al., 2010).
probably allows Mycoplasmas to evade their host immune response and cause chronic infection (Browning et al., 2011). Recent studies on genomic comparison of different pathogenic Mycoplasmas have provided new insights into the role of lipoproteins in pathogenicity (Browning et al., 2011).

According to the results of immunoblotting analysis utilizing a monospecific and polyclonal anti-P30-His serum, P30 has been considered as a specific, stable, and highly immunogenic *M. agalactiae* antigen and therefore can be used to detect, identify, and subtype *M. agalactiae* (Fleury et al., 2001). Furthermore, based on the outcomes of Southern blot analysis and PCR technique using P30 specific primers, this gene is present in all *M. agalactiae* strains, while being absent in other species of *Mycoplasma* (Fleury et al., 2001).

A number of molecular studies have been previously performed on the isolation, detection and identification of *M. agalactiae* by amplification of 375 bp of P80 lipoprotein gene (for instance, the studies conducted by: Khezri et al. (2012) on 69 sheep samples in Kurdistan province, Kheirkhah et al. (2013) on 57 samples of Iranian goats, Pooladgar et al. (2014) on 91 sheep samples affected by contagious agalactia in Khuzestan province and Momen et al. (2014) on 367 samples in Hameden province.

In this study, detection and identification of *M. agalactiae* were carried out by new specific primers based on conserve and specific P30 gene.

**MATERIALS AND METHODS**

**Cultural method and DNA extraction.** 125 Samples were collected from eye, ear, joint exudate and milk secretions of sheep and goats exhibiting clinical signs of probable infection with
Mycoplasma. Samples were obtained from 15 Provinces of Iran (namely, East Azerbaijan, West Azerbaijan, Ardabil, Isfahan, Kerman, Qom, Khuzestan, Semnan, Kermanshah, Golestan, Gilan, Yazd, Sistan and Baluchestan, Kurdistan and Ilam). After transferring samples to the medium and incubation on ice, they were taken to the Mycoplasma Reference Laboratory of Razi Vaccine and Serum Research Institute, Karaj, Iran, for subsequent studies.

All samples were attenuated, filtered into fresh PPLO broth and then inoculated into PPLO agar (BBL, Becton Dickinson and company, Cockeysville, Sparks, MD). The inoculated agar and broth were incubated at 37°C, 5% CO₂ and 98% humidity.

The broths and plates were daily observed for signs of growth and typical Mycoplasma colonies, respectively. To extract DNA, we used the phenol-chloroform method previously described by Tola et al. (1997).

Detection of Mycoplasma genus. In this study, a previously published primer set was used for specific detection of Mycoplasma genus (i.e. M1F: 5’ GCTGCCTGATACCTTCT-3’, M3R: 5’- TCCCCACGTTCTCAGGG-3’) (Van Kuppeveld et al., 1992). PCR was performed using 25 µl samples, each containing 2.5 µl of 10 X PCR buffer (CinnaGen, Iran), 2 mM MgCl₂, 5 mM dNTPs, 2 µl of each primer (10 pm) and 0.5 U Taq DNA polymerase (CinnaGen, Iran). Consequently 15.5 µl of deionized distilled water and 50 ng of extracted DNA as the template were added. PCR was carried out using a Gradient Mastercycler instrument (Eppendorff, Germany) according to the following protocol (Zendulkova et al., 2007): 7.5 minutes at 94 °C, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 56 °C and 1 minute at 72 °C, with a final extension cycle for 5 minutes at 72 °C. M-PCR assay was performed for Mycoplasma genus detection in 163bp fragment of 16S rRNA gene.
**Detection of *Mycoplasma agalactiae* based on P30 gene.** One pair of primers (i.e. P30F: 5’-GCA GTT TTA AAT AAC ACA GG-3’ and P30R: 5’ AAA TCT TGC GCG CAG CAA GA-3’) were designed in ORF region of P30 gene by Oligo 5.0 software. The P30 coding sequence was amplified via PCR technique using 50 µl samples, each containing 5 µl of 10 X PCR buffer (CinnaGen, Iran), 2 mM MgCl₂, 10mM dNTPs, 2 µl of each primer (10 pm), 0.2 µl (1 unit) of Taq DNA polymerase (CinnaGen, Iran), 37.8 µl of deionized distilled water and finally, 100 ng of extracted DNA as the template. PCR was performed in a Gradient Master cycler (Eppendorff, Germany) based on the following protocol: 3 minutes at 95°C, followed by 35 cycles of 1 minute duration at 93°C, 30 seconds at 54°C and 40 seconds at 72°C, with a final 5-minute extension cycle at 72°C. In P30-PCR assay, 800bp fragment of P30 membrane lipoprotein gene was amplified to detect *M. agalactiae* species.

**Detection of *Mycoplasma agalactiae* based on P80 lipoprotein.** The following *M. agalactiae*-primer set was used for specific detection of *M. agalactiae* species: FS₁: 5’AAA GGT GCT TGA GAA ATG GC-3’ and FS₂: 5’-GTT GGC AGA AGA AAG TCC AAT CA-3’, as suggested in a previous study (Tola et al., 1997). The prepared 25 µl samples for PCR assay contained 2.5 µl of 10 X PCR buffer (CinnaGen, Iran), 2 mM MgCl₂, 5 mM dNTPs, 2µl of each primer (10 pm), 0.5 U Taq DNA polymerase (CinnaGen, Iran), 15.3 µl of deionized distilled water and 50 ng of extracted DNA as the template. PCR was conducted in a Gradient Mastercycler (Eppendorff, Germany) based on Tola et al. (1997) reported protocol with some modification: 5 minutes at 95 °C, followed by 34 cycles of 1 minute duration at 94 °C, 1 minute at 50 °C and 1
minute at 72 °C, with a final 5-minute extension cycle at 72 °C. In MA-PCR, a fragment of P80 lipoprotein gene with 375 base pair length was amplified to detect *M. agalactiae* species.

Visualization of amplified products (M-PCR, MA-PCR and P30-PCR) was performed through UV illumination following electrophoresis (using agarose gel (1%) in TAE buffer) and SYBR Safe staining (Tola et al., 1997).

**RESULTS**

According to the cultural method typical *Mycoplasma* colonies were observed in 43 of 125 sheep and goat samples (34.4%), while 82 samples (65.6%) were negative for this pathogen. The positive *Mycoplasma* samples in PPLO broth media were also cultured on PPLO agar media and the resulting *Mycoplasma* colonies exhibited distinctive "fried egg" morphology under a light microscope.

61 positive samples (48.8%) for *Mycoplasma* presence and 64 negative samples (51.2%) from 125 total samples were identified by *Mycoplasma* genus PCR (M-PCR) assay. For detection of *Mycoplasma* genus, a DNA fragment with approximately 163 bp from 16SrRNA was amplified successfully in all *Mycoplasma* culture-positive samples and 18 other specimens (Figure 1).

In the P30-PCR method, 20 samples (16%) were positive and 105 samples (84%) were negative. The complete coding sequence of P30 gene with 800bp length was successfully amplified in all 20 isolates of *M. agalactiae* (Figure 2).

The results of MA-PCR were similar to those of P30-PCR exactly. The complete coding sequence of P80 gene with 375 bp length was amplified in 20 samples (out of 125 samples) which were positive in P30-PCR method as shown in Figure 3.
*M. agalactiae* positive samples from a total of 125 samples was successfully detected by using two P30-PCR & MA-PCR *M. agalactiae* specific PCR methods.

According to both P30-PCR&MA-PCR results, milk samples were the most affected specimens, whiles joint exudates and ear samples were the least influenced samples by *M. agalactiae* (Table 1).

We proved that 20 samples (16%) were infected with *M. agalactiae* based on cultural and PCR methods and successfully detected this pathogen using both methods.

The results of P30-PCR and MA-PCR methods were similar exactly and hence, P30-PCR method using the new designed primers (P30-F, P30-R) can be a good alternative to the routine MA-PCR method using commonly used primers (FS1-FS2) in *M. agalactiae* detection (Table 1).

**DISCUSSION**

In the present study, we used samples of mammary gland (milk), conjunctivae (eye), external canal of ear and joint exudate of sheep and goats for *M. agalactiae* detection, based on P30 gene. All 125 samples (isolated from 15 provinces of Iran) were cultured in PPLO broth media to diagnosis of *Mycoplasma*. M-PCR was also performed to detect *Mycoplasma* genus based on 16S rRNA gene. P30-PCR method was used to identify *M. agalactiae*, based on P30 gene coding for a membrane and immunodominant lipoprotein.

To verify our results for *M. agalactiae* detection by P30-PCR method and gain a better understanding of Iranian isolates of this pathogen, the presence of P80 lipoprotein gene was checked in all samples using MA-PCR method. On the other hand, we performed a comparative analysis between two P30-PCR and MA-PCR methods to examine the specificity of P30 gene for *M. agalactiae* isolates. According to the results, the expected 800 bp fragment belonging to P30
gene was amplified from chromosomal DNA of all *M. agalactiae* isolates used in this study, while it was not observed for other tested *Mycoplasma* species. Therefore, it can be concluded that P30 gene is highly specific to *M. agalactiae* isolates.

In order to determine the sensitivity of P30-PCR assay, a serial dilution of extracted DNA was prepared (100ng- 10ng- 1ng- 100pg- 10pg). Finally based on the results, the sharp band belonging to P30 gene was appeared in >1ng dilution of extracted DNA from *M. agalactiae* isolates.

As a molecular detection method, PCR is a very sensitive and commonly-used approach which can be considered as an early and rapid warning system for clinical samples. PCR allows performing a full assessment in the case of positive results, though negative outcomes can't be regarded definitive. The detection limit of cultural method for *M. agalactiae* has been suggested to be $2 \times 10^2$ CCU/ml (Dediu et al., 1995; Tola et al., 1997; Nicolas, 2008; Bergonier et al., 1997). Our findings were in agreement with the results reported by Kheirkhah et al. (2011) in Iran and Amores et al. (2010) in Spain, indicating that PCR is a more reliable and successful technique for *M. agalactiae* detection, compared to the cultural method. Furthermore, the results of the present study were consistent with those observed by Tola et al. (1997), proving that PCR is much sensitive and faster than the cultural method and reduces the required time for detection.

Based on the results of this study, 12% of eye samples and 26% of milk samples were positive in P30-PCR and MA-PCR, while P30 and P80 genes were not detected in ear samples and joint exudates. Khierkhah et al. (2011) have detected *M. agalactiae* in milk and joint exudates of goats and observed the highest number of *Mycoplasma* colonies in joint exudates. Our data were partially in agreement with their findings, as *M. agalactiae* was detected in the milk and eye samples, though we did not detect this pathogen in joint exudates (and also ear) samples. The
obtained results might have been dependent on the initial number of clinical samples obtained from different sampling sites. As you can see in the table, the total numbers of joint and ear samples were less than those of milk and eye specimens, because of the difficulty of sampling. The present study was conducted on suspected samples. It was associated with more reliable results, compared to other studies such as those performed by De La Fe et al. (2007) and Zendulkova et al. (2007) on samples with or without signs of C.A. In the above-mentioned studies, *M. agalactiae* has been detected based on DNA fragment of P80 gene with 375bp length. Based on previous studies, the prevalence of contagious agalactia in sheep and goat herds in Iran has been significantly increased in recent years such that since 2011, the incidence rate of this disease has reached 33.3% (Pirali Kheirabadi et al., 2007, Kheirkhah et al., 2011). Many variable genes in *M. agalactiae* play prominent roles in inducing host immunity to infection and facilitating the immune evasion (Browning et al., 2011). Therefore, detection and identification of conserve and specific genes in *M. agalactiae* are among important strategies for controlling *M. agalactiae* infections. One of the conserve genes in *M. agalactiae* is P40 which encodes an immunodominant lipoprotein that play role in cytoadhesion and pathogenicity (Oravcova et al., 2009). Mahdavi et al. (2011) have studied P40 gene in three Iranian vaccine strains of *Mycoplasma agalactiae* and reported the homology percentage of this gene among these strains, though that study was limited to vaccine strains and involved no field isolates. Furthermore, based on a study by Fleury et al. (2002), P40 is associated with a functional analogy in *Mycoplasma bovis* and *Mycoplasma hominis* species. They found that the P40 gene has no satisfactory specialty for *M. agalactiae*. However, according to previous studies conducted by Fleury et al. (2001), the P30 is a specific, stable and highly immunogenic *M. agalactiae* antigen, inducing a strong and persistent immune response.
To analyze the specificity of the P30 gene for *M. agalactiae*, we designed a primer pair, P30F & P30R, being complementary to the extremities of P30. For the first time in Iran, the expected 800bp complete coding sequence was amplified from chromosomal DNA of all *M. agalactiae* isolates used in this study (Figure 1), but other *Mycoplasma* species were not tested. Based on the results, the P30 gene was present in all tested *M. agalactiae*, being specific for this pathogen species. The findings of this study were in agreement with the data reported by Fleury et al. (2001) in Switzerland, in which the P30 gene was specific for *M. agalactiae* species according to PCR outcomes.

According to the findings of our study, the P30 gene was specific to *M. agalactiae* and no particularly similar analogues to P30 was found in related species. Moreover, we indicated that the new PCR assay designed based on the P30 gene was useful in detecting *M. agalactiae* as a MA-PCR approach.

A similar study is required for phylogenetic analysis of the P30 gene of *M. agalactiae* in field and vaccine isolates via applying this lipoprotein gene sequence in GenBank to identify the source of infection. Furthermore, it is necessary to conduct a complete P30 molecular typing study so as to obtain genomic diversity and genomic homogeneity in Iranian *M. agalactiae* isolates as candidates for application in diagnostic ELISA kits and recombinant vaccines against contagious agalactia.

*Color Chaining Unite*
Acknowledgements

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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.
Table 1. The results of cultural and three PCR methods in all samples, based on the clinical sampling sites.

<table>
<thead>
<tr>
<th>Tests</th>
<th>Result</th>
<th>Milk sample</th>
<th>Ear sample</th>
<th>Eye sample</th>
<th>joint sample</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>24</td>
<td>3</td>
<td>15</td>
<td>1</td>
<td>43</td>
</tr>
<tr>
<td>Culture</td>
<td>Negative</td>
<td>30</td>
<td>5</td>
<td>36</td>
<td>11</td>
<td>82</td>
</tr>
<tr>
<td>M-PCR</td>
<td>Positive</td>
<td>31</td>
<td>4</td>
<td>25</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>23</td>
<td>4</td>
<td>26</td>
<td>11</td>
<td>64</td>
</tr>
<tr>
<td>P30-PCR</td>
<td>Positive</td>
<td>14</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>40</td>
<td>8</td>
<td>45</td>
<td>12</td>
<td>105</td>
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<tr>
<td>MA-PCR</td>
<td>Positive</td>
<td>14</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>40</td>
<td>8</td>
<td>45</td>
<td>12</td>
<td>105</td>
</tr>
</tbody>
</table>
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Figure 1. Mycoplasma genus PCR (M-PCR): PCR electrophoresis analysis in 1% gel agarose. M: Marker (100bp DNA ladder). Lane C+: Positive control (163bp band, Mycoplasma genus). Lane C-: Negative control (uncultured PPLO broth) and Lane 1 to 4 are the Mycoplasma isolates in this study.

Figure 2. P30-PCR: PCR electrophoresis analysis in 1% gel agarose. M: Marker (100bp DNA ladder). Lane C-: Negative control (uncultured PPLO broth), Lane C+: Positive control (800bp band, P30 gene of Mycoplasma agalactiae). and Lane 1 to 4 are the Mycoplasma isolates in this study.

Figure 3. Mycoplasma agalactiae PCR (MA-PCR): PCR electrophoresis analysis in 1% gel agarose. M: Marker (100bp DNA ladder). Lane C+: Positive control (375bp band, Mycoplasma agalactiae). Lane C-: Negative control (uncultured PPLO broth) and Lane 1 to 4 are the Mycoplasma isolates in this study.