

**Original Article**

# Molecular Identification of *Mycoplasma agalactiae* in Iran Based on *P30* Gene

Babazadeh, M<sup>1</sup>, Pourbakhsh, S. A<sup>2</sup> \*, Noormohammadi, Z<sup>1</sup>, Esmaelizad, M<sup>3</sup>, Goudarzi, H<sup>2</sup>

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

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Corresponding Author: a.pourbakhsh@rvsri.ac.ir

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## Abstract

*Mycoplasma agalactiae* (*M. agalactiae*) is known as the main etiological agent of contagious agalactia (CA). The CA is a disease affecting dairy sheep and goats, the main characteristics of which include keratoconjunctivitis, arthritis, and mastitis. This pathogen results in milk production reduction and suppression, thereby leading to serious economic loss. In the present study, 125 sheep and goat samples were collected from 15 provinces of Iran. Cultural and molecular methods were used for sample characterization. After extracting genomic DNAs using the phenol/chloroform method, the 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 clinical samples. Finally, to validate the experimental approach, a 375 bp amplicon of *P80* lipoprotein was amplified using the MA-PCR. Out of 125 samples under investigation, 43 cases were positive, and *Mycoplasma* colonies were observed in the pleuropneumonia-like organisms agar culture. Based on the results of the M-PCR method, 61 specimens (out of 125 samples) were scored positive for *Mycoplasma* presence. Furthermore, 20 samples were positive according to the P30-PCR data. It should be mentioned that the MA-PCR was performed based on the *P80* gene on 125 total samples to further verify the results for *M. agalactiae* detection. Based on the obtained data, *P30* and *P80* genes were presented and amplified in all Iranian *M. agalactiae* isolates (n=20). Our results indicated that the *P30* gene was conserved and specific to all Iranian *M. agalactiae* isolates and this new P30-PCR method (as an MA-PCR technique) might be useful in the detection of this pathogen.

**Keywords:** Contagious agalactia, Culture, *Mycoplasma agalactiae*, PCR, *P30* gene

## Identification moléculaire de *Mycoplasma agalactiae* en Iran sur la base du gène *P30*

**Résumé:** *Mycoplasma agalactiae* (*M. agalactiae*) est connu comme le principal agent étiologique de l'agalactie contagieuse (AC). L'AC est une maladie affectant les ovins et caprins laitiers, dont les principales caractéristiques comprennent la kératoconjunctivite, l'arthrite et la mammite. Cet agent pathogène entraîne une réduction et une suppression de la production laitière, entraînant ainsi de graves pertes économiques. Dans la présente étude, 125 échantillons de moutons et de chèvres ont été collectés dans 15 provinces d'Iran. Des méthodes culturelles et moléculaires ont été utilisées pour la caractérisation des échantillons. Après avoir extrait les ADNs génomiques à l'aide de la méthode phénol/chloroforme, la technique de PCR a été utilisée pour détecter le genre *Mycoplasma* dans un fragment de 163pb du gène de l'ARNr 16S (M-PCR) et *M. agalactiae* dans un fragment de 800pb de la conservation et le gène de la lipoprotéine *P30* spécifique (P30-PCR) dans des échantillons culturels et cliniques. Enfin, pour valider l'approche expérimentale, un amplicon de 375 pb de

lipoprotéine *P80* a été amplifié à l'aide de la MA-PCR. Sur 125 échantillons étudiés, 43 cas étaient positifs et des colonies de *Mycoplasmas* ont été observées dans la culture sur gélose d'organismes de type pleuropneumonie. Sur la base des résultats de la méthode M-PCR, 61 échantillons (sur 125 échantillons) ont été marqués positifs pour la présence de *mycoplasmas*. De plus, 20 échantillons étaient positifs selon les données P30-PCR. Il convient de mentionner que la MA-PCR a été réalisée sur la base du gène *P80* sur 125 échantillons au total pour vérifier davantage les résultats de détection de *M. agalactiae*. Sur la base des données obtenues, les gènes *P30* et *P80* ont été présentés et amplifiés dans tous les isolats iraniens de *M. agalactiae* (n=20). Nos résultats ont indiqué que le gène *P30* était conservé et spécifique de tous les isolats iraniens de *M. agalactiae* et que cette nouvelle méthode P30-PCR (en tant que technique MA-PCR) pourrait être utile dans la détection de ce pathogène.

**Mots-clés:** Agalactie contagieuse, Culture, *Mycoplasma agalactiae*, PCR, gène *P30*

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## Introduction

*Mycoplasma agalactiae* has been recognized as the main agent of contagious agalactia (CA) disease, which affects dairy sheep and goats; moreover, it is mainly identified by keratoconjunctivitis, arthritis, and mastitis (Office International des Epizooties, 2008). This pathogen results in milk yield reduction and suppression, and consequently, serious economic loss (Oravcova et al., 2009). This syndrome is produced by *Mycoplasma agalactiae* subsp. *agalactiae* though other *Mycoplasmas* (e.g., *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., 2009).

Contagious agalactia has been observed in several countries and is considered 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 an important adaptive strategy in this family of pathogens (Nouvel et al., 2009). It 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 a specific, stable, and

highly immunogenic *M. agalactiae* antigen, and therefore, can be used to detect, identify, and subtype *M. agalactiae* (Fleury et al., 2002). 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., 2002).

Several molecular studies have been previously performed on the isolation, detection, and identification of *M. agalactiae* by the amplification of 375 bp of *P80* lipoprotein gene (the studies conducted by Khezri et al. [2012] on 69 sheep samples in Kurdistan province, Kheirkhah et al. [2011] on 57 samples of Iranian goats, Ashtari et al. [2015] on 91 sheep samples affected by contagious agalactia in Khuzestan province, as well as Momen and Roshdi Maleki [2014] on 367 samples in Hamadan province).

This study aimed to detect and identify *M. agalactiae* using new specific primers based on conserve and specific *P30* gene.

## Material and Methods

**Cultural Method and DNA Extraction.** In total, 125 samples were collected from the eye, ear, joint exudate, and milk secretions of sheep and goats exhibiting clinical signs of probable infection with *Mycoplasma*. It should be noted that the samples were obtained from 15 Provinces of Iran (East Azerbaijan, West Azerbaijan, Ardabil, Isfahan, Kerman, Qom, Khuzestan, Semnan, Kermanshah, Golestan, Gilan, Yazd, Sistan and Baluchestan, Kurdistan, and Ilam). After transferring

the samples to the medium and incubating them 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 pleuropneumonia-like organisms (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% CO<sub>2</sub>, and 98% humidity. The broths and plates were daily observed for the signs of growth and typical *Mycoplasma* colonies, respectively. To extract DNA, the phenol-chloroform method previously described by Tola et al. was used in this study (1997).

**Detection of *Mycoplasma* Genus.** In this study, a previously published primer set was utilized for specific detection of *Mycoplasma* genus (*i.e.* M1F: 5'-GCTGCGGTGAATACGTTCT-3', M3R: 5'-TCCCCACGTTCTCGTAGGG-3') (van Kuppeveld et al., 1992). Following that, the PCR was performed using 25 µl samples, each containing 2.5 µl of 10 X PCR buffer (CinnaGen, Iran), 2 mM MgCl<sub>2</sub>, 5 mM dNTPs, 2 µl of each primer (10 pm), and 0.5 U Taq DNA polymerase (CinnaGen, Iran). Eventually, 15.3 µl of deionized distilled water and 50 ng of extracted DNA as the template were added. The PCR was carried out using a Gradient Master cycler instrument (Eppendorf, Germany) according to the following protocol (Zendulkova et al., 2007): 7.5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 56°C, and 1 min at 72°C with a final extension cycle for 5 min at 72°C. The 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') was designed in the open reading frame region of P30 gene by Oligo 5.0 software. The P30 coding sequence was amplified via the PCR technique using 50 µl samples, each containing 5 µl of 10 X PCR buffer (CinnaGen, Iran), 2

mM MgCl<sub>2</sub>, 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. The PCR was performed in a Gradient Master cycler (Eppendorff, Germany) based on the following protocol: 3 min at 95°C, followed by 35 cycles of 1 min duration at 93°C, 30 sec at 54°C, and 40 sec at 72°C with a final 5-min extension cycle at 72°C. In the 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 the specific detection of *M. agalactiae* species: FS<sub>1</sub>: 5'AAA GGT GCT TGA GAA ATG GC-3' and FS<sub>2</sub>: 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 MgCl<sub>2</sub>, 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. The PCR was conducted in a Gradient Master cycler (Eppendorf, Germany) based on Tola et al. (1997) reported protocol with some modification: 5 min at 95°C, followed by 34 cycles of 1 min duration at 94°C, 1 min at 50°C, and 1 min at 72°C with a final 5-min extension cycle at 72°C. In MA-PCR, a fragment of the P80 lipoprotein gene with 375 base pair length was amplified to detect *M. agalactiae* species.

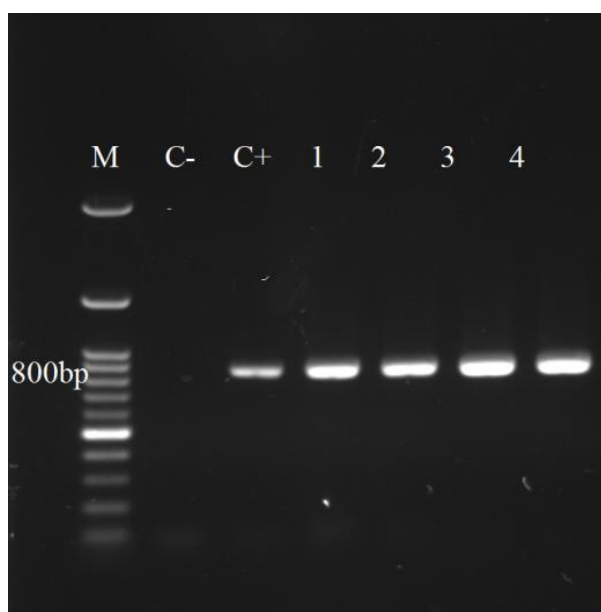
Visualization of amplified products (M-PCR, MA-PCR, and P30-PCR) was performed through ultraviolet illumination following electrophoresis (using agarose gel [1%] in TAE buffer) and SYBR Safe staining (Tola et al., 1997).

## Results

According to the cultural method, typical *Mycoplasma* colonies were observed in 43 (34.4%) sheep and goat samples (out of 125 samples), while 82

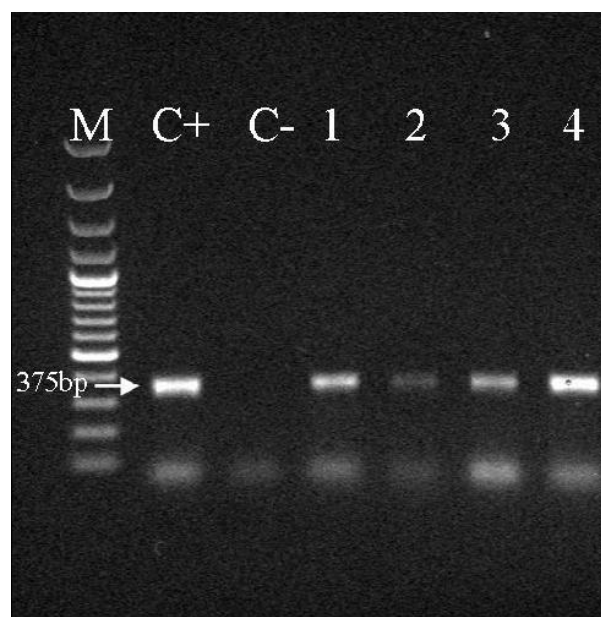
(65.6%) samples were negative for this pathogen. The positive *Mycoplasma* samples in the PPLO broth media were also cultured on PPLO agar media, and the resulting *Mycoplasma* colonies exhibited distinctive "fried egg" morphology under a light microscope.

Out of 125 total samples, 61 (48.8%) and 64 (51.2%) positive and negative samples for *Mycoplasma* presence were identified by *Mycoplasma* genus PCR (M-PCR) assay, respectively. For the 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).

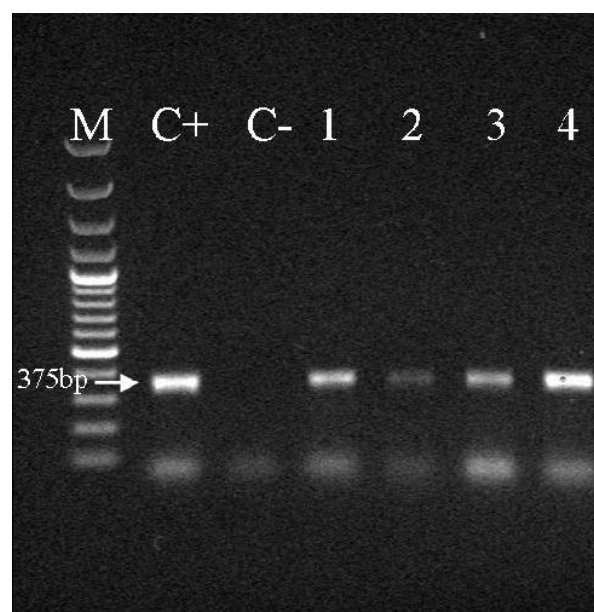


**Figure 1.** Mycoplasma genus PCR (M-PCR): PCR electrophoresis analysis in %1 gel agarose. M: Marker (100bp DNA ladder). Lane C+: Positive control (163bp band, Mycoplasma genus), Lane C-: Negative control (uncultured PPLO broth), and Lane 1 to 4 are the Mycoplasma isolates in this study.

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



**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.

The results of MA-PCR were similar to those of P30-PCR exactly. The complete coding sequence of the P80 gene with 375 bp length was amplified in 20 samples (out of 125 samples) which were positive in the P30-PCR method as shown in Figure 3. Furthermore, *M. agalactiae* positive samples from a total of 125 samples were successfully detected using two P30-PCR and MA-PCR *M. agalactiae* specific PCR methods. According to both P30-PCR and MA-PCR results, milk samples were the most affected specimens, while joint exudates and ear samples were the least influenced

samples by *M. agalactiae* (Table1). In addition, it was proved that 20 (16%) samples were infected with *M. agalactiae* based on cultural and PCR methods, and this pathogen was successfully detected using both methods.

The results of P30-PCR and MA-PCR methods were similar exactly, and therefore, the P30-PCR method using the newly 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).

**Table 1.** Results of cultural and three PCR methods in all samples based on the clinical sampling

Tests	Result	Milk sample	Ear sample	Eye sample	joint sample	Total
<b>Culture</b>	Positive	24	3	15	1	43
	Negative	30	5	36	11	82
<b>M-PCR</b>	Positive	31	4	25	1	61
	Negative	23	4	26	11	64
<b>P30-PCR</b>	Positive	14	0	6	0	20
	Negative	40	8	45	12	105
<b>MA-PCR</b>	Positive	14	0	6	0	20
	Negative	40	8	45	12	105

## Discussion

The samples of the mammary gland (milk), conjunctivae (eye), external canal of ear, and joint exudate of sheep and goats for *M. agalactiae* detection were used in this study based on the *P30* gene. All 125 samples (isolated from 15 provinces of Iran) were cultured in the PPLO broth media for the diagnosis of *Mycoplasma*. The M-PCR was also performed to detect *Mycoplasma* genus based on the *16S rRNA* gene. Furthermore, the 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 the P30-PCR method and gain a better understanding of Iranian isolates of this pathogen, the presence of the *P80* lipoprotein gene was checked in all samples using the MA-PCR method. On the other hand, a comparative analysis was performed between two P30-PCR and MA-PCR methods to examine the specificity of the *P30* gene for *M. agalactiae* isolates. According to the

results, the expected 800 bp fragment belonging to the *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 the *P30* gene is highly specific to *M. agalactiae* isolates.

In order to determine the sensitivity of the 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 the *P30* gene 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 an early and rapid warning system for clinical samples. This method allows performing a full assessment in the case of positive results though negative outcomes cannot be regarded as definitive. The detection limit of the cultural method for *M. agalactiae* has been suggested to be  $2 \times 10^2$  CCU\*/ml (Bergonier et al., 1997; Tola et al., 1997). Our findings were in line with the results reported by Kheirkhah et al. (2011) in Iran and Amores et al. (2010) in Spain, indicating that PCR was 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 was much sensitive and faster than the cultural method and reduced 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, respectively. However, *P30* and *P80* genes were not detected in ear samples and joint exudates. Kheirkhah 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. The obtained data in the present study were partially consistent with their findings since *M. agalactiae* was detected in the milk and eye samples; however, this pathogen was not detected in joint exudates and ear samples. The results might have been

dependent on the initial number of clinical samples obtained from different sampling sites. As can be observed in Table 1, the total numbers of joint and ear samples were fewer than those of milk and eye specimens due to difficulty in sampling. The present study was conducted on suspected samples, and it was associated with more reliable results, compared to other studies (De la Fe et al., 2007; Zendulkova et al., 2007) on samples with or without signs of CA. In the above-mentioned studies, *M. agalactiae* has been detected based on the DNA fragment of the *P80* gene with 375bp length.

Based on previous studies, the prevalence of contagious agalactia (*agalactiae*) in a herd of sheep/goat in Iran has been significantly increased in recent years such that since 2011, the incidence rate of this disease has reached 33.3% (Kheirkhah et al., 2011). Many variable genes in *M. agalactiae* play prominent roles in inducing host immunity to infection and facilitating immune evasion (Browning et al., 2011). Therefore, detection and identification of conserved and specific genes in *M. agalactiae* are among important strategies for controlling *M. agalactiae* infections. One of the conserved genes in *M. agalactiae* is *P40*, which encodes an immunodominant lipoprotein that plays a role in cytoadherence and pathogenicity (Oravcova et al., 2009). Mahdavi et al. (2009) have studied the *P40* gene in three Iranian vaccine strains of *Mycoplasma agalactiae* and reported the homology percentage of this gene among these strains; however, the aforementioned study was limited to vaccine strains and involved no field isolates. Furthermore, based on a study carried out 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. (2002), 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*, a primer pair (*P30F* and *P30R*) was

designed that was 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); however, other *Mycoplasma* species were not tested in this regard. Based on the results, the *P30* gene was present in all tested *M. agalactiae*, and it was specific to this pathogen species. The findings of this study were in agreement with the data reported by Fleury et al. (2002) in Switzerland, in which the *P30* gene was specific to *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 analogs to *P30* were found in related species. Moreover, it was indicated in this study that the new PCR assay designed based on the *P30* gene was useful in detecting *M. agalactiae* as an 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 to obtain genomic diversity and genomic homogeneity in Iranian *M. agalactiae* isolates as candidates for the application in diagnostic ELISA kits and recombinant vaccines against contagious agalactia (*agalactiae*).

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#### Authors' Contribution

Study concept and design: S. A. P.

Acquisition of data: M. B.

Analysis and interpretation of data: M. E.

Drafting of the manuscript: M. B. and S. A. P.

Critical revision of the manuscript for important intellectual content: Z. N.

Statistical analysis: H. G.

Administrative, technical, and material support: RVSRI

#### Ethics

It is declared that all ethical standards have been respected in the preparation of the submitted article.

#### Conflict of Interest

The authors declare that they have no conflict of interest.

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