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*Original Article*

# Detection of *Mycoplasma agalactiae* in Small Ruminants of Southeast Iran

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**ABSTRACT**

Agalactia is an infectious and contagious disease of small ruminants caused by *Mycoplasma agalactiae* (*M. agalactiae*). Although different microorganism strains contribute to this disease, *M. agalactiae* is known as the most prominent causative agent. Therefore, this study aimed to investigate the rate of *M. agalactiae* involvement in contagious agalactia in the southeast region of Iran. Sampling was performed from milk, conjunctiva, ear lesions, and joints exudate of suspicious sheep and goat flocks according to the reports of Iran Veterinary Organization. The presence of Mycoplasma and its species, namely *M. agalactiae*, was evaluated through microbial culture and polymerase chain reaction (PCR) techniques. The detected microorganisms were confirmed to be Mycoplasma and *M. agalactiae* by the PCR amplification of 16S rRNA and lipoprotein target genes. According to the findings of present study, 14.8% and 36.0% of the samples were diagnosed as positive for Mycoplasma by culture and PCR, respectively. Moreover, the incidence of *M. agalactiae* was determined as 6.1% using the specific PCR method. Therefore, it is recommended to identify the other species of Mycoplasma in small ruminant samples involved with contagious agalactiae disease.

***Keywords:*** Agalactia, *Mycoplasma agalactiae*, PCR, Southeast of Iran

**Détection de *Mycoplasma agalactiae* chez les petits ruminants du le Sud-est de l'Iran**

**Résumé:** L'agalaxie est une maladie infectieuse et contagieuse chez les petits ruminants engendrée notamment par le *Mycoplasmaagalactiae* (*M. agalactiae*). Différents facteurs contribuent à l'apparition del'agalaxie , mais *M. agalactiae* a été reconnu comme l'un des agents étiologiques les plus importants. Dans cetteétude, l'incidence de *M. agalactiae*à l'origine de l'agalaxie contagieuse a été évaluéedans le Sud-est de l'Iran. Des prélèvements ont été menés en respectant les consignes de l'organisation vétérinaire iranienne sur le lait, la conjonctive, leslésionsauculairesainsi que sur la la ponction du liquide synovial des troupeaux de brebis et chèvres suspectés d'être contaminés. La présence de *Mycoplasma* et de *M. agalactiae* a été détectée en utilisant les techniques de culture microbienne et de PCR. *Mycoplasma* et *M. agalactiae* ont été identifiés par PCR via l'amplification de la séquence 16S-rRNA desgènes cibles et par la caractérisation des lipoprotéines. Le taux de contamination par le genre *Mycoplasma*a été respectivement estimé a14.8% et 36.0% par culture et par PCR. Les infections causées par *M. agalactiae*constituées 6.1% des échantillons. Cette étude souligne l'importance d'identifier d'autres espèces de Mycoplasma dans les échantillons de petits ruminants engagé avec maladie de l'agalaxie contagieuse.

**Mots-clés:** Agalaxie, *Mycoplasma agalactiae*, Culture, PCR, Sud-est de l'Iran

## INTRODUCTION

Agalactia is an infectious and contagious disease of sheep and goats with a wide spread throughout Europe, North Africa, and some parts of Asia in a non-sex-linked pattern. This disease usually occurs after parturition and presents in three forms, including mastitis, arthritis, and conjunctivitis, in addition to abortion, which may be observed in pregnant animals. This disease could be fatal in young animals due to the induced pulmonary complications (Khezri et al.; Al-Momani et al., 2008)OIE, 2013; (Quinn et al., 2011; Shamsaddini Bafti, 2015). While *Mycoplasma agalactiae (M. agalactiae)* is considered as the main agent for this disease, researchers have also reported some other pathogenic strains as the causative agents involved in the pathogenesis of agalactia in Iran (Khezri et al.; Kheirkhah et al., 2011b; Khezri et al., 2012). According to the official statistics of Iran Veterinary Organization, agalactia is found in some livestock-breeding areas and infects both goats and sheep. Although the mortality rate of this disease is low, the resultant economic losses are significant. Accordingly, vaccines for the prevention and control of agalactia due to *M. agalactiae* are widely applied in the Mediterranean Europe and Western Asia countries (OIE, 2013). Different methods have been used to test for Mycoplasma infections, namely microbial culture, immunological methods (e.g., enzyme-linked immunosorbent assay, immunofluorescence, and immunoblotting), and molecular techniques, such as polymerase chain reaction (PCR) (Uphoff and Drexler, 2002). The PCR method is based on the direct detection of 16S rRNA genes specific for most of the important Mycoplasmas (Dvorakova et al., 2005) (Hopert et al., 1993; Pruckler et al., 1995; Nissen et al., 1996; Garner et al., 2000) (Wirth et al., 1994; Rawadi and Dussurget, 1995; Tang et al., 2000; Uphoff and Drexler, 2002). However, *M. agalactiae* cannot be detected by a single test due to the limited sensitivity and specificity of these techniques leading in time-consuming isolation and characterization. With this background in mind, the present study attempted the rate of *M. agalactiae* involvement in contagious agalactia in the southeast region of Iran.

## MATERIALS AND METHODS

**Sampling.** This study examined the small ruminants (i.e., fat-tailed sheep and Raeini goats) of the southeast region of Iran. The samples (n=264) were taken from 70 suspected herds with the most obvious signs in different parts of the region. Depending on the affected organs, sampling was performed through milking (n=161), conjunctival swabs (n=52), ear swabs (n=39), joint puncture for exudate (n=6), and venipuncture (n=6) in the sick animals. Joint exudate samples were kept and transferred in microtubes without transport medium. Other samples were immediately transferred to the Mycoplasma culture transport medium (Hesarak, Razi Vaccine and Serum Research Institute, Iran), and kept at 4 °C until being sent to the laboratory within 24 h.

**Culture and Isolation.** The culture and isolation assays were carried out as previously described with slight modifications (Shamsaddini Bafti, 2015). Briefly, the samples were incubated at 37 °C in the presence of $CO_2$ for 24 h. In order to investigate the formation of the unique colonies of Mycoplasma, the cultured PPLO agar and broth mediums were incubated at 37 °C in a $CO_2$ incubator for 21 days. The growth and specific colonies formed on the agar medium could discriminate between the infection with the intended microorganisms and contamination in case of discoloration or opacity of the PPLO broth medium.

**Molecular Techniques.** DNA extraction was performed according to the previously described protocol (Tola et al., 1997). Furthermore, the PCR technique was carried out as defined by Shamsaddini Bafti et al. (2015). All the primers used in the PCR are listed in Table 1. When the reaction was completed, 10 µL of the amplified product was separated by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide. Finally, the amplified bands were visualized and photographed under the UV illumination. The DNA of the standard *M. agalactiae*

bacterium sample (NCTC 10123) and the uncultured PPLO broth medium were utilized as the positive and negative controls, respectively.

## RESULTS

Microbial culture and PCR were performed on the 264 samples collected from 70 farms of 10 different provinces, the results of which are summarized in tables 2 and 3. As the findings demonstrated, 39 (14.8%) samples were detected as positive with culture. In the PCR technique executed based on 16S rRNA gene, 95 (36.0%) Mycoplasma isolates were identified (Figure 1), 16 (6.1%) cases of which were *M. agalactiae* (Figure 2). In addition, the presence of *M. agalactiae* species in the ear swabs of the studied small ruminants was confirmed by the molecular methods. Overall, 29 (10.9%) of the secreted milk, conjunctiva swab, ear swab, and joint exudate samples were revealed to be positive in both culture and PCR results. The lowest frequency belonged to the milk, conjunctiva and ear swabs, as well as joint exudate samples with positive culture and negative PCR results. In addition, negative culture despite positive PCR results was observed in 25% of the samples (Table 4). Furthermore, a total of 16 milk, conjunctiva, and ear swab samples were diagnosed as positive by the three techniques of culture, Mycoplasma PCR, and *M. agalactiae* PCR (Table 5).

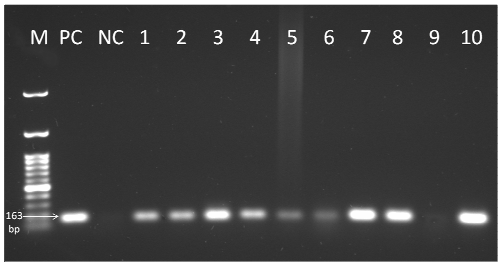


**Figure 1**. Polymerase chain reaction gel electrophoresis for 16S rRNA gene in Mycoplasma genus Line M: marker 100bp, Line PC: positive control (Mycoplasma genus, NCTC 10123l), Line NC: negative control (uncultured PPLO broth) and Line 1-10: samples

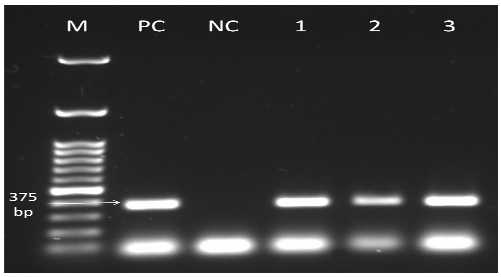


**Figure 2.** Polymerase chain reaction gel electrophoresis for lipoprotein gene in *Mycoplasma agalactiae*; Line M: marker 100bp, Line PC: positive control (*Mycoplasma agalactiae*, NCTC 10123l), Line NC: negative control (uncultured PPLO broth), and Line 1-3: samples

## DISCUSSION

**Table 1**. Sequences of primers used for the detection of Mycoplasma and *M. agalactiae* by polymerase chain reaction

| Primer | Target gene | Sequence | Amplicon (bp) | Reference |
|---|---|---|---|---|
| M1, M3 | 16S rRNA | F: 5′-GCTGCGGTGAATACGTTCT-3′<br>R: 5′-TCCCCACGTTCTCGTAGGG-3′ | 163 | Kojima et al. 1997 |
| FS1, FS2 | Lipoprotein | F: ′5-AAAGGTGCTTGAGAAATGGC-3′<br>R: 5′-GTTGCAGAAGAAAGTCCAATCA-3′ | 375 | Tola et al. 1997 |

**Table 2.** Culture and polymerase chain reaction results for Mycoplasma and *M. agalactiae* in study population

| Species | No. | Results<br>Number (percent within sample) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Culture | | | | *Mycoplasma*-PCR | | | | *M. agalactiae- PCR* | | | |
| | | Positive | | Negative | | Positive | | Negative | | Positive | | Negative | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Goat | 176 | 25 | 14.2 | 151 | 85.8 | 62 | 35.2 | 114 | 64.8 | 11 | 6.3 | 165 | 93.8 |
| Sheep | 88 | 14 | 15.9 | 74 | 84.1 | 33 | 37.5 | 55 | 62.5 | 5 | 5.7 | 83 | 94.3 |
| **Total** | **264** | **39** | **14.8** | **225** | **85.2** | **95** | **36.0** | **169** | **64.0** | **16** | **6.1** | **248** | **93.9** |

**Table 3.** Culture and polymerase chain reaction results of Mycoplasma and *M. agalactiae* in collected samples

| Sample Type | No. | Results Number (percent) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Culture | | | | *Mycoplasma*-PCR | | | | *M. agalactiae*-PCR | | | |
| | | Positive | | Negative | | Positive | | Negative | | Positive | | Negative | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| **Milk** | **161** | 19 | 11.8 | 142 | 88.2 | 50 | 31.1 | 111 | 65.7 | 9 | 5.6 | 152 | 94.4 |
| **Conjunctiva** | **52** | 10 | 19.2 | 42 | 80.8 | 28 | 53.8 | 24 | 46.2 | 4 | 7.7 | 48 | 92.3 |
| **Ear** | **39** | 9 | 23.1 | 30 | 76.9 | 14 | 35.9 | 25 | 64.1 | 3 | 7.7 | 36 | 92.3 |
| **Joint exudate** | **6** | 1 | 16.7 | 5 | 83.3 | 2 | 33.3 | 4 | 66.7 | 0 | 0.0 | 6 | 100 |
| **Blood** | **6** | 0 | 0.0 | 6 | 100 | 1 | 16.7 | 5 | 83.3 | 0 | 0.0 | 6 | 100 |
| **Total** | **264** | **39** | **14.8** | **225** | **85.2** | **95** | **36.0** | **169** | **64.0** | **16** | **6.1** | **248** | **93.9** |

**Table 4.** Distribution of the culture and PCR results for Mycoplasma genus

| Results | | Number (percent within sample) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Milk | | Conjunctiva | | Ear | | Joint exudate | | Blood | | Total |
| Culture | PCR | No. | % | No. | % | No. | % | No. | % | No. | % | |
| + | + | 11 | 4.2 | 9 | 3.3 | 8 | 3.0 | 1 | 0.4 | 0 | 0.0 | **29** |
| + | - | 8 | 3.0 | 1 | 0.4 | 1 | 0.4 | 0 | 0.0 | 0 | 0.0 | **10** |
| - | + | 39 | 14.8 | 19 | 7.2 | 6 | 2.2 | 1 | 0.4 | 1 | 0.4 | **66** |
| - | - | 103 | 39.1 | 23 | 8.7 | 24 | 9.1 | 4 | 1.5 | 5 | 1.9 | **159** |

**Table 5.** Comparison of polymerase chain reaction results for Mycoplasma and *M. agalactiae*

| Results | | Number (percent within sample) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Milk | | Conjunctiva | | Ear | | Joint exudate | | Blood | | Total |
| Genus | Species | No. | % | No. | % | No. | % | No. | % | No. | % | |
| + | + | 9 | 3.4 | 4 | 1.5 | 3 | 1.6 | 0 | 0.0 | 0 | 0.0 | **16** |
| + | - | 41 | 21.6 | 24 | 12.7 | 11 | 5.5 | 2 | 1.0 | 6 | 0.6 | **79** |
| - | - | 111 | 32.8 | 24 | 7.1 | 25 | 7.5 | 4 | 1.2 | 5 | 1.5 | **169** |

Contagious agalactia is a disease of small ruminants with a complicated distribution pattern. The causative agent of this disease has unique characteristics. It should be noted that poor management practice in sheep and goat farms plays a critical role in the occurrence of the disease (Kumar et al., 2014). The results of the current study showed that small ruminants suspicious of contagious agalactia in Iran were infected with *M. agalactiae* as the major agent. The PCR techniques are of value for the direct identification of the organisms in clinical samples, including nasal and conjunctival swabs, synovial exudates, milk, and tissue samples. This technique is a rapid, reliable, and simple method for detecting Mycoplasma infection in small ruminants and has been shown to be successful in recognizing *M. agalactiae* in various samples. Both culture and PCR methods are accurate in the identification of Mycoplasma genus. On the other hand, microbial culture not only is almost costly and time consuming, but also has presented false negative results in some cases. Accordingly, positive culture results should be confirmed by PCR using 16s rRNA genes, especially in the areas with low contagious agalactia prevalence. However, early and timely diagnosis is of great importance regarding the control of the diseases caused by Mycoplasma (Nicholas et al., 2008). In the present study, all the samples with positive PCR results for Mycoplasma genus were tested for species diagnosis. Our findings demonstrated that only 6.1% of the samples diagnosed as Mycoplasma in the study area were *M. agalactiae*; in other words, 83.0% of the detected Mycoplasma infections were the species other than *M. agalactiae*. Khezri et al. (2014) reported that Mycoplasma and *M. agalactiae* were found in 93.1%

and 12.4% of the Kurdish sheep with the signs of agalactia, respectively. In this regard, 86.6% of the detected *Mycoplasma* infections in the mentioned study seems to be species other than *M. agalactiae*. Kheirabadi and Ebrahimi (Kheirabadi and Ebrahimi, 2007) revealed the presence of *M. agalactiae* in 22.2% and 17% of the sheep eye swabs and milk samples using PCR technique, respectively. Pooladgar et al. (Pooladgar et al., 2011) reported 19.1% of *M. agalactiae* presence in the samples taken from Khuzestan region by PCR method. The sensitivity of the PCR technique has been indicated to be higher than that of the culture method. When the *Mycoplasma* load is low in the sample, or the microorganism is lost for different reasons (e.g., inappropriate maintenance conditions or use of antibiotics in the treatment period), it cannot be detected and tracked by the culture method. However, PCR technique facilitates the identification of this species (Amores et al., 2010). As shown by the results of different samples used in the present study, conjunctiva was a less appropriate specimen than the other samples for the approval of *Mycoplasma* and *M. agalactiae* presence. All of the aforementioned locations have been used to isolate the disease agent in similar studies (Kheirkhah et al., 2011a; Khezri et al., 2012). *Mycoplasma* was detected in only one blood sample, which could be caused by the septicemia resulting from the proliferation and spread of Mycoplasma by blood. The generated septicemia plays an important role in the development of acute lesions with poor prognosis (Gutierrez et al., 1999; MR et al., 2011). In Iran, more than 90% of the sheep and goat population is kept as mixed herds, which facilitates the transmission of Mycoplasmas from one animal species to the other. Clinical signs in the studied herds mostly entailed mastitis in goats and sheep, arthritis in young goats, and pneumonia in different age groups (Kheirkhah et al., 2011a; Khezri et al., 2012); (Pooladgar et al., 2011). Animals may suffer from acute, sub-acute, chronic, or asymptomatic forms of the disease, which depends on various factors, such as maternal antibodies titer, immune-compromised state, transportation stress, pregnancy, or extreme climatic conditions. Rapid spread and multiple infection sources, along with vertical and horizontal modes of transmission, are the reasons for immense concern and affect the local economy severely. Moreover, the presence of asymptomatic carriers, which carry the infectious agent in a herd, is a grave concern in endemic regions. Therefore, the successful diagnosis of carrier animals is considered as an essential step in taking appropriate control measures. Although few Mycoplasma vaccines are presently available, they might be an influential and cost-efficient way for the prevention of the disease spread (Al-Momani et al., 2008). Live vaccines can prevent symptoms; however, they do not prevent animals from becoming infected or shed the organism. On the other hand, inactivated vaccines generally provide short-term protection.

As the findings of the present study revealed, *M. agalactiae* was detected in small ruminants in the southeast region of Iran. Furthermore, the results of the current study suggested that in addition to *M. agalactiae* as the main etiological agent of contagious agalactia in this area, other species of Mycoplasma are also involved in this disease. Consequently, it is recommended to investigate the presence and prevalence of the other species, such as *Mycoplasma capricolum*, *Mycoplasma mycoides*, and *Mycoplasma putrefaciens* in sheep and goat population.

## 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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## References

Al-Momani, W., Nicholas, R.A., Abo-Shehada, M.N., 2008. Risk factors associated with *Mycoplasma agalactiae* infection of small ruminants in northern Jordan. Prev Vet Med 83, 1-10.

Amores, J., Corrales, J.C., Martín, Á.G., Sánchez, A., Contreras, A., de la Fe, C., 2010. Comparison of culture and PCR to detect *Mycoplasma agalactiae* and Mycoplasma mycoides subsp. capri in ear swabs taken from goats. Vet Microbiol 140, 105-108.

Dvorakova, H., Valicek, L., Reichelova, M., 2005. Detection of mycoplasma contamination in cell cultures and bovine sera. J Vet Med 50, 262-268.

Garner, C., Hubbold, L., Chakraborti, P., 2000. Mycoplasma detection in cell cultures: a comparison of four methods. Br J Biomed Sci 57, 295.

Gutierrez, C., Rodriguez, J., Montoya, J., Fernandez, A., 1999. Clinico-pathological and haematological findings in goat kids experimentally infected simultaneously with *Mycoplasma mycoides* subsp. capri and Mycoplasma mycoides subsp. mycoides (large colony-type). Small Rumin Res 31, 187-192.

Hopert, A., Uphoff, C.C., Wirth, M., Hauser, H., Drexler, H.G., 1993. Specifity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasma contamination in cell lines. J Immunol Methods 164, 91-100.

Kheirabadi, K., Ebrahimi, A., 2007. Investigation of *Mycoplasma agalactiae* in milk and conjunctival swab samples from sheep flocks in west central, Iran. Pak J Biol Sci: PJBS 10, 1346-1348.

Kheirkhah, B., Pourbakhsh, S., Ashtari, A., Amini, K., 2011a. Detection of Mycoplasma agalactiae by culture and Polymerase Chain Reaction (PCR) methods from affected sheep to contagious agalactiae in Baft County.

Kheirkhah, B., Pourbakhsh, S.A., Nadalian, M.-g., Banani, M., Ashtari, A., 2011b. Detection of Mycoplasma agalactiae by culture and polymerase chain reaction (PCR) methods from Iranian goats. Afr J Microbiol Res 5, 1668-1972.

Khezri, M., Pourbakhsh, S.A., Ashtari, A., Rokhzad, B., Investigation of *Mycoplasma agalactiae* by molecular methods in goat bucks in West of Iran.

Khezri, M., Pourbakhsh, S.A., Ashtari, A., Rokhzad, B., Khanbabaie, H., 2012. Isolation and prevalence of Mycoplasma agalactiae in Kurdish sheep in Kurdistan, Iran. Vet World 5.

Kumar, A., Rahal, A., Chakraborty, S., Verma, A.K., Dhama, K., 2014. *Mycoplasma agalactiae*, an etiological agent of contagious agalactia in small ruminants: a review. Vet Med Intl 2014.

Mokhber Dezfouly, M., Sadeghian, S., Javanbakht, J., Lakzian, A., 2011. A study of occurrence and histopathology of Mycoplasma infection in sheep in Tehran suburb, Iran. J Infect Dis Immun 3, 106-111.

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

Nissen, E., Pauli, G., Vollenbroich, D., 1996. Comparison of PCR detection methods for mycoplasma in cell cultures. In Vitro Cell Dev Biol Anim 32, 463-464.

Pooladgar, A., Rahimilarki, E., Hossieni, S., 2011. Application of PCR for diagnosis of contagious Agalactia in Khuzestan Province-Iran. Afr J Microbiol Res 5, 5097-5101.

Pruckler, J.M., Pruckler, J.M., Ades, E.W., 1995. Detection by polymerase chain reaction of all common Mycoplasma in a cell culture facility. Pathobiology 63, 9-11.

Quinn, P.J., Markey, B.K., Leonard, F.C., Hartigan, P., Fanning, S., FitzPatrick, E., 2011. Vet Microbiol Microb Dis, John Wiley & Sons.

Rawadi, G., Dussurget, O., 1995. Advances in PCR based Detection of Mycoplasmas Contaminating Cell Cultures. prokaryotes 20, 20.

Shamsaddini Bafti, M., Pourbakhsh, S.A., Ezatkhah, M. and Ashtari, A., 2015. 16S rRNA PCR test for mycoplasma spp in sheep. Online J Vet Res 19, 704-708.

Tang, J., Hu, M., Lee, S., Roblin, R., 2000. A polymerase chain reaction based method for detecting Mycoplasma/Acholeplasma contaminants in cell culture. J Microbiol Methods 39, 121-126.

Uphoff, C.C., Drexler, H.G., 2002. Comparative PCR analysis for detection of mycoplasma infections in continuous cell lines. In Vitro Cell Dev Biol Anim 38, 79-85.

Wirth, M., Berthold, E., Grashoff, M., Pfützner, H., Schubert, U., Hauser, H., 1994. Detection of mycoplasma contaminations by the polymerase chain reaction. Cytotechnology 16, 67-77.