

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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Received 12 July 2016; Accepted 25 December 2016
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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 detected 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

(29,6%) étaient négatifs. 40 échantillons positifs (80%) ont été détectés, en termes de Mycoplasma, dans le test de réaction en chaîne, mais 34 (68%) des échantillons étaient positifs en culture. 11 échantillons (27,5%) sur 40 échantillons identifiés de Mycoplasma, étaient de genre mycoplasma agalactiae. Les échantillons positifs de mycoplasma étaient plus fréquents dans les échantillons auriculaires et vaginaux, puis dans les échantillons d'oreille, du lait en culture et réaction en chaîne par polymérase. Mycoplasma / Mycoplasma Agalactiae étaient plus fréquemment détectés dans les échantillons provenant de l'oreille et du vagin. Mycoplasma agalactiae est l'une des principales causes d'Agalaxie contagieuse, qui a été identifiée pour la première fois dans les troupeaux de brebis dans la province de Guilan. Les résultats ont démontré que la méthode de la chaîne polymérase pourrait être une bonne alternative à la méthode de culture en raison d'un temps de diagnostic plus court.

Mots-clés: Mycoplasma agalactiae, chaîne polymérase, brebis, province de Guilan

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 diseases 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 18000 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 18000 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 μ l of 70% ETOH, centrifuged for 5 min at 18000 g. After discarding ETOH and drying tubes, 50 μ l 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'-TCCCCACGTTCTCGT AGGG-3') (Van Kuppeveld *et al.*, 1992). Additionally, the *M. galactiae*-specific amplification primers were FS1 (5'-AAAGGTGCTTGAGAAATGGC-3') and FS2 (5'-GTTGGCAGAAGAAAGTCCAATCA-3') as described by Tola *et al.* (1997). The PCR master mix was performed in a total volume of 25 μ l per sample, containing 2.5 μ l of 10 XPCR buffer (CinnaGen), 2 μ l of 50 mM MgCl₂, 5 mM dNTPs, 10 pm of each primer and 0.5 U *Taq*DNA polymerase (CinnaGen). Consequently, 15.3 μ l of deionized distilled water and 2 μ l 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

Samples	Culture	MPCR	MAPCR
50 (70.4%) +	34 (68%) +	40 (80%) +	11 (27.5%) +
21 (29.6%) -	16 (31.3%) -	10 (20%) -	29 (72.5%) -
71 (100%)	50 (100%)	50 (100%)	40 (100%)

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

Table 2. Results of culture and polymerase chain reaction methods in different samples

Samples	NO	MAPCR +	MPCR +
Eye	13	2 (15.4%)	3 (23%)
Ear	9	2 (22.2%)	8 (88.8%)
Ear and vagina	30	6 (16.6%)	20 (70%)
Milk	17	1 (5.8%)	9 (52.9%)
Joint exudates	2	0	0
	71	11	40

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 NC: negative control (uncultured PLO broth), Lanes 1-8: *Mycoplasma* isolates.

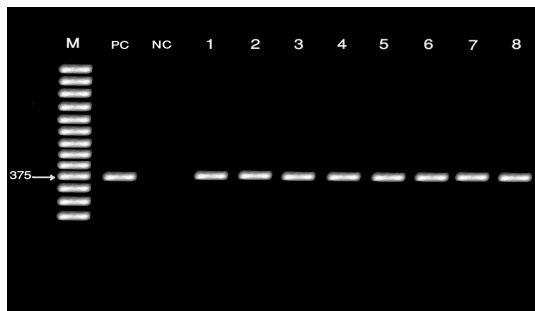


Figure 2. Mycoplasma *agalactiae*-specific polymerase chain reaction (PCR): PCR electrophoresis analysis in %1 gel agarose, M: marker (100 bp DNA ladder), lane PC: positive control (375 bp, *Mycoplasma agalactiae*, NCTC 10123), lane NC: negative control (uncultured PLO broth), lanes 1-8: *Mycoplasma*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

- 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.
- Azevedo, E.O.d., Alcântara, M.D.B.d., Nascimento, E.R.d., Tabosa, I.M., Barreto, M.L., Almeida, J.F.d., *et al.*, 2006. Contagious agalactia by *Mycoplasma agalactiae* in small ruminants in Brazil: first report. *Braz J Microbiol* 37, 576-581.
- Dedieu, L., Mady, V., Lefevre, P.-C., 1995. Development of two PCR assays for the identification of mycoplasmas causing contagious agalactia. *FEMS Microbiol Lett* 129, 243-249.
- Greco, G., Corrente, M., Martella, V., Pratelli, A., Buonavoglia, D., 2001. A multiplex-PCR for the diagnosis of contagious agalactia of sheep and goats. *Mol Cell Probes* 15, 21-25.
- Hajizadeh, A., Moazeni, J., Akhlagi, F., Naserirad, A., Motarez, B., Soleimani, S., *et al.*, 2012. Isolation and identification of *Mycoplasmas* causing contagious Agalactia syndrome in sheep & goats by using culture method and PCR in Ardebil province. *J Vet Lab Res* 4, 271-281.
- Kheirabadi, K., Ebrahimi, A., 2007. Investigation of *Mycoplasma agalactiae* in milk and conjunctival swab samples from sheep flocks in west central, Iran. *Pak J of Biol Sci* 10, 1346-1348.
- Kheirkhah, B., Pourbakhsh, S.A., Nadalian, M.-g., Banani, M., Ashtari, A., 2011. Detection of *Mycoplasma agalactiae* by culture and polymerase chain reaction (PCR) methods from Iranian goats. *Afr J Microbiol Res* 5, 1668-1972.
- Madanat, A., Zendulkova, D., Pospíšil, Z., 2001. Contagious agalactia of sheep and goats. A review. *Acta Vet Brno* 70, 403-412.
- Nicholas, R., Ayling, R., McAuliffe, L., 2008. *Mycoplasma diseases of ruminants*, CABI.
- Ruffin, D.C., 2001. *Mycoplasma infections in small ruminants*. *Veterinary Clinics of North America: Food Anim Pract* 17, 315-332.
- Subramaniam, S., Bergonier, D., Poumarat, F., Capaul, S., Schlatter, Y., Nicolet, J., *et al.*, 1998. Species identification of *Mycoplasma bovis* and *Mycoplasma agalactiae* based on the *uvrC* genes by PCR. *Mol cell Probes* 12, 161-169.
- Tola, S., Angioi, A., Rocchigiani, A., Idini, G., Manunta, D., Galleri, G., *et al.*, 1997. Detection of *Mycoplasma agalactiae* in sheep milk samples by polymerase chain reaction. *Vet Microbiol* 54, 17-22.
- Zendulkova, D., Madanat, A., Lany, P., Rosenbergova, K., Pospíšil, Z., 2007. Detection of *Mycoplasma agalactiae* by Polymerase Chain Reaction in Jordanian sheep and goat herds. *Acta Vet Brno* 76, 71-77.