



**Full Article**

**Isolation and identification of *Mycoplasma agalactiae* by culture and polymerase chain reaction (PCR) from sheep of Qom province, Iran**

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Received 18 Jan 2012; accepted 26 Dec 2012

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**ABSTRACT**

Contagious agalactia (C.A) is an infectious syndrome of sheep that is characterized by mastitis and subsequent failure of milk production, arthritis, abortion and keratoconjunctivitis. *Mycoplasma agalactiae* (*M. agalactiae*) is the main cause of the disease in sheep. The aim of this study was isolation and identification of *M. agalactiae* with culture and polymerase chain reaction (PCR) assay from sheep of Qom province in Iran. A total of 102 samples were collected from milk secretion, eye, ear and joint exudates of sheep. All samples were cultured in PPLO broth supplemented for *M. agalactiae* isolation. The bacteria DNAs were extracted by phenol/chloroform method and the PCR assay was applied for detecting of *Mycoplasma* genus in 163bp fragment of 16S rRNA gene and *M. agalactiae* in 375bp fragment of lipoprotein gene from culture as same as in clinical samples. Out of the 102 samples, 19(18.63%) cultures were shown positive and typical *Mycoplasma* colonies in PPLO agar culture diagnostic method and 59(57.8%) were scored positive by *Mycoplasma* genus PCR, 19(18.62%) of the samples were scored positive by using *M. agalactiae* PCR as diagnostic method. Out of the 102 samples, 19 samples were shown both positive in the culture and PCR, 42 samples were shown both negative in the culture and PCR. 40 samples were negative in the culture and positive in PCR whereas only one sample was positive in culture and negative in PCR. The results showed that the more isolations of *M. agalactiae* were taken from milk and less in joint samples. *M. agalactiae* was one of the main factors of contagious agalactia that was detected for the first time from sheep in Qom province.

**Keywords:** *Mycoplasma agalactiae*, Culture, PCR, 16S rRNA, Lipoprotein gene, Sheep, Qom province

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**INTRODUCTION**

Contagious agalactia (C.A.) is a significant infectious disease in dairy sheep and goats. It is identified by mastitis, arthritis, pneumonia and keratoconjunctivitis

(Nicolas 2008). It may be caused by any of the five *Mycoplasma* species associated with this disease, namely, *M. agalactiae*, *Mycoplasma mycoides* subsp *mycoides* LC (large colony), *Mycoplasma capricolum* subsp *capricolum*, *Mycoplasma mycoides* subsp *Capri* and *Mycoplasma putrefaciens* (Nicolas 2008,

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Zendulkova *et al* 2007, Bergnioer *et al* 1997). However *M. agalactiae* is still regarded, particularly in sheep as the “classical” etiological agent of the C.A (Bergnioer *et al* 1997). *M. agalactiae* can be very contagious in goats and sheep. It appears to acute, sub acute or chronic disease (Greco *et al* 2001).

C.A has been reported in southern Europe (Bergonier *et al* 1997) and south of America and north of Africa and serious problem exist in Iran where over 1300 cases were reported in 2006 (OIE 2008). It often appears in a herd in the spring soon after lactation begins and probably represents the activation of latent infection and females transmitted infectious factor to lambs by milk, so the main target of C.A is mammary gland, where a fall in or complete loss of milk production sometimes within 2 or 3 days (Nicolas 2008). This infection occurred in the herds at any ages but the pregnant and lactating females are more susceptible (Aytu *et al* 1990). The latency of this disease is 1-2 weeks but they carry it out for 7 months after spending the period of the incubation (Aytu *et al* 1990). Since 1981 several *Mycoplasma* species have been identified in the external ear canal of goats, including all species involved in the etiology of CA syndrome (Amores *et al* 2010). The sign of keratoconjunctivitis is usually of short duration and it was seen in about 50% of infected animals and it may occasionally develop in to a chronic infection, occasionally resulting in unilateral or bilateral blindness (Mebuss 1998).

Culture can be costly and time-consuming, and can also be inconclusive (Kheirkhah *et al* 2011). Molecular diagnostic tests have been used to identify the *M. agalactiae* since last decade too, PCR assay have been one of those tests for detecting of *M. agalactiae* in milk samples directly (Tola *et al* 1997). *M. agalactiae* was isolated and identified by culture and PCR assays from goats and sheep of provinces of Iran (Kheirkhah *et al* 2011, Moradi Bidhendi *et al* 2011, Pirali *et al* 2007). Because of the Qom province is located in central part of Iran and it is one of the most important provinces in terms of goats and sheep breeding. The incidence of

C.A in this province has increased during these years. There is not any investigation for detecting the agent of this syndrome in that province so this leads to isolate and detect of *M. agalactiae* which is one of the main etiological agent of the C.A. The aim of the present study was designed to isolate and identify of *M. agalactiae* by culture and PCR assays from sheep of Qom provinces in Iran with clinical signs of the disease.

## MATERIALS AND METHODS

**Sampling and cultures.** Samples were collected from eye, ear, milk secretion and joint exudates. Most of the samples were obtained from herds with clinical signs of a probably infection by *Mycoplasma*. All the sheep which were taken samples form them in this study had been previously examined to confirm that they had clinical signs of C.A. Following this clinical examination, the samples (eye, ear, milk secretion and joint exudates) were taken and placed in to the transport medium then samples transported on ice to the *Mycoplasma* reference laboratory of Razi Vaccine and Serum and Research Institute, Karaj, Iran.

The specimens were diluted and filtered into the fresh PPLO broth and then inoculated on to PPLO agar medium (BBL, Becton Dickinson and company, Cockeysville, Sparks, MD, USA). Inoculated agar and broth incubated at 37°C in 50% Co<sub>2</sub> and 98% humid atmosphere. The broths were observed daily for signs of growth and the plates were considered for the typical appearance of *Mycoplasma* colonies. *M. agalactiae* reference strain (NCTC 10123) have used in this study as a positive control and uncultured PPLO broth as a negative control.

**DNA extraction.** DNA was extracted from samples using a previously described method by Pourbakhsh *et al* (2010) with some modifications. 0/5ml of each sample was transferred to Eppendorf tube and centrifuged for 15 min at 13000 rpm. The supernatant fluid was discarded and add lysis buffer (Tris-HCl 50 mM pH=8, SDS 1%, NaCl 100mM, EDTA 50 mM, proteinase K 20 µl to 200µl) to the tube equal volume

of the pellet in it and incubate for at least 4 hrs at 56 °C. Equal volume of the material in the tube added phenol and mixed well by vortex. Centrifuged at 13000 rpm for 15 min. removed all aqueous layer (top layer) and transferred in a new tube. Added phenol: chloroform (1:1) in tube equal volume of the tube containing. Centrifuged at 13000 rpm for 15 min and transferred all aqueous layer in a new tube. Added chloroform in the tube, equal volume of the tube containing mix them well by vortex and centrifuged at 13000 rpm for 15 min. Transferred all aqueous layer in a new tube and sodium acetate was added 1:10 volume 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 13000 rpm. Discarded liquid containing of tube softly and 200 µl of 70% ETOH, centrifuged for 5 min at 13000 rpm. Discarded ETOH and drying tubes then add 50 µl distilled water to them.

**Amplification with specific primers.** In this study published primers set were used for the specific detection of genus and species of *M. agalactiae*. For genus *Mycoplasma* as follow: M1F: 5'-GCTGCGGTGAATACGTTCT-3', M3R: 5'-TCCCCCACGTTCTCGTAGGG-3'. (Pourbakhsh et al 2010). *M. agalactiae*-specific amplification primers set FS1: 5'-AAAGGTGCTTGAGAAATGGC-3' and FS2: 5'-GTTGGCAGAAGAAAGTCCAATCA-3' used that described by Tola et al (1997).

The PCR mix was performed in a total volume of 25µl per sample, containing 2.5 µl of 10 X PCR buffer (Sinagen), 2 µl of 50 mM MgCl<sub>2</sub>, 5 mM dNTPs, 10pm each primer, 0.5 U Taq DNA polymerase (Sinagen). Consequently 15.3 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) as follows: In genus: 7.5 minuets at 94 °C, followed by 30 cycles of 30 second at 94 °C, 30 second at 56 °C and 1 minuets at 72 °C, with a final extension cycle of 5 minuets at 72 °C. For *M. agalactiae* PCR 5 min at 95 °C, followed by 34 cycles of 1 min at 94 °C, 1 minuets at 50 °C and 1 minuets at 72 °C, with a final extension cycle of 5 minuets in at 72 °C. Visualization of amplified products was done by UV illumination after electrophoresis (1% agarose gel in 1×Tris-acetic acid-EDTA (TAE) buffer) and ethidium bromide staining.

## RESULTS

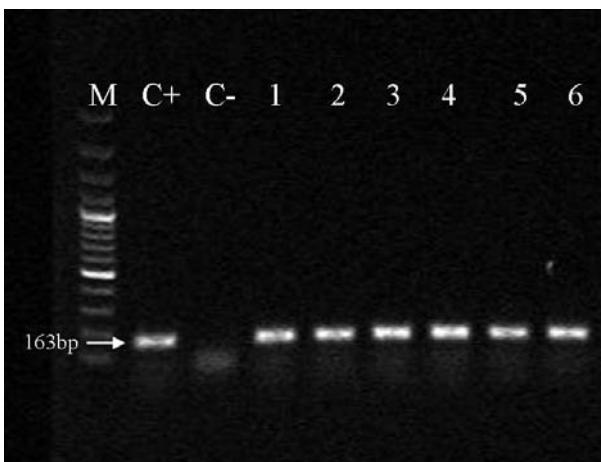
The 102 samples collected from sheep herds which analyzed simultaneously by culture and *Mycoplasma* genus PCR (MPCR) and *M. agalactiae* PCR (MAPCR). 19(18.63%) samples were positive and showed typical *Mycoplasma* colonies and 83 (81.37%) samples scored negative for using culture method. 59(57.8%) samples were scored positive for the

**Table 1.** The results of the culture and PCR methods in different samples.

Tests	Result	Milk sample	Ear sample	Eye sample	joint sample	Total
<b>Culture</b>	Positive	3(16.6%)	2(7%)	14(27%)	0	<b>19</b>
	Negative	15(86.4%)	27(93%)	38(73%)	3(100%)	<b>83</b>
<b>PCR</b>	Positive	8(44%)	12(41.4%)	39(75%)	0	<b>59</b>
	Negative	10(66%)	17(58.6%)	13(25%)	3(100%)	<b>43</b>
<b>MAPCR</b>	Positive	5(28%)	2(7%)	12(23%)	0	<b>19</b>
	Negative	13(72%)	27(93%)	40(77%)	3(100)	<b>83</b>

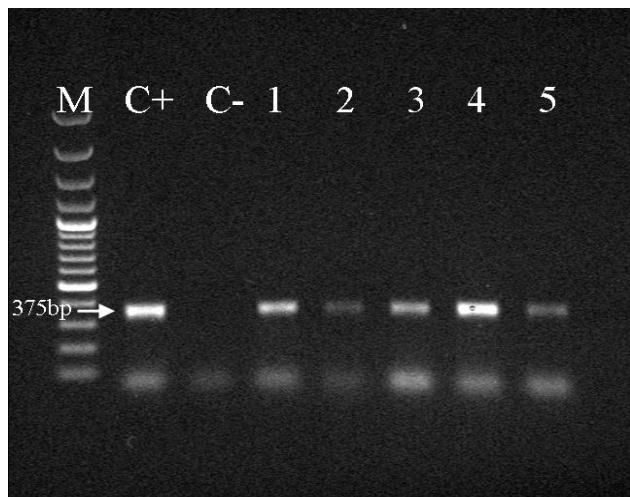
presence of *Mycoplasma* and 44 samples (43.2%) were scored negative by PCR method. 19(32.2%) samples were positive in *M. agalactiae* specific PCR, and 40(67.8%) were negative for using PCR method for *M. agalactiae*. *M. agalactiae* was detected in 19(32%) of the samples tested with both methods. 40 samples were MPCR positive-culture negative and there was only one sample that MPCR negative-culture positive, also 40 of the samples scored negative for using MPCR and MAPCR and no one of samples are MAPCR positive-MPCR negative (Table 1).

In this study after clinical examinations, samples were collected from the most significant lesions that were observed in each herd, eye samples were the most lesions that *Mycoplasma* was isolated and diagnosed by culture and PCR and joint exudates were the least lesions that *Mycoplasma* was isolated and diagnosed by culture and PCR, whereas milk samples were the most lesions that *M. agalactiae* was identified by MAPCR and joint exudates also were the least samples that *M. agalactiae* was detected by MAPCR (Table 1). In PCR the DNA fragment of *Mycoplasma* genus with approximately 163 bp was amplified (Figure 1).



**Figure 1.** *Mycoplasma* genus PCR (MPCR): PCR electrophoresis analysis in %1 gel agarose. M: Marker (100bp DNA ladder).Lane C+: Positive control (163bp band, *Mycoplasma* genus, NCTC 10123).Lane C-: Negative control (uncultured PPLO broth) and Lane 1 to 6 are the *Mycoplasma* isolates in this study.

The *M. agalactiae* PCR product was 375 bp in length (Figure 2).



**Figure 2.** *Mycoplasma agalactiae* PCR (MAPCR): PCR electrophoresis analysis in %1 gel agarose. M: Marker (100bp DNA ladder).Lane C+: Positive control (375bp band, *Mycoplasma agalactiae*, NCTC 10123).Lane C-: Negative control (uncultured PPLO broth) and Lane 1 to 5 are the *Mycoplasma* isolates in this study.

## DISCUSSION

In this study, *M. agalactiae* was detected from mammary gland, conjunctive, ear and joint exudates samples of sheep herds of Qom province, Iran by using PCR and culture methods. PCR can be used more reliably on *Mycoplasma* growing in culture with 24 hour enrichment of the *Mycoplasma* in the appropriate medium greatly facilities PCR detection even in the presence of bacterial contamination (Nicolas 2008). PCR as a molecular detection method is routinely used in many laboratories and is extremely sensitive, it can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when the results are positive, However the negative results should not be considered definitive and the detection limit for *M. agalactiae* in cultures has been brought to  $2 \times 10^2$  CCU/ml (Dediu et al 1995, Tola et al 1997, Nicolas 2008, Bergonier 1997). The result of this study showed that PCR was more successful than the culture in detecting of *M. agalactiae* and was in agreement with the result of the recent reports Kheirkhah et al (2011) from Iran, Amores et al (2010) from Spain and Tola et al (1997) from Italy.

This study showed that, 23% of eye samples were positive in PCR with *M. agalactiae* primers, 7% of ear samples were positive in MAPCR, 28% of milk samples were positive in MAPCR and *M. agalactiae* was not detected from joint exudates in present study. Bidhendi et al (2011) showed that *M. agalactiae* was detected from milk samples of the healthful and suspected sheep herd of C.A from Kordestan province in Iran. In the peresent study, *M. agalactiae* was detected from milk samples of suspected herd to C.A so the results of this study were more reliable. Khierkhah et al (2011) have detected *M. agalactiae* from milk and joint exudates of goats; they have declared that the highest number of *Mycoplasma* colony was obtained from joint exudates. This study was in agreement with them to detect of *M. agalactiae* from milk samples, but it was contrary in detection of *M. agalactiae* from joint exudates. However, *M. agalactiae* was not detected from joint exudates. The isolation of *M. agalactiae* from joint exudates might be related to special maintenance condition of goats herd. It causes that joint exudates of sheep were not suitable for detection and identification of *M. agalactiae*. Amores et al (2010) have detected *M. agalactiae* from ear swab samples of goats. Therefore, this study was in agreement with that study in detection of *M. agalactiae* from ear samples.

Zendulkova et al (2007) claimed that the PCR method was not efficient for detecting of *M. agalactiae* from sheep and goats of Jordan, also the eye samples and ear samples were not suitable for isolation and identification of *M. agalactiae*. This study was contrary with them to show that the PCR could be efficient for detecting of *M. agalactiae*, as well as ear and eye samples were one of the best useful for diagnosing of *M. agalactiae*. Pirali et al (2007) have also detected *M. agalactiae* from eye and milk samples of sheep and goats. Therefore, this study was in agreement with detecting of *M. agalactiae* from eye and milk samples of sheep.

This study was conducted on the suspected samples. It has more reliable result than the other studies such as Moradi Bidhendi et al (2011) that conducted on the

samples with or without signs of C.A. Eventually the most and the least isolations of *M. agalactiae* were taken respectively from milk and joint exudates samples. The similar study in goats is required for phylogenetic analysis of *M. agalactiae* in field samples by applying the lipoprotein gene sequence in GeneBank to identify source of the infection.

In conclusion, *M. agalactiae* was detected for the first time from sheep of Qom province and the result of our finding confirmed that *M. agalactiae* was one of the main etiological agents of the C.A in this province. In order to complete this study, it is necessary to identify other species of *Mycoplasma* such as *Mycoplasma mycoides* or *Mycoplasma putrefaciens* or *Mycoplasma capricolum* in samples which were positive with genus using *Mycoplasma* PCR.

### Acknowledgements

We thank all the staff of the *Mycoplasma* reference laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran. This study was supported by a project of Razi Institute and Education and Research Deputy of Jihad-Agriculture Ministry with the grant No. 2-18-18-89067.

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