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Original Article

Isolation and identification of *Mycoplasma agalactiae* by culture and polymerase chain reaction (PCR) from affected sheep to Contagious agalactia of Khuzestan province, Iran

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ABSTRACT

Mycoplasma agalactiae (*M. agalactiae*) is one of the main causes of contagious agalactia, an infectious syndrome of sheep and goats in Khuzestan province –southwest of Iran that is characterized by mastitis and subsequent failure of milk production, arthritis, abortion and keratoconjunctivitis. This study was carried out to isolation and identification of *M. agalactiae* with culture and polymerase chain reaction (PCR) method from sheep in Khuzestan province, Iran. A total of 91 samples were collected from milk secretion, eye, ear, nose and joint exudates of sheep. All samples were cultured in PPLO broth supplemented for isolation of *M. agalaciae*. Extraction of the DNA of bacteria was done by phenol/chloroform method and the PCR assay was applied for detection 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 91 samples, 34(37.36%) cultures were shown positive and typical *Mycoplasma* colonies in PPLO agar culture diagnostic method and 47(51.65%) were scored positive by *Mycoplasma* genus PCR, 8(8.79%) of the samples were scored positive by using *M. agalactiae* PCR as diagnostic method. Out of the 91 samples, 26 samples were shown both positive in the culture and PCR, 5 samples were shown both positive in the culture, MPCR and MAPCR. 15 samples were negative in the culture and positive in PCR whereas only 3 samples were positive in culture and negative in PCR. The results showed that the more isolations of *M. agalactiae* were taken from eye and less in ear and nose samples. *M. agalactiae* was one of the main factors of contagious agalactia that was detected for the first time from sheep in Khuzestan province.

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

INTRODUCTION

Contagious agalactia is a mycoplasmal disease of sheep and goats that can cause an important infectious syndrome of sheep and goats (Bergonier *et al* 1997). The disease is characterized by primarily affects the mammary glands, joints, eyes and to a lesser degree respiratory tract in male and female. It is clinically manifested as mastitis (and subsequent failure of milk production), polyarthritis, keratocojunctivitis, abortion

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and pneumonia(Nicolas 2008, Kheirkhah 2011 *) and* in kids, losses due to septicemia and pneumonia can be high. *Mycoplasma agalactia* is one of the main causes of the disease in sheep and goats. However similar clinical and pathological changes in these animals can also be caused by mycoplasma species (Nicolas 1996, Sarris 1996, Bolske 1994) included into the "*Mycoplasma mycoides cluster" or* by of the five *Mycoplasma* species associated with this disease, namely, M. agalactiae, *Mycoplasma mycoides subsp mycoides LC* (large colony*), Mycoplasma capricolum* subsp *capricoum*, *Mycoplasma mycoides* subsp *Capri* and *Mycoplasma putrefaciens* (Nicolas 2008, *et al* 2007, Bergonier *et al* 1997). However *M. agalactiae* is still regarded, particularly in sheep as the "classical" etiological agent of the Contagious agalactia (Bergonier *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). Contagious agalactia has been reported in southern Europe (Bergonier *et al* 1997) and south of America and north of Africa , Mediterranian region and western Asia including serious problem exist in Iran where over 1300 cases were reported in 2006 (OIE 2008, Zendulkova *et al* 2004). 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 agent to lambs by milk, so the main target of Contagious agalactia is mammary gland, where a fall in or complete loss of milk production sometimes within 2 or 3 days (Nicolas 2008, Kheirkhah 2011). 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 Contagious agalactia 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 into a chronic infection, occasionally resulting in unilateral or bilateral blindness (Mebuss 1998). Culture as a diagnostic method can be costly and time-consuming, and can also be inconclusive (Kheirkhah *et al* 2011, Zendulkova *et al* 2004). Molecular diagnostic tests have been used to identify the *M. agalactiae* since last decade too. PCR method have been one of those tests for detection of *M. agalactiae* in milk samples directly (Tola *et al* 1997). *M. agalactiae* was isolated and identified by culture and PCR method from goats and sheep in provinces of Iran (Kheirkhah *et al 2011*, Moradi Bidhendi *et al* 2011, Pirali *et al* 2007*).* Because of the Khuzestan province is located in southwest of Iran, the site traffic and settlement of nomads and their animals from adjacent provinces and it is one of the most important provinces in terms of goats and sheep breeding ,the incidence of Contagious agalactia in this province has increased during these years. There is not any investigations 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 contagious agalactia. The aim of this study was to isolation and detection of *M. agalactiae* by culture and PCR method from affected sheep with or without clinical signs of the disease in Khuzestan province - Iran.

MATERIALS AND METHODS

Sampling and cultures. The samples included swabs from eye and nasal conjunctivae and mucosae and also from the external canal of ear, milk secretion and joint exudates. A total of 91 swabs and samples were collected and immediately placed in test tubes containing transport medium for *mycoplasma culture.* Most of the samples were obtained from herds with clinical signs of a probably infection by *Mycoplasma*. All the sheep had been previously examined before sampling to confirm for clinical signs of *contagious agalactia*. Then the samples transported on ice to the *Mycoplasma* reference laboratory of Razi Vaccine and Serum Research Institute, Karaj, Iran. At the

laboratory, the specimens were diluted and filtered into the fresh PPLO broth, then inoculated on to PPLO agar medium (BBL, Becton Dickinson and company, Cockeysville, Sparks, MD, USA). Inoculated agar and broth were incubated at 37 °C in 50% Co2 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 been used in this study as a positive control and uncultured PPLO broth as a negative control.

DNA extraction. DNA was extracted 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 incubated for at least 4 hrs at 56 ºC.
- Equal volume of phenol added to the tube containing, and mixed well by vortex.
- Centrifuged at 13000 rpm for 15 min.
- Removed all aqueous layers (top layer) and transferred in another tube.
- Added equal volume of phenol: chloroform (1:1) in tube.
- Centrifuged at 13000 rpm for 15 min and transferred all aqueous layer in another tube.
- Added equal volume of chloroform in the tube and mixd well by vortex then centrifuged at 13000 rpm for 15 min.
- All aqueous layer Transferred in another tube and sodium acetate was added 1:10 volume of the tube containing and mixed well.
- Added ethanol (ETOH) to them, two fold of the tube containing.
- This solution was placed on -20 for 20 min then centrifuged for 15 min at 13000 rpm.
- Discarded softly liquid containing of tube and added 200 µl of 70% ETOH, then centrifuged for 5 min at 13000 rpm.
- Discard 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 of Mycoplasma as follow:

M1F: 5'-GCTGCGGTGAATACGTTCT-3', M3R: 5'-TCCCCACGTTCTCGTAGGG-3'. (Pourbakhsh *et al* 2010). *M. agalactiae*-speccific 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 MgCl2, 5 mM dNTPs, 10ppm 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: For genus of mycoplasma: 7.5 minutes at 94 °C, followed by 30 cycles of 30 second at 94 °C, 30 second at 56 °C and 1 minute at 72 °C, with a final extension cycle of 5 minutes at 72 °C. For species of *M. agalactiae* PCR: 5 min at 95 °C, followed by 34 cycles of 1 min at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C, with a final extension cycle of 5 minutes 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 samples collected from sheep herds which analyzed simultaneously by culture and *Mycoplasma* genus PCR (MPCR) and *M. agalactiae* PCR (MAPCR). Out of 91 samples, 34(37.36%) cultures samples were shown positive and typical *Mycoplasma* colonies in PPLO agar culture diagnostic method and 47(51.65%) were scored positive by *Mycoplasma*

genus PCR, 8(8.79%) of the samples were scored positive by using *M. agalactiae* PCR as specific diagnostic method. Out of 91 samples, 26 (28.57%) were shown both positive in the culture and PCR, 5 (5.49%) were shown both positive in the culture, MPCR and MAPCR. 15 (16.48%) were negative in the culture and positive in PCR whereas only 3 (3.29%) was positive in culture and negative in PCR. The results showed that the more isolations of *M. agalactiae* were related to eye samples and less isolations were related to ear and nose samples. *M. agalactiae* was one of the main causes of contagious agalactia that was detected for the first time from sheep in Khuzestan province (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 (Table1). In PCR, the DNA fragment of *Mycoplasma* genus with approximately 163 bp was amplified (Figure 1). The *M. agalactiae* PCR product was 375 bp in length (Figure 2).

DISCUSSION

Contagious agalactia is a permanent health and economic problem (Belaid *et al* 1990). In our previous study we demonstrated the presence of *M. agalactiae* in Khuzestanian sheep and goats (Pooladgar *et al* 2011).In present study, *M. agalactiae* was detected from

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

Tests		Milk sample	Ear sample	Eye sample	joint sample	Nose sample	Total
Culture	Positive	4 (4.39%)	4 (4.39%)	14 (15.38%)	9 (9.89%)	3 (3.29%)	**34** (37.36%)
	Negative	15 (16.48%)	4 (4.39%)	19 (20.88%)	6 (6.59%)	15 (16.48%)	**59** (64.83%)
PCR	Positive	9 (9.89%)	5 (4.90%)	19 (20.88%)	10 (10.99%)	4 (4.39%)	**47** (51.65%)
	Negative	10 (9. 9%)	3 (3.29%)	16 (17.58%)	5 (5.49%)	17 (18.68%)	**51** (56 %)
MAPCR	Positive	1 (1.1%)	2 (2.19%)	3 (3.3%)	2 (2.19%)	0	**8** (8.79%)
	Negative	6 (6.59%)	2 (2.19%)	9 (9. 9%)	13 (14.28%)	5 (5.49%)	**35** (38.46%)

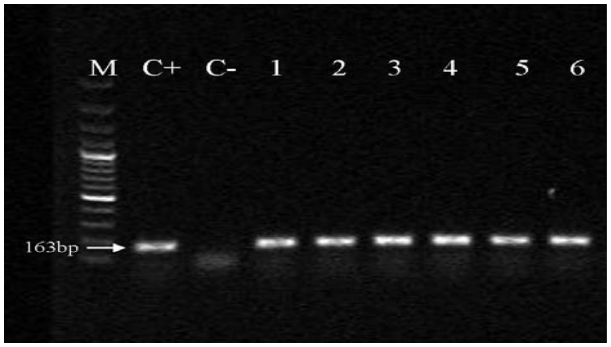

Figure 1. *Mycoplasma* genus PCR (MPCR): PCR electrophoresis analysis in %1 gel agarose. M*:* Marker (100bp DNA ladder). *Lane C+*: Positive control (163bp band, *Mycoplasma* genus, NCTC 10123). *Lane C-:* Negative control (uncultured PPLO broth) and *Lane 1 to 6* are the Mycoplasma isolates in this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

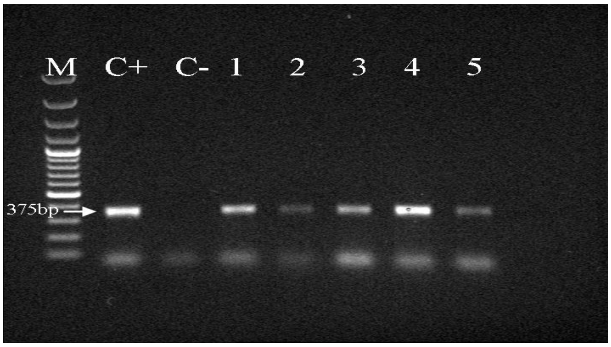

Figure2. *Mycoplasma agalactiae* PCR (MAPCR): PCR electrophoresis analysis in %1 gel agarose. M: Marker (100bp DNA ladder).*Lane C+*: Positive control (375bp band, *Mycoplasma agalactiae*, NCTC 10123). *Lane C-:* Negative control (uncultured PPLO broth) and *Lane 1 to 5* are the *Mycoplasma* isolates in this study.

mammary gland (milk), conjunctivae(eye), external canal of ear, respiratory tract (nose mucosa) and joint exudates samples of sheep herds in Khuzestan 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). The detection of *Mycoplasma* spp. is so far most frequently carried out in culture. However, this method is time consuming and laborious. The use of molecular diagnostic methods improves laboratory diagnosis of infectious agalactia also in terms of reliability, accuracy and time, which is very important because some infected sheep and goats have shown atypical forms of the disease (Nicolet 1994b); in addition, asymptomatic disease carriers have been reported (Bergonier 1996ab, Lillini *et al* 1996, Sanchis *et al* 2000) which are difficult to detect by less sensitive laboratory methods. 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 2×102 CCU/ml (Dediu *et al* 1995, Tola *et al* 1997, Nicolas 2008, Bergonier 1997). The results of our study confirmed that Khuzestanian sheep herds are currently very infected by M. *agalactiae*(up to 51.65) in most areas of province especially in Ramshir(near to 90%) and Mahshahr in coastal areas of Khuzestan . By PCR we can use of a more successful than the culture in detecting of *M. agalactiae* and it 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. Our results showed that, all tested samples were positive in PCR with *M. agalactiae* primers: the maximum rate were 19(20.88%) of eye samples, and the minimum rate were 4(4.39%) of nose samples ,as well as ,3 (3.3%) of eye samples and 1 (1.1%) of milk samples were positive in MAPCR and *M. agalactiae* was not detected from nose samples in MAPCR. Bidhendi *et al* (2011) showed that *M. agalactiae* was detected from milk samples of the healthful and suspected sheep herd of Contagious agalactia from Kordestan province in Iran. In our study, *M. agalactiae* was detected from milk samples of suspected herd to Contagious agalactia by culture, PCR and MAPCR methods 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 and especially eye samples (Abtin *et al* 2013). 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 in three methods mentioned as above. 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 samples of suspected and healthful sheep herds. It has more reliable results than the other studies such as Moradi Bidhendi et al. (2011) that conducted on the samples with or without signs of Contagious agalactia. Eventually the most and the least isolations of *M. agalactiae* were taken respectively from eye 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 and confirmed for the first time from sheep of Khuzestan province and the results of our finding confirmed that *M. agalactiae* was one of the main etiological agents of the Contagious agalactia in this province. In order to complete this study, it is necessary to identify other species of Mycoplasma such as *M. mycoides*, *M. putrefaciens* or *M. capricolum* in samples which were positive with genus using Mycoplasma PCR.

Ethics

I hereby declare all ethical standards have been respected in preparation of the article.

Conflict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

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