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Full Length Research Paper

Isolation and identification of *Mycoplasma agalactiae* by polymerase chain reaction (PCR) in suspected sheep samples in Kerman Province, Iran

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The infectious agalactia syndrome occurs worldwide but mainly in the Mediterranean regions, north, central and east Africa, and western Asian countries such as Iran. *Mycoplasma agalactiae* is the chief causal agent of contagious agalactia. The aim of this research was to use the polymerase chain reaction (PCR) method to isolate and identify *M. agalactiae* in suspected sheep samples in Iran. They cultured samples from 142 diseased animals and screened them with genus specific PCR oligomers. 59 samples were mycoplasma positive and subsequent testing of these samples with species specific oligomers identified 17 samples that were positive for the agalactiae species of mycoplasma. The derived results established that the detection of *M. agalactiae* as one of the agents of contagious agalactia in sheep and the prevalence of bacteria in Iranian sheep is lower than that in goats.

Key words: *Mycoplasma agalactiae*, contagious agalactia, identification, sheep, Iran.

INTRODUCTION

Mycoplasmas consist of wall-less prokaryotes which are the smallest fastidious bacteria and have unusually small genomes (Tully, 1989). *Mycoplasma agalactiae* belongs to the mycoplasmataceae family and is a facultative anaerobic germ (Carter et al., 2004). *M. agalactiae* was the first mycoplasma isolated from sheep and goats and it is the etiological agent of a bacterial disease often described as “contagious agalactia of sheep and goats” (Madanat et al., 2002). Other important mycoplasmas, namely *M. mycoides* subsp. mycoides large colony and *M. capricolum*, are claimed to cause similar syndromes (Carter et al., 2004). The disease occurs mainly in the Mediterranean region, but is also reported in many other areas of the world (Zendulkova et al., 2007) including Iran.

Among the prominent Iranian sheep diseases, mycoplasmal infections result in significant losses. This disease is endemic in Iran and characterized by agalactia, mastitis, arthritis, keratoconjunctivitis, and sometimes abortion and pneumonia (Hasani-Tabatabayi et al., 2005). Because infection spreads quickly, it is relevant to apply a specific, sensitive and rapid diagnostic procedure for its detection (Tola et al., 1997).

The Polymerase Chain Reaction (PCR) with mycoplasmal 16S ribosomal RNA has been applied for detecting a variety of mycoplasma species (Zendulkova et al., 2007; Kojima et al., 1997). Computer alignment studies of these rRNA sequences have revealed the existence of regions with highly conserved sequences and zones, which display sequence variability at the genus and species levels, allowing the selection of genus- and species-specific primers for PCR (Van Kuppeveld et al., 1992). The rRNA is naturally present in high-copy numbers (up to 10 000 molecules per cell) (Waters and McCutchan, 1990).

The PCR method has been applied for the detection of a variety of mycoplasma species that cause other

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Table 1. Nucleotide sequences and primers used for identification of *M. agalactiae* by PCR.

Primer	Target gene	Sequence	Length (bp)	Reference
	16S rRNA	F: 5′-GCTGCGGTGAATACGTTCT-3′ R: 5′-TCCCCACGTTCTCGTAGGG-3′	163	Kojima et al. (1997)
FS1 FS2	lipoprotein	F: 5′-AAAGGTGCTTGAGAAATGGC-3′ R:5′-GTTGCAGAAGAAAGTCCAATCA-3′	375	Tola et al. (1997)

mycoplasmal diseases in some research works (Zendulkova et al., 2007; Kojima et al., 1997; Azevedao et al., 2006; Blanchard et al., 1991). *M. agalactiae* was reportedly detected by the PCR method in Iranian goats. In one report, this bacterial agent was identified through PCR in sheep only from milk sample in Kordestan Province, Iran (Moradi et al., 2011) while in another one, the P40 gene of vaccine *M. agalactiae* strain in Iran was amplified, cloned and sequenced. This gene sequence was compared with other strains in the gene bank with the BLAST program (Mahdavi et al., 2009). Thus, the use of PCR for the detection of animal mycoplasmas has been reported previously but there is no investigation so far on applying PCR for direct isolation of mycoplasmal agents of contagious agalactiae in sheep samples in Iran. The aim of this study was to isolate and identify *M. agalactiae* by PCR from suspected Iranian sheep samples in Kerman Province.

MATERIALS AND METHODS

Mycoplasma isolation and enrichment

142 suspected samples collected from infected conjunctival swabs, milk secretion and leg joint exudates from sheep herds in different geographical areas of Kerman Province, Iran, within one year, stored at 4°C and transported to the Reference Mycoplasma Laboratory in Razi Vaccine and Serum Research Institute, Karaj, Iran for examination. These samples were grown and enriched in humid air with 5% CO_2 at 37°C for 24 to 48 h in liquid mycoplasma medium (pH 7.6-8) which contained 10ml of basal medium (2.1% (W/V) PPLO broth (Biolife), 0.01% (W/V) glucose, and 0.002% (W/V) phenol red) supplemented with additive (20 ml of equine serum, 1 ml of 25% (W/V) b-nicotinamid-adenine dinucleotide, 0.5 ml of 4% (W/V) thallous acetate and 0.5 ml of 50 000 units of penicillin G potassium).

DNA extraction

DNA was extracted from the enriched samples according to the procedures of Kojima et al. (1997). As per European Pharmacopoeia 2005, negative and positive controls were PPLO broth media and a standard strain of *M. agalactiae* (NCTC 10123), respectively. 500 µL of the samples were placed in a 1× 5 µL Eppendorf tube and micro centrifuged at 13 000rpm for 15 min. 100 µL of lyses buffer was added to 100 µL of precipitate, which the tube was placed in a 56°C bath for 4 h. Then, 200 µL saturated phenol was added and the tube was centrifuged at 13 000 rpm for 20 min. The upper phase was transferred to another tube and an equal volume of mixed phenol/cholorophorm (1:1) was added. After centrifuging at 13 000rpm for 20 min, the aqueous phase was transferred to another tube to which an equal volume of pure cholorophorm was added, and then centrifuged at 13 000 rpm for 5 min. The upper phase was transferred to a new tube, mixed with 1/10 volume of acetate sodium (3 M) and was precipitated in a -20°C refrigerator with a 2-fold volume of cool and pure ethanol (20 min). Next the tube was centrifuged at 13 000 rpm for 15 min. 200 µL of 70% ethanol was added and the tube was centrifuged at 13 000 rpm for 5 min, and finally the DNA was dried and re-suspended in DDW at 4°Cto be used for PCR (Kojima et al., 1997).

PCR

From the work on mycoplasma genus amplification, two primers (forward and reverse), designed by Kojima et al. (1997) were used; a 163bp region of 16S *rRNA* gene was amplified. For the *M. agalactiae* species the primer sets FS1 and FS2 had already been designed by Tola et al. (1997) who amplified a 375bp region of lipoprotein gene. The primers' sequences with their corresponding genes are shown in Table 1. DNA amplification was done in a total volume of 35.25 µL containing 17.5 µL DNA, 0.1 µL of each primer, 0.5 µL dNTP mix (10 mM) (Cinnagen Inc.), 4 µL $Mgcl_2$ (25 mM) (Cinnagen Inc.), 2.5 µL PCR buffer (10X) (Cinnagen Inc.),and 0.25 µL Tag DNA polymerase (5 units/µL) (Cinagen Inc.). The reaction mixture was thermocycled 30 times beginning with an initial denaturation step of minimum 94°C. The temperature and time profile of each cycle was as follows: 94°C for 1 min (Annealing) and 72°C for 1 min (Extension), while PCRs were finished with a final extension step at 72°C for 5 min. PCR products were stored at 4°C. PCRs were carried out using two programmable, thermal cyclers (Primus and Master gradient). Positive and negative controls were included in all tests. Each µL aliquot of every PCR product was mixed with a 2 µL loading buffer (6X). The PCR products and 100bp DNA ladder were then separated by electrophoresis on 1% agarose gel and stained with 0.5 µL/ml ethidium bromide (100 volts for 1h) following a UV Translluminator.

RESULTS

The growth of mycoplasmas in PPLO broth media was demonstrated by changes in color or turbidity due to the bacteria's biochemical activity and metabolism. A total of 142 transmitted suspected samples collected from sheep were analyzed with a PCR test. Mycoplasma which tested positive to the PCR test was isolated from samples of 59 sheep and all isolates showed a specific amplicon at 163bp to the mycoplasma genus (Figure 1). This confirms that 41% of suspected sheep were infected with mycoplasma. All mycoplasma-positive samples were analyzed for *M. agalactiae* infection by PCR method. In

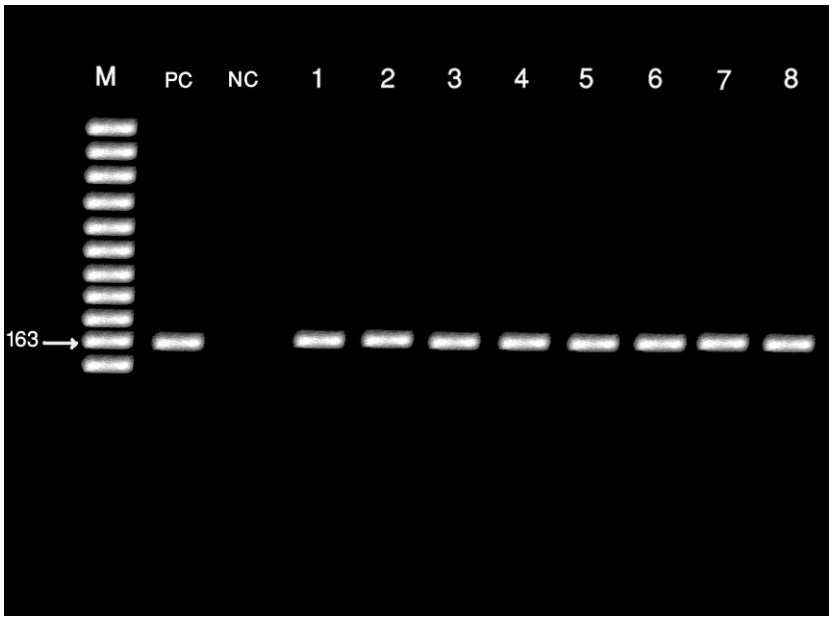

Figure 1. Specificity of the PCR detection assay using the specific primers. M, Marker 100 bp; PC, Positive Control [*M. agalactiae* (NCTC 10123)]; NC, Negative Control;1 to 8 suspected samples. 163 bp bands formed in 8 genus positive samples.

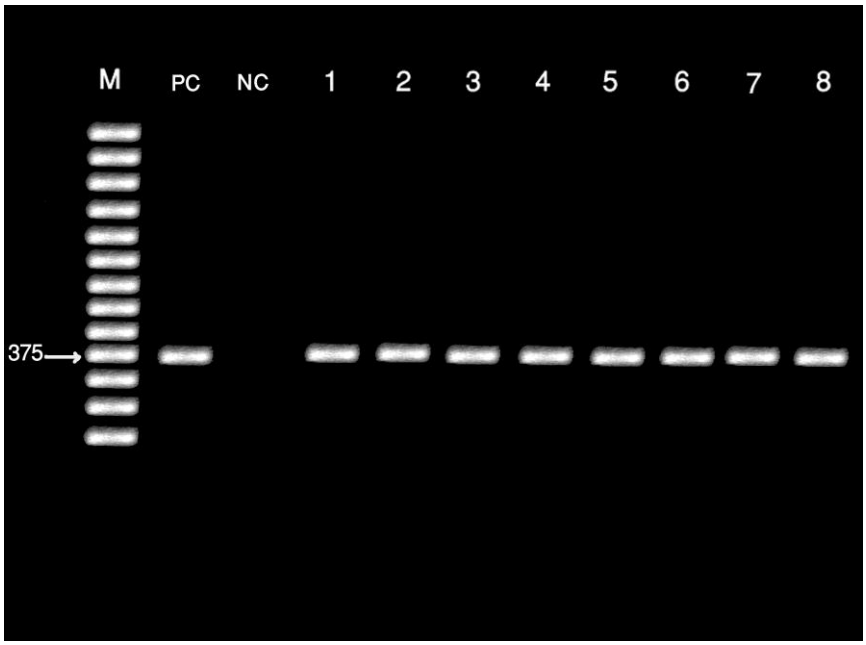

Figure 2. Specificity of the PCR detection assay using the primers FS1 and FS2. M: Marker 100bp; PC: Positive Control (*M. agalactiae* (NCTC 10123)); NC: Negative Control. 1 to 8 positive Mycoplasma genus samples; 375bp formed in 8 positive species.

the amplification of the lipoprotein gene, we were able to detect a *M*. *agalactiae*-specific amplicon (375bp) in 29% of mycoplasma-positive samples (Figure 2) and established that 17 out of all samples were positive for *M. agalactiae* species. The results of the PCR tests are presented in Table 2.

Table 2. Distribution of samples for Mycoplasma-PCR and *M agalactiae*-PCR results.

Control	Mycoplasma –PCR		*M. agalactiae* –PCR	
	%	No.	%	No.
+	41	59	29	17
-	59	83	71	42
Total	100	142	100	59

DISCUSSION

In this study, Isolation and Identification of *M. agalactiae* by PCR was done on suspected sheep samples in Kerman Province, Iran. It is one of the etiological agents of contagious agalactia in suspected sheep samples (conjunctival swabs, milk secretion and joint exudates) for the first time in Iran. *Mycoplasma* and other species are common in the udder, eyes and joints of sheep, where they may act as pathogens; however, *M. agalactiae* is one of the most important pathogens in sheep. The results reported in this paper confirm the detection of *M. agalactiae*. Also, one isolate of *M. agalactiae* was detected in a sheep milk sample in Kerman Province. Moradi et al. detected *M. agalactiae* in sheep and goat milk by culture and PCR in Kordestan Province, Iran (Moradi et al., 2011). Pirali and Ebrahimi used the PCR method to show that 8 out of 47 (17%) milk samples were positive with *M. agalactiae* primers in west and central Iran (Pirali and Ebrahimi 2007).

In recent research works, routine identification of *Mycoplasma* species has usually been based on PCR. In the present study, we detected bacteria via the PCR method from enriched samples by transport to a culture medium for primary enrichment. This technique is successful in the detection of *M. agalactiae* in sheep samples (Tola et al., 1997), proves faster than other tests, is trustworthy and supersedes in finding *M. agalactiae* in affected goats (Moradi et al., 2011; Kojima et al., 1997). In the present study, the aforementioned technique was applied to detect infectious *M. agalactiae* in sheep. These results report the occurrence of *M. agalactiae* in Iran among the various countries with intensive rearing of sheep and goats (Kusiluka et al., 2000). The previous study conveyed that *M. agalactiae* is a major agent of contagious agalactia in goats in Iran, while in a PCR test, 61% of examined samples tested positive for *M*. *agalactiae* from all mycoplasma-positive samples. On the other hand, the findings in this research confirm that only 29% of mycoplasma-positive sheep samples were *M. agalactiae*–positive species. This establishes that *M*. *agalactiae* is associated with infectious agalactiae in sheep, but the main agent of this disease is not *M*. *agalactiae* and that other strains may be responsible for infection in sheep. Although *M. agalactiae* is a significant pathogen in goats, it does not seem to be as widely spread in Iranian sheep. These results are confirmed by other studies as well. Al-Momani et al reported that *M. putrefaciens* was found to be key agent of contagious agalactia in sheep (48%) and *M. capricolum* subsp. *capricolum* (*Mcc*) (30%), *M. mycoides* subsp. *mycoides* large colony type (*Mmm LC*) (20%) and *M. agalactiae* (2%) were other detected mycoplasma species (Al-Momani et al., 2006). *M. putrefaciens, M. capricolum* or *M. mycoides* were not examined in the present study.

In conclusion, this report demonstrates that the PCR method is potentially useful for the detection of *M. agalactiae* in samples of all pathological lesions of contagious agalactia in sheep. The results established the detection of *M. agalactiae* as one of the agents of contagious agalactia in sheep in Iran. The prevalence of *M. agalactiae* in Iranian sheep is lower than that in goats although, these bacteria were identified as being carried by sheep, limited information is available on other mycoplasma species associated with this infection. Future research on the molecular epidemiology of *M. agalactiae* in both sheep and goats of the region will be necessary to fully understand the epidemiology, risk factors and other bacterial agents of this disease in sheep in Iran.

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