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Full Length Research Paper

Detection of *Mycoplasma agalactiae* by culture and polymerase chain reaction (PCR) methods from Iranian goats

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Mycoplasma agalactiae* is the etiological agent of contagious agalactia that is a serious disease affecting sheep and goats. It is characterized by mastitis and subsequent failure of milk production, arthritis and keratoconjunctivitis. The aim of the study was to detection of *M. agalactiae* by culture and polymerase chain reaction (PCR) methods from infected Iranian goats. For detection, a total of 57 samples were taken from conjunctiva (n=11), joint exudates (n=35) and milk secretion (n=11). After enrichment in PPLO broth media, identification of the *Mycoplasma* species was carried out by cultured and PCR method. Of the 57 samples in 9 samples (16%) fried egg colony appeared on the agar media. The PCR with mycoplasmal 16S rRNA was applied for detection of a variety of *Mycoplasma* species. PCR identification of genus was successful in 31 isolates (54%) and showed specific amplicon at 163 bp. From this positive samples, 19 isolates were examined (61%) were positive for *M. agalactiae* that showed specific amplicon at 375 bp. Of the total samples, only in 3 samples both culture and genus PCR tests were positive and in 20 samples were negative. Whereas in 6 PCR negative samples *Mycoplasma* colony appeared on the agar media and this colonies did not observed in 28 PCR positive samples. This paper reported detection of *M. agalactiae* from goats for the first time in Iran. PCR can be used as trusty and supersede test in the detection of *M. agalactiae* from affected goats. Among different collecting sites, milk secretion samples are suitable for PCR detection of *M. agalactiae.

Key words: *Mycoplasma agalactiae*, goat, polymerase chain reaction, culture, Iran.

INTRODUCTION

The genus *Mycoplasma* consists of wall-less prokaryotes which are small in size and have unusually small genomes (Tully, 1989). *Mycoplasma agalactiae* is considered to be the classic agent of contagious agalactia, which occur worldwide (Contreras et al., 2003) and is one of the principal mycoplasmas of ovine and caprine ruminants (Bashiruddin et al., 2005). It is a fastidious and facultative anaerobic germ and required protein with serum factor and yeast extract for growing (Carter et al., 2004; Hasani et al., 2005). Contagious agalactia is an acute, sub acute, or chronic disease caused by *M. agalactiae* (other mycoplasmas namely, *Mycoplasma mycoides* subsp. *mycoides* LC and *Mycoplasma capricolum*, are claimed to cause similar syndromes) (Carter et al., 2004). It causes important loss due to decreased milk production, death of animals and cost of treatment and prevention. The disease is characterized by mastitis, agalactia, polyartheritis, keratoconjunctivitis, and occasionally abortion (Azevedo et al., 2006). It

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occurs mainly in countries with intensive small animal husbandry, such as the Mediterranean region, north, central and east Africa, and western Asia (Zendulkova et al., 2007), such as Iran. In Iran, contagious agalactia is widespread in most regions and caused major economic losses.

The detection of *M. agalactiae* with culture procedure includes the culturing of samples onto agar media plates for 7 days at 37°C is time-consuming and hard to achieve for mycoplasmas. It is relevant to apply a specific, sensitive and rapid procedure for its detection (Kojima et al., 1997). The polymerase chain reaction (PCR) with mycoplasmal 16S rRNA and specific primers have been applied for detection of a variety of *Mycoplasma* species (Zendulkova et al., 2007; Kojima et al., 1997; Azevedao et al., 2006; Blanchard et al., 1991). The rRNA is naturally present in high copy numbers (up to 10,000 molecules per cell) (Waters and McCutchan, 1990). There is not any investigation to detect the contagious agalactia causes on goats by culture and PCR method in Iran. Although, some research find about detection of the species that cause other mycoplasmal disease. The aim of this paper is for the detection of *M. agalactiae* in affected goats by PCR and culture methods in Iran.

MATERIALS AND METHODS

Within two years of this study (2008-2010) 57 samples collected with purposive sampling in goat herds in Kerman Province, Iran. All goats tested had been previously examined to confirm they had clinical signs of contagious agalactia. Clinical signs were observed in 17 goat herds. Following this clinical examination, samples were collected from conjunctival swab, milk secretion, and joint exudates. The samples were immediately placed in test tube with transport *Mycoplasma* culture medium and were kept at 4°C until they have been transported to the Razi Vaccine and Serum Research Institute laboratory in 24 h. Transport media contain thallous acetate (250 mg/liter), which is toxic and inhibitory to some mycoplasmas but not those causing contagious agalactiae and reduce bacterial contamination from clinical sample (Buonavoglia et al., 2009). The collected samples in transport culture medium were then incubated on PPLO broth in order for the primary *Mycoplasma* to be propagated and enriched in a humid air with 5% CO_2 at 37°C over night in laboratory. According to Pharmacopoeia (2005) negative and positive controls were PPLO broth and standard strain of *M. agalactiae* (NCTC, 10123) respectively.

Culture method

In this stage, at first, Membrane filtration method (Millipore co.) of 45 µm diameter and 0.1 µm pore size were used. Enriched samples in incubated medium were inoculated at 0.5 ml amounts into test tube with 5 ml liquid *Mycoplasma* medium (pH 7.6 to 8) which contained 10 ml 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 50000 units of penicillin G potassium]. All the PPLO broth medium tubes were incubated in a humid air with 5% CO_2 at 37°C. The tubes were examined every other day for a change in turbidity and color (Sakhaei et al., 2009). After 24 to 48 h, 0.2 ml of all the broth medium were cultured in Petri dish which contained 10 ml of 3.5% (W/V) PPLO agar medium (Biolife) (pH 7.6 to 8) at 37°C for 21 days. At 4 day intervals they were checked for *Mycoplasma* colony (fried egg colony) appearing on the agar media.

PCR method

The enriched samples were used in PCR method. DNA was extracted from enriched samples using a previously described method (Kojima et al., 1997). In this method 500 µl of samples were placed in 1 × 5 µl Eppendorf tube and micro centrifuged at 1300 rpm for 15 min. 100 µl of lyses buffer was added to 100 µl of precipitated and tubes were placed in 56°C bath for 4 h. Then 200 µl saturated phenol was added and tubes was centrifuged at 13000 rpm for 20 min. Upper phase was transferred to another tube and equal volume of mixed phenol/cholorophorm (1:1) was added. After centrifuged at 13000 rpm for 20 min the aqueous phase was transferred to another tube and added equal volume of pure cholorophorm and was centrifuged at 13000 rpm for 5 min. Upper phase was transferred to a new tube and mixed with 1/10 volume of acetate sodium (3 M) and were precipitated in -20°C refrigerator with 2 fold volume of cool and pure ethanol (20 min), then 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, the DNA was dried and resuspended in DDW at 4°C and used for PCR (Kojima et al., 1997).

In this study two primers (forward and reverse) which have been already designed by Kojima et al. (1997) and amplify a 163 bp region of 16S rRNA gene of *Mycoplasma* genus and two primers have been already designed by Tola et al. (1997) and amplify 375 bp region of 16S rRNA gene of *M. agalactiae* species were used. The primers sequence and their corresponding genes are shown in Table 1.

DNA amplification were carried out in a total volume of 35.25 µl containing 17.5 µl DNA, 0.1 µl of each primers, 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 unit /µl) {Cinagen Inc.}. Reaction mixture were thermocycled 30 times beginning with an initial denaturation step of minute at 94°C. The temperature and time profile of each cycle was as following: 94°C for 1 min (Annealing) and 72°C for 1 min (Extension), 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 cycler (Primus and Master gradient). Positive and negative controls were included in all tests. Each microlitre aliquot of each PCR products was mixed with 2 µl loading buffer (6x) . The PCR products and 100 bp DNA ladder were then separated by electrophoresis on 1% agarose gel and stained with 0.5 µl/ml ethidium bromide (100 volts for 1 h) following UV Transllluminator.

RESULTS

At first, a total of 57 samples were enriched in PPLO broth media. After culture in 9 samples (16%) fried egg colony appeared on the agar media and detected *Mycoplasma* species (Figure 1). PCR identification of *Mycoplasma* species in total enriched samples was successful in 31 strains and showed specific amplicon at 163 bp (Figure 2). This proved that 54% suspected goats were infected with *Mycoplasma* in PCR test. The results of cultivation and PCR of samples are presented in

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

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

Figure 1. Colonies of *M. agalactiae* on the PPLO agar (x 40).

Tables 2 and 3 shows distribution of samples for culture, *Mycoplasma*-PCR and *Mycoplasma agalactiae*-PCR results. Then all *Mycoplasma* positive samples were analyzed for *M. agalactiae* infection by PCR method. On the PCR test, 19 isolates (61%) examined were positive for *M*. *agalactiae* that showed specific amplicon at 375 bp (Figure 3). This method established that 19 goats out of 57 were infected with *M. agalactiae.* The highest number of *Mycoplasma* colony on solid medium was obtained from joint exudates, then from conjunctiva and milk samples. PCR results of total enriched samples 14 strains (25%) came from the joint and 5 strains (8.9%) from milk samples.

DISCUSSION

The results reported in this paper confirmed the detection of *M. agalactiae* as major agent of contagious agalactia in goats for the first time in Iran. These results reported occurrence of *M. agalactiae* in Iran such as other countries with intensive rearing of sheep and goats (Kusiluka et al., 2000). Microorganism with characteristics of *Mycoplasma* was isolated from goats. The identification of the isolates was performed by their culture characteristics as well as by the PCR test. Routine identification of *Mycoplasma* species is usually based on classic methods, that is, biochemical tests and immunofluorescence examination (Zendulkova et al., 2007). PCR is faster than the routine tests and it can be used as trusty and supersede test in the detection of *M. agalactiae* from affected goats. In this present study this technique showed a distinct advantage over the culture method of identification. The collection sites were chosen in accordance with those preferred by other authors (Zendulkova et al., 2007). In this study, examination of 11 milk samples by PCR, provide 5 *Mycoplasma* species. All of *Mycoplasma* positive milk samples that analyzed for *M. agalactiae* infection by PCR method was positive (100%). On the other hand, 11 conjunctival swab samples were collected that detected 6 *Mycoplasma* species and none of them were *M*. *agalactiae* positive (0%). This study suggests that, among different collecting sites, milk secretion samples are suitable for detection of *M. agalactiae* and the number of mycoplasmas in milk may vary considerablely. This established that main agent of keratoconjunctivitis was not *M*. *agalactiae* and other strains may infect goats.

In our examination all *Mycoplasma* positive samples were analyzed for *M. agalactiae* infection by PCR method and 12 isolates (39%) examined were negative for *M*. *agalactiae.* This etiological isolation is an important finding. The finding of other mycoplasmas with significant epidemiology challenges existing plans for the control of contagious agalactia in Iranian goats. In one study *M. capriculum* isolated from caprine contagious agalactiae outbreak (De La Fa et al., 2007) and in other study *M. capriculum* and *M. Putrefaciens* isolated from respiratory signs and symptoms in goats (Awan et al., 2009). Mahdavi et al. (2009) reported high homology between vaccine strains in Iran (Shiraz strain with Lorestan strain 99.7% and Shiraz strain with Taleghan strain 74.6%). This vaccine used in Iranian herds and detection of *M. agalactiae* in this herds suggests that the nucleotide sequences should be compared between vaccine strains and detected *M. agalactiae* in the next studies. Detected strains can be used in future vaccine production if poor homology has been observed between vaccine strains

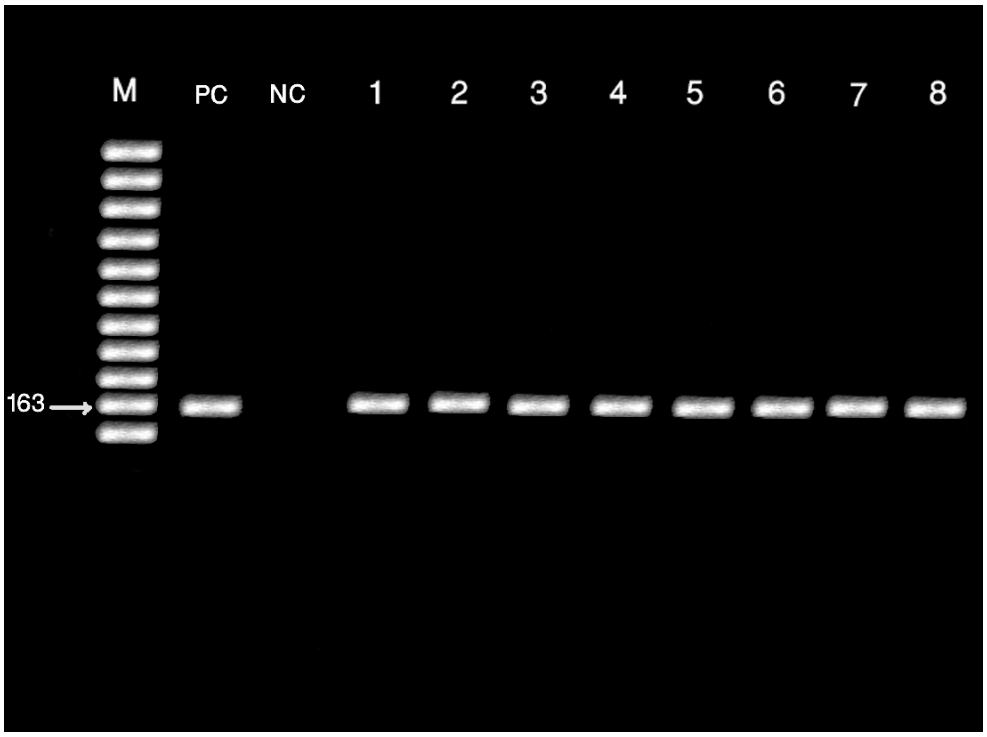

Figure 2. Specificity of the PCR detection assay using the specific primers. M: Marker 100 bp; PC: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control, 1-8 suspected samples. The formation of 163 bp bands in 8 genus positive sample.

Table 2. Distribution of samples for culture and *Mycoplasma*-PCR methods results.

Culture	PCR	No. Sample
+	+	3
-	-	20
+	-	6
-	+	28
Total		57

Table 3. Distribution of samples for culture, *Mycoplasma*-PCR and *M. agalactiae*-PCR results.

Samples	Culture	*Mycoplasma* -PCR	*Mycoplasma agalactiae* –PCR
+	9	31	19
-	48	26	12
Total	57	57	31

and detected *M. agalactiae.*

In conclusion, this report demonstrates that, as both culture and PCR methods are accurate identification of *Mycoplasma* contamination but the culture method not only can be costly and time consuming but also may show some false negative results. The PCR method is rapid, reliable, and simple method for the detection of *Mycoplasma* contamination in goats and PCR technique can be successful for the detection of *M. agalactiae* in milk samples.

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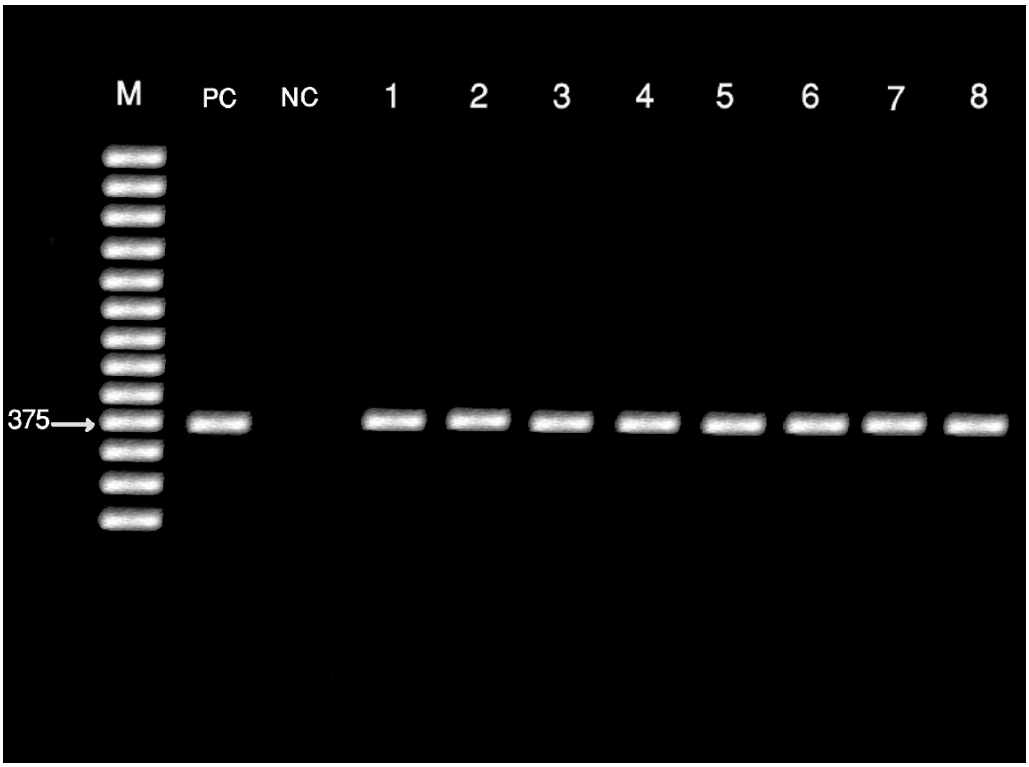

Figure 3. Specificity of the PCR detection assay using the primers FS1 and FS2. M: Marker 100bp; PC: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control. 1-8 positive *Mycoplasma* genus samples. The formation of 375bp in 8 positive species.

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