Isolation And Molecular Detection of Mycoplasma Mycoides Cluster In Goats With Clinical Signs Of Contagious Agalactia In Kerman Province, Iran

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ABSTRACT

Background: Contagious agalactia (C.A) is an infectious syndrome of sheep and goat characterized by mastitis, arthritis, keratoconjunctivitis and abortion. Mycoplasma agalactiae is the main cause of the disease in small ruminants. Objective: The aim of this study was to isolate and identify Mycoplasma mycoides cluster using PCR and culture from goats of Kerman city in Iran. Results: A total of 298 samples were collected from ear, eye and nose swab and milk secretion. All samples were cultured in PPLO broth and agar. After culture 56 samples (18.79%) were positive and showed fried egg colony on the agar media. At the same time the bacterial DNAs were extracted by Phenol-Chloroform method and the PCR assay was applied for detection of Mycoplasma genus in 163bp fragment of 16SrRNA gene, M.agalactiae in 375bp fragment of lipoprotein gene and M.mycoides cluster in 259bp fragment of 16SrRNA gene in all clinical samples. Out of 298 samples, 127 samples (42.6%) were positive for Mycoplasma-genus, 52(40.94%) of the samples were positive for M.agalactiae and 14 samples (22.95%) were positive for M.mycoides cluster PCR Conclusion: This is the first report of isolation and identification of M.mycoides cluster in goat with clinical signs of C.A in Kerman, Iran. This study showed that M.mycoides cluster was the main factors in etiology of the C.A in goats from Iran. This study showed that M.agalactiae was mostly isolated from milk samples and M.mycoides cluster was isolated from nose swab samples of goat herds in Kerman province, Iran, using PCR and culture method. The results of this study showed that PCR was more successful than culture in detecting Mycoplasma.

INTRODUCTION

The genus Mycoplasma consists of wall-less prokaryotes which are small in size and usually have small genomes. [1, 2, 3]. Contagious Agalactia(C.A) is an acute, subacute or chronic disease caused by Mycoplasma agalactiae and Mycoplasma mycoides cluster(M.mycoides cluster)too. The M.mycoides cluster consists of six pathogenic mycoplasmas causing disease in ruminants that share many genotypic and phenotypic traits. The M.mycoides cluster comprises six recognized taxa: Mycoplasma mycoides subsp. mycoides Small Colony (MmmSC), Mycoplasma mycoides subsp. mycoides Large Colony (MmmLC), Mycoplasma mycoides subsp. capri (Mmc), Mycoplasma capricolum subsp. capricolum (Mcc) and Mycoplasma capricolum subsp.capripneumoniae (Mccp). The group of strains known as Mycoplasma subsp. bovine group 7 of Leach (MBG7) has remained unassigned due to conflicting data obtained by different classification methods [4, 5, 6 and 7]. However M.agalactiae is still regarded, particularly in sheep and goat, as the ‘classical’ etiological agent of the C.A [7] but other M.mycoides cluster species are claimed to cause similar syndromes [2]. It causes important loss due to decreased milk production, death of animals and cost of treatment and prevention [8].C.A is characterized by mastitis, poly arthritis, pneumonia, keratoconjunctivitis and occasionally abortion [4, 8, 9, 10 and 11], mainly in countries with intensive small animal husbandry, such as in southern Europe [8, 12].North, Central and East Africa and South America, the USA and Western Asia [6, 13, 14, 15 and 16]. C.A is a serious problem existing in Iran where over 1300 cases were reported in 2006 [8, 16, 17 and 18]. Detection of Mycoplasma using culture procedure includes the culturing of samples onto agar media plates for 7 days at 37°C which is time consuming.

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and hard to achieve for Mycoplasma. Therefore, applying a specific, sensitive and rapid procedure for the
detection of mycoplasma is necessary [8, 17, 18 and 19]. The polymerase chain reaction (PCR) with
Mycoplasma 16S rRNA and specific primers has been used to detect a variety of Mycoplasma species [6, 8,
10,17, 18 and 19]. The rRNA is naturally present in a high copy number (up to 10,000 molecules per cell) [17].
Mycoplas maycoides cluster was isolated and identified by culture and PCR assay from goats of provinces in
Iran. [8, 17, 18, 20, 21 and 22]. Kerman Province is one of the 31 provinces of Iran. Kerman is in the southeast
of Iran with its administrative center in the city of Kerman. In 2014 it was placed in Region 5.Mentioned in
ancient times as the Achamenid satrapy of Carmania. It is the second largest province of Iran with an area of
180,726 km², that encompasses nearly 11 percent of the land area of Iran. Kerman provinces located in the
southern part of Iran and are one of the most important locations in terms of goat breeding. The incidence of
C.A in this province has increased during recent years. There has been no report on the detection of the
respective agent in that province, so this prompted us to isolate and detect M.mycoides cluster, the etiological
agent of the C.A.

2- Objective:
The aim of this study was to isolate and identify M.mycoides cluster by culture and PCR assay from goats
of Kerman province in Iran based on the sign of disease.

MATERIALS AND METHODS

3.1. Sampling method:
Within one year of this study (2012 - 2013) 298 samples were collected with purposive sampling in goat
herds in Kerman city, Iran. All the goats tested had been previously examined to confirm they were with C.A
clinical signs. Following the clinical examination, samples were collected from milk secretion, eye, ear, and
nose swab. The samples were immediately placed in test tubes with transport Mycoplasma culture medium and
they were then transferred on ice to the Mycoplasma Reference Laboratory of Razi Vaccine and Serum
Research Institute, Karaj, Iran, in 24 hours. Transport media contained Thallous Acetate (350mg/lit), which is
toxic and inhibitory to the same Mycoplasma which cause C.A and reduce the bacterial contamination. The
specimens were diluted and filtered into the fresh PPLO broth and then, inoculated onto PPLO agar medium
(BBL, becton Dickinson and Company, Cockeysville, Sparks, MD, USA). Inoculated agar and broth were
incubated at 37 °C in 5% Co2 and 98% humid atmosphere overnight in the laboratory. The broth was observed
daily for signs of growth and the plates were considered for the typical appearance of Mycoplasma colonies
[18]. According to Pharmacopoeia (2005), negative and positive controls were uncultured PPLO broth and
standard strain of M.agalactiae (NTCC, 10123), respectively [17].

3.2. DNA extraction:
DNA was extracted from the samples using a previously described method by Pourbakhsh et al (2010), with
some modifications [19], briefly 0.5 ml of each sample was transferred to Eppendorf tube and centrifuged for 15
min at 13000 rpm. The supernatant fluid was discarded and lysis buffer (Tris – HCl 50 mM PH = 8, SDS 1%,
NaCl 100 mM, EDTA 50 mM, Proteasine K 20 µl to 200 µl ) was added to the tube in equal volume to the
pellets in it and incubated for at least 4 hours at 56 °C. Equal volume of phenol was added to the material in the
tube and the contents mixed well by vortex. The mixture was centrifuged for 15 min at 13000 rpm. The aqueous
layer (top layer) was removed and transferred to a new tube. Phenol: chloroform (1:1) was added to the tube in
an equal volume and the tube was then centrifuged for 15 min at 13000 rpm and all of the aqueous layer was
transferred into a new tube. Chloroform was then added in an equal volume of the tube containing the mixture
and then mixed well by vortex. Ethanol (ETOH) was added twofold the material in tube. This solution was
placed on -20 C for 20 min and centrifuged for 15 min at 13000 rpm. All liquid contents of tube were discarded
carefully and 70% EDTA was added to the tube to200 µl and then the tube was centrifuged for 15 min at 13000
rpm. ETOH was discarded and the tubes allowed to dry, then 50µl distilled water was added to them.

3.3. Amplification with specific primers:
In this study two primers (forward and reverse) were previously designed by Pourbakhsh [22] and amplify a
163 bp region of 16S rRNA gene of Mycoplasma genus. For M. agalactiae species two primers were used that
had already been designed by Tola [23] and amplify 375bp region of 16S rRNA gene of M. agalactiae and two
primers have been designed by Hotzel [24] and amplify 259 bp region of 16S rRNA gene of M.mycoides cluster.
For genus Mycoplasma, amplification is as follows:
M1F: 5′ - GCTCGGTTGAATACGTCT - 3′
M3R: 5′ - TCCCCACGTTCGTAAGG - 3′ [22]
For *Mycoplasma agalactiae*, amplification is as follows:

M1F: 5′ - AAAGGTGCTTGAGAAATGGC - 3′
M3R: 5′ - GTTGCGAGAGAAATGCTCA - 3′ [23]

*Mycoplasma mycoides* cluster amplification primers set is:
P1: 5′ - GCTGGCTGAGATAAGTTCT - 3′
P2: 5′ - AATGCACTCAAAATATTG - 3′ [24]

The PCR mix was performed in a total volume of 25 µl per sample, containing 2 – 5 µl of 10X PCR buffer (Cinnagen), 2µl of 30mM MgCl\(_2\), 5mM dNTPs, 10 pm each primer, 0.5 U TaqDNApolymerase (Cinnagen). Consequently 15.3µl of deionized distilled water and 2µl of extracted DNA as template were carried out. The PCR assay was conducted in a Gradient Mastercycler (Eppendorf, Germany) as follows:

In genus: 7.5 min at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 56°C and 60 sec at 72°C, with a final extension cycle of 5 min at 72°C. For *M. agalactiae* PCR Reaction mixture, thermocycle beginning with an initial denaturation step of 5 min at 95°C, followed by 34 cycles of 94°C for 60 sec, 1 min. at 50°C and 72°C for 1 min, PCRs were finished with a final extension step at 72°C for 5 min and for *M. mycoides* cluster PCR; 3 min at 94°C, followed by 33 cycles of 45 sec at 94°C, 60 sec at 46°C and 90 sec at 72°C, with a final extension cycle of 5 min at 72°C. PCR products were stored at 4°C. Visualization of amplified products was done by UV illumination after electrophoresis (1% agarose gel in 1X tris-acetic acid–EDTA (TAE) buffer) and ethidium bromide staining.

4. Results:

4.1. Culture results:

A total of 298 samples were enriched in PPLO broth media then all of the samples were analyzed simultaneously by culture and PCR. After culture 56 samples (18.79%) were positive and showed fried egg colony about 0.5 to 2.0 mm in diameter appearing after 7 days on the agar media (Figure 1), 242 (81.21%) samples scored negative for using culture method. The highest number of *Mycoplasma* colony on solid medium was obtained from milk samples, followed by nose, eye and ear samples.

![Fig. 1: Colonies of *M. agalactiae* on the PPLO agar (×40)](image)

4.2. PCR results:

121 samples (40.61%) were scored positive in *Mycoplasma* genus PCR (MG-PCR) and showed specific application at 163 bp (Figure 2) while 177 samples (59.39%) were negative by MG-PCR method.

![Fig. 2: *Mycoplasma* genus PCR (M-PCR). M:100bp DNA ladder. C+: Positive control (163bp band, Mycoplasma genus, NCTC 10123). C-:Negative Control (uncultured PPLO broth) and 1 to 5 are the *Mycoplasma* isolates in this study.](image)
52 samples (40.94%) were positive in *M. agalactiae* PCR (MA-PCR) and showed specific application at 375 bp (Figure 3), 69 (59.06%) samples were negative by MA-PCR. 14 (22.95%) samples were positive in *M. mycoides* cluster Specific PCR (MC-PCR). 55 (87.05%) were negative with MC-PCR. All 56 samples which were positive in culture were MG-PCR positive too. 65 samples were culture negative and MG-PCR positive. All 177 samples which were MG-PCR negative were also culture negative. There was no culture positive, MG-PCR negative sample. All 121 *Mycoplasma* positive samples were analyzed for *M. agalactiae* by PCR method. On the PCR test, 52 (40.94%) isolates examined were positive for *M. agalactiae*.

![Figure 3](image1.png)

**Fig. 3:** *Mycoplasma agalactiae* PCR (MA-PCR): M: 100bp DNA ladder. C+: Positive control (375bp band, *Mycoplasma mycoides* cluster). C-: Negative control (uncultured PPLO broth) and 1 - 8 are the *Mycoplasma agalactiae* isolates in this study.

All 69 *M. agalactiae* negative samples were then analyzed for *M. mycoides* cluster by PCR method and 14 of the examined isolates (22.95%) were positive for *M. mycoides* cluster that showed specific applicant at 259 bp (Figure 4). All the *M. mycoides* cluster positive samples were detected from nose swabs. 177 samples scored negative in MG-PCR, 69 (59.06%) samples scored negative in MA-PCR and 55 (87.05%) samples were negative for MC-PCR, too.

![Figure 4](image2.png)

**Fig. 4:** *Mycoplasma mycoides* cluster PCR (Mmcl-PCR): M: 100bp DNA ladder. C+: Positive control (259bp band, *Mycoplasma mycoides* cluster). C-: Negative control (uncultured PPLO broth) and 1 - 2 are the *Mycoplasma mycoides* clusters isolates in this study.

Table 1 shows the distribution of samples for culture, MG-PCR, MA-PCR and MC-PCR results.

<table>
<thead>
<tr>
<th>TEST</th>
<th>RESULT</th>
<th>MILK SAMPLE</th>
<th>EAR SWAB</th>
<th>EYE SWAB</th>
<th>NOSE SWAB</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CULTURE</td>
<td>POSITIVE</td>
<td>18</td>
<td>10</td>
<td>12</td>
<td>16</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>NEGATIVE</td>
<td>143</td>
<td>31</td>
<td>40</td>
<td>28</td>
<td>242</td>
</tr>
</tbody>
</table>
In this study, after clinical examination, samples were collected from the most significant lesions that were observed in each herd. Milk samples had the most common mycoplasma lesions isolated and ear swabs had the least common mycoplasma lesions isolated and diagnosed by culture and MG-PCR. Milk samples had the most common \textit{M. agalactiae} lesions and ear swab had the least common \textit{M. agalactiae} lesions by MA-PCR. All 14 samples in which \textit{M. mycoides} cluster was positive were detected from nose swabs.

**Discussion:**

This study showed that \textit{M. agalactiae} was mostly isolated from milk samples and \textit{M. mycoides} cluster was isolated from nose swab samples of goat herds in Kerman city, Iran, using PCR and culture method. The results of this study showed that PCR was more successful than culture in detecting \textit{Mycoplasma}, consistent with the results of some recent reports, Kheirkhah [17], Moslemi [8] and Abtin [18] from Iran, Azevedao [10] and De La Fe [14] from Spain, and Tola [23] from Italy. Previous studies in Iran were focused on the C.A in sheep herds and reported isolation of \textit{M. agalactiae}, the main etiological agent of this disease. Moslemi [8] detected \textit{M. agalactiae} from milk samples and \textit{M. mycoides} cluster from conjunctiva (34.7\%) and also nose swabs (32.4\%), the difference in the results can be explained by varying etiological agents and geographical locations. Kheirkhah [20] detected \textit{M. agalactiae} from milk and joint exudate of goat. Moradi [20] detected \textit{M. agalactiae} in sheep and goat milk by culture and PCR method in Kordestan province, Iran [20]. Abtin [18] detected \textit{M. agalactiae} in sheep eye samples by culture and PCR in Qom province, Iran [18]. Pirali and Ebrahimi [21] used the PCR method to show that 17\% of milk samples in sheep were positive with \textit{M. agalactiae} primers in the west and central regions of Iran [21]. This study was in agreement with them in detection of \textit{M. agalactiae} from milk samples, but it was contrary in detection of \textit{M. agalactiae} from joint exudate. This could be because joint exudate was not a suitable site for detection and identification of \textit{M. agalactiae}. This pattern of findings shows that determination of exact etiological agent for C.A has eluded the researchers in different parts of Iran. Interestingly, no other species of \textit{Mycoplasma} has been reported to be studied as a possible cause of C.A in goats of Iran. This study, therefore, is indicative of the possible role of \textit{M. mycoides} cluster in the causation of C.A in goats in Iran.

In an earlier study carried out in Gran Canario in Spain wherein C.A is endemic, Mmm.LC was the most isolated species from the C.A affected goats’ samples. Al-Momeni (2006) reported that \textit{M. putrefaciens} (46\%) was found to be a key agent of C.A in sheep and Mcc(30\%) Mmm.LC (20\%) and \textit{M. agalactiae} (2\%) were other detected \textit{Mycoplasma} species. The results of these studies indicate that \textit{M. agalactiae} is not the major cause of C.A in goats in those areas.[25]

Further research would be needed to confirm if \textit{M. mycoides} cluster does actually have a role in etiology of C.A in goats of Iran. Gene sequencing and phylogenetic analysis are suggested wherever \textit{M. mycoides} cluster is isolated from C.A affected goats. A comparative analysis of the reports on the \textit{Mycoplasma} isolated from the other countries could be tremendously helpful in developing preventive strategies in future.

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