# Isolation and prevalence of *Mycoplasma agalactiae* in Kurdish sheep in Kurdistan, Iran

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## Abstract

Aim: Ruminant Mycoplasmosis are important diseases worldwide and several are listed by the World Organization for Animal Health (OIE) to be of major economic significant. The aim of this study was to isolation mycoplasmas from sheep presenting contagious agalactiae (CA) in Kurdistan in the West of Iran.

Materials and Methods: Sixty-nine samples included (milk, conjuctiva swabs, synovial fluid and ear canal swabs) were examined by PCR assay during 2011-2012. DNA was extracted from enriched samples. Two primers (forward and reverse) amplify a 163bp region of 16S rRNA gene of Mycoplasma genus and two primers amplify 375bp region of 16S rRNA gene of *Mycoplasma agalactiae* (*M. agalactiae)* species were used.

Results: This proved that 46 samples (66.7%) were infected with Mycoplasma in culture and PCR test, respectively. On the PCR test, 15 isolates (32.6%) examined were positive for *M. agalactiae* that showed specific amplicon at 375bp. All Mycoplasma positive samples were analyzed for *M. agalactiae* infection by PCR method and 31 isolates (67.4%) examined were negative for *M. agalactiae.* The finding of other mycoplasmas with significant epidemiology challenges existing plans for the control of CA in sheep population in Iran.

Conclusion: The results of the present study show that *M. agalactiae* in CA disease in Kurdistan Province, 32.6% involved. In Iran, only *M. agalactiae* vaccines are commercially available, thus, the animals are not protected against these other species.

Key word: Contagious agalactiae, Iran, *Mycoplasma agalactiae*, PCR

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## Introduction

*Mycoplasma agalactiae* (*M. agalactiae)* is the principal etiological agent of contagious agalactiae (CA) in small ruminants. In male and female sheep, this syndrome is produced mostly by *Mycoplasma agalactiae* subsp. *agalactiae*, but other mycoplasmas are also implicated (*M. capricolum* subsp. *capricolum*, *M. mycoides* subsp. *capri*, *M. mycoides* subsp. *mycoides* LC, and *M. putrefaciens*). CA is a serious infectious process characterized by three major signs: mastitis, arthritis, and keratoconjunctivitis [1]. It causes the reduction and even the suppression of milk production and occasionally causes abortion and death in up to 70% of young animals, resulting in serious economic loss [2]. *M. agalactiae* is one of the principal mycoplasmas of ovine and caprine ruminants [3]. The principal sources of *M. agalactiae* infection are the ingestion of contaminated feed, water, or milk and the urine, feces, and nasal or ocular liquids of infected animals. Ewes can be infected through the udder, and lambs can be infected by the consumption of colostrums. Thus, it is recommended that newborn animals be removed from the dam immediately after birth and fed only by using pasteurized colostrums. The period of incubation of this syndrome varies between 7 and 56 days. Most cases of infection occur in the summer, during birth and peak lactation periods. Several mycoplasmas can be isolated from milk and blood for a short period during the infectious process [4].

Nowadays, this syndrome affects animals in most countries that have a high production of sheep and goats. It is found across several continents, including mainly North America, western Asia, North Africa, and Europe, and is endemic in most Mediterranean countries [5]. To work and perform culture, biochemical and serological tests would take long times at least two

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

| Primer | Target gene | Sequence | Length(bp) |
|---|---|---|---|
| FS1 | 16S rRNA | F: 5'-GCTGCGGTGAATACGTTCT-3'<br>R: 5'-TCCCCACGTTCTCGTAGGG-3' | 163 |
| Fs2 | 16S rRNA | F: 5'-AAAGGTGCTTGAGAAATGGC-3'<br>R: 5'-GTTGCAGAAGAAAGTCCAATCA-3' | 375 |

weeks while by PCR due to no need of purification and cloning, pathogenic Mycoplasma are easy to detected even within the mixed organisms in a sample, without spending long times (It requires at least 2 to 3h) [6]. Several different methods of PCR have been described and used on the bases of different gene by researchers [7,8]. The polymerase chain reaction (PCR) with mycoplasmal 16S rRNA and specific primers have been applied for detection of a variety of Mycoplasma species [9-11]. The rRNA is naturally present in high copy numbers (up to 10,000 molecules per cell) [12]. The aim of this study was to isolation and prevalence of *M. agalactiae* from sheep presenting CA in Kurdistan in the West of Iran**.**

## Materials and Methods

Samples collection and processing: A total, 69 samples included (Milk, Conjunctiva swabs, Synovial fluid, and Ear canal swabs) were examined by PCR assay during 2011-2012. All sheep tested had been previously examined to confirm they had clinical signs of CA. On average, flocks selected to participate in the study constituted about 5% of all herds in the study area. 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 24h. Transport media contain thallous acetate (250mg/liter), which is toxic and inhibitory to some mycoplasmas but not those causing CA and reduce bacterial contamination from clinical sample [13]. 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% CO2 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.

PCR method: The enriched samples were used in PCR method. DNA was extracted from enriched samples using a previously described method [10]. In this method 500µl of samples were placed in 1 × 5µl Eppendorf tube, micro centrifuged at 13000 rpm for 15min. 100µl of lyses buffer was added to 100µl of precipitated, and tubes were placed in 56°C bath for 4h. Then 200µl saturated phenol was added and tubes was centrifuged at 13000 rpm for 20min. Upper phase was transferred to another tube and equal volume of mixed phenol/Cholorophorm (1:1) was added. After centrifuged at 13000 rpm for 20min the aqueous phase was transferred to another tube and added equal volume of pure cholorophorm and was centrifuged at 13000 rpm for 5min. Upper phase was transferred to a new tube and mixed with 1/10 volume of acetate sodium (3M) and were precipitated in -20°C refrigerator with 2 fold volume of cool and pure ethanol (20min), then the tube was centrifuged at 13,000 rpm for 15min. 200µl of 70% ethanol was added and the tube was centrifuged at 13,000 rpm for 5min, the DNA was dried and resuspended in DDW at 4°C and used for PCR [10]. In this study two primers (forward and reverse) which have been already designed by Kojima et al. [10] and amplify a 163bp region of 16S rRNA gene of Mycoplasma genus and two primers have been already designed by Tola et al. [14] and amplify 375bp 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 35.25µl containing 17.5µl DNA, 0.1µl of each primers, 0.5µl dNTP mix (10mM) {Cinnagen Inc.}, 4µl Mgcl2 (25mM) {Cinnagen Inc.}, 2.5µl PCR buffer (10x) {Cinnagen Inc.}, and 0.25µl Tag DNA polymerase (5unit/µl) {Cinnagen 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 1min (Annealing) and 72°C for 1min (Extension), PCRs were finished with a final extension step at 72°C for 5min. 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 micro liter aliquot of each PCR products was mixed with 2µl loading buffer (6×). The PCR products and 100bp DNA ladder were then separated by electrophoresis on 1% Agarose gel and stained with 0.5µl/ml Ethidium bromide (100 volts for 1h) following UV Transllluminator.

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

| Samples | No. | Culture | | | | Mycoplasma-PCR | | | | *M. agalactiae*-PCR | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Positive | | Negative | | Positive | | Negative | | Positive | | Negative | |
| | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Conjunctiva swabs | 24 | 11 | 45.8 | 13 | 54.2 | 17 | 70.8 | 7 | 29.2 | 3 | 17.6 | 14 | 82.4 |
| Ear canal swabs | 12 | 3 | 25 | 9 | 75 | 7 | 58.3 | 5 | 41.7 | 0 | 0 | 7 | 100 |
| Milk | 19 | 8 | 42.1 | 11 | 57.9 | 14 | 73.7 | 5 | 26.1 | 10 | 71.4 | 4 | 28.6 |
| Synovial fluid | 14 | 2 | 14.3 | 12 | 85.7 | 8 | 57.1 | 6 | 42.9 | 2 | 25 | 6 | 75 |
| Total | 69 | 24 | 34.8 | 45 | 65.2 | 46 | 66.7 | 23 | 33.3 | 15 | 32.6 | 31 | 67.4 |

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

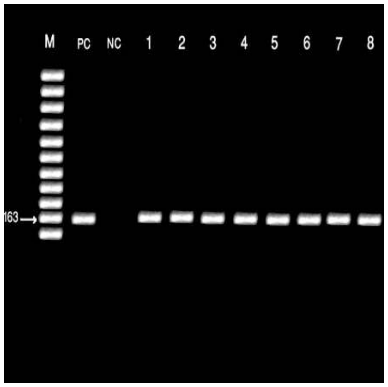


Figure-2. Specificity of the PCR detection assay using the specific primers. M: Marker 100bp; PC: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control, 1-8 suspected samples. The formation of 163bp bands in 8 genus positive sample

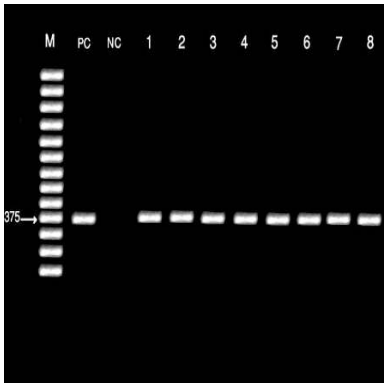


Figure-3. Specificity of the PCR detection assay using the primers FS1 and FS2. M: Marker 100bp; PC: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control. 1-8 positive Mycoplasma genus samples. The formation of 375bp in 8 positive species

## Results

At first, 69 samples were enriched in PPLO broth media. After culture in 24 samples (34.8%), 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 163bp (Figure-2). This proved that 46 samples (66.7%) were infected with Mycoplasma in PCR test. Then all Mycoplasma positive samples were analyzed for *M. agalactiae* infection by PCR method. On the PCR test, 15 isolates (32.6%) examined were positive for *M. agalactiae* that showed specific amplicon at 375bp (Figure-3). This method established that 3 out of 17 (17.64%) of conjunctiva swab, zero percent of ear canal swab, 10 out of 14 (71.43%) of milk and 2 out of 8 (25%) synovial fluid samples were positive. Our results suggested that milk secretion and conjunctiva samples are suitable for PCR detection of *M. agalactia.*

## Discussion

PCR assay is routinely used in many laboratories and are extremely sensitive. It can provide a rapid early warning system when carried out on clinical samples, enabling a full investigation to take place when results are positive. However, negative results should not be considered definitive. PCR has been accepted as a valuable tool for the diagnosis of Mycoplasma infections, has the advantage of easy use, rapid availability of results, and standardization, and is more suitable for processing of a large number of specimens [15]. In this study, 15 isolates were positive for *M. agalactiae* that showed specific amplicon at 375bp. In one study, using the PCR method, the presence of *M. agalactiae* was detected in 20 out of 101 animals examined (19.80%). In 12 animals out of 54 (22.22%) the conjunctival swabs samples were positive for *M. agalactiae* and in 8 out of 47 animals (17%) milk samples were positive in west central, Iran [16]. In Jordan, Mycoplasma was isolated from eight (13%) of the 62 milk samples and seven (2.3%) of the 310 nasal swabs collected from sheep, unusually *M. agalactiae* was not isolated of the sheep samples [17]. In our study showed that 3 out of 17 (17.64%) of Conjunctiva swab, zero percent of ear canal swab, 10

out of 14 (71.43%) of milk and 2 out of 8 (25%) synovial fluid samples were positive. Result of PCR with 367 milk samples with *M. agalactiae* primers showed that 11(3%) of them were positive in west of Iran [18]. A total of 240 random raw milk samples were collected from cows, buffaloes, sheep and goats in Assiut and Qena cities, Egypt (30 samples each). Fifteen strains of Mycoplasma were isolated from raw milk. The strains were biochemically characterized followed by PCR assay for confirmation. On the basis of biochemical characterization the strains were divided into 5 species as follow *M. arginini*, *M. bovirhinis*, *M. bovis*, *M. species* group 7, and M. dispar, but *M. bovis* could not be detected [19]. In the Mediterranean region, CA alone is estimated annually to cost at least US$ 30 million mainly as the result of milk production losses but mortality and poor growth in the young may also be significant [20]. In Iran over 90% of sheep and goats are kept as mixed flocks, which must facilitate the transmission of mycoplasmas from one animal species to the other. The clinical signs in the flocks, which have been studied, are mostly mastitis in sheep and goats, arthritis mainly in young goats, pneumonia in different age groups. In our examination all Mycoplasma positive samples were analyzed for *M. agalactiae* infection by PCR method and 31 isolates (67.4%) examined were negative for *M. agalactiae*. The finding of other mycoplsmas with significant epidemiology challenges existing plans for the control of CA in sheep population in Iran. The treatment of mycoplasmoses is currently based on antibiotics, such as the Tetracyclines, Macrolides, Chloramphenicol, Tylosin, and Fluoroquinolones [5].

However, results of treatment may be poor or ineffective against mycoplasmas if the therapeutic dose is not well defined and the antibiotic is not administered for a sufficiently long period [2]. Meanwhile after occurrence of an agalactiae infection on 200 animals of a herd, gradually the cure will happen among the animals of that herd, but sometimes *M. agalactia* may be isolated even after three months of its clinical signs being recorded [21]. In Iran, only *M. agalactiae* vaccines are commercially available, so that in endemic areas, in which all species are present, the animals are not protected against these other species. Vaccines may be an efficient and cost effective way of preventing the spread of disease although few mycoplasmas vaccines are presently available. Another important epidemiological factor in the high percentage of isolation from samples collected from the ear canal. The mixing of animals of different origins is a major factor favoring Mycoplasmosis [22] and healthy carriers are important in Iran, where the interchange of animals is a common practice. The peculiarity and the risk of this carrier state is that it may occur independently of any clinical expression, and the most frequent location of infection identified to date is the external ear canal [5]. In Kurdistan, carrier sheep introduced the disease from the other areas. These high percentages could explain the fast diffusion of contagious agalactiae, and we recommend that practicing veterinarians should include the external ear as a primary culture site for determining the presence or absence of mycoplasmas in sheep. The results reported in this paper confirmed the detection of *M. agalactiae* modest agent of CA in sheep in Kurdistan province in the West of Iran.

## Author's contributions

MK, BR and HK prepared samples. SAP and AA analyzed samples. MK analyzed the data. MK drafted and revised the manuscript. All the authors read and approved final manuscript.

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## Competing interests

Authors declare that in Kurdistan province in the West of Iran, other *Mycoplasma;* have been isolated in sheep with pneumonia, mastitis, arthritis or keratoconjunctivitis, but their economic importance remains unknown.

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