A SURVEY OF *MYCOPLASMA AGALACTIAE* IN SMALL RUMINANTS WITH CONTAGIOUS AGALACTIAE SYNDROME IN IRAN

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ABSTRACT

Contagious agalactia (CA) is one of the major animal health problems in small ruminants. It has economic effect and is caused by *Mycoplasma agalactiae*, the 'classic' etiological agent in sheep and goats. The significance of the different *Mycoplasma spp* causing CA varies depending on the geographic area. This study conducted between 2012 and 2013 on 189 small ruminants with CA signs in the west of Iran, an area where CA is endemic. All samples (milk, synovial fluid, ear swabs, conjunctival swabs and nasal swabs) were examined by PCR method. *Mycoplasma spp* was detected in 76.2% and *M. agalactiae* isolated from 16% of positive samples. *M. agalactiae* were isolated from 7 conjunctival swabs, 15 milk samples and one synovial fluid sample. Results showed that *M. agalactiae* was found to be the non-main cause of CA in small ruminants in Iran.

Keywords: *Mycoplasma spp*, *Mycoplasma agalactiae*, contagious agalactia, Iran, small ruminant

INTRODUCTION

Mycoplasma (M.) *agalactiae* is the main causative agent of CA, a syndrome that causes mastitis, arthritis, conjunctivitis and pneumonia in sheep and goats (Bergonier *et al*., 1997). It may be caused byany of the five Mycoplasma species associated with this disease, namely, *M. agalactiae*, *Mycoplasma mycoides* subspecies *mycoides* LC (large colony), *Mycoplasma capricolum* subspecies *capricolum*, *Mycoplasma mycoides* subspecies *Capri* and *Mycoplasma putrefaciens* (Corrales *et al*., 2007; Ariza-Miguel *et al*., 2012). CA has its major impact on the Mediterranean countries, where the disease is considered to be endemic, however, it is also widely distributed in West Asiancountries, central, north and east African countries, the USA, and Brazil (De la Fe *et al*., 2005; Chazel *et al*., 2010). Interestingly, the significance of the different *Mycoplasma spp* causing CA varies depending on the geographic area. In the United States, *M. mycoides* subsp. *capri* is the most prevalent caprine Mycoplasma, although *M. agalactiae* has been recently isolated (Kinde*et al*., 1994). In Spanish dairy sheep farms *M. agalactiae* was the only species detected that showing a high frequency of presence (Ariza-Miguel *et al*., 2012). In Northern Jordan, *M. agalactiae* and *M. mycoides* subspecies *capri* play the major role in both, sheep and goats (Al-Momani *et al*., 2011). Although most reports in the literature describe the classic lesions in the mammary glands, joints, eyes, or respiratory tract as the pathologic findings of CA (Bergonier*et al*., 1997; Corrales *et al*., 2007; De la Fe *et al*., 2009). As *M. agalactiae* shows differing prevalence across the world, it is currently absent from some countries, notably the UK (McAuliffe *et al*., 2008). In most cases, infected hosts spontaneously recover from acute clinical signs within a few weeks, but develop a chronic infection accompanied by shedding of *M. agalactiae* in milk and/or other body secretions for years without presenting any clinical signs (Bergonier *et al*., 1997); these (asymptomatic) carriers can transmit the bacteria to other susceptible animals and cause acute disease (Sanchis *et al*., 2000). Preventive and therapeutic strategies remain very inefficient in the control of CA, most likely because of both pathogen-specific features and the lack of epidemiological data. Thus far management strategies have been the most satisfactory method for controlling this disease (De la Fe *et al*., 2012). In Iran, sheep and goats are usually kept together mainly under free roaming transhumance husbandry with a small number of flocks reared under semi-intensive husbandry methods (Ministry of Agriculture, 2011). The routinely recommended control measure for infectious disease is vaccination. Currently, vaccines are used against: enterotoxaemia, brucellosis, anthrax, pox, and foot and mouth disease. Vaccines are available for mycoplasma diseases, this vaccine is available commercially including a monovalent preparation containing *M. agalactiae*, and there is few data on their efficacy.

The aim of present study was to assess the presence and geographic distribution of *Mycoplasma spp* and *M. agalactiae* species causing CA, by analyzing conjunctival, nasal, ear and milk samples from sheep and goats with CA singe by PCR based methods which have been demonstrated to be specific and sensitive.

MATERIALAND METHODS
Samples collection
All 189 samples were collected and subjected to PCR procedures to detect the presence of *Mycoplasma spp* and *M. agalactiae*. All the sheep and goats which selected have been previously examined to confirm that they have clinical signs of CA. Following clinical examination, samples were taken, placed in transport medium, immediately refrigerated (4°C) and transported to the laboratory.The samples were twirled and left in 2 ml of PH culture medium (De la Fe *et al.*, 2005) for 30 minutes at room temperature. After discarding the samples, aliquots of the remaining fluid were taken for PCR and the rest was stored at-80°C.

DNA extraction and PCR
DNA was extracted from enriched samples using Kojima *et al.* (1997) method. 500μl of samples were placed in 1×5μl Eppendorf tube, micro centrifuged at 13,000 g 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 13,000 g for 20 min. Upper phase was transferred to another tube and equal volume of mixed Phenol/ Chloroform (1:1) was added. After centrifuged at 13,000 g for 20 min the aqueous phase was transferred to another tube and added equal volume of pure Chloroform and was centrifuged at 13,000 g for 5 min. 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 (20 min), then the tube was centrifuged at 13,000 g for 15 min. 200μl of ethano l70% was added and the tube was centrifuged at 13,000 g 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) amplify 163bp region of 16S rRNA gene of Mycoplasma genus (Kojima *et al*., 1997) and amplify 375bp region of 16S rRNA gene of *M. agalactiae* species (Tola *et al*., 1997) were used (Table 1).

Table 1. Nucleotide sequences and primers used for identification of *Mycoplasma spp* and *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

DNA amplification was carried out in a total volume of 35.25μl containing 17.5μl DNA, 0.1μl of each primer, 0.5μl dNTP mix (10mM) {Cinnagen Inc.}, 4μl Mgcl2 (25mM) {Cinnagen Inc.}, 2.5μl PCR buffer (10×) {Cinnagen Inc.}, and 0.25μl Tag DNA polymerase (5unit/μl) {Cinnagen Inc.}. Reaction mixture was thermo cycled (Bio-Rad, Hercules, CA, USA) 30 times 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 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 gels and stained with 0.5μl/ml ethidium bromide (100 volts for 1h) following UV trans illuminator (BioRad, Hercules, CA, USA). Sequences were aligned using MEGA 4.0 software (Tamura *et al*., 2007).

RESULTS AND DISCUSSION
CA of sheep and goats has been known for about two centuries (Zavagli, 1951). For first time, Borry *et al*. (1963) reported the presence of CA disease in sheep and goats in Iran. Over 90% of sheep and goats are kept as mixed flocks in Iran (Ministry of Agriculture, 2011), which 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 (Hasani-Tabatabayi and Firouzi, 2005). In Iran, the disease has been controlled either by vaccination or antibiotic therapy. However, these strategies are not successful for prophylaxis or eradication program. Antibiotic therapy can result in symptomatic improvement, but treated animals may remain carriers due to antibiotic resistance, and inactivated vaccines generally provide short-term protection (MoradiBidhendi *et al.*, 2011). Our findings showed that the rate of isolation of *Mycoplasma spp* from sheep (65.7%) and goat (76.8%) indicates that goats are susceptible to mycoplasmosis than sheep. This findingis in agreement with previous studies (Al-Momani*et al*., 2006; Azevedao *et al*., 2006). Results showed that the prevalence of *M. agalactiae* was 16%which is in agreement with results obtained by Khezri *et al*. and Moslemi *et al*. (Khezri *et al*., 2012 and Moslemi *et al.*, 2013*)* in which *M. agalactiae* was found to be the non-main cause of CA in small ruminants in Iran.All 36 milk samples were tested for *Mycoplasma spp* and *M. agalactiae* 26 out of 36 samples were positive for *Mycoplasma spp* (Fig. 1), and of these samples, *M. agalactiae* (Fig. 2) detected in 15 samples by PCR (Table 2).
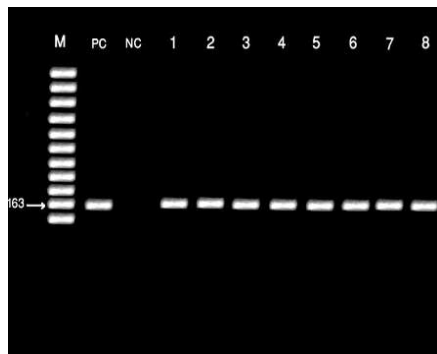

Fig 1. Specificity of the PCR detection assay using the specific primers. M: Marker 100bp; C: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control, 1-8 suspected samples. The formation of 163bp bands in 8 genus positive sample.
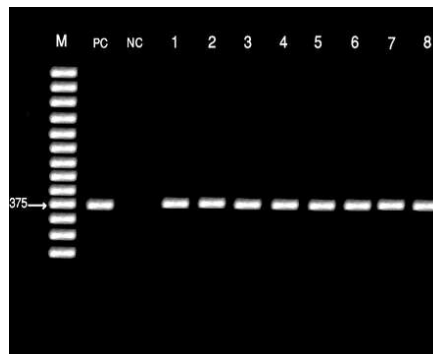
Fig. 2. Specificity of the PCR detection assay using the primers FS1 and FS2. M: Marker 100bp; PC: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control. 1-8 positive Mycoplasma genus samples. The formation of 375bp in 8 positive species.

Table 2. Results of PCR test used to confirm infection by *Mycoplasma spp.* and *M. agalactiae* in the examined samples

Samples	n	*Mycoplasma spp*	%	*M. agalactiae*	%
Milk	36	26	72.2	15	57.7
Ear swap	26	20	77	0	0.0
Nasal swap	27	26	96.3	0	0.0
Synovial fluid	18	10	55.5	1	10
Conjunctival swap	82	62	75.6	7	11.3
	189	144	76.2	23	16

Asobserved, detection frequency of *M. agalactiae* increased from 0.0% (Sotoodehnia and Arabi, 1986) in 1986 to 57.7% in milk samples, suggesting that the pathogen has been spread in the sampling area during the present study period. *M. agalactiae* is a highly persistent pathogen remaining in the animals for years (Bergonier *et al*., 1997; Corrales *et al*., 2007).The treatment of mycoplasmosis is currently based on antibiotics, such as the Tetracycline, Macrolides, Chloramphenicol, Tylosin and Fluoroquinolones (Bergonier *et al*., 1997; Madanat *et al*., 2001). Antibiotics can result in symptomatic improvement, but they may not be effective in chronic joint infections or Keratoconjunctivitis. Treatment may not eliminate the infection from carriers; however, many authors feel that bacteriological treatment is an illusory objective. If the therapeutic dose is not exactly defined and the relevant antibiotic is not administered for a sufficiently long period, the resulting effect may be very poor or none at all. The causal agents continue to be shed into the environment and there is a possibility that resistant

strains will develop (Madanat *et al*., 2001). The presence of asymptomatic carriers in a herd appears to be a serious health risk. These animals carry the infectious agent in their genital tracts; the carrier state is less obvious in males than females (Bergonier *et al*., 1997). As *M. agalactiae* is a highly persistent pathogen, remaining in the animals for years (Corrales *et al.*, 2007; Bergonier *et al.*, 1997), and that it is very difficult to eliminate from infected herds. It has been shown that other animal species, such as cattle, camels or small wild ruminants, can function as infection reservoirs for mycoplasmas (Perrin *et al*., 1994). Cottew and Yeats first reported that the goat's ear canal can be an unusual source of mycoplasmas, including pathogenic species. Gil *et al*. (1999) reported *M. agalactiae* and *M. mycoides* subspecies *mycoides* LC type were the species most frequently isolated from ear canals (70% and 25.5%, respectively). *Mycoplasma spp* were isolated from 26 (96.3%) of the 27 nasal swab, 20 (77%) of the 26 ear swab and 10 (55.5%) of the 18 synovial fluid samples. *M. agalactiae* were only isolated from 1 (10%) of the 10 synovial fluid samples. Overall, 76.2% of samples were detected *Mycoplasma spp*, whereas, 23 of these samples (16%) were positive for *M. agalactiae* (Table 2). In present study*M. agalactiae* was not detected from ear canals. Our study was designed to determine the prevalence of *M. agalactiae* in CA infected animals, the number of samples scoring positive for mycoplasmas was high. 16% of the samples taken proved positive for *M. agalactiae* based on PCR. In Iran, programs based on *M. agalactiae* inactivated vaccine have resulted in the reduction of clinical signs associated with CA outbreaks. Vaccination strategies against contagious agalactia of sheep and goats are based on bothlive attenuated or inactivated vaccines (Tola *et al*., 1999; Madanat *et al*., 2001). Live CA vaccines provide an excellent immunity witha longer period of protection (Madanat *et al*., 2001; Nicholas, 2002) and can be used in infected animals (Macun *et al*., 2010). However they can produce a transient infection with shedding of Mycoplasma (Sotoodehnia *et al*., 2005; OIE, 2013). Live vaccines should not beused in lactating animals andshould be part of a regional plan inwhich all flocks from which animals are likely to come into contact be vaccinated at the same time (OIE, 2013).Vaccines may be an efficient and cost effective way of preventing the spread of disease although few mycoplasma vaccines are presently available (Al-Momani *et al*., 2006).Vaccines may be available for some organisms in some area. Inactivated vaccines generally provide short-term protection. Live vaccines can prevent symptoms, but do not prevent animals from becoming infected or shedding the organism. Vaccines organisms may also be shed in the milk (OIE, 2013). Vaccines for the prevention of CA due to *M. agalactiae* are used widely in the Mediterranean countries, Europe and in western Asia. No single vaccine has been universally adopted (OIE, 2013). The use of inactivated CA vaccines in endemic areas has been reported to reduce the number of animals developing clinical CA but fails to prevent natural infection (Leon-Vizcaino *et al*., 1995; De la Fe *et al*., 2007; Amores *et al*., 2012) and seems to indicate that current CA vaccines only serve to reduce clinical symptoms but not to prevent new infections or reduce the prevalence of infected animals with infected herd (Amores *et al*., 2012).

Based on above facts and our findings it is concluded that *M. agalactiae* have not a primary role in etiology of CA in small ruminants and other Mycoplasmas infected these animals.

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