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Originall Article

Investigation of *Mycoplasma agalactiae* in sheep in Kurdistan province by PCR

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ABSTRACT

Contagious agalactia (CA) is a serious disease syndrome of sheep and goats that is characterized by mastitis, arthritis, keratoconjunctivitis and, occasionally, abortion. *Mycoplasma* (M.) *agalactiae* is the main cause of the disease in sheep and goats. The aim of the present study was to detect *M. agalactiae* in conjunctival, synovial fluid, nasal, ear and milk samples in sheep herds with or without CA sign in Kurdistan province. One hundred and seventy three samples analyzed were taken from sheep herds reared in a CA endemic area. Of the samples tested, 130 were positive by PCR for *Mycoplasma spp.* and of these, 19 showed a positive result for *M. agalactiae*. *M. agalactiae* was detected in conjunctival (7/59), synovial fluid (1/8) and milk (11/21) samples. Ear and nasal swap samples were free of *M. agalactiae*. The results of 60 samples in sheep without signs of CA syndrome showed that *Mycoplasma spp.* and *M. agalactiae* were detected in 25 and 6 samples, respectively. Our findings indicate that in Kurdistan province; *M. agalactiae* was not the main etiological agents of the CA syndrome. Also, this species can be isolated from animals without clinical signs of disease. Our results suggested that milk secretion is suitable for PCR detection of *M. agalactiae*.

Keywords: Contagious agalactia, *Mycoplasma agalactiae*, PCR, Sheep, Kurdistan province

INTRODUCTION

CA is an infectious syndrome caused by several species of mycoplasma, which affects small ruminants. *M. agalactiae* is considered as the classic etiological agent of CA, but other species are now known to cause the disease, especially in goats: *Mycoplasma mycoides* subsp. M*ycoides* large colony type (Mmm LC), *Mycoplasma capricolum* subsp. *capricolum* (Mcc) and occasionally *Mycoplasma putrefaciens* (Mp). The clinical signs of CA are the typical triad of mammary, joint and eye symptoms, although respiratory symptoms and occasionally reproductive or other symptoms may appear (Bergonier *et al* 1997, Da Massa *et al* 1992, Gil *et al* 2003). CA appears to acute, sub-acute or chronic form (Greco *et al* 2001). However, in endemic areas, clinical outbreaks of the disease are sporadic (Contreras *et al* 2008). In addition to clinical consequences, CA generates economic costs due to treatments, veterinary assistance and vaccinations,

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combined with the effects of genetic resource losses via mortality or culling of selected animals affected by the disease (Bergonier *et al* 1997, Corrales *et al* 2007). Asymptomatically infected animals can shed mycoplasma for many years after infection, therefore, they play a very important role in the epidemiology of CA, making unsuccessful both prophylaxis and eradication programs (Moradi Bidhendi *et al* 2011). In chronically infected carriers, the presence of *M. agalactiae* and M. Mmc at sites other than the external ear canal has also been observed. Moreover, in one of artificial insemination center in Spain, in which *M. agalactiae* and *M. mycoides subsp. capri* (Mmc) were detected in conjunctival and preputial swabs, infection spread to the rest of the animals, confirming the fact that carriers actively participate in transmitting the disease (De la Fe *et al* 2010). Despite evidence that some vaccinated goats shed mycoplasmas in their milk (Amores *et al* 2012, De la Fe *et al* 2007). Moreover, *M. agalactiae* was detected in the semen of asymptomatic goat bucks, pointing to a real risk of venereal transmission of CA (Amores *et al* 2011, De la Fe *et al* 2009). This situation is not totally unjustifiable since CA is not considered a disease of the reproductive tract of goat males and females despite sporadic clinical cases observed after natural or experimental infection (Amores *et al* 2011, Gil *et al* 2003, Nayak & Bhowmik, 1990, Singh *et al* 1974). Vaccines for the prevention of contagious agalactia due to *M. agalactiae* are used widely in the Mediterranean countries of Europe and in western Asia. No single vaccine has been universally adopted, and no standard methods of preparation and evaluation have been applied (OIE 2013). In Iran, where live vaccines for *M. agalactiae* are not acceptable, attention has focused on the use of killed organisms. Between vaccine strains in Iran were high homology [Shiraz strain with Lorestan strain 9.7% and Shiraz strain with Taleghan strain 74.6%] (Mahdavi *et al* 2009). Live attenuated vaccines against *M. agalactiae* have been used in Turkey for many years and have been reported to provide better protection in ewes and their lambs than inactivated vaccines (Nicholas, 2002). The present study was designed to detection of *Mycoplasma spp.* and *M. agalactiae* in ear, conjunctival, nasal, synovial fluid and milk samples taken from sheep herds in a CA endemic with or without clinical signs of the disease.

MATERIALS AND METHODS

Sample collection. The study was conducted on people herds with or without signs of CA syndrome. Vaccination in herds was unknown. A total of 173 samples were collected from conjunctival swap (n=77), nasal swap (n=23), ear swab (n=26), synovial fluid (n=16) and milk (n=31), in 2012-2013 (Table 1). The samples were taken and immediately placed in the test tube with Mycoplasma culture transport medium, and stored at 4 °C till transport to Mycoplasma reference laboratory of Razi Vaccine and Serum Research Institute, Karaj, Iran within 24 hours. The transport medium contained thallous acetate (250mg/liter) which toxic to some Mycoplasma but not those causing CA and reduce the bacterial contamination (Buonavoglia *et al* 2010).

Polymerase Chain Reaction. DNA was extracted from enriched samples using a previously described method (Kojima *et al* 1997). In this 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 20min. 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 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 g for 15min. 200µl of 70% was added and the tube was centrifuged at 13,000 g for 5min, 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 2). 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 $MgCl_2$ (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 program 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

In total, 130 of the samples (75.1%) were positive for the presence of mycoplasmas (Table 3). A positive result for *M. agalactiae* was obtained in 19 (14.6%) samples [Figures 1 & 2]. In the conjunctivas, *Mycoplasma spp.* was the most detected with 59 samples proving positive for these microorganisms (76.6%). In 21 of the milk positive samples, *M. agalactiae* was detected in 11 samples (52.4%). Table 4 and 5 shows the presence of mycoplasmas detected in the two types of sheep samples (with and without signs of CA syndrome). *Mycoplasma spp.* and *M. agalactiae* were isolated and identified in 41.7% and 24.0% analyzed samples in sheep without signs of CA syndrome. Considering all milk samples analyzed, 47.1% and 75% of the samples (with and without signs of CA syndrome) were positive for *M. agalactiae.*

DISCUSSION

CA is a highly infectious disease of small ruminants in Iran (Sotoodehnia *et al* 2005). The first isolation of *M. agalactiae*, the causative agent of CA was reported from ewe milk (Bory & Entessar 1959). Little information is available on mycoplasma detection in the ear, conjunctival, nasal, synovial fluid and milk samples for sheep and goat herds in Kurdistan province. Sotoodehnia et al. (1986) cultured 490 sheep and goats milk. Ninety six isolates were biochemical identified as *M. agalactiae* of which only 23 were confirmed by serological test. There was not any isolation from milk samples were taken from Kurdistan province. Moradi Bidhendi et al. (2011) reported that twenty (5.5%) out of 367 milk samples were positive in PPLO agar. 16 (80%) out of 20 isolated mycoplasma were from sheep and the others were from goat. Result of PCR with 367 milk samples with *M. agalactiae* primers showed that 11(3%) of them were positive. Khezri et al. (2012) reported that *M. agalactiae* was detected in 15 out of 46 sample examined (32.6%), PCR method established that 17.64% of conjunctival, 71.43% of milk and 25% of synovial fluid samples were positive. Moslemi et al. (2013) shown that 113 (49.77%) out of 227 conjunctival, nasal and milk goat samples were positive in culture method. 158(69.6%) samples were positive for the presence of *Mycoplasma spp.* by PCR. As well as, the presence 51(32.27%) and 4(2.53%) positive samples of *M. Mycoides* cluster and *M. agalactiae* were demonstrated, respectively. Our findings indicate that *M. agalactiae* is not the mycoplasma species that most frequently infects sheep herds in our geographical region (Table 3). In other province, reports were agreement with prior observation (Abtin *et al* 2013, Kheirkhah *et al* 2013, Pirali Kheirabadi & Ebrahimi, 2007). Interestingly, the significance of the different Mycoplasma species causing CA varies depending on the geographic area.

Table 1. Distribution of samples in sheep with and without sings of CA syndrome in herds

Samples	N	with signs of CA syndrome	without signs of CA syndrome
Conjunctival swap	77	52	25
Synovial fluid	16	10	6
Nasal swap	23	15	8
Ear swap	26	16	10
Milk	31	20	11
Total	173	113	60

Table 2. 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′-TTGCAGAAGAAAGTCCAATCA-3′	375	(Tola et al., 1997)

Table 3. The results of samples for *Mycoplasma spp.*-PCR and *M. agalactiae* –PCR

	N	*Mycoplasma spp.*-PCR		*M. agalactiae*-PCR	
		N	%	N	%
Conjunctival swap	77	59	76.6	7	11.9
Synovial fluid	16	8	50.0	1	12.5
Nasal swap	23	23	100	0	0.0
Ear swap	26	19	70.1	0	0.0
Milk	31	21	67.7	11	52.4
Total	173	130	75.1	19	14.6

Table 4. The results of samples for *Mycoplasma spp.*-PCR and *M. agalactiae*-PCR in sheep with signs of CA syndrome

samples	N	with signs of CA syndrome	*Mycoplasma spp.*-PCR		*M. agalactiae*-PCR	
			N	%	N	%
Conjunctival swap	77	52	50	96.1	4	8.0
Synovial fluid	16	10	7	70.0	1	14.3
Nasal swap	23	15	15	100	0	0.0
Ear swap	26	16	16	100	0	0.0
Milk	31	20	17	85.0	8	47.1
Total	173	113	105	93.0	13	12.4

Table 5. The results of samples for *Mycoplasma spp.*-PCR and *M. agalactiae*-PCR in sheep without signs of CA syndrome

samples	N	without signs of CA syndrome	*Mycoplasma spp.*-PCR		*M. agalactiae*-PCR	
			N	%	N	%
Conjunctival swap	77	25	9	36.0	3	33.3
Synovial fluid	16	6	1	16.7	0	0.0
Nasal swap	23	8	8	100	0	0.0
Ear swap	26	10	3	30.0	0	0.0
Milk	31	11	4	36.4	3	75.0
Total	173	60	25	41.7	6	24.0

In the United State and in Northern Jordan Mmc and *M. agalactiae* play the major role in both, sheep and goats (Al-Momani *et al* 2011, De la Fe *et al* 2005). In France, *M. agalactiae* has reemerged in sheep flocks (Chazel *et al* 2010). Although our study was designed to determine the prevalence of carriers in CA infected herds, the number of samples scoring positive for *M. agalactiae* was low. However, 75.1% of the samples taken proved positive for *Mycoplasma spp.* based on PCR. These results are in agreement with other obtained in different regions of the Iran (Khezri *et al* 2012, Moradi Bidhendi *et al* 2011, Moslemi *et al* 2013). 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). There are reports of excretion of organisms in milk even after 8 years of infection with mild and with or without clinical signs (Bergonier *et al* 1996, Da Massa & Brooks 1991). Animal species other than homologous hosts as cattle, camel and many other wild small ruminants can also act as reservoir of the infection. These carrier states are more frequently observed in females particularly in their genital tracts (Bergonier *et al* 1997, Verbisck *et al* 2010, Cokrevski *et al* 2001). In Iran, inactivated vaccine was prepared and used in limited area since 1971(Baharsefat *et al* 1971). The use of inactivated CA vaccines in endemic areas has been reported to reduce the number of goats and kids developing clinical CA but fails to prevent natural infection (Amores *et al* 2012, De la Fe *et al* 2007), 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 within an infected herd (Amores *et al* 2012). The vaccination against *M. agalactiae* in sheep induce both specific and non - specific, humoral and cellular response irrespective of type of vaccine, however, duration of persistence of antibodies depend upon multiple factor viz., strain used, adjuvant incorporated, dose of vaccine, routes of inoculation, physiological status of animal etc., Live attenuated vaccines are more effective and have been reported to provide better protection in ewes and their lambs than the inactivated vaccines but can produce a transient infection with shedding of mycoplasma through milk. Importantly, the live vaccines should be part of a regional plan in which all flocks from which animals are likely to come into contact be vaccinated at the same time. Inactivated vaccines are much safer with no side effects but have shorter period of protection with doubted efficacy (FAO 1992, Buonavoglia *et al* 2012, Nicolet, 1994). Moreover, it shows that some animals in the vaccinated herd may shed mycoplasmas in the milk, semen and thus in theory poses a risk for any measures implemented to control the disease (De la Fe *et al* 2009, Pepin *et al* 2001).

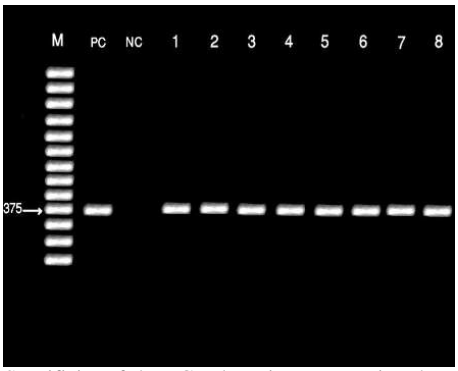

Figure 1. Specificity of the PCR detection assay using the specific primers. M: Marker 100bp; C: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control, 1-8 suspected samples. The formation of 163bp bands in 8 genus positive sample.

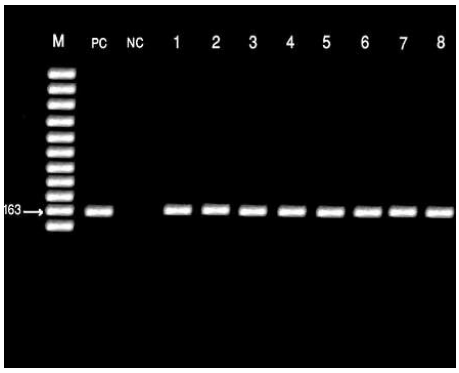

Figure 2. Specificity of the PCR detection assay using the primers FS1 and FS2. M: Marker 100bp; PC: Positive Control [*M. agalactiae* (NCTC 10123)]; NC: Negative Control. 1-8 positive Mycoplasma genus samples. The formation of 375bp in 8 positive species.

In conclusion, CA is considered as a neglected disease of small ruminants because of the complex disease distribution pattern, ubiquitous nature of the causal agent and poor sheep and goat farm management practices, especially in developing and under developed countries like Iran. Rapid spread, multiple sources of infection along with vertical and horizontal mode of transmission are matter of immense concern and severely affect the local economy. Depending upon

conditions like deprivation of maternal antibodies, immunocompromised state, stress due to transportation, pregnancy or extreme climatic conditions; animals may suffer from acute, sub-acute, chronic or asymptomatic forms of disease. Our findings indicate that in Kurdistan province; *M. agalactiae* was not the main etiological agents of the CA. Moreover, in CA endemic regions, the presence of asymptomatic carriers in a herd which carry the infectious agent is of major concern.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted review article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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