Species of Mycoplasma *causing contagious agalactia in small ruminants in Northwest Iran*

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Sheep,
Goat.

Summary

Contagious agalactia (CA) is a serious disease of small ruminants that occurs in many countries, and is usually characterized by mastitis, arthritis, keratoconjunctivitis, pleuropneumonia, and septicemia. *Mycoplasma agalactiae* (Ma) is the main causative agent in sheep and goats but other pathogens including *Mycoplasma mycoides* subsp. *capri* (Mmc, which incorporates the former *M. mycoides* subsp. *mycoides* Large Colony type), *Mycoplasma capricolum* subsp. *capricolum* (Mcc), and *Mycoplasma putrefaciens* (Mp) might be involved. They are all usually associated with infections in goats and may cause similar clinical signs. A total of 116 sheep and 16 goats suffering from the acute form of the disease were included in this study. They were recruited following a number of outbreaks suspected to be CA in the Ardebil province of Iran. Milk, lachrymal or synovial fluid were collected exclusively from the affected animals in order to identify the pathogen involved. Of the 132 collected samples, 33 (25%) were positive for *Mycoplasma* species by culture in PPLO broth and agar. The polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR/DGGE) method identified 18 (12 sheep and 6 goats) of the 33 *Mycoplasma* positive samples with mixed *Mycoplasma* population. In particular, 25 Ma (47.2%), 23 Mp (43.4%), 4 Mcc (7.5%), and 1 Mmc (1.9%) were identified. This confirms that the several *Mycoplasma* species rather than the Ma only are in circulation, and are able to cause CA in sheep and goats in Iran. This is the first report on the isolation and identification of Mp, Mmc and Mcc in infected small ruminant flocks in Iran.

Specie di Mycoplasma *responsabili dei casi di agalassia contagiosa dei piccoli ruminanti nel nord-ovest dell'Iran*

Parole chiave
Agalassia contagiosa,
Mycoplasma agalactiae,
Mycoplasma mycoides subsp. *capri,*
Mycoplasma capricolum subsp. *capricolum*,
Mycoplasma putrefaciens,
Pecora,
Capra.

Riassunto

L'agalassia contagiosa (AC) è una malattia seria che colpisce i piccoli ruminanti di molti Paesi e si manifesta solitamente con mastite, artrite, keratocongiuntivite, pleuropolmonite e setticemia. Il *Mycoplasma agalactiae* (Ma) è la principale causa di infezione in pecore e capre; *Mycoplasma mycoides* subsp. *capri* (Mmc, che comprende anche il *M. mycoides* subsp. *mycoides* Large Colony type), *Mycoplasma capricolum* subsp. *capricolum* (Mcc) e *Mycoplasma putrefaciens* (Mp) possono dare forme cliniche simili ma sono di solito associati a infezioni nelle capre. Per questo studio sono stati prelevati campioni da116 pecore e 16 capre, scelte in seguito a epidemie sospette nella provincia di Ardebil in Iran, affette dalla forma acuta della malattia. Latte, liquido lacrimale e liquido sinoviale sono stati raccolti, esclusivamente dagli animali colpiti, per identificare la causa principale della malattia. Dopo la coltura in brodo e agar PPLO, 33 dei 132 campioni raccolti (24,2%) sono risultati positivi a specie di *Mycoplasma*. In 18 (12 pecore e 6 capre) il metodo PCR / DGGE ha rilevato infezioni miste con

il coinvolgimento di più specie di *Mycoplasma*. In totale sono stati identificati 25 Ma (47,2%), 23 Mp (43,4%), 4 Mcc (7,5%) e 1 Mmc (1,9%). Questo è il primo rapporto sull'isolamento e l'identificazione di Mp in pecore infette e di Mmc e Mcc nelle capre in Iran. I dati confermano inoltre che oltre al *Mycoplasma agalactiae* anche altre specie di *Mycoplasma* sono responsabili dell'agalassia contagiosa nelle pecore e capre dell'Iran.

Introduction

Contagious agalactia (CA) has been known for nearly 200 years (Anonymous 2008). The clinical disease was first described by Metaxa in Italy in 1816 and was given the name contagious agalactia by Brusasco in 1871 (Madanat *et al.* 2001). Many countries in the world report CA, but countries in the Middle East and Mediterranean are probably most affected (Kumar *et al.* 2014). The main cause of the disease is *Mycoplasma agalactiae* (Ma), but *Mycoplasma mycoides* subsp. *capri* (Mmc), which now incorporates *M. mycoides* subsp. *mycoides* Large Colony type (Shahram *et al.* 2010, Vilei *et al.* 2006), *Mycoplasma capricolum* subsp. *capricolum* (Mcc), and *Mycoplasma putrefaciens* (Mp) are considered as other infectious agents of this syndrome (Anonymous 2008, Hotzel *et al.* 2003).

Contagious agalactia has previously been included in the List B of diseases by the Office International des Epizooties (OIE) (Anonymous 2008). CA is a severe infectious disease of small ruminants, with Ma being found in sheep and goats, and Mmc, Mcc, and Mp usually detected in goats. Some reports state that goats are more susceptible to this disease than sheep (Maré 2014, Smith and Sherman 2009). CA is most commonly transmitted through oral, respiratory, and mammary routes. The incubation period varies between 1 week and up to 2 months. Clinical signs in infected animals can be acute or in more severe cases can lead to bacteremia and fever with sub-acute or chronic disease. CA originating agents are carried by circulating blood to susceptible organs such as eyes, mammary gland, and joints (Madanat *et al.* 2001). The infection of susceptible flocks can cause high morbidity (30-60%) and mortality. Mortality rates can be as high as 40-70% in lambs and kids, but is generally lower in adults, although it may still be as high as 20% depending on the *Mycoplasma* species. The clinical signs of CA include: fever, mastitis, arthritis, keratoconjunctivitis, pleuropneumonia, and septicemia (MAKePS Syndrome) with lameness and keratoconjunctivitis affecting between 5-10% of infected animals (Anonymous 2008). The organisms can persist for more than 1 year and up to 8 years after clinical recovery in infected animals, which may then serve as a source of infection (Bergonier *et al.* 1997).

According to the latest Food & Agriculture Organization (FAO), Iran has 72.5 million small ruminants (68% sheep and 32% goats) of several breeds that are mostly kept together[1]. This is similar to many other countries in the Middle East (Awan *et al.* 2010). Bory and Entessar first described Ma as a causative agent of CA in Iran (Bory and Entessar 1959). Further studies revealed that Ma is endemic all over the country (Kheirabadi and Ebrahimi 2007, Sotoodehnia and Aerabi 1986). Although an inactivated monovalent vaccine adjuvanted with saponin against Ma is used to try to control the disease (Sotoodehnia *et al.* 2007), CA persists in Iran.

Despite the current vaccination programme in Iran, other CA causing agents have been identified, including Ma, Mcc, Mmc and Mp. This study therefore aimed to determine if other *Mycoplasma* species are involved in the occurrence of the disease.

Materials and methods

Standard strains

Four standard isolates were obtained from Animal and Plant Health Agency (APHA), UK and used as controls: *Mycoplasma agalactiae* NCTC 10123, *Mycoplasma mycoides* subsp. *mycoides* Large Clony (MmmLC) type NCTC 10137, *Mycoplasma capricolum* subsp. *capricolum* NCTC 10154, *Mycoplasma putrefaciens* NCTC 10155.

Sample size and sampling method

Despite frequent reports of CA in Northwest Iran and the high population of small ruminants, no previous studies into the presence of CA have been carried out. Six cities in the Ardebil province located in the Northwest of Iran, which has a moderately cold mountain climate, were selected as the region for investigation. CA is frequently present in this region, and neighbouring countries may present a risk of disease incursion. A total of 18 flocks with clinical signs of CA were identified, and samples were collected from 132 animals (116 sheep, 16 goats)

[1] http://www.fao.org/faostat/en/#country/102.

Table I. *Sample size and location.*

City	Animal species	Total samples			*Mycoplasma* identified positive samples		
		Milk	Lachrymal fluid	Joint fluid	Milk	Lachrymal fluid	Joint fluid
Bileh Savar	Sheep	10	3	3	3 (Ma 2, Mp 1)	0	0
Parsabad	Sheep	12	3	0	2* (Ma 1, Mp 2)	0	0
	Goat	3	0	0	2* (Mcc 2, Mp 2)	0	0
Germi	Sheep	10	6	4	5* (Ma 5, Mp 5)	2 (Ma 2, Mp 2)	1 (Ma 1, Mp 1)
	Goat	4	0	0	3* (Ma 2, Mp 2, Mcc 1)	0	0
Ardebil	Sheep	16	4	1	6* (Ma 5, Mp 6)	0	0
	Goat	5	0	0	2* (Mmc 1, Mcc 1, Ma 1, Mp 1)	0	0
Meshgin Shahr	Sheep	12	4	1	3 (Ma 3)	0	0
	Goat	4	0	0	1 (Ma)	0	0
Khalkhal	Sheep	14	10	3	3 (Ma 2, Mp 1)	0	0
Total		**90**	**30**	**12**	**30**	**2**	**1**

*samples contained more than one *Mycoplasma* species.
Ma = *Mycoplasma agalactiae;* Mp = *Mycoplasma puterfaciens;* Mcc = *Mycoplasma capricolum* subsp. *capricolum;*
Mmc = *Mycoplasma mycoides* subsp. *capri* includes the former *M. mycoides* subsp. *mycoides* large colony trype.

that hadn't received either CA vaccination in the previous year or recent antimicrobial treatment. Samples were collected between October 2010 and November 2012. According to clinical conditions, milk samples were aseptically collected from 90 mastitis cases, lachrymal fluid from 12 animals with weeping eyes, and synovial fluid from 30 cases with arthritis (Table I). Samples were transported to the laboratory for examination (Tola *et al.* 1997) within 24 hours at 2-8°C, following the required safety and biosecurity regulations (WHO 2008).

Isolation and identification procedures

All of the samples were cultured in Pleuropneumonia-Like Organism (PPLO) broth and incubated at 37 °C in a humidified incubator with 5% CO_2 for up to 3 days. Cultures were observed daily and examined for any indications of *Mycoplasma* growth, including turbidity and changes in colour to the culture medium. Those showing signs of growth were used to inoculate fresh PPLO agar plates for up to 2 weeks at 37 °C (Anonymous 2008) and any cultures obtained were subjected to further testing and also freeze-dried for later examination and identification.

The DNA of the cultures was extracted using the DNA extraction kit (Cinnagen, Iran) and stored in micro-tubes at -20°C until they were required as template DNA in the polymerase chain reaction (PCR) tests. Six different PCR's were performed, generic PCR to detect the *Mycoplasma* species. Components for 12-µl reactions contain 6 µl PCR master mix (Ampliquor, Denmark), 0.7 µl of working

Table II. *Sequences of primers.*

Targeted species	Denomination	Sequence (5'-3')	bp size	Reference
General	Myc23F1729	CTAAGGTDAGCGAGWDAACTATAG*	102-110	Hotzel *et al.* 2003
	Myc23R1837	CCCCYCWTSYTTYACTGMGGC		
Mycoides cluster	P1	TATATGGAGTAAAAAGAC	253-265	Hotzel *et al.* 1996
	P2	AATGCATCATAAATAATTG		
Ma	FS1	AAAGGTGCTTGAGAAATGGC	375	Tola *et al.* 1996
	FS2	GTTGCAGAAGAAAGTCCAATCA		
Mp	Mput 1	AAATTGTTGAAAAATTAGCGCGAC	316	Peyraud *et al.* 2003
	Mput 2	CATATCATCAACTAGATTAATAGTAGCACC		
MmmLC**	MMMLC2-L	CAATCCAGATCATAAAAAACCT	1049	Le Grand *et al.* 2004, Hotzel *et al.* 2003, Maigre *et al.* 2008
	MMMLC1-R	CTCCTCATATTCCCCTAGAA		
Mcc	MCCPL1-L	AGACCCAAATAAGCCATCCA	1356	Le Grand *et al.* 2004, Maigre *et al.* 2008
	MCCPL1-R	CTTTCACCGCTTGTTGAATG		

*Degenerate nucleotides: D = A, G, T; W = A, T; Y = C, T; S = G, C; M = A, C.

solution (5 pM/µl) from each flanking primer (Table II), and 2.25 µl of DNA template plus 2.35 µl of molecular-grade PCR water. PCR reactions were run on an Eppendorf PCR system (Eppendorf, Germany) where an initial 2-minute denaturation at 95 °C was followed by 35 cycles of denaturation (93 °C for 45 seconds), annealing (48 °C for 60 seconds), and extension (72 °C for 60 seconds), with a final elongation step of 72 °C for 10 minutes. In addition, more specific PCRs were used to detect Ma and the *M. mycoides* cluster: Mp, Mmc, and Mcc. Details of the PCR methods and primers are provided in Table II. All primers were synthesised by MWG Biotech (Germany). A total of 33 lyophilised samples were sent to the Office International des Epizooties (OIE) Contagious Agalactia Reference Laboratory, Animal and Plant Health Agency (APHA) Weybridge, UK for confirmatory testing by PCR and analysis using denaturing gradient gel electrophoresis (PCR/DGGE). The McAuliffe method (McAuliffe *et al.* 2005) was followed for this process.

Results

The most frequent clinical signs observed among flocks were mastitis, arthritis, and keratoconjunctivitis in descending order. Of the 132 collected samples, 33 (25%) were positive for *Mycoplasma* species by culture in PPLO broth and agar. Twenty six (22.4%) were from sheep samples and 7 (43.8%) from goat samples, The most successful isolation rate (33.3%) was obtained from milk samples.

Using the PCR/DGGE method, 18 of the 33 samples were mixed *Mycoplasma* cultures comprised of isolates from 12 sheep and 6 goats. Of the 53 cultures considered, 25 were identified as Ma (21 from sheep, 4 from goats) (47.2%), 23 as Mp (18 from sheep, 5 from goats) (43.4%), 4 as Mcc from goats (7.5%), and 1 Mmc from a goat (1.9%). These results were also confirmed in Iran by using species-specific PCRs (Tables III and IV, Figures 1 and 2).

Table III. *Specific PCRs to detect the* M. mycoides *cluster.*

Mmc	Ma	Mcc	Mp	PCR Stages
Lid Temp.	C °110	C °110	C ° 110	C ° 110
Initial denaturation	C/ 300 sec °94	C/300 sec °94	C/300 sec °95	C/120 sec °94
Denaturation	C/30 sec °94	C/30 sec °94	C/60 sec °94	C/30 sec °94
Annealing	C/30 sec °64	C/30 sec °49	C/60 sec °55	C/15 sec °48
Extension	C/30 sec °72	C/60 sec °72	C/60 sec °65	C/15 sec °72
Cycles	25	35	35	33
Final extension	C/420 sec °72	C/600 sec °72	C/600 sec °67	C/600 sec °72
Final product (bp)	360	1049	375	1356

Discussion

This study was conducted to determine if *Mycoplasma* species other than Ma can be involved in CA in Iran. Iran is a large country that exceeds 1,648,195 km^2 and has a diverse climate. Traditional food regimes have led Iranian farmers to rear sheep and goats together in common units. Considering the close phylogenetic relationships of these 2 ruminant species and the potential for shared *Mycoplasma* flora that produce the clinical signs of CA, knowledge of the organisms causing this disease is required to improve control measures.

The Ardebil province (17,800 km^2) was selected for this study as it has a large population of 2.5 million sheep and goats. A high morbidity rate caused by CA is common in this region. Annual vaccinations against Ma including an indigenous strain in an inactivated monovalent vaccine adjuvanted with saponin, is in progress. However, CA continues to be reported as an issue across this province and more broadly across Iran.

There are many reports and articles about the isolation and identification of causative agents of CA from around the world. Culture and PCR methods are most commonly used in these studies (Anonymous 2008), although more recently real-time PCR methods for Ma have been described (Oravcová *et al.* 2009). We used conventional PCR to detect *Mycoplasma* species, although this method may be less sensitive than the newer developed real-time PCR methods. In this study, out of 33 positive cultures, a total of 53 different isolates were identified. We isolated 4 Mcc and 1 Mmc from goats, which is similar to that which has been reported in other studies (Al-Momani *et al.* 2006), and 18 Mp from sheep. Al-Momani and colleagues (Al-Momani *et al.* 2006) also found mixed populations of *Mycoplasma* species in sheep and goats in Jordan.

In this study, we targeted animal sites where clinical signs were being observed in order to maximize our chances of detecting and recovering relevant organisms. However, if a more comprehensive sampling regime with multiple sites was used, more isolates may have been obtained. Our findings indicated that the milk was the best and most

Table IV. *Results of PCR tests with specific primers.*

Genus and species of isolates	Sheep	Goat	Total	Percent
Mycoplasma agalactiae	21	4	25	47.2%
Mycoplasma putrefaciens	18	5	23	43.4%
Mycoplasma capricolum subsp. *capricolum*	0	4	4	7.5%
Mycoplasma mycoides subsp. *capri*	0	1	1	1.9%

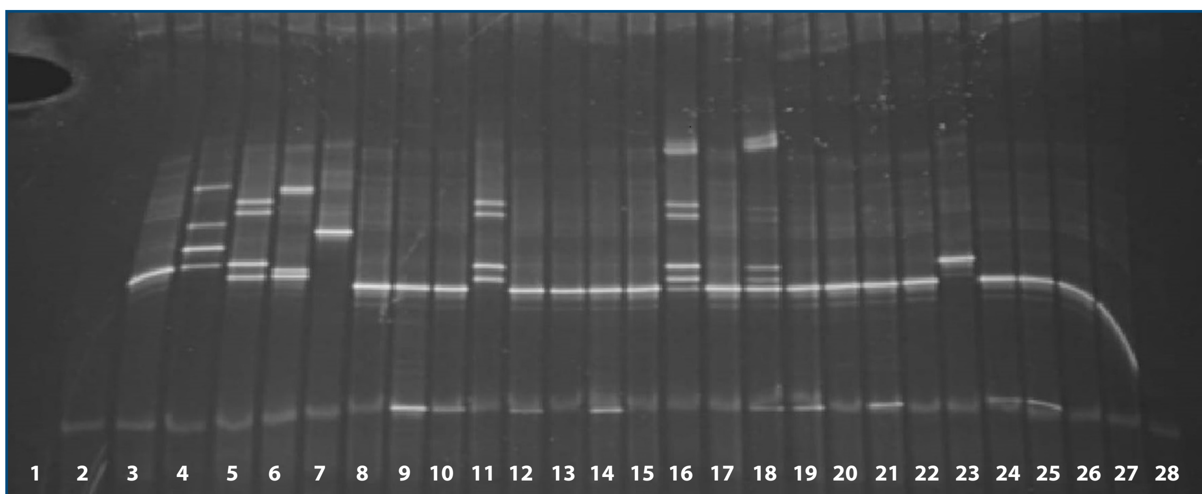

Figure 1. *DGGE method for identifying the* Mycoplasmas *among the samples.* Lane1 = Negative control; lane 2 = Ma control; lane 3 = Mmc; lane 4 = MmmSc control; lane 5 = Mcc control; lane 6 = *M. capripneomonia* control; lane7 = *M. ovipneomonia* control; lanes 8-28 = our samples.

successful sample, which accounted for 90% (30 out of 33) of the positive results. Future studies may therefore consider to limit the sampling to milk samples. Bacteriological culturing of *Mycoplasma* species requires specialist media and laboratory skills, and is complicated by the potential overgrowth by other bacterial species, it can also select for the more rapid growing *Mycoplasma* species. The use of molecular tests directly on samples, rather than on the culture selection may also increase the detection rate and show a higher prevalence of CA than detected in this study. The PCR/DGGE method is useful as it is sensitive and detects mixed infections and identifies the *Mycoplasma* species within a single test, but the equipment and controls that are required with this method mean that the test is not available in many laboratories. In contrast, standard PCR tests can now be performed in many laboratories, but the tests are usually species-specific and multiple tests are required to detect the organisms that are being targeted. The isolation of Mp from both goats and sheep demonstrated that Mp was the second most commonly isolated organism for CA in Iran, and is therefore a major contributor to CA in both sheep and goats. In this study no Mcc or Mmc was detected in sheep.

This is the first report on the isolation and identification of Mp, Mmc and Mcc in small ruminant infected flocks in the Ardebil Province of Iran. Working from the samples collected for this study, the identification of all the causative agents of contagious agalactia (Ma, Mp, Mcc, and Mmc) by PCR and PCR/DGGE indicates and confirms the presence of these agents in small ruminants. This study relied on the culture and identification of *Mycoplasma* species, which gave 33 positive results out of a total of 132 samples collected. Had molecular methods been used instead of, or in addition to, culture methods, more positive results may have been obtained. These findings suggest that new approaches toward controlling CA are of great importance. Currently, vaccination with a monovalent inactivated vaccine (Ma) is performed in Iran. Considering the multiple *Mycoplasma* species isolated here, a polyvalent vaccine could be developed as it potentially may offer a better protection.

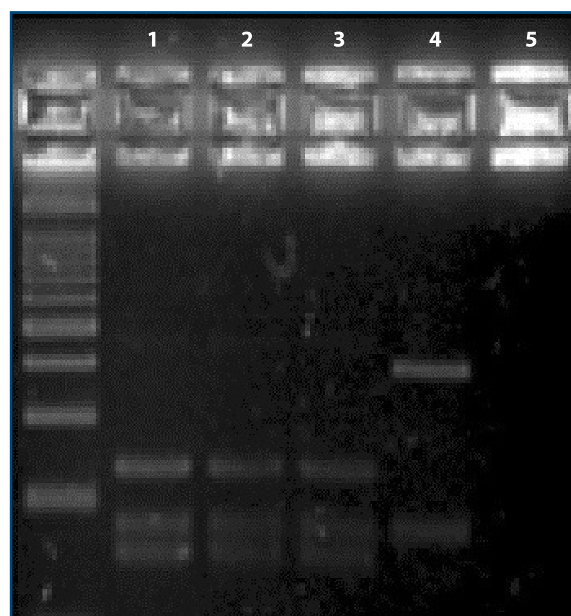

Figure 2. *PCR products with specific primers, amplified from* Mycoplasma mycoides *sub.* capri. Lane1 = positive sample; lane 2, 3 = Mmc control; lane 4 = MmmSC control; lane 5 = negative control.

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