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

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Keywords
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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 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 together1. 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 (Mcc), and Mycoplasma putrefaciens (Mp) are considered as other infectious agents of this syndrome (Anonymous 2008, Hotzel et al. 2003).

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)

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% CO₂ 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 I. Sample size and location.

<table>
<thead>
<tr>
<th>City</th>
<th>Animal species</th>
<th>Total samples</th>
<th>Milk</th>
<th>Lachrymal fluid</th>
<th>Joint fluid</th>
<th>Mycoplasma identified positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bileh Savar</td>
<td>Sheep</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2’ (Ma 1, Mp 2)</td>
</tr>
<tr>
<td>Parsabad</td>
<td>Sheep</td>
<td>16</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>6’ (Ma 5, Mp 6)</td>
</tr>
<tr>
<td>Germi</td>
<td>Sheep</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2’ (Mcc 1, Ma 1, Mp 1)</td>
</tr>
<tr>
<td>Germi</td>
<td>Goat</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>3’ (Ma 2, Mcc 1)</td>
</tr>
<tr>
<td>Germi</td>
<td>Goat</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>2’ (Mcc 1, Ma 1, Mcc 1)</td>
<td></td>
</tr>
<tr>
<td>Ardebil</td>
<td>Sheep</td>
<td>12</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1 (Ma)</td>
</tr>
<tr>
<td>Meshghin Shahr</td>
<td>Sheep</td>
<td>14</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Khalkhal</td>
<td>Sheep</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>90</td>
<td>30</td>
<td>12</td>
<td>30</td>
<td>2</td>
</tr>
</tbody>
</table>

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

Table II. Sequences of primers.

<table>
<thead>
<tr>
<th>Targeted species</th>
<th>Denomination</th>
<th>Sequence (5’-3’)</th>
<th>bp size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>General</td>
<td>Myc23F1729</td>
<td>CTAAGGTGGGAGAGGDAACTATAG*</td>
<td>102-110</td>
<td>Hotzel et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Myc23R1837</td>
<td>CCCYCWYSYTGACTGMGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycoides cluster</td>
<td>P1</td>
<td>TATATGGTAGAAAAAGAC</td>
<td>253-265</td>
<td>Hotzel et al. 1996</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>AATGCATCAAATAATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ma</td>
<td>FS1</td>
<td>AAGGTTGCTTGGAAATGGC</td>
<td>375</td>
<td>Tola et al. 1996</td>
</tr>
<tr>
<td></td>
<td>FS2</td>
<td>GGTGCAGAAGAAAGTCACTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp</td>
<td>Mput 1</td>
<td>AAATTGTTGAAAAATTAGCGC</td>
<td>316</td>
<td>Peyraud et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Mput 2</td>
<td>CATACATCAACTAGATTAATGAGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MmmLC**</td>
<td>MMMLC2-L</td>
<td>CAATCAGATCAAAAATCCT</td>
<td>1049</td>
<td>Le Grand et al. 2004, Hotzel et al. 2003, Maigre et al. 2008</td>
</tr>
<tr>
<td></td>
<td>MMMLC1-R</td>
<td>CTTCTTATCCCTGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCCPL1-R</td>
<td>CTTCACCGCTTGGAAATG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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 45 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).

**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 km² 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 km²) 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

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

<table>
<thead>
<tr>
<th>Lid Temp.</th>
<th>Mmc</th>
<th>Ma</th>
<th>Mcc</th>
<th>PCR Stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>C °110</td>
<td>C °110</td>
<td>C °110</td>
<td>C °110</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Genus and species of isolates</th>
<th>Sheep</th>
<th>Goat</th>
<th>Total</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mycoplasma agalactiae</em></td>
<td>21</td>
<td>4</td>
<td>25</td>
<td>47.2%</td>
</tr>
<tr>
<td><em>Mycoplasma putrefaciens</em></td>
<td>18</td>
<td>5</td>
<td>23</td>
<td>43.4%</td>
</tr>
<tr>
<td><em>Mycoplasma capricolum</em></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>7.5%</td>
</tr>
<tr>
<td><em>Mycoplasma mycoides</em> subsp. capri</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1.9%</td>
</tr>
</tbody>
</table>

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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.

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References


