Isolation and identification of *Mycoplasma mycoides* cluster strains from goats in Chongqing, China

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Received: November 21, 2013  Accepted: February 21, 2014

Abstract

In order to evaluate the prevalence of the *Mycoplasma mycoides* cluster in goats in Chongqing, China, an epidemiological survey in this area was carried out. A total of 68 samples were subjected to bacteria isolation on Hartley’s medium. Four isolates (three from lung tissue and one from nasal discharges) were recovered from the samples and identified as the *Mycoplasma* species by their morphological and biochemical characteristics. They were further confirmed by PCR using 16S rRNA specific primer pairs and by restriction enzyme analysis. *In vitro* antimicrobial susceptibility of the isolates indicated that some strains had developed resistance to the antibiotics tested. This is the first report on the isolation, identification, and molecular characterisation of *Mycoplasma* species isolated from goats in Chongqing. This study also revealed a prevalence of *Mycoplasma* species infection in goats in this area.

Key words: goat, *Mycoplasma mycoides* cluster, isolation, biochemical characterisation, antimicrobial resistance.

Introduction

The *Mycoplasma mycoides* cluster consists of five closely related *Mycoplasmas*, which share many genotypic and phenotypic traits. The cluster includes *M. mycoides* subsp. *Mycoides* Small Colony (MmmSC), *Mycoplasma leachii* (MI), *M. mycoides* subsp. *Capri* (Mmc), *M. capricolum subsp. capricolum* (Mcc), and *M. capricolum subsp. caprineumoniae* (Mccp). According to previous data and phylogenetic analyses, *Mycoplasma leachii*, which was previously called *Mycoplasma* sp. bovine group 7 and revealed a close relatedness either to *M. mycoides* or to *M. capricolum* (9), has had to be designated a new separate species. This pathogen causes cattle diseases (19). Another *M. mycoides* cluster member, Mmc, was considered as two subspecies previously, *M. mycoides* subsp. *Mycoides* Large Colony and *M. mycoides* subsp. *Capri*. When they were regarded as two subspecies, numerical analysis of one-dimensional sodium dodecyl sulphate-polyyacrylamide gel electrophoresis (SDS-PAGE) protein patterns (6, 16), two-dimensional PAGE protein patterns (21, 22), and serological analysis (20) showed them to be very similar antigenically. They have consequently now been grouped into a single subspecies (19, 28).

Both MmmSC and *M. leachii* are pathogenic for cattle. MmmSC is responsible for contagious bovine pleuropneumonia (CBPP), which is considered to be the most important threat to cattle farming in affected countries, and one which impacts farmers directly through mortality and poor productivity of their animals. *M. leachii* is associated with polyarthritis in calves (12, 23, 24) and with mastitis (6), pneumonia (2), and abortion (12) in cows. Whereas, three *M. mycoides* cluster members, Mccp, Mmc, and Mcc, are the caprine pathogens. The most important and prevalent pathogen is Mccp, the aetiologic agent of contagious caprine pleuropneumonia (CCPP), which is a significant disease of goats in Africa, the Middle East, and Western Asia, and causes mortality of up to 80%. Mmc and Mcc are reported to cause a pattern of disease called contagious agalactia syndrome (CA) including mastitis, arthritis, pulmonary diseases, and sepsicaemia especially in goats (25, 26, 29). All three caprine pathogens can be identified and distinguished by both PCR and biochemical characterisation of their different nucleotide sequences and biochemical activity (14, 15, 19).
The aim of this study was to isolate the pathogens from dead goats and identify them by PCR, restriction enzyme digestion, and biochemical identification, as well as to test the susceptibility of the pathogens to antimicrobials.

**Material and Methods**

**Sample collection.** A total of 68 samples from dead goats were collected from five different farms in Chongqing municipality, China (Table 1). Forty-five goats were less than one year old, 22 goats were between one and two years, and only one goat was two years of age. Thirty-five were male and 33 were female. Before death, the goats had exhibited clinical signs including mild fever, laboured breathing, coughing, and nasal drops. The nasal discharge was mucous and serous in the early stage, and then became purulent and rust-coloured in the later stage. When the thorax wall was pressed, the goat demonstrated a pain reaction. Some goats showed swollen eyelids and lacrimation with slime and purulent exudate. The nasal discharge was mucous and purulent and rust coloured in the later stage. When the thorax wall was pressed, the goat demonstrated a pain reaction. Some goats showed swollen eyelids and lacrimation with slime and purulent exudate. The major pathological changes were limited to the thorax. There was some pale yellow liquid in the pericardium. Yellowish-white fibrinous exudate was adhered to the pleura, pericardium, and lungs. Some of the lung lobes displayed hepatisation. The pulmonary lesions were surrounded by connective tissue with necrosis, and on some areas of lung surface abscesses with black pus were observed. Some grey needle-like focal necrosis appeared in the surface of the swollen liver.

![Table 1. The samples and isolates taken in Chongqing Municipality](image)

**Mycoplasma isolation.** The following samples were taken: nasal discharges (six), lungs (55), liver (one), spleen (one), kidneys (one), lymph nodes (one), pleural fluid (two), and blood (one). The samples were placed directly in Hartley’s medium and kept at 37°C in a 5% humid CO₂ incubator for 6-10 d until the colour became orange-yellow. Positive cultures were passaged twice until the cultured media became orange during about 4-6 d, passed through 0.45 μm filter, and then cultured in Hartley’s medium again. When the media became orange, the inocula were screened through a 0.22 μm filter again. The filtered inocula were cultured on a Hartley’s plate at 37°C in a humid 5% CO₂ incubator. All the plates were examined daily with a 4 × microscope because the colonies were too small to be seen clearly with the naked eye. If growth was sparse (1 to 10 colonies per plate), the plates were flooded with 1.5 mL of sterile broth and reincubated for an additional 4-6 d to increase the number of colonies.

**DNA extraction and 16S rRNA amplification.** Genomic DNA of the isolates was extracted from 1 mL of the late exponential growth phase by using a Bacteria DNA Extract Kit (Tiangen, China). The purity and concentration of DNA was checked on 1% agarose gel.

Specific primers were designed according to the *Mycoplasma mycoides* cluster 16S rRNA to amplify a 548 bp expected fragment (4). The upstream primer MmF was 5'CGAAAGCGGCTTACTGGCTTGTTG-3' and the downstream primer MmR was 5'TTGAGATTAGCTCCCTTCACAG-3'. The amplification was carried out using the following programme: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 40 s, and a final extension step at 72°C for 6 min, in a Biometra T1 Thermocycler (Biometra). Mmc type strain PG3 was used as the positive control. PCR amplified fragments were analysed on a 1.5% agarose gel, stained with ethidium bromide, and visualised under UV illumination.

**Analysis of the amplified isolated strains.** The amplified isolated strains were analysed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). PCR products were recycled with a purification kit (Promega). Each amplified product was digested with restriction endonuclease Pst I for 6 h in a final volume of 20 μL. The resulting DNA fragments were analysed by electrophoresis in 2% agarose gel.

**Biochemical characterisation.** Biochemical characterisation of the isolates and PG3 strain was performed as described by Al-Aubaidi et al. (3). The assays included metabolism of glucose, maltose, lactose, sucrose, fructose, sorbitol, mannitol, decarboxylation of arginine, urease activity, formation of film and spots, fermentation, and reduction of tetrazolium chloride and methylene blue.

**Standardisation of inocula.** A panel of 20 Eppendorf tubes each containing 0.9 mL of glucose-Hayflick's broth (pH 7.6) was prepared for each isolate, and 0.1 mL of the isolates was added to the first tube. A 10-fold dilution was made up to the 20th tube by transferring 0.1 mL of the suspension at each step. The
tubes were incubated at 37°C until an acidic reaction (colour change from pink to yellow) was observed. The time of this reaction was recorded, and the lowest dilution showing a colour change represented the reciprocal of the number of colour changing units (ccu) in the undiluted Mycoplasma culture. The inoculum size was determined to be 10³ ccu/mL for all strains tested. The acceptable number of organisms for minimum inhibitory concentration (MIC) tests was 10³ to 10⁴ ccu/mL (13).

**Determination of minimum inhibitory concentrations (MIC).** The MIC assay was done as described by Kidanemariama et al. (13) with minor modification. The tested antibiotics included: florfenicol, tylosin, ceftiofur, sulphamonomethoxine, erythromycin, lomefloxacin, and floraxcin. The MICs were determined by a glucose metabolism inhibition method performed in Eppendorf tubes. Two-fold dilutions of each antibiotic were made. To each tube 0.1 mL of diluted culture containing 10⁴ ccu/mL was added. The tubes were sealed with transparent self-adhesive tape to prevent evaporation, and then incubated at 37°C. The incubation time was controlled by observing the colour changes equivalent to the growth control well, and the tubes were monitored twice daily until the required colour change was observed. The MIC was recorded as the lowest concentration of antibiotic that inhibited visible colour change of the medium at the time when a colour change could be observed in the growth control without antibiotic. MICs were obtained after 24 to 48 h depending on the strains tested. All MICs were determined three times to confirm results.

**Results**

**Prevalence of Mycoplasma mycoides cluster-positive samples.** Sixty-eight clinical samples were cultured in Hartley’s medium, and the Mycoplasma organisms were screened by changes in the media colour indicating the Mycoplasma growth. After following the protocol of 2-3 reversal and forward passages, the possibility of an “L-phase variant” was excluded. Only four (5.9%) samples yielded colonies of 1 to 2 mm size exhibiting the typical fried egg appearance on Hartley’s solid medium. The results are listed in Table 1. The growth characteristics of the bacteria were indicative of the Mycoplasmas.

Of these four isolates, one was obtained from Farm 001, Yubei County, and the other three were obtained from Farm 002, Beibei County. However, no isolate was recovered from the remaining farms. Positive results were obtained in two out of six (33%) nasal discharge samples tested, and in two out of 55 (3.6%) lung samples. No strain was isolated from the liver, spleen, kidney, lymph node, pleural fluid, or blood.

To further confirm the presence of the cluster specifically, the PCR method was used to amplify the 16S rRNA gene of the Mycoplasma mycoides cluster. Specific primers, MmF/MmR, were used to differentiate the M. mycoides cluster members from non-cluster Mycoplasma. All four isolates of Mycoplasma yielded amplified products of 548 bp (Fig. 1). The M. mycoides cluster isolates were further classified by PstI digestion. All four isolates' fragments could be digested and generate two bands (data not shown). These are fragments of 420 bp and 128 bp deriving from the digestion of 548 amplicon with PstI. This indicates their belonging to Mmc and/or Mcc.

**Fig. 1.** PCR amplification products. M. D2000 DNA marker (Tiangen, China); 1. PG3; 2. BB3; 3. BB7; 4. BB42; 5. YB2; 6. E. coli DH5α

**Biochemical characterisation of mycoplasmal isolates.** All four isolates initially identified as Mycoplasmas on the basis of their morphological and colony characteristics were subjected to various biochemical tests (Table 2).

The strain PG3 and all isolates grew fast (the media became orange after 3-4 d), reduced methylene blue, hydrolysed glucose, maltose, lactose, sucrose, fructose, sorbitol and mannitol, did not use urease, and did not form film and spots. Therefore, Mccp could be excluded, because Mccp grew slowly and could not use maltose and mannose, neither did the PCR-RFLP result support the evidence of Mccp.

The strain PG3 and isolates BB3, BB7, and BB42 did not catabolise arginine, whereas strain YB2, isolated from Yubei, utilised it. According to the molecular clues and biochemical results, BB3, BB7 and BB42 could be preliminarily identified as Mycoplasma mycoides subsp. capri (Mmc), and YB2 could be identified as Mycoplasma capricolum subsp. capricolum (Mcc). Interestingly, the YB2 strain did not reduce tetrazolium chloride.

**Susceptibility of Mycoplasma mycoides cluster isolates to antibiotics.** The MIC of the antibiotics to which the field isolates were susceptible is shown in Table 3. Triplicate tests did not vary by more than one serial two-fold dilution. All isolates showed the MIC value ≤1.2 μg/mL for ceftiofur, but ≥6.7 for ofloxacin. The MIC of lomefloxacin ranged from 3.3 to 6.7 for the four isolates and this indicated that the wild type strains were resistant to lomefloxacin. The MIC values of tylosin
and sulphamonomethoxine were low in the case of BB3 and BB7 and high as regards BB42 and YB2, while the MIC values of florfenicol and erythromycin were low concerning BB3, BB7 and BB42 and high regarding YB2. Moreover, BB3 was susceptible to all tested antibiotics except ofloxacin, and YB2 was resistant to all tested antibiotics except ceftriaxone.

Table 2. Biochemical characteristics of the examined strains

<table>
<thead>
<tr>
<th>Biological reagents</th>
<th>PG3</th>
<th>BB3</th>
<th>BB7</th>
<th>BB42</th>
<th>YB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene blue</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Lactose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Fructose</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Urease activity</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Tetrazolium chloride</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Film and spots</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

+: Positive; -: Negative

Discussion

*Mycoplasma mycoides* cluster pathogens are important aetiological agents and they are reported to cause a pattern of diseases. There were only a few reports related to ruminant mycoplasmal pathogens in China, especially the caprine pathogens (17, 18, 27). From 68 tissue and fluid samples, a total of four *Mycoplasma* isolates were obtained in our studies. Three of these isolates were *M. mycoides* subsp. *capri* and one was *M. capricolum* subsp. *Capricolum*. They were identified by various biochemical tests and the PCR-RFLP method. This indicates and confirms the presence of *Mycoplasma* infection in Chongqing area. Moreover, the isolation rate (5.9%) was found to be in agreement with Kumar et al. (14, 15); however, our isolation rate seems to be quite low in contrast to the 25% - 71% obtained by Gil et al. (10).

The isolated strain cannot belong to MmmSC and *M. leachii* because both of these cause disease in cattle, whereas the strains were isolated from sick goats. The amplification of *Mycoplasma mycoides* by cluster-specific PCR was achieved in all isolates, yielding 548 bp amplicon. This PCR was specific to the 16S rRNA region of the *Mycoplasma mycoides* cluster (4).

However, none of the species-unspecific PCR product was amplified (Figure 1) except *Mycoplasma capricolum* cluster-specific strains. Thus, the presence of any other species (*M. putrefaciens, M. agalactiae*) was excluded, although they are also known to be associated with similar clinical signs. This finding was in agreement with that of Bascuñana et al. (4) and Kumar et al. (14), who also designed the primers to amplify the 16S rRNA fragment specifically.

Digestion of the 548 bp amplicons obtained from all four *Mycoplasma* isolates with the enzyme *Pst I* resulted in two fragments only: 420 bp and 128 bp, which is the characteristic pattern of *M. mycoides* subsp. *Capri* and *M. capricolum* subsp. *capricolum*. This finding was in agreement with Böliske et al. (5) and Hernandez et al. (11). The PCR-RFLP result confirmed that the isolates are Mmc and/or Mcc, but not *M. capricolum* subsp. *Caprineumoniae*, because in the case of *Caprineumoniae* the 548 bp amplicon of Mccp should be digested into three fragments, 548 bp, 420 bp, and 128 bp.

The isolates BB3, BB7, and BB42, did not catalyse arginine, did not form a film and spot, reduced tetrazolium, and hydrolysed some carbohydrates. These isolates were preliminarily identified as *Mycoplasma mycoides* subsp. *capri*. These results agree with Damassa et al. (8) and Kumar et al. (14), who reported that isolates of *Mycoplasma* were characterised biochemically as *M. mycoides* subsp. *capri* on the basis of fermentation of glucose, reduction of tetrazolium, and lack of arginine catabolism. The fourth isolate, YB2, was identified as *Mycoplasma capricolum* subsp. *capricolum* based on its biochemical activity. YB2 hydrolysed several carbohydrates, did not form a film and spot, and catalysed arginine. These results are in agreement with the findings of Adehan et al. (1), Damassa et al. (8), and Kumar et al. (14). Surprisingly, this isolate did not reduce tetrazolium.

The aim of quantitative studies of antimicrobial sensitivity is to indicate the most effective antimicrobial to control the infection. *In vitro* antimicrobial activity does not always correlate with the *in vivo* efficacy. Sometimes, an antibiotic showing little or no activity *in vitro* is effective *in vivo* by enhancement of the body’s defences to eliminate the infection (13). Although the MICs of most antibiotics were not very large and the antibiotics might be effective for the treatment of diseases, some isolates proved to be more resistant to several of them. For example, YB2 strain was much more resistant than other isolates to all tested antibiotics except ceftriaxone. This result was not surprising in light of the broad indiscriminate use of antibiotics in animal husbandry in China. However, the susceptibility test of *Mycoplasma mycoides* cluster isolates to antibiotics can guide the selection of medication and alert veterinarians to the increasing antimicrobial resistance of *Mycoplasma*.

In conclusion, our present study demonstrated that *Mycoplasma mycoides* cluster infection occurs in goats.
in Chongqing. PCR is a rapid and simple method of detection and identification of the *Mycoplasma mycoides* cluster. RFLP and biochemical characterisation are effective methods for the identification of *Mycoplasma* species. Drug resistance should also be considered.

Acknowledgements: This work was supported by the grants from the Rural Areas’ Scientific and Technological National Project during the 12th five year plan period, the Key Technologies R & D Program of Chongqing, and the Major State Basic Research Development Program of China (973 program).

We thank Miss Yuanxiao Li for the review of the manuscript’s grammar.

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