Isolation, identification and molecular characterization of *Mycoplasma* isolates from goats of Gujarat State, India

Pankaj Kumar¹, Ashish Roy¹, Bharat B. Bhanderi*, and Bhik C. Pal²

¹Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, Anand Agricultural University, Gujarat, India
²Department of Veterinary Epidemiology, Veterinary University, Mathura (U. P.), India

**ABSTRACT**

A total of 358 samples [nasal discharges (215), lung tissue (6), pleural fluid (4), joint fluid (5), milk (60), preputial swabs (22), conjunctival swabs (16), ear swabs (5) and vaginal discharges (25)] were subjected for cultural isolation on MBHS-L and MBHS-A media. Of these, a total of 30 isolates (8.35%) of *Mycoplasma* species, (20 from nasal discharges, 4 from lung tissue, 2 from pleural fluid, 3 from milk and 1 from joint fluid) were isolated and characterized biochemically and identified as *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* by PCR and PCR-RFLP. The isolates were identified as the *Mycoplasma* species by their cultural, morphological, biochemical characteristics and this was further confirmed by PCR and PCR-RFLP using different cluster, group and species specific primer pairs and by restriction enzyme respectively. This is a rare report on the isolation, identification and molecular characterization of goat *Mycoplasma* species in Western India. These studies have revealed a high prevalence of *Mycoplasma* species infection in Western India.

**Key words:** India, mycoplasmas, goats, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*

**Introduction**

Mycoplasmas are the smallest fastidious bacteria, which can cause diseases in major species of animals, including humans. In small ruminants, they are known for respiratory disease, arthritis, eye lesions, genital disease and mastitis (Nicholas, 2002; Sharif and Muhammad, 2009). Most of the members of the *Mycoplasma mycoides* cluster group are important pathogens for small ruminants. This group comprises six species and subspecies. Some of these *Mycoplasma* species can cause severe and contagious diseases in goats, with significant economic impact. Of the many *Mycoplasma* diseases, contagious
caprine pleuropneumonia (CCPP) is a highly fatal disease that occurs in Eastern Europe, the Middle East, Africa and Asia (KOPCHA, 2005).

The M. mycoides cluster members are closely interrelated both serologically as well as genetically and include: M. mycoides subsp. mycoides small colony type (MmmSC), M. mycoides subsp. capri (Mmc), M. capricolum subsp. capricolum (Mcc), M. capricolum subsp. capripneumoniae (Mccp) and M. leachii (MANSO-SILVÁN et al., 2009). Apart from MmmSC and Mccp, the etiological agents of CBPP and CCPP respectively, and M. leachii which causes bovine arthritis and mastitis, other members have been implicated in the MAKePS syndrome (Mastitis, Arthritis, Keratoconjunctivitis, Pneumonia, Septicaemia) along with M. agalactiae and M. putrifaciens in goats (THIAUCOURT and BOLSKE, 1996).

Looking at the paucity of the literature in India, the present work was carried out to isolate, identify, and undertake molecular characterization of Mycoplasma from caprine infection in Gujarat State, India.

**Materials and methods**

*Sample collection.* During this study, all 358 samples (comprising nasal discharges (215), lung tissue (6), pleural fluid (4), joints fluid (5), milk (60), preputial swabs (22), conjunctival swabs (16), ear swabs (5) and vaginal discharges (25)) were collected aseptically on Modified Hank’s Balanced Salt Solution Liquid Media (MBHS-L) and transported to the laboratory in a cold chain.

*Processing of samples.* Samples were placed directly in MBHS-L medium and kept at 37 °C for 1 hour and then cultured by inoculating 0.2 mL amounts of sample into 3 mL of fresh MBHS-L media. The inoculated liquid medium was incubated at 37 °C up to 10 days and was observed daily for signs of growth. Positive cultures were kept at 4 °C and those with no signs of growth were discarded after 10 days of incubation.

The samples that were positive for growth in MBHS-L broth were inoculated into Modified Hank’s Balanced Salt Solution Agar Media (MBHS-A) and incubated anaerobically at 37 °C for up to 10 days under humid conditions in 5-10% CO₂ tension to obtain optimum growth. All the plates were examined daily with a conventional microscope at 4× magnification and growth and colony morphology were recorded. If growth was sparse (1 to 10 colonies per plate) the plates were flooded with 1.5 mL sterile broth and reincubated for an additional 48 to 72 hours to increase the number of colonies. If no growth occurred after 10 days of incubation, then the plates were discarded.

*Purification (cloning) of primary culture.* Pure stock cultures were prepared by transferring a small agar block containing a single colony of *Mycoplasma* to MBHS-L broth and incubated at 37 °C for 3-5 days and stored at 4 °C. The subculturing of each isolate from the liquid broth culture was done every 30th day.

P. Kumar et al.: Molecular characterization of *Mycoplasma*
Examination of the isolated colonies. To study the morphology of the suspected colonies of Mycoplasma, they were stained by Diene’s method, Giemsa’s and acridine orange stain as described by GUPTA (2005).

Characterization of Mycoplasma. To determine the biochemical activity of Mycoplasma isolates, tests such as catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion, digitonin sensitivity test and film and spot formation were carried out as per the technique adopted by GUPTA (2005).

Molecular characterization. DNA extraction. The template DNA from the broth culture was prepared according to the method of JOHANSSON et al. (1998) with slight modifications. About 1 mL of the broth culture of each isolate was centrifuged at 12000 rpm in a microcentrifuge at 4 °C for 10 minutes and the pellet washed in 0.5 mL of PBS twice. The pellets were resuspended in 100 μL of 0.3X TE and treated with proteinase K (Genei, Cat no. 105973) at 60 °C for 30 minutes and finally kept at 97 °C for 10 minute. Cell debris was removed by centrifugation and 5 μL of the supernatant was used as a DNA template in the PCR reaction mixture.

Identification of Mycoplasma mycoides ‘Cluster’. PCR was performed using the M. mycoides cluster specific primers MC 323 (TAG AGG TAC TTT AGA TAC TCA AGG) and MC 358 (GAT ATC TAA AGG TGA TGG T) for an expected amplified product of 1.5 kb long sequence homologous to the CAP-21 probe of the M. mycoides cluster members (BASHIRUDDIN et al., 1994). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation, annealing and extension (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

Similarly PCR was performed using the M. mycoides cluster specific primers CA (F) (CGA AAG CGG CTT ACT GCC TTT TTG T) and CA (R) (TTG AGA TTA GCT CCC CTT CAC AG) for an expected amplified product of 548 bp long sequence of the 16S rRNA gene of the the M. mycoides cluster members (BOLSKE et al., 1996). The amplification was carried out using the following programme: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation, annealing and extension (95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 10 min followed by 10 °C hold at infinity.

Identification of mycoides group members. Mycoides group specific PCR was performed using the primers MM 450 (GTA TTT TCC TTT CTA ATT TG) and MM 451 (AAA TCA AAT TAA TAA GTT TG) for an expected amplified product of 574 bp of the CAP-21 sequence of the 16S rRNA gene of the Mycoides group members MmmSC, Mmc and MmmLC (BASHIRUDDIN et al., 1994). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of
denaturation, annealing and extension (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

Identification of Mycoplasma isolates up to species level. PCR using the Mycoplasma capricolum subsp. capricolum specific primers P4 (ACT GAG CAA TTC CTC TT) and P8 (GTAAAC GTG GTA TAT CAA AT) for an expected amplified product of 192 bp long sequence of the 16S rRNA gene of the Mcc (HERNANDEZ et al., 2006). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation, annealing and extension (94 °C for 30 sec, 56 °C for 1 min, 72 °C for 1.5 min), and final extension at 72 °C for 5 min followed by 10 °C hold at infinity.

PCR using the Mycoplasma mycoides subsp. capri specific primers P4 (ACT GAG CAATTC CTC TT) and P6 (TTAATAAGT CTC TAT ATG AAT) for an expected amplified product 194 bp long sequence of the 16S rRNA gene of the the Mmc (HERNANDEZ et al., 2006). The amplification was carried out using the following programme: initial denaturation at 94 °C for 1 min followed by 30 cycles of denaturation, annealing and extension (94 °C for 1 min, 46 °C for 1 min, 72 °C for 2 min), and final extension at 72 °C for 5 min followed by hold at infinity.

PCR using Mycoplasma agalactiae specific primers [Mga F (CCT TTT AGA TTG GGA TAG CGG ATG / MgaR (CGG TCA AGG TAG CGT CAT TTC CTA C)] and M. putrefaciens specific primers [SSF1(GCG GCA TGC CTA ATA CAT GC ) / SSR1 (AGC TGC GGC GCT GAT GAG TTT A)] for an expected amplified product of 360 bp (DE AZEVEDO et al., 2006) and 540 bp (SHANKSTER et al., 2002) long amplified product of the 16S rRNA gene of the respectively.

To confirm the targeted PCR amplification, 5 μL of PCR product from each tube was mixed with 1 μL of 6X gel loading buffer from each tube and electrophoresed on 1.5 % agarose gel along with 100 bp DNA Ladder (GeneRuler- Fermentas) and stained with ethidium bromide (1 % solution at the rate of 5 μL/100 mL) at a constant 80 V for 30 minutes in 0.5X TBE buffer. The amplified product was visualized as a single compact band of the expected size under UV light and documented by the gel documentation system (Syn Gene, Gene genius bioImaging System, UK).

PCR-RFLP analysis. Restriction digestion of the PCR products of 16S rRNA gene was carried out to confirm the identity of PCR products by using the restriction enzymes PstI (BOLSKE et al., 1996) and MboII. The restriction enzymes were selected from a restriction map created using the sequences available in GenBank and NEBcutter V2.0 software. A reaction mixture was prepared in a 200 μL capacity PCR tube for 16S rRNA gene using MboII and incubated in a water bath or heat block for 1 hour at 37 °C and inactivated the MboII by incubation at 65 °C for 20 minute. For Pst I (Fast Digest) reaction mixture was
All the 30 isolates obtained were subjected to various biochemical tests. Characteristics as well as digitonin sensitivity tests, primarily identifying characteristics and on cultural isolation and on preliminary screening by morphological and colony methods. The light blue periphery of the suspected colonies of Mycoplasma were stained by Diene’s and Acridine orange method. The typical fried egg appearance of colonies of Mycoplasmas was observed on solid media.

Results

An outbreak of respiratory disease occurred in a herd of 1000 goats in the State of Gujarat, India, in March, 2009. The first cases were seen in March, but the number of affected animals peaked during April. Initially, 300 animals died over a period of 10 days and, after 1 month, the flock mortality had reached 80%. Animals of all ages were affected, although they were mostly adults. Kids usually died in less than 1 week after the onset of clinical signs, whereas adults survived longer, up to 2 weeks.

The clinical picture was characterized by high fever, bilateral nasal discharge, difficult breathing, conjunctivitis, prostration, ear drop, and very low milk production. Arthritis was observed in some animals. Six animals on necropsy showed grey, red areas of consolidation in the lungs, affecting more than one lobe and animals showed marked pleuritis and pleural effusion. The pleural fluid was thick and yellowish. The cut surface of some affected lungs revealed a fine granular texture with hepatization.

Isolation. Modified Hank’s Balanced Salt Solution Liquid (MBHS-L) medium and Modified Hank’s Balanced Salt Solution Agar (MBHS-A) medium was used as a primary broth and solid medium respectively for isolation of Mycoplasma organisms. The typical yellow orange colonies with a deep blue central growth under a low power microscope after the dienes staining procedure, while Mycoplasma colonies stained with acridine orange showed a light orange periphery and deep orange central growth under a low power microscope.

Digitonin sensitivity. To specifically identify and differentiate isolates of Mycoplasma from Acholeplasma, digitonin sensitivity test was done. All the isolates were found to be sensitive for digitonin, revealing a zone of inhibition of more than 2 mm, thus indicating that they belong to the Mycoplasma species.

Biochemical characterization of Mycoplasmal isolates. All the 30 isolates obtained on cultural isolation and on preliminary screening by morphological and colony characteristics as well as digitonin sensitivity tests, primarily identified as Mycoplasmas, were subjected to various biochemical tests.
### Table 1. Overall biochemical reactions of goat Mycoplasma isolates

<table>
<thead>
<tr>
<th>Groups</th>
<th>Strains examined</th>
<th>Glucose hydrolysis</th>
<th>Arginine catabolism</th>
<th>Serum digestion</th>
<th>Phosphatase activity</th>
<th>Tetrazolium reduction</th>
<th>Films and spot</th>
<th>No. isolated</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>y</td>
<td>11/30</td>
<td>36.66</td>
</tr>
<tr>
<td>II</td>
<td>19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>y</td>
<td>19/30</td>
<td>63.33</td>
<td></td>
</tr>
</tbody>
</table>

(+): positive, (-): negative, (v): variable. (All the isolates were negative for film and spot except ML 25 and ML 27). Mcc (Mycoplasma capricolum subsp. capricolum); Mmc (Mycoplasma mycoides subsp. capri)

### Table 2. Result of PCR using various primers for detection of mycoides cluster, mycoides group and species identification of Mycoplasma species CA (F)/CA(R), MC323/MC358, MM450/MM451, P4/P8, P4/P6, Maga(F)/Maga(R) and SSF1/SSR1

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of isolates</th>
<th>Cluster specific primers</th>
<th>Gene (16S rRNA gene) and Primers</th>
<th>Species specific primers</th>
<th>Species identified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MC 323/MC 358</td>
<td>CA (F)/CA (R)</td>
<td>MM 450/MM 451</td>
<td>P4/P8</td>
</tr>
<tr>
<td>1</td>
<td>MN1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>MN2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>MN3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>MN4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>MN5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>MN6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>MN7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>MN8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>MN9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>MN10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>MN11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>MN12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>MN13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>MN14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>MN15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>MN16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. Result of PCR using various primers for detection of mycoides cluster, mycoides group and species identification of *Mycoplasma* species CA (F)/CA(R), MC323/MC358, MM450/MM451, P4/P8, P4/P6, Maga(F)/Maga(R) and SSF1/SSR1 (Continued)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of isolates</th>
<th>Gene (16S rRNA gene) and Primers</th>
<th>Cluster specific primers</th>
<th>Group specific primer</th>
<th>Species specific primers</th>
<th>Species identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>MN17</td>
<td>+ + + + - - - -</td>
<td>MC 323/MC 358</td>
<td>CA (F)/CA (R)</td>
<td>P4/P8</td>
<td>Mmc</td>
</tr>
<tr>
<td>18</td>
<td>MN18</td>
<td>+ + + + - - - -</td>
<td>MM 450/MM 451</td>
<td>- + + - - - -</td>
<td>Mmc</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>MN19</td>
<td>+ + + + - - - -</td>
<td>P4/P6</td>
<td>- + + - - - -</td>
<td>Mmc</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>MN20</td>
<td>+ + + + - - - -</td>
<td>Maga(F)/Maga(R)</td>
<td>- + + - - - -</td>
<td>Mmc</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>ML21</td>
<td>+ + + + - - - -</td>
<td>SSF1/SSR1</td>
<td>- + + - - - -</td>
<td>Mmc</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>ML22</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>ML23</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>ML24</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>MP25</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>MP26</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>MJ27</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>MM28</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>MM29</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>MM30</td>
<td>+ + + + - - - -</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mmc (*Mycoplasma mycoides* subsp. *capri*), Mcc (*Mycoplasma capricolum* subsp. *capricolum*)
The isolates were preliminary characterized by using different biochemical tests: catabolism of glucose, hydrolysis of arginine, phosphatase activity, tetrazolium reduction, serum digestion and film and spot formation, for preliminary identification of the species.

**Preliminary identification of species.** On the basis of the above findings, the 30 isolates of *Mycoplasma* obtained by cultural isolation could be divided into two groups as follows: The details are given in Table 1.

**Group I.** Consists of 11 isolates comprising: 8 isolates (nasal discharges) from goats with a clinical history of respiratory distress and 3 isolates (milk) from apparently healthy goats. They hydrolyzed glucose, catabolized arginine, did not form a film and spot, reduced tetrazolium, digested serum and did not produce phosphatase. These isolates were preliminarily identified as *Mycoplasma capricolum* subsp. *capricolum* (McC).

**Group II.** Consists of 19 isolates comprising: 12 isolates (nasal discharges), 4 isolates (lungs), 2 isolates (pleural fluid), one isolate (joints) from goats with a clinical history of respiratory distress, arthritis, conjunctivitis and heavy mortality. Isolated *Mycoplasma* strains hydrolyzed glucose, did not catabolized arginine, did not form a film and spot (except 2 isolates of lung tissue, ML25 and ML27 were positive), reduced tetrazolium, digested serum and did not produce phosphatase. These isolates were preliminary identified as *Mycoplasma mycoides* subsp. *capri* (Mmc).
P. Kumar et al.: Molecular characterization of Mycoplasma

Fig. 2. Agarose gel showing PCR amplified product (548 bp) for 16S rRNA gene of mycoides cluster using primer pair CA (F) / CA (R). La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples.

Fig. 3. Agarose gel showing PCR amplification product (574 bp) for CAP-21 probe using primer pair MM 450 / MM 451 for mycoides group members. La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples (isolates).

Confirmation of mycoplasma isolates by PCR using various sets of primer and PCR-RFLP. Two sets of cluster specific primers, MC 323 / MC 358 and CA(F) / CA(R), were used to differentiate the M. mycoides cluster members from non cluster Mycoplasmas by PCR in which all 30 isolates of Mycoplasma yielded amplified products of 1.5 Kbp and 548 bp respectively. Further, the members of the mycoides group (M. mycoides subsp. capri) were differentiated from those of the capricolum group (M. capricolum subsp. capricolum) by mycoides group specific PCR, using the primers MM 450 and MM 451 yielded 574 bp long amplicons were only observed in the mycoides group members (19 isolates of M. mycoides subsp. capri) (Figs. 1, 2 and 3).
P. Kumar et al.: Molecular characterization of *Mycoplasma*

Fig. 4. Agarose gel showing PCR amplification product (129 bp) for 16 S rRNA gene using primer pair P4 and P8 of *M. capricolum* subsp. *capricolum*. La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples (isolates).

Fig. 5. Agarose gel showing PCR amplification product (194 bp) for 16 S rRNA gene using primer pair P4 and P6 of *M. mycoides* subsp. *capri*. La: DNA molecular weight ladder 100 bp; -Ve: negative control; 01-06: field samples (islates).

Fig. 6. Agarose gel showing restriction fragmentation pattern of the mycoides cluster by using the enzyme *Pst*I on 548 bp amplicons (specific PCR product amplifield with primer CA (F)/ CA (R). La: DNA molecular weight ladder 100 bp; -Ve: negative control; Lane 01, 03, 05, 07, 08, 11: field samples (548 bp); Lane 02,04,06,08,10,12 are their restriction fragments (420 bp and 128 bp) after digestion with *Pst*I.
Out of the 358 different samples processed, a total of 30 *Mycoplasma* isolates were obtained. Among the isolates, 11 were *Mycoplasma capricolum* subsp. *capricolum* and 19 were *M. mycoides* subsp. *capri*, identified by various biochemical tests and by the PCR method. This indicates and confirms the presence of *Mycoplasma* infection in Western India.

**Discussion**

PCR using the *Mycoplasma capricolum* subsp. *capricolum* specific primers P4 and P8 resulted in an approximately 192 bp long amplified product for 11 isolates, identified as *M. capricolum* subsp. *capricolum*, while none of the 19 isolates identified as *M. mycoides* subsp. *capri* yielded any amplified products (Fig. 4).

PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers P4 and P6 resulted in an approximately 194 bp long amplified product of the16S rRNA gene for all the 19 isolates identified as *M. mycoides* subsp. *capri*, but none of the 11 isolates were identified as *M. capricolum* subsp. *capricolum* (Fig. 5). The overall results are given in Table 2.

Digestion of the 548 bp product yielded by using the primer pair CA(F) and CA(R) amplicons obtained from all the 30 *Mycoplasma* isolates, with the enzyme *Pst* I, resulted in 2 fragments only, 420 bp and 128 bp, which is a characteristic pattern of *M. capricolum* and *M. mycoides* subsp. *capri*. Digestion of the 548 bp amplicons obtained from all the 30 *Mycoplasma* isolates with the enzyme *Mbo* II resulted in only 2 fragments of 295 bp and 253 bp for all the 30 *Mycoplasma* isolates (Figs. 6 and 7).

**Fig. 7.** Agarose gel showing restriction fragmentation pattern of the mycoides cluster by using the enzyme *Mbo* II on 548 bp amplicons (specific PCR product amplifield with primer CA (F)/CA (R). La: DNA molecular weight ladder 100 bp; -Ve: negative control; Lane 01: 548 bp field samples; lane 02 - 06: restriction fragments (295 bp and 253 bp) after digestion with *Mbo* II
Ten samples (6 lung tissue and 4 pleural fluids) after processing yielded 6 (60%) *Mycoplasma* isolates (4 from lungs, 2 from pleural fluid), and were identified as *M. mycoides* subsp. *capri*. Similar reports regarding isolation have been documented by IKHELOA et al. (2004), RODRIGUEZ et al. (1995) and ADEHAN et al. (2006).

215 nasal discharges yielded 20 (9.30 %) *Mycoplasma* (8 *Mycoplasma capricolum* subsp. *capricolum* and 12 *M. mycoides* subsp. *capri*) isolates. Isolation from nasal discharges has been reported earlier by GUPTA et al. (1988), AL-MOMANI et al. (2006) and AWAN et al. (2009).

Three out of 60 milk samples (5.0%) were found positive for *Mycoplasma capricolum* subsp. *capricolum* isolates. Similar reports with a higher percentage of recovery have been reported by AL-MOMANI et al. (2006) and DELAFE et al. (2009).

5 samples processed from an outbreak of *Mycoplasmosis* in goats, resulted in isolation of one *Mycoplasma* isolate (*Mycoplasma mycoides* subsp. *capri*) from the animals showing typical symptoms of lameness. SINGH et al. (2004) also isolated *Mycoplasma mycoides* from cases of caprine arthritis.

The first group consisting of 11 isolates of *Mycoplasma*, were identified as *Mycoplasma capricolum* subsp. *capricolum* based on the biochemical activity, that is they hydrolyzed glucose, catabolized arginine, did not form a film and spot, reduced tetrazolium, digested serum and did not produce phosphatase. These results are in agreement with the findings of ADEHAN et al. (2006) and DAMASSA et al. (1992) although, IKHELOA et al. (2004) reported similar findings but found that *Mycoplasma capricolum* subsp. *capricolum* isolates showed phosphatase activity.

The second group consists of 19 isolates of *Mycoplasma*. They hydrolyzed glucose, did not catabolize arginine, did not form a film and spot (except two isolates of lung tissue- ML 25 and ML 27), reduced tetrazolium, digested serum and did not produce phosphatase. These isolates were preliminarily identified as *Mycoplasma mycoides* subsp. *capri* (Mmc). These results agree with DAMASSA et al. (1992), AJUWAPE et al. (2003) and NICHOLAS (2002), who reported that isolates of *Mycoplasma* were characterized biochemically as *M. mycoides* subsp. *capri* on the basis of fermentation of glucose, reduction of tetrazolium and serum digestion.

PCR was performed using genomic DNA of all the 30 isolates of *Mycoplasma* using the *M. mycoides* cluster specific primers MC 323 and MC 358, and yielded as expected a 1.5 kbp long sequence, homologous to the CAP-21 analogues from the *M. mycoides* cluster members for all the isolates. This finding was in agreement with that of TAYLOR et al. (1992) and BASHIRUDDIN et al. (1994), who designed these oligos complementary to each end of the sequence of the CAP-21 probe.
A second set of primers CA (F) and CA (R) was also used for all the 30 isolates, and yielded an amplified product of approximately 548 bp long sequence of the 16S rRNA gene of the *M. mycoides* cluster members for all the isolates. This finding was in agreement with that of BOLSKE et al. (1996) and PETTERSSON et al. (1996), who developed a CCPP specific PCR using primers amplifying a 548 bp sequence of the 16S rRNA genes from all the six members of the cluster.

Digestion of the 548 bp amplicons (the product yielded by using primer pair CA(F) and CA(R)) were obtained from all 30 *Mycoplasma* isolates with the enzyme *Pst* I resulting in 2 fragments only, 420 bp and 128 bp, which is the characteristic pattern of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *capri*. This finding was in agreement with BOLSKE et al. (1996) and HERNADEZ et al. (2006). Digestion of the 548 bp amplicons obtained from all the 30 *Mycoplasma* isolates with the enzyme *Mbo* II, resulted in only 2 fragments of 295 bp and 253 bp for all the 30 *Mycoplasma* isolates.

PCR using the *Mycoides* group specific primers MM 450 and MM 451, resulted in an approximately 574 bp long amplified product of the CAP-21 probe of the *Mycoides* group members *M. mycoides* subsp. *capri* (19 isolates), this finding was in agreement with that of BASHIRUDDIN et al. (1994).

PCR using the *Mycoplasma capricolum* subsp. *capricolum* specific primers P4 and P8 resulted in an approximately 192 bp long amplified product of the 16S rRNA gene for 11 isolates, identified as *M. capricolum* subsp. *capricolum*, while none of the 19 isolates identified as *M. mycoides* subsp. *capri* yielded any amplified products. This finding was in agreement with that of HOTZEL et al. (1996), KUMAR (2000) and HERNANDEZ et al. (2006). They used the Mcc specific primer P8 in combination with another primer P4, which was common to all cluster members except MmmSC, to amplify approximately 192 bp product from only Mcc strains.

PCR using the *Mycoplasma mycoides* subsp. *capri* specific primers P4 and P6 resulted in an approximately 194 bp long amplified product of the 16S rRNA gene for all the 19 isolates identified as *M. mycoides* subsp. *capri*, but none of the 11 isolates identified as *M. capricolum* subsp. *capricolum*. This finding was in agreement with that of HOTZEL et al. (1996); KUMAR, (2000) and HERNANDEZ et al. (2006), who used Mmc specific primers (P4 and P6) and yielded approximately 194 bp product with Mmc strains.

**Conclusions**

The analysis of the findings from the present study implies that there is indeed a prevalence of *Mycoplasma* infections in India. Isolation and identification was conducted of Mycoplasmas from goats presenting a range of clinical signs, including respiratory disease, conjunctivitis, arthritis, mastitis and loss in milk production. PCR based detection of *mycoides* cluster, group and species is a rapid and simple method of
P. Kumar et al.: Molecular characterization of Mycoplasma
detection and identification of the Mycoplasma organism and can be an effective tool for epidemiological surveys. Restriction enzyme fragmentation assay can be a useful method for differentiation and identification of Mycoplasma isolates.

Acknowledgements
The authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Anand (Gujarat), for providing the facilities to pursue this work.

References
presence of *Mycoplasma* spp. in herds without symptoms of contagious agalactia. J. Dairy Res. 76, 562-567.


P. Kumar et al.: Molecular characterization of *Mycoplasma*


**SAŽETAK**

Ukupno je 358 uzoraka različitog materijala koza bilo nacijepljeno na hranjive podloge MBHS-L and MBHS-A radi izdvajanja mikoplazama. Pretraženo je bilo 215 uzoraka nosnog iscjetka, šest uzoraka plućnog tkiva, četiri uskog pleuralnog tkiva, pet pleuralnih tekućina, pet uzoraka zglobne tekućine, 60 uzoraka mlijeka, 22 uzorka prepucijskog ispirka, 16 uzoraka obrisa spojnice, pet uzoraka obrisa uške i 25 uzoraka vaginalnog ispirka. Mikoplazme su bile izdvojene iz 30 (8,35%) uzoraka, od čega iz 20 uzoraka nosnog iscjetka, četiri uzorka plućnog tkiva, dva pleuralne tekućine, tri uzorka mlijeka i jednog uzorka zglobne tekućine. Izolati su bili identificirani kao *M. mycoides* subsp. *capri* i *M. capricolum* subsp. *capricolum* i to na osnovi kulturalnih, morfoloških i biokemijskih svojstava te dodatne potvrde lančanom reakcijom polimerazom i PCR-RFLP rabeći različite skupine specifičnih početnica i restrikcijskih enzima. Ovo je rijetko izvješće o izdvajanju, identifikaciji i molekularnim svojstvima mikoplazama u Zapadnoj Indiji, koje ujedno pokazuje da je na tom području velika učestalost mikoplazmalnih zaraza u koza.

**Ključne riječi:** Indija, mikoplazme, koze, *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum*

Received: 10 June 2010
Accepted: 17 March 2011