Research Article

Isolation and Characterization of *Mycoplasma mycoides* Subspecies *capri* from Milk of Natural Goat Mastitis Cases

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1. Introduction

*Mycoplasma mycoides* subsp. *capri* (*Mmc*) belongs to the “Mycoplasma mycoides” cluster (*M. capricolum* subsp. *capricolum, M. capricolum* subsp. *capripneumoniae, M. mycoides* subsp. *mycoides* large colony type (LC), *M. mycoides* subsp. *mycoides* small colony type (SC), *Mycoplasma* spp. bovine group 7, and *Mmc*) and is reported to cause a pattern of disease (mastitis, arthritis, keratoconjunctivitis, and pleuropneumonia) in goats, similar to those induced by the rest of the species of the mycoides cluster and other mycoplasmas, namely, *M. agalactiae* and *M. putrefaciens* [1, 2]. Mastitis is one of the manifestations of contagious agalactia (CA) [3] and is characterized by clinical signs like heat, pain, swelling, and redness in the udder besides alteration in milk (clot, flakes, discoloration, and reduction or complete cessation of milk yield). CA is prevalent in several regions of the world [4] by causing high morbidity (26.1–100%) in adult goats and 36.5 to 100% in kids [5] along with 25% and 90% mortality in adult goats and kids, respectively [6]. In goat-rearing units the economic loss may reach up to 15–20% [4].

Although *M. agalactiae* is known as the classical etiological agent of CA and/or mastitis, other species of the mycoides cluster have also been found to be associated with goat mastitis in different countries [7–9]. Very recently, Amores et al. [9] have detected *Mmc* using polymerase chain reaction (PCR) from bulk tank milk which was collected from goats exhibiting clinical signs of mastitis from a CA endemic area. However, there is no report about the isolation of *Mmc* from natural goat’s mastitis except for the experimental study of Misri et al. [2] and D'Angelo et al. [10].

In view of the dearth of information on the association of *Mmc* with goat mastitis on culture bases, the present study was carried out to determine the involvement of *Mmc* as well as intraspecific strain variation by isolation, characterization based on species specific PCR and 16S amplified rDNA restriction analysis (16S ARDRA) pattern.

2. Materials and Methods

2.1. Classification of Goats for Sampling. Goats exhibiting the clinical signs of mastitis, that is, swollen udder with pain, secreting altered milk, fever, lethargy, and labored breathing, and goats either living in close proximity to goats suffering from mastitis or in herds having a history of CA, were selected...
for sampling. Goats not exhibiting clinical mastitis were
taken as asymptomatic ones and suspected for carriers of
mycoplasmas.

2.2. Goat Herd, Sample Collection, and Isolation of Mycoplasmas. A total of 171 goats were sampled for milk from five
different goat herds of Mathura region (CIRG, Makhdoom;
Jhandipur, Chattar Singh Ka Nagla, Keetham and Agra)
that facing the problems of CA and/or mastitis. Out of 171
milk samples, 102 were from clinical mastitis and 69 from
asymptomatic goats. Milk samples were collected aseptically
from the isolates was extracted from late exponential growth
phase using the phenol-chloroform method described by van
Kuppeveled et al. [14], and purity and concentration of DNA
was checked on 0.7% agarose gel and spectrophotometric
analysis according to Sambrook et al. [15].

2.3. Biochemical Characterization. A preliminary charac-
terization of the isolates was performed by digitonin sensitivity
and growth inhibition tests as per the method described
elsewhere [12] and Giemsa method of staining. This was
followed by biochemical tests, namely, glucose fermentation,
phosphate reduction, gelatin hydrolysis, and film and spot
formation test as described earlier [13].

2.4. Confirmation of Isolates by PCR. Genomic DNA of
the isolates was extracted from late exponential growth
phase using the phenol-chloroform method described by van
Kuppeveled et al. [14], and purity and concentration of DNA
was checked on 0.7% agarose gel and spectrophotometric
analysis according to Sambrook et al. [15].

Mycoplasma isolates were confirmed by employing Mmc-
specific PCR and the presence of other mycoplasmas was
ruled out by conducting the respective species-specific PCRs
according to respective protocols described elsewhere as
referred in Table 1. The Qiagen PCR core kit was used to
perform all the PCRs and consequent PCR products were
checked on 2% agarose gel.

2.5. Characterization of Intraspecies Strain Variation Using 16S
ARDRA. The 16S rDNA of all isolates was amplified by using
the universal primer pair pA (5'-AGAGTTTGATCCTG-G
CTCAG-3') and pH (5'-AAGGAGTTGACGCGAAGCCAG-
AGTTGGAATG-3') for 30 cycles (20 sec. 94°C; 15 sec.
57°C; and 30 sec. 72°C) using Qiagen PCR core kit as per Edwards et al.
[21]. The resultant amplicon (1500 bp) was purified by using
purification kit (Bangalore Genei, India). It was subsequently
digested with restriction enzyme Alu I (Fermentas, sequence:
AG'C) and the restriction fragments were separated on 3% NuSieve
3:1 agar by using the method of Stakenborg et al.
[22].

3. Results and Discussion

Out of 171 clinical and asymptomatic samples, a total of
45 samples showed fine turbidity and pH shift (acidic)
imparting a yellow color to the broth medium within 3 to
10 days indicating the mycoplasma growth. After following
the protocol of 4-5 reversal and 3-4 forward passages, the
possibility of “L phase variant” was ruled out. Only 6 (3.5%)
samples yielded colonies of 1 to 2 mm size exhibiting typical
fried egg appearance on HBSS-S medium. Their growth
characteristics were indicative of the mycoplasmas. Of six
isolates, 5 were recovered from clinical mastitis milk, whereas
one (isolate number 6) was from subclinical mastitis milk.
These growth evidences were in accordance with Razin and
Freundt [23] and Sori et al. [24]. In the study, the isolation
rate (3.5%) was found to be in agreement with Ikhloea et al.
[25], who obtained similar results of 3.7 to 11%; however, our

Table 1: Details of primers used in study.

| Primer       | Specificity to species | Sequence 1 | Target gene region | Annealing temperature | Reference |
|--------------|------------------------|------------|--------------------|-----------------------|----------|
| P4/P6        | Mmc*                   | 5'-ACTGACGAAAATTCCTCCT-3' | CAP-21              | 46°C, 90 sec          | [16]     |
|              | PCR                    | 5'-TTAATAAGTCTCTATAGAATT-3' |                     |                       |          |
| P4/P5        | Mmm LC*                | 5'-ACTGACGAAATTTCTTTCT-3'  | CAP-21              | 54°C, 30 sec          | [16]     |
|              |                        | 5'-TTAATAAGTTTGTATATGAAAT-3' |                     |                       |          |
| Mag-F/Mag-R  | M. ag*                 | 5'-CTTTTATAGGTGAGGACATG-3'  | 16S rRNA            | 60°C, 60 sec          | [17]     |
|              |                        | 5'-CCGTCAGGTCGTCATTGTTGCTAC-3' |                 |                       |          |
| MputF/MputR  | M. put*                | 5'-AAATTGTGAAATTACCGCGAC-3'  | Arc B               | 52°C, 15 sec          | [18]     |
|              |                        | 5'-CATATCTCAACTGAATAAGTGACACC-3' |                   |                       |          |
| MCCPL1-L/MCCPL1-R | Mcc*            | 5'-AGACCCAAAATAAGGCGATCGA-3'  | LppA                | 51°C                 | [19]     |
|              |                        | 5'-CTTTCCGCGCTTGTGAATG-3' |                     |                       |          |
| P67BG7-L/P67BG7-R | Mbg7*         | 5'-GGTAAATTGAAATGATTCCT-3'  | P67 gene            | 46°C                 | [20]     |

* Mmc: M. mycoides subsp. capri, Mmm LC: M. mycoides susp. mycoides large colony type, M. ag: M. agalactiae, Mcc: M. capricolum subsp. capricolum, Mbg7: M. bovine group 2.
isolation rate seems to be quite low in contrast to the 25 to 70% obtained by Gil et al. [26].

All the isolates showed purplish-pinkish coccobacillary bodies with pleomorphic shape and size upon Giemsa staining. The isolates passed filtration test through 0.45 μm filter and found to be sensitive to digitonin. Biochemical tests, namely, glucose fermentation and gelatin hydrolysis tests gave positive results, while, film and spot formation test and phosphatase test were negative for all isolates. The isolates exhibited positive growth inhibition test using anti-Mmc PG-3 antiserum. On the basis of these results all the isolates were suspected to be of Mmc.

The PCR amplification in Mmc-specific PCR was found positive in all isolates by yielding 195 bp amplicon (Figure 1) the specificity of this PCR was for CAP-21 genomic region [16]. However, none of the species-specific PCR (mentioned in Table 1) except Mmc PCR was amplified against any isolate. Thus the presence of any other species (M. putrefaciens, M. agalactiae, M. capricolum subsp. capricolum, Mmm LC, and M. bovine group 7) was ruled out, although they are also known to be associated with goat mastitis milk.

Mmc isolates were further studied for any intraspecific strain variation using 16S ARDRA. The 16S rDNA upon digestion with Alu I exhibited strain variation in Mmc isolates by revealing three types of ARDRA patterns (Figure 2). The isolate numbers 1, 2, 3, and 4 showed a similar band pattern as that of Mmc PG-3 by yielding 5 bands (236, 186, 147, 105, and 85 bp), while isolate numbers 5 and 6 showed different and unique band patterns by yielding 3 (620, 473, and 413 bp) and 7 (620, 473, 413, 236, 186, 147, and 105 bp) bands, respectively, which were different than the standard strain PG-3. The similarity in band pattern with that of standard strain PG-3 was in agreement with the observations of Stakenborg et al. [22], who observed the same pattern for PG-3 using the same primer and restriction enzyme. However, the different band pattern observed in isolates numbers 5 and 6 was not in agreement with their observation. Our results, that is, different band patterns within species are supported by Monnerat et al. [19] who also found intraspecific strain variation in lppA gene of Mmc strains by using Alu I enzyme. The band pattern different from the reference strain (PG-3) observed by us may be attributed to the presence of different Alu I cutting sites in both of the operons (rrnA and rrnB) as described by Bascunana et al. [27].

Although M. agalactiae is known as the main causative agent of mastitis [28] along with other species reported earlier, in our case Mmc was isolated from goats having clinical mastitis as well as from asymptomatic goats. The isolation of Mmc in the present study has also been supported by the detection of Mmc from milk collected from clinical mastitis cases in CA endemic area in Spain [9]. Our findings are experimentally supported by Misri et al. [2], who observed the involvement of Mmc in development of goat mastitis after following the Koch’s postulate. But the present findings are contradictory to earlier reports describing the association of goat mastitis with other mycoplasma species like M. capricolum subsp. capricolum, M. putrefaciens, M. arginini and Mmm LC [6, 28–31]. Since the present study does not cover a wide geographic area, therefore an isolation work needs to be carried out at a wider level.

In conclusion, our finding reports the isolation of Mmc having intra specific strain variation (in 16S rDNA) from natural mastitis in goats which have not been reported ever and consequently indicates the association and dually favors the earlier report of development of mastitis in goats after the experimental infection of Mmc. Also, it reports that, in India, the occurrence of mycoplasmal mastitis in goats may be due to Mmc infections as no other mycoplasmal species could be isolated from goat mastitis.

**Conflict of Interests**

The authors have no conflict interest to declare.

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of members of mycoides cluster and their monospecific antisera.

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