Hindawi Publishing Corporation
ISRN Veterinary Science
Volume 2013, Article ID 593029, 5 pages
http://dx.doi.org/10.1155/2013/593029

Research Article

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

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Received 4 April 2013; Accepted 24 April 2013

Academic Editors: P. Butaye, J. Cabaret, and I. Nsahlai

Association of *Mycoplasma mycoides* subspecies *capri* (*Mmc*) with natural goat mastitis has been studied earlier largely by detecting the *Mmc* DNA using molecular methods. However, report on detection of cultivable *Mmc* isolates from natural goat-mastitis milk is still very rare. In this study, *Mmc* was isolated from milk samples ($n = 171$) of goats with or without clinical signs of mastitis. *Mmc* isolates were further characterized by biochemical and species-specific PCR methods. Intra species strain variation was also studied by 16S amplified rDNA restriction analysis (16S ARDRA). The study recovered a total of 6 *Mmc* isolates (3.5%). Three types of intraspecies variants among the recovered *Mmc* isolates were found by 16S ARDRA. The study concluded that *Mmc* may be an etiological agent of mycoplasmal mastitis in Indian goat herds.

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

Table 1: Details of primers used in study.

Primer	Specificity to species	Sequence	Target gene/region	Annealing temperature	Reference
P4/P6	*Mmc** PCR	5′-ACTGAGCAATTCCTCTT-3′ 5′-TTAATAAGTCTCTATATGAAT-3′	CAP-21	46°C, 90 sec	[16]
P4/P5	*Mmm* LC*	5′-ACTGAGCAATTCCTCTT-3′ 5′-TTAAATAAGTTTGTATATGAAT-3′	CAP-21	54°C, 30 sec	[16]
Mag-F/Mag-R	*M. ag**	5′-CCTTTTAGATTGGGATAGCGGATG-3′ 5′-CCGTCAAGGTAGCGTCATTTCCTAC-3′	16S rRNA	60°C, 60 sec	[17]
MputF/MputR	*M. put**	5′-AAATTGTTGAAAAATTAGCGCGAC-3′ 5′-CATATCATCAACTAGATTAATAGTAGCACC-3′	*Arc B*	52°C, 15 sec	[18]
MCCPL1-L/MCCPL1-R	*Mcc**	5′-AGACCCAAATAAGCCATCCA-3′ 5′-CTTTCACCGCTTGTTGAATG-3′	*LppA*	51°C	[19]
P67BG7-L/P67BG7-R	*Mbg7**	5′-GGTAATTCGAATAATGATCCT-3′ 5′-TAAGTTTATTGAATTAAAGCG-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 7.

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 in sterile vials, that is, before milking affected teats were cleaned with 70% ethanol and then the first 2-3 streams were discarded.

Isolation of mycoplasmas from milk samples was performed as described by Carmichael et al. [11] with slight modifications. Briefly, milk samples were inoculated into liquid Hank's balanced salt solution (HBSS-L; pH 7.6–7.8) medium and incubated at 37°C under 5% CO_2 for up to 14 days and subsequently transferred on solid Hank's balanced salt solution (HBSS-S) medium. The probability of L phase variants was ruled out by forward and reversal passages in the artificial medium.

2.3. Biochemical Characterization. A preliminary characterization 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′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAGCCGCA-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^CT) 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

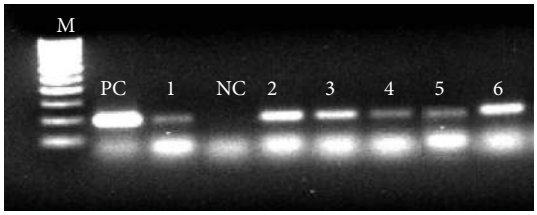

Figure 1: *M. mycoides* subsp. *Capri*-specific PCR exhibiting 195 bp amplicon. M: 100 bp DNA ladder; PC: positive control (*M. mycoides* subsp. *capri* PG-3); NC: negative control; Lanes 1–6: respective isolates.

isolation rate seems to be quite low in contrast to the 25 to 71% 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].

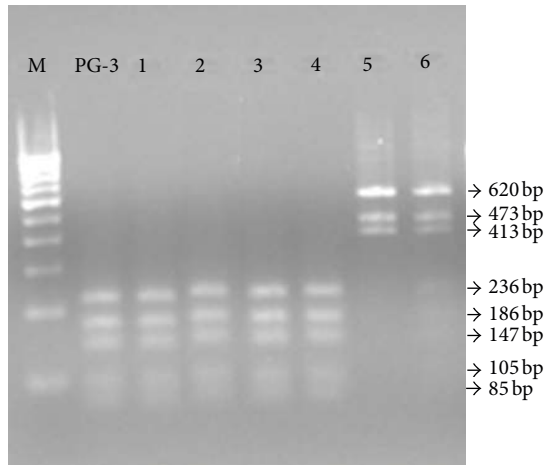

Figure 2: Intra specific strain variation in 16S ARDRA profile among *Mmc* isolates. M: 100 bp DNA ladder; PG-3: *M. mycoides* subsp. *capri* PG-3; Lanes 1–6: respective isolates. The band, that is, 236 bp, 186 bp, 147 bp, and 105 bp are visible very faintly in lane number 6.

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.

Acknowledgment

The authors are thankful to Dr. R. A. J. Nicholas, Mycoplasma Group, Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey, UK, for providing reference strains

of members of mycoides cluster and their monospecific antisera.

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