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Mycoplasma mycoides subsp. mycoides SC identification by PCR in sperm of seminal vesiculitis-affected bulls

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Abstract – In this study, by using the polymerase chain reaction (PCR) diagnosis for the detection and identification of Mycoplasma, we investigated mycoplasmas contaminating the semen of yearling bulls affected by seminal vesiculitis. The bulls presented neither subclinical nor clinical contagious bovine pleuropneumonia signs and the complement fixation test for specific antibodies was negative. Furthermore, we have investigated mycoplasmas isolated from semen of healthy breeding bulls of several breeds and origins, which routinely underwent breeding soundness examinations and presented no clinical signs of seminal vesiculitis. We were able to demonstrate mycoplasma infection in all tested samples by i) growth on mycoplasma-specific media and ii) a PCR-based method using a mycoplasma-specific MGSO/GPO1 primer set to amplify the 16S fragment rDNA. In addition, the identification of Mycoplasma species was made by PCR using the MSC1/MSC2 primer set that specifically amplifies \textit{M. mycoides} subsp. mycoides SC or the MM450/MM451 primer set followed by AsnI digestion analysis in order to identify \textit{M. mycoides} subsp. mycoides LC. The data presented herein clearly show that \textit{M. mycoides} subsp. mycoides SC infection was associated with seminal vesiculitis while \textit{M. mycoides} subsp. mycoides LC was only found in bull semen from healthy control animals. Our findings confirm that the \textit{M. mycoides} subsp. mycoides SC is shed in the sperm making the ejaculate a valuable biological sample for the isolation of these bacteria from serologically negative animals. Although the pathogenic role of \textit{M. bovigenitalium} in bull seminal vesiculitis has been established, our clinical findings, semen characteristics, microbiological and bacterial genomic analysis strongly suggest that \textit{M. mycoides} subsp. mycoides SC may contribute to induce vesicular adenitis in the bull. © Inra/Elsevier, Paris.

Keywords: bovine / sperm / seminal vesiculitis / Mycoplasma / PCR

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bovin / sperme / vésiculite séminale / *Mycoplasma* / PCR

1. INTRODUCTION

The seminal vesiculitis syndrome affects bulls of all ages. The pathogenesis has not been clearly established; however, several pathogenic organisms such as *Brucella abortus* [5], *Pseudomonas aeruginosa* and *Actinobacillus actinomycetemcomitans* [17], *Corynebacterium pyogenes* [12], *Proteus* [28], *Haemophilus somnus* [16], *Mycoplasma* spp. [1] and *Ureaplasma diversum* [41] have been associated with seminal vesiculitis.

Mycoplasma organisms are widely distributed in animals and human beings and have been observed in different tracts of the reproductive system in cattle [13, 23]. A high incidence of *Mycoplasma* and *Ureaplasma* contamination in fresh and frozen bull semen has been reported [2, 29, 35] by the Artificial Insemination Industry. Some species of mycoplasma have been associated with both natural and experimentally induced genital infections in cattle [18, 24, 39].

The *mycoides* cluster includes six *Mycoplasma* species or subspecies that are all significant pathogens in small and large ruminants: *M. mycoides* subsp. *mycoides* with two biotypes, small-colony (SC) and large-colony (LC): *M. mycoides* subsp. *capri*; *M. capricolum* subsp. *capricolum*; *M. capricolum* subsp. *capripneumoniae* (type F38) and *M. sp.* group 7 of Leach (type PG50) [8, 25]. *Mycoplasma mycoides* subsp. *mycoides* SC is responsible for contagious bovine pleuropneumonia (CBPP), a sporadic infection in Europe [26]. The complement fixation test for the detection of this pathogen is highly specific in approximately 70 % of infected animals, while it is poorly responsive in asymptomatic animals in the early stages of infection and in chronically infected ones [31]. In affected animals, *M. mycoides* subsp. *mycoides* SC and its antigens have been found in the lungs, thoracic...
lymph nodes, blood and extrathoracic organs such as the kidney and liver [27, 36, 37]. M. mycoides subsp. mycoides SC has recently been identified in bull semen, although clinical findings associated with genital infections were not observed [14].

In the present study, we investigated bacteria isolated from semen of seminal vesiculitis-affected yearling bulls, derived from an outbreak of CBPP in Italy in 1994 and from healthy control bulls presenting no clinical signs of seminal vesiculitis.

The data reported herein, using specific PCR assays for the identification of bovine Mycoplasma species, has clearly shown the infection of seminal vesiculitis bull semen with M. mycoides subsp. mycoides SC and the semen from control bulls with M. mycoides subsp. mycoides LC.

2. MATERIALS AND METHODS

2.1. Animals and sample collections

The study was carried out on four yearling fresian bulls (from 11 to 13 months of age) affected by seminal vesiculitis derived from an outbreak of contagious bovine pleuropneumonia in 1994 in Italy (bulls A, B, C, D). The bulls presented neither subclinical nor clinical CBPP signs and the complement fixation test [15] for specific antibodies proved to be negative.

Furthermore, we included mycoplasma-infected semen samples from healthy breeding bulls of several breeds and origin, which routinely underwent breeding soundness examinations, presenting no clinical signs of seminal vesiculitis in our investigation (bulls E, F, G, H).

The clinical evaluation of the genital tract, including transrectal ultrasound examination of the accessory sexual glands (Medison, SONACE 88P, 5 MHz linear array transducer), were carried out. Seminal vesicle secretions were harvested using a sterile catheter via the urethra by transrectal massage, as described by Parsonson et al. [30].

In addition, two consecutive ejaculates were collected by the artificial vagina method. The volume of each ejaculate was determined. Progressive motile spermatozoa were evaluated in diluted semen according to the World Health Organization [42]. The concentration (10^9 spermatozoa/mL) was measured by means of the Bürker haemocytometer. The spermatozoa morphology was evaluated with the eosin-nigrosin stain [6] and defective cells were grouped into primary and secondary abnormalities [7]. Additional smears were also stained with Giemsa for leukocyte identification.

2.2. Microbiological analysis

Seminal vesicle secretions and semen samples from affected animals were diluted with PBS solution pH 7.2 and were centrifuged at 1 500 g for 30 min. The supernatant (0.5 mL) was plated onto agar, blood agar and Gäsner media and then incubated at 37 °C for 24 h. For mycoplasma isolation, 0.5 mL of the supernatant was added to 6 mL PPLO broth (Difco, Milano, Italy) supplemented with 0.5 % yeast extract (Difco, Milano, Italy), 20 % heat-inactivated horse serum (Difco, Milano, Italy), 10 g of tryptose (Difco, Milano, Italy), 1 % glucose, 500 000 IU of Penicillin (Carlo Erba, Milano, Italy), thallium acetate (Carlo Erba, Milano, Italy) and distilled water pH 7.6–7.8. Four 10-fold dilutions (10⁻¹ to 10⁻⁸) were titrated from this in the same broth and incubated at 37 °C for 3 days. The 10⁻⁴ dilution of the PPLO broth was routinely subcultured on the PPLO agar. Agar plates were incubated in an aerobic and anaerobic environment and observed for colony appearance on the 3rd and 6th days of in vitro culture. Mycoplasma colonies were isolated into liquid culture medium and maintained at –80 °C until genomic analysis.

In order to identify Mycoplasma spp. by biochemical tests, glucose fermentation [33], arginine hydrolysis [3], film and spot formation, casein and serum digestion and tetrazolium salt reduction were performed [11].

2.3. DNA preparation from mycoplasma colonies

DNA was prepared from mycoplasma samples as described by Kellog and Kwok [21]. Briefly, mycoplasmas were centrifuged at 17 000 g for 30 min at 4 °C, and the cell pellet was resuspended in 500 mL of proteinase K (Gibco BRL, France) solution (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.5 %
Tween 20, 0.5 % Triton X-100, and 60 mg/mL proteinase K) and incubated at 37 °C for 2 h. Heating at 95 °C for 10 min denatured proteinase K, and the samples were cooled in ice prior to use in PCR amplification. At this stage the samples could be stored at −20 °C for several months.

2.4. PCR assay for mycoplasma detection

MGSO and GPO1 PCR primers (table I) [32] were used to amplify mycoplasma 16S rDNA from samples as described by Roulland-Dussoix et al. [34]. Here, 40 mM of each primer were mixed in 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 0.2 mM dNTP and 1 unit of Taq DNA polymerase (Appligène Oncor, Montreuil, France). DNA sample (2 μL) was added to 48 μL of PCR mix, then heated for 15 min at 95 °C and amplification was carried out for 35 cycles as follows: 95 °C for 30 s, 58 °C for 1 min and 30 s, 72 °C for 1 min and 30 s.

2.5. PCR product sequencing

Mycoplasmal 16S rDNA fragments amplified from DNA samples were purified by phenol/chloroform extraction and precipitated with ethanol. Purified DNA fragments were sequenced using the automated ABI sequencer employing 373 software. Sequencing was performed with MGSO or GPO1 primers.

2.6. PCR assay for mycoplasma species identification

The identification of Mycoplasma mycoides subsp. mycoides SC was performed as described by Dedieu et al. [9]. Mycoplasma mycoides subsp. mycoides SC-specific amplification using MSC1 and MSC2 primers (table I) was performed on DNA samples as described above, except that the annealing temperature was 53 °C.

The identification of Mycoplasma mycoides subsp. mycoides LC was performed as described by Bashiruddin et al. [4]. Amplification from DNA samples was performed using MM450 and MM451 primers (table I) as described above except that the annealing temperature was 50 °C. Amplified DNA fragments using the MM450/MM451 primer set were extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNA was digested with 10 units of AscI in a total volume of 20 mL. The digests were then electrophoresed in 2.5 % Metaphor (FMC, Le Perray en-Yvelines, France) gel.

M. agalactiae identification was performed as described by Subramaniam et al. [38] by specific amplification of the uvrC gene. MAGAUVRC1-L and MAGAUVRC1-R primers were used to carry out M. agalactiae identification in

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequences 5’–3’</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGSO (S)</td>
<td>TGCACCATCTCTGTCACCTCTGTTAA</td>
<td>Roulland-Dussoix et al. [34]</td>
</tr>
<tr>
<td></td>
<td>CCTC</td>
<td>Van Kuppeveld et al. [40]</td>
</tr>
<tr>
<td>GPO1 (AS)</td>
<td>ACTCTCTACGGAGGCGAGAGTA</td>
<td>Roulland-Dussoix et al. [34]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Van Kuppeveld et al. [40]</td>
</tr>
<tr>
<td>MSC1 (S)</td>
<td>ATACTTCTGTGCTAATATAT</td>
<td>Dedieu et al. [9]</td>
</tr>
<tr>
<td>MSC2 (AS)</td>
<td>AAAAAAGTAGAGATTCATCT</td>
<td>Dedieu et al. [9]</td>
</tr>
<tr>
<td>MM450 (S)</td>
<td>GTATTTTCTTTCTTATTTTG</td>
<td>Bashiruddin et al. [4]</td>
</tr>
<tr>
<td>MM451 (AS)</td>
<td>AAATCCAATTAAAGGTTT</td>
<td>Bashiruddin et al. [4]</td>
</tr>
<tr>
<td>MBOUVR2C-L (S)</td>
<td>TTACGCAAGAGAATGCTTCA</td>
<td>Subramaniam et al. [38]</td>
</tr>
<tr>
<td>MBOUVR2-R (AS)</td>
<td>TAGGAAAAGCACCCTATTGAT</td>
<td>Subramaniam et al. [38]</td>
</tr>
<tr>
<td>MAGAUVRC1-L (S)</td>
<td>CTCAAAAAATATCAACAGC</td>
<td>Subramaniam et al. [38]</td>
</tr>
<tr>
<td>MAGAUVRC1-R (AS)</td>
<td>CCTCAACTGTGACATCAAATA</td>
<td>Subramaniam et al. [38]</td>
</tr>
</tbody>
</table>

S: sense primer; AS: anti-sense primer.
Table II. Semen characteristics of seminal vesiculitis-affected bulls versus breeding bulls.

<table>
<thead>
<tr>
<th>Semen characteristics</th>
<th>Seminal vesiculitis bulls (n = 4)</th>
<th>Breeding bulls (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>5.17 ± 2.47</td>
<td>4.02 ± 0.43</td>
</tr>
<tr>
<td>$10^9$ spermatozoa/mL</td>
<td>0.67 ± 0.55</td>
<td>0.69 ± 0.43</td>
</tr>
<tr>
<td>Progressive motile spermatozoa (%)</td>
<td>59.67 ± 13.05</td>
<td>60.07 ± 11.69</td>
</tr>
<tr>
<td>Total sperm abnormalities (%)</td>
<td>29.21 ± 3.57</td>
<td>23.34 ± 16.39</td>
</tr>
<tr>
<td>Abnormal tail (%)</td>
<td>5.49 ± 2.25</td>
<td>6.37 ± 4.56</td>
</tr>
<tr>
<td>Abnormal midpiece (%)</td>
<td>15.24 ± 1.82</td>
<td>10.22 ± 12.11</td>
</tr>
<tr>
<td>Detached head (%)</td>
<td>2.97 ± 1.88</td>
<td>3.43 ± 2.9</td>
</tr>
<tr>
<td>Abnormal head (%)</td>
<td>5.51 ± 1.94</td>
<td>3.31 ± 2.94</td>
</tr>
</tbody>
</table>

2.7. Analysis of amplified PCR products

Each amplified DNA (20 mL) was subjected to electrophoresis with 1.5 % agarose (Sigma, St. Quentin Fallavier, France) gel. AsnI DNA digestion was carried out for *M. mycoides* subsp. *mycoides* identification and the digestion products were analysed by electrophoresis on 2.5 % Metaphor agarose (FMC, Le Perray en-Yvelines, France). DNA was detected by UV fluorescence after ethidium bromide staining. OX174 digested with *Hae*III (Boehringer Mannheim GmbH, Germany) was used as a marker to estimate the molecular weight of the amplified bands.

3. RESULTS

3.1. Clinical findings

Neither congenital nor acquired abnormalities of the external genitalia were observed while internal genital examination was characterized by moderate enlargement, loss of lobulation and fluctuation areas of the seminal vesicles. The animals presented a monolateral enlargement of the glands, except bull D in which both seminal glands were affected. Upon transrectal ultrasound examination, the glandular lumen appeared dilated, presenting an irregularly echogenic content due to abnormal fluid accumulation (figure 1).

The ejaculates were stringy, creamy-yellow and contained clots of pus. Semen characteristics, compared to breeding bulls, are reported in table II. No significant differences were observed in semen characteristics, although total defective sperm cells were more than 6 % higher in the seminal vesiculitis-affected bulls. Clusters of agglutinated sperm cells were observed. Further-
more, the Giemsa-stained smears presented polymorphonuclear leukocytes and epithelial cells.

Biochemical data showed that isolated mycoplasmas fermented glucose, reduced tetrazolium salts, were negative for arginine and did not digest serum and casein.

3.2. Mycoplasma detection in semen samples

No significant bacterium was isolated from the LB agar, blood agar and Gassner media. However, on PPLO agar plates the typical mycoplasma colonies called ‘fried-egg’, were shown in all seminal fluids and ejaculate samples. These data clearly indicated that the specimens were infected with mycoplasmas.

In order to confirm mycoplasma contamination in these semen samples, DNA was extracted from samples and subjected to PCR amplification using the primer set, MGSO/GPO1, that specifically amplifies the 16S rRNA gene from the mycoplasma genus. All samples were found to be positive for mycoplasma based on the amplification results obtained with the mycoplasma genus-specific PCR as indicated in table III. These data indicate that the specimens were contaminated with mycoplasmas and confirmed earlier findings.

Table III. Identification of the Mycoplasma species contaminating semen and seminal fluid samples.

<table>
<thead>
<tr>
<th>Growth on PPLO plates</th>
<th>Mycoplasma detection by PCR</th>
<th>MSC1/ MSC2</th>
<th>MM450/MM451(^a)</th>
<th>MBOUVR/C2-L/ MBOUVR/C2- R(^b)</th>
<th>MAGAUVR/C1-L/ MAGAUVR/C1- R(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PCR ampl.</td>
<td>Asnl Digest</td>
<td></td>
</tr>
<tr>
<td>Affected bulls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Healthy bulls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>3</td>
<td>–</td>
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<tr>
<td>F</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>3</td>
<td>–</td>
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<tr>
<td>G</td>
<td>+</td>
<td>+</td>
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<td>3</td>
<td>–</td>
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<tr>
<td>H</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>3</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\) Amplification of both *M. mycoides* subsp. *mycoides* SC and LC.

\(^b\) Specific amplification of *M. mycoides* subsp. *mycoides* SC.

\(^c\) Number of fragments generated by Asnl digestion; two fragments for *M. mycoides* subsp. *mycoides* SC and three fragments for *M. mycoides* subsp. *mycoides* LC.

\(^d\) Specific amplification of *M. bovis*.

\(^e\) Specific amplification of *M. agalactiae*.
3.3. Identification of mycoplasma contamination

We further pursued our investigation to identify the *Mycoplasma* species that had been isolated from the semen samples. PCR products obtained from the MGSO/GPO1 amplification were purified and sequenced. The sequences obtained were compared with those of the Genebank database by using a blast and fast program. The sequence comparison data showed that the amplified 16S rRNA gene from the semen samples was related to the mycoides cluster and highly similar to the *M. mycoides* subsp. *mycoides* LC and SC (98% similarity; data not shown). Yet, given the high similarity (up to 97%) between these two subspecies, it was inaccurate to clearly identify the *Mycoplasma* species or subspecies based on a 16S rRNA sequence comparison.

From these data, we could predict that our samples were most likely contaminated with a mycoides SC or LC subtype; therefore, we set up and performed specific PCR assays for the identification of these subtypes. The data are summarized in *table III*. When PCR was carried out using the MSC1/MSC2 primer set, only samples from the affected bulls were found positive, indicating that the mycoplasma contaminating these samples was *M. mycoides* subsp. *mycoides* SC. Sequencing of PCR products confirmed our results (data not shown).

In addition, when PCR was carried out using the MM450/MM451 primer set, all samples were found to be positive (*table III*). Digestion of the PCR products from samples from bulls B, C and D with *Ase*1 yielded two bands allowing the identification of subsp. *mycoides* SC and thus confirmed the previous identification. Furthermore, the digestion of PCR products from samples from bulls F and G yielded three bands indicating that the *Mycoplasma* species contaminating these samples was *M. mycoides* subsp. *mycoides* LC. Identification of the LC subtype was confirmed by sequencing of PCR products (data not shown). Finally, in order to determine whether these samples were contaminated with other *Mycoplasma* species related to the *mycoides* cluster, such as *M. agalactiae* or *M. bovis*, we carried out a specific PCR for these two species. No amplification was obtained from our samples, thus ruling out this possibility (data not shown).

4. DISCUSSION

The aetiology of seminal vesiculitis has never been completely elucidated. Overfed and growing yearling bulls appear to be more susceptible to seminal vesiculitis, although some affected bulls have presented a history of pneumonia or septicemia. The bulls that were the object of our study had, however, no previous history of disease. Lowered fertility has been related to the seminal vesiculitis syndrome; however, young affected bulls may overcome the infection spontaneously [10].

Although the occurrence of such contaminants as *U. diversum*, *M. bovis* and *M. bovigenitalium* has been reported in the reproductive tract and bull semen, our study demonstrates semen contamination by *M. mycoides* subsp. *mycoides* SC in yearling bulls shown to be negative by the CBPP complement fixation test, as previously observed in Portugal by Gonçalves [14].

Within the last few years, *M. mycoides* cluster identification has been dramatically improved through the use of species-specific DNA probes and PCR. Several PCR assays have been previously described for the identification of *M. mycoides* subsp. *mycoides* LC [4] and SC [9]. The MSC1/MSC2 primer set described by Dedieu et al. [9] allows the specific identification of *M. mycoides* subsp. *mycoides* SC. On the contrary, the MM450/MM451 primer set described by Bashiruddin et al. [4] allows the specific detection of both *M. mycoides* subsp. *mycoides* SC and LC. Digestion of the PCR products using *Ase*1 makes a clear distinction between these two
subtypes. Therefore, we have used primer sets described in these studies to investigate the contamination of seminal fluid by the subspecies mycoides SC and LC. To ensure that no other contamination than mycoides, such as M. bovigenitalium and U. diversum, are present in our samples, 16S rRNA amplicons were cloned and several clones were sequenced from each and all gave the same result indicating that their mycoide was the only contaminant. However, this does not include contaminants that did not grow in the culture before PCR.

Away from the conventional serological diagnosis, we confirmed that M. mycoides subsp. mycoides SC is shed in sperm, allowing the ejaculate to be a valuable biological sample for the isolation of these bacteria from serologically negative animals.

M. mycoides subsp. mycoides LC contamination has been detected in naturally aborted bovine foetuses [19] and in frozen and fresh bull semen [22], as also observed in the present study. The experimental pathogenicity of M. mycoides subsp. mycoides LC has been demonstrated in vivo in the rat mammary gland and in vitro in rabbit and hamster organ cultures [20]. We need further studies, however, to elucidate the biological significance of this bacterium in the breeding bull reproductive efficiency.

Although the pathogenic role of M. bovigenitalium in bull seminal vesiculitis has been established, our clinical findings, semen characteristics, microbiological and bacterial genomic analysis, strongly suggest that M. mycoides subsp. mycoides SC may contribute to induce vesicular adenitis in the bull.

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Bull seminal vesiculitis by *M. mycoides* ssp. *mycoides* SC


