Assessment of PCR for routine identification of species of the *Mycoplasma mycoides* cluster in ruminants

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Abstract – DNA amplification techniques offer considerable promise for the identification of *Mycoplasma mycoides* cluster members. They avoid antigenic cross-reactivity and variability that hamper serological methods. Many sets of primers, specific of these different members and of *Mycoplasma putrefaciens*, have been proposed. To assess the reliability of some of these PCR tests in routine laboratory diagnostic use, 230 field strains supposed to belong to this group were simultaneously identified by PCR and an antigenic method. The results were well correlated to antigenic identification for *M. putrefaciens*, but PCR failed to identify respectively 74% and 52% of *M. mycoides* subsp. *mycoides* Large Colony type and *M. capricolum* subsp. *capricolum* strains. Any identification of *M. mycoides* subsp. *mycoides* Small Colony type must be confirmed by two different tests. Difficulties in defining the *M. species* bovine serogroup 7 were also encountered with both the PCR and immunological methods. The occurrence of putative variable antigen(s) on the mycoplasma surface may explain part of the identification difficulties encountered with the immunological methods.

*Mycoplasma mycoides* cluster / PCR / ruminants / identification / variable antigen

1. INTRODUCTION

The so-called *Mycoplasma mycoides* (M. *mycoides*) cluster [7] consists of very closely related mycoplasmas, belonging phylogenically to the spiroplasma group [35], and that are of great concern in ruminant production. This group comprises the following species, subspecies and types: *M. mycoides* subsp. *mycoides* Small Colony type (*MmmSC*), *M. mycoides* subsp. *mycoides* Large Colony type (*MmmLC*), *M. mycoides* subsp. *capri* (*Mmc*), *M. capricolum* subsp. *capricolum* (*Mcc*), *M. capricolum* subsp. *capripneumoniae* (*Mccp*), and *M. species* bovine serogroup 7 (*Msp7*). These organisms are known to produce various diseases in cattle, goats and sheep, the most prominent of which being Contagious Bovine Pleuropneumonia (CBPP) (causative agent: *MmmSC*) and Contagious Caprine Pleuropneumonia (CCPP) (causative agent: *Mccp*). CBPP is the most important animal disease in Africa and recently reappeared in Europe.
affecting several countries between 1980 and 1999 [22]. CCPP is currently causing major economical losses in Africa [31]. Both diseases are included respectively in the A and B lists of the International Office of Epizooties and are thereby subjected to international control measures.

Mcc, MmmLC and Mmc affect sheep and goats. Mastitis is usually the most prominent symptom but adults may also suffer from arthritis and keratitis, and kids may experience arthritis, pleuropneumonia and septicemia (MAKePS syndrome) [31].

Msp7 is as of yet an unassigned mycoplasma represented by strain PG50. Recent outbreaks of mastitis, arthritis and abortion in Australian dairy cattle [10] have attracted further attention to the pathogenic importance of this mycoplasma, until now sporadically isolated. M. putrefaciens (causing mastitis and arthritis), M. cottewii, and M. yeatsii (pathogenic activity unknown) [8] are three antigenically distinct species found in goats and are not regarded as members of the classical M. mycoides cluster. However phylogenetic analysis of the 16S rRNA genes has revealed that all three species belong to the M. mycoides cluster. M. cottewii is closely related to M. yeatsii (99.7% similarity), and that both show a 98.9% similarity with M. putrefaciens [15].

A precise diagnosis of these mycoplasmoses must be established, since the control strategies and consequences differ widely depending on the etiological agent, especially for CBPP and CCPP. Due to the small number of biochemical or physiological properties available in Mycoplasmas, the definition of species has been based mainly on serological methods. But classifying the members of the M. mycoides cluster has always been problematic: (i) Extensive and complex cross-reactions occur between subspecies or types, and acute antigenic identification of CBPP and CCPP agents has been achieved only through the use of specific monoclonal antibodies [4, 32]; (ii) High antigenic heterogeneity within some types or subspecies (MmmLC, Mcc) hampers identification [6, 18, 27]; (iii) The definitive taxonomy of the M. mycoides cluster has not yet been established and discrepancies have recently appeared between their taxonomy and phylogeny. Msp7 strain PG50 produces significant cross-reactions with Mcc and, to some extent, with MmmSC [13, 27], and should be phylogenetically included as a subspecies of the M. capricolum species [1, 14, 26, 33]. The 16S rRNA genes of Mmc and MmmLC are 99.9% similar, suggesting they should be considered as two phenotypes of the same species, distinct from MmmSC [21, 26]; (iv) Animal host specificity, previously thought as very specific and used as a clue to identification, has proven to be unreliable [23].

DNA amplification techniques offer considerable promise for the identification of M. mycoides cluster members. They avoid the antigenic cross-reactivity and variability that hinder serological methods, and they allow easier standardization between laboratories. As of today, arrays of primers specific to different members of the M. mycoides cluster and of M. putrefaciens have been proposed [1–3, 9, 13, 16, 19–21, 24, 28, 30]. But the reliability of these PCR tests needs to be assessed further before being routinely used.

With this goal in mind, we decided against using amplification techniques followed by an analysis of amplicon restriction profiles since these cumbersome techniques are incompatible with routine diagnosis.

To evaluate the selected primers, 230 field strains presumed belonging to, or closely related to, the M. mycoides cluster were identified using both PCR and antigenic methods.

2. MATERIALS AND METHODS

2.1. Strains

The PCR assays were evaluated on:

- 31 reference strains representative of the mycoplasma species isolated from ruminants (Tab. I);
PCR identification in the *M. mycoides* cluster

Table I. The 31 reference strains representative of the mycoplasma species, subspecies or types commonly and occasionally isolated from ruminants and used to test the specificity of selected primers.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference strain</th>
<th>Species</th>
<th>Reference strain</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. axanthum</em></td>
<td>S-743</td>
<td><em>M. canadense</em></td>
<td>275C</td>
</tr>
<tr>
<td><em>A. laidlawii</em></td>
<td>PG8</td>
<td><em>M. canis</em></td>
<td>PG 14</td>
</tr>
<tr>
<td><em>A. modicum</em></td>
<td>PG49</td>
<td><em>M. conjonctivae</em></td>
<td>HRC581</td>
</tr>
<tr>
<td><em>M. adleri</em></td>
<td>G-145</td>
<td><em>M. gallisepticum</em></td>
<td>PG 31</td>
</tr>
<tr>
<td><em>M. agalactiae</em></td>
<td>PG 2</td>
<td><em>M. ovipneumoniae</em></td>
<td>Y98</td>
</tr>
<tr>
<td><em>M. alkalescens</em></td>
<td>PG 51</td>
<td>*M. species ovine serogroup 11</td>
<td>2D</td>
</tr>
<tr>
<td><em>M. arginini</em></td>
<td>G230</td>
<td><em>M. verecundum</em></td>
<td>107</td>
</tr>
<tr>
<td><em>M. auris</em></td>
<td>UIA</td>
<td><em>M. species</em></td>
<td>3990</td>
</tr>
<tr>
<td><em>M. bovigenitalium</em></td>
<td>PG 11</td>
<td><em>U. diversum</em> serotype A</td>
<td>A 417</td>
</tr>
<tr>
<td><em>M. bovirhinis</em></td>
<td>PG 43</td>
<td><em>U. diversum</em> serotype B</td>
<td>D 48</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>PG45</td>
<td><em>U. diversum</em> serotype C</td>
<td>T 74</td>
</tr>
<tr>
<td><strong>M. mycoides subspecies</strong></td>
<td></td>
<td><strong>M. mycoides subspecies</strong></td>
<td></td>
</tr>
<tr>
<td><em>M. mycoides</em> subsp. <em>capri</em></td>
<td>PG 3</td>
<td><em>M. capricolium</em> subsp. <em>capricolium</em></td>
<td>California Kid</td>
</tr>
<tr>
<td>(Mmc)</td>
<td></td>
<td>(Mcc)</td>
<td></td>
</tr>
<tr>
<td><em>M. mycoides</em> subsp. <em>mycoides</em></td>
<td>Y Goat</td>
<td><em>M. capricolium</em> subsp. <em>capripneumoniae</em></td>
<td>F38</td>
</tr>
<tr>
<td>LC (MmMLC)</td>
<td></td>
<td>(Mccp)</td>
<td></td>
</tr>
<tr>
<td><em>M. mycoides</em> subsp. <em>mycoides</em></td>
<td>PG1</td>
<td><em>M. species</em> bovine serogroup 7</td>
<td>PG 50</td>
</tr>
<tr>
<td>SC (MmSC)</td>
<td></td>
<td>(Msp7)</td>
<td></td>
</tr>
</tbody>
</table>

**Species closely related to the M. mycoides cluster**

*M. putrefaciens* (Mp) | KS1
*M. cottewii* | VIS
*M. yeatsii* | GIH

*A. Acholeplasma; M. Mycoplasma; U. Ureaplasma.*

- 5 field strains, isolated from goats and bovines, unclassifiable by current immunological assays;
- 225 field strains, isolated from bovines, goats and sheep.

These field isolates were preliminarily identified by Membrane Filtration dot immunobinding (MF dot) [27] using polyclonal rabbit anti-sera against the reference strains of *M. mycoides* subsp. *mycoides* LC (Y Goat), *M. mycoides* subsp. *mycoides* SC (PG1), *M. mycoides* subsp. *capri* (PG3), *M. capricolum* subsp. *capricolium* (California Kid), *M. capricolum* subsp. *capripneumoniae* (F38), *M. species* bovine serogroup 7 (PG50), *M. putrefaciens* (KS1). These strains came from France (154); Portugal (19); Switzerland (5); USA (1); Italy (8); Greece (1); Spain (24); Africa (7); Australia (2) and 4 were of unknown origin. They were isolated between 1972–2001 from various anatomical sites (ear, lung, udder, joint, spleen, liver, kidney, eyes, vagina) or biological material (milk, pleural fluid, synovial fluid, sperm, lymph, uterine mucus).

The mycoplasma medium and growth conditions used were previously described by Poumarat et al. [27]. For the reliability of the study, genetic (PCR) and antigenic (MF dot) identification were simultaneously performed on the same subculture of these isolates.
2.2. Membrane Filtration dot immunobinding (MF dot)

Antigenic identification of the strains was performed by MF dot following the procedure described by Poumarat et al. [27]. Briefly, mycoplasmas from broth cultures were directly trapped onto the surface of low protein-binding affinity membranes by vacuum filtration. Then, specific polyclonal or monoclonal anti-mycoplasma sera were applied and the mycoplasma-antibody complexes were revealed by an enzyme conjugated anti-immunoglobulin. A positive reaction was indicated by the development of a color reaction when the substrate was added.

2.3. Oligonucleotide primers

Only primers amplifying sequences of known and presumably conserved genes were used. As a first step, the primers described by Persson et al. [24] (REAP), targeting highly conserved 16S rRNA genes, were used to characterize the strains belonging to the *M. mycoides* cluster. Subsequently, all 230 strains were tested using primers specific for the subspecies of *Mmm*SC (SC3 NEST), *Mm*LC, and *Mmc* (MMMLC), *Mps*7 (P67 BG7), *Mcc* (MCCPL), *Mp* (Ssf2, Ssr1) (Tab. II).

2.4. PCR reactions

Working temperatures were standardized based on the reference strains, to ensure perfect specificity of the primers with the reference strains of the *M. mycoides* cluster and affiliates (Tab. II). Following centrifugation for 25 min at 12 000 rpm at 4 °C, the mycoplasmas were washed once in a Tris buffer (Tris 0.05 M; NaCl 0.2 M; pH 7.4), and resuspended in a lysis buffer (Tris-HCl pH 8.8 at 0.015 M; Tween 20 0.05%; proteinase K 0.24 mg/mL (Roche Diagnostic, Meylan, France)). The samples were then incubated for 1 h at 57 °C, followed by 15 min
PCR identification in the *M. mycoides* cluster

5°-TCTGGGTATTCAACCCATAT-3°, using an annealing temperature of 53 °C.

2.5.2. Insertion Sequence (IS) 1296 fingerprinting with non-radioactive hybridization

The strains were analyzed with respect to the polymorphism of distribution of the IS 1296 on their chromosome. Fingerprints were obtained by Southern blot with a specific IS 1296 probe, following the procedure described by Frey et al. [12]. After extraction, DNA of each Mycoplasma strain was digested with the restriction enzyme *Hind*III, separated electrophoretically on a 0.8% agarose gel, transferred onto positive charged nylon membrane and denaturated using standard protocols. IS 1296 DNA was labeled by hybridization with an IS 1296 specific probe labeled with digoxigenin-11-dUTP (Boehringer-Mannheim, Paris, France).

2.5.3. PCR restriction endonuclease analysis based on the 16S rRNA gene

This analysis was performed as described by Persson et al. [24] using the primers REAP (Tab. II). This PCR amplifies a segment in the 16S rRNA genes of the two operons *rrnA* and *rrnB* specific for species belonging to the *M. mycoides* cluster. To differentiate *MmmSC* from the other members of the *M. mycoides* cluster, the PCR products were digested with *Alu*I and analyzed by agarose gel electrophoresis. Due to the polymorphism between *rrnA* and *rrnB* operons, the biotype *MmmSC* lacks one of the *Alu*I restriction sites. Thereby, *Alu*I restriction of the amplicon from *MmmSC* gives a 370 bp fragment in addition to the five fragments that are shared by all members of the *M. mycoides* cluster.

2.5.4. Distribution of vmm-like genes on the chromosome

The occurrence and distribution of the *vmm*-like genes was assessed by Southern blot hybridization with a *vmm* gene family.
specific probe using the procedure described by Persson et al. [25]. Chromosomal DNA was digested with the restriction enzyme HindIII and the fragments were separated by electrophoresis on 0.8% agarose gels. After southern blotting, a hybridization with the digoxigenin-labeled 5G1-insert probe [25] was carried out on the membrane.

2.5.5. Characterization of mycoplasmas by PCR and sequence analysis with universal 16S rRNA primers

These analyses were performed on 20 field strains following the standard procedures described by Johansson et al. [17]. Briefly, in vitro amplification of the 16S rRNA genes was performed by seminested PCR, using the four universal primers (U1 (F), U8 (R), U2 (F), U5 (R)) described by Johansson et al. [17], and was followed by sequence determination of the PCR products (Genome Express, Meylan, France). Evaluation of sequence data was performed on-line using a Basic Local Alignment Search Tool.

2.6. Colony immunostaining

A strain with the double serological valence Mcc and Msp7 (Afssa 11670) and a strain with the double serological valence MmmLC and Mmc (Afssa 4149) were cloned and analyzed with colony immunostaining following the technique described in Persson et al. [25]. Briefly, circles of nitrocellulose membrane filters (0.45 µm pore size; Schleicher & Schuell, Ecquevilly, France) were placed on mycoplasma colonies on the surface of agar plates and were left for 5 min. They were then gently removed and placed in Petri dishes with transferred colonies facing up. Colony blots were immunostained as described by Persson et al. [25] using polyclonal antibodies anti-Msp7 (reference strain PG50) for the Afssa strain 11670 and anti-Mmc (reference strain PG3) for the Afssa strain 4149. Positive colonies (specific staining) were revealed by an enzymatic reaction that gives a blue color (which appears dark in the figures). The membranes were then stained with a Ponceau S solution (Sigma), which unspecifically stains proteins red (grey on the figures), to reveal the negative colonies.

3. RESULTS

3.1. Antigenic identification of the field strains

Based on the MF dot results, the 230 field strains tested were classified as follows (Tab. III):
- 5 strains isolated from goats and bovines but not belonging to the M. mycoides cluster and currently serologically unclassifiable;
- 3 strains with atypical cross-reacting schemes with the different M. mycoides cluster polyclonal anti-sera used;
- 23 strains identified as MmmSC with a polyclonal sera (anti-PG1) and the monoclonal antibody 3F3;
- 65 strains with the double serological valence Mcc/Msp7 or strictly similar to the Msp7 strain PG50;
- 96 strains with the double serological valence MmmLC/Mmc;
- 38 strains of M. putrefaciens.

3.2. Specificity of the various primers relative to the reference strains of the main species isolated in ruminants

PCR-REAP, targeting the entire M. mycoides cluster, is specific. However, we found a weak amplification in M. conjunctivae (Fig. 1, lane 8), and an amplicon of different molecular weight in M. putrefaciens (Fig. 1, lane 7). With the annealing temperatures used (Tab. II), all other primers were found to be strictly specific for their respective subspecies or biotype with the exception of the MMMLC primers, which identify MmmLC and Mmc indistinctly.
3.3. Assessment of the primers on 230 field isolates (Tab. III)

3.3.1. Primers REAP for the M. mycoides cluster

Out of the 230 strains tested, 187 strains were positive, 33 yielded doubtful reactions, and 10 strains were negative. Four strains out of the 187 PCR-REAP positives were not classified antigenically in the *M. mycoides* cluster (3 *Mp* and 1 unclassifiable), yielding a relative PPV of 98%.

Thirty three strains, most of which were antigenically *Mp* (29/33), were also amplified but the amplicon size differed, allowing for easy differentiation. Among the 187 antigenic strains of the *M. mycoides* cluster, four were not detected by the REAP primers (three were antigenically classified *MmmmLC/MMc* and one is atypical). This resulted in a relative intra-species sensitivity of 98%.

The repeatability of the results obtained from the primers was good with the strains from the *M. mycoides* cluster, but average with the *Mp* strains.

3.3.2. Primers SC3 NEST for MmmmSC

Amongst the 230 field isolates tested, 23 strains yielded a positive reaction, 13 a doubtful reaction, and 194 were negative. Taking in account the doubtful strains, the relative PPV was 64%. The 23 PCR-SC3 NEST positive strains were antigenically of the *MmmmSC* biotype. The 13 strains which
yielded unclear to very ambiguous reactions were not antigenically of the MmmSC biotype. They were subjected to the following additional tests (Tab. IV): (i) amplification using the inner primers SC3 VII/IV; (ii) analysis of a profile obtained using the distribution of the insertion sequence IS 1296 on their chromosome; (iii) analysis of the profile of the PCR-REAP products after digestion with the AluI endonuclease (Fig. 2); (iv) sequence analysis of the 16S rRNA genes. These different methods clearly established that the doubtful strains did not belong to the MmmSC biotype and were genetically and antigenically diverse (5 MmmLC/Mmc, 1 Mcc/Msp7, 3 Mp, 1 Msp7, 2 atypical and 1 unclassifiable).

The relative intra-species sensitivity was 100%. The repeatability of the results obtained with the primers was good with the positive strains but, with the doubtful strains, the intensity of the reaction was variable within the assays.

### 3.3.3. Primers P67 BG7 for Msp7

Nine of the 230 strains tested yielded a positive reaction with the P67 BG7 primers. Among these nine strains, the five strains belonging to the cluster of Mcc/Msp7 strains exhibited antigenic, IS1296 and vmm-like profiles that were strictly identical to that of the reference strain PG50. Based on these results, these five strains could be strictly related to the Msp7.

The four remaining strains, which were antigenically MmmLC/MMc, had no vmm-like genes (like all strains of the LC type) and had distinct IS1296 profiles from the PG50 reference strain. They were identified as MmmLC by sequence analysis of the 16S rRNA genes (Tab. V, strains Afssa 4234, 5475, 10253, 10277).

Thus, the relative PPV of P67 BG7 primers was estimated at 55%.

According to the great number of field strains antigenically intermediary to Mcc/Msp7, the relative intra-species sensitivity of this specific primer may have been missetimated. However, it is noteworthy that the five PCR-P67 BG7 positive strains corresponded to the five ones that were previously supposed strictly related to Msp7 (see above).

### 3.3.4. Primers MCCPL for Mcc

Out of the 230 strains tested, 31 were positive and 4 doubtful.

All the 31 positive strains were classified antigenically in the Mcc/Msp7 group, bringing the relative PPV to 100%. However, 3 antigenically classified MmmLC/Mmc strains were also amplified, but the difference in the size of the amplicons made them easily distinguishable.
**Table IV.** The results of sequencing and complementary methods used to identify the sample of 13 strains yielding doubtful reactions with PCR tests specific for the *MmmSC* biotype (SC3-NEST).

<table>
<thead>
<tr>
<th>Afssa strains number</th>
<th>Antigenic identification by MF dot (M. mycoides cluster)</th>
<th>Identification with primers REAP (M. mycoides cluster)</th>
<th>Identification with primers SC3-NEST</th>
<th>Identification by sequence analysis of the 16S rRNA genes</th>
<th>Identification with inner primers SC3 VI (MmmSC)</th>
<th>Profile obtained with the IS 1296 probe</th>
<th>Profile obtained by PCR REAP followed by restriction with <em>Alu</em>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>4117</td>
<td><em>Msp7</em> positive</td>
<td>doubtful</td>
<td>negative</td>
<td>nt</td>
<td>nt</td>
<td>negative</td>
<td>not <em>MmmSC</em></td>
</tr>
<tr>
<td>4119</td>
<td>atypical</td>
<td>doubtful</td>
<td>negative</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>4120</td>
<td>unclassifiable</td>
<td>negative</td>
<td>doubtful</td>
<td>nt</td>
<td>negative</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>10252</td>
<td><em>M</em></td>
<td>positive</td>
<td>doubtful</td>
<td>nt</td>
<td>negative</td>
<td>not <em>MmmSC</em></td>
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<tr>
<td>11164</td>
<td><em>M</em></td>
<td>doubtful</td>
<td>nt</td>
<td>negative</td>
<td>not <em>MmmSC</em></td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>11184</td>
<td><em>M</em></td>
<td>doubtful</td>
<td>nt</td>
<td>negative</td>
<td>not <em>MmmSC</em></td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>12586</td>
<td><em>Mcc/Msp7</em> positive</td>
<td>doubtful</td>
<td>nt</td>
<td>positive</td>
<td>not <em>MmmSC</em></td>
<td>nt</td>
<td>not <em>MmmSC</em></td>
</tr>
<tr>
<td>12587</td>
<td>atypical</td>
<td>positive</td>
<td>doubtful</td>
<td>nt</td>
<td>positive</td>
<td>nt</td>
<td>not <em>MmmSC</em></td>
</tr>
<tr>
<td>12645</td>
<td><em>MmmLC</em> positive</td>
<td>doubtful</td>
<td><em>MmmLC</em></td>
<td>positive</td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
</tr>
<tr>
<td>12649</td>
<td><em>MmmLC</em> positive</td>
<td>doubtful</td>
<td>nt</td>
<td>negative</td>
<td>not <em>MmmSC</em></td>
<td>nt</td>
<td>nt</td>
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<tr>
<td>12678</td>
<td><em>MmmLC</em> positive</td>
<td>doubtful</td>
<td><em>MmmLC</em></td>
<td>positive</td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
</tr>
<tr>
<td>12584</td>
<td><em>MmmLC</em> positive</td>
<td>doubtful</td>
<td>nt</td>
<td>positive</td>
<td>nt</td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
</tr>
<tr>
<td>12595</td>
<td><em>MmmLC/Mmc</em> positive</td>
<td>doubtful</td>
<td><em>MmmLC</em></td>
<td>positive</td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
<td>not <em>MmmSC</em></td>
</tr>
</tbody>
</table>

nt: not tested.
Out of the 65 strains that were classified antigenically in the Mcc/Msp7 group, only 31 strains were PCR-MCCPL positive. With the set of primers used in this study, 29 strains, among these 65 strains, remained unidentified and 5 were identified as Msp7. Thus, relative intra-species sensitivity was assessed at 48%. The repeatability of the results obtained with these primers was good.

### 3.3.5. Primers MMMLC for MmmLC/Mmc

Out of the 230 strains tested, 25 were positive, 2 doubtful, and 203 negative. All positive strains belong antigenically to the MmmLC/Mmc group (relative PPV of 100%).

Only 25 strains, among the 96 classified antigenically as MmmLC/Mmc, were PCR-MMMLC positive. The 71 negative or doubtful strains were not identified with the other primers, except for 4 strains identified as Msp7. Thus, relative intra-species sensitivity was assessed at 26%. The repeatability of the results from these primers was good.

### 3.3.6. Primers for M. putrefaciens

Out of the 230 strains tested, 44 were positive and 186 negative.

Among the 44 positive strains, 38 were classified antigenically Mp (relative PPV of 86%). The remaining six positives belonged either to the MmmLC/Mmc group (3/6), or to the Mcc/Msp7 group (3/6).

All strains classified antigenically as Mp were Mp positive by PCR (a relative intra-species sensitivity of 100%). The repeatability of the results from these primers was good.

### 3.4. Sequence analysis of genes coding for 16S rRNA

This analysis was performed on a sample of 16 strains representative of the discrepancies sometimes observed between the results of antigenic identification by MF dot and the results of genetic identification with the selected set of specific primers (Tab. V). In all cases (except two strains), sequence analysis confirmed the antigenic identification.
Table V. The results of sequence analysis of genes coding for 16S rRNA used to identify a sample of 16 strains showing discrepancies between their antigenic (MF dot) and genetic (PCR) identification.

<table>
<thead>
<tr>
<th>Afssa strains number</th>
<th>Identification with primers REAP (M. mycoides cluster)</th>
<th>Identification with the specific primers</th>
<th>Antigenic identification by MF dot</th>
<th>Identification by sequence analysis of the 16S rRNA genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3266</td>
<td>positive indeterminate</td>
<td>indeterminate</td>
<td>M. yeatsii</td>
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<td>Mcc</td>
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3.5. Colony immunostaining

Afssa strain 11670 was classified antigenically intermediary between Mcc and Msp7 and genetically Mcc by PCR and sequence analysis of the 16S rRNA genes. Afssa strain 4149 was classified antigenically intermediary between MmmLC and Mmc, genetically belonging to the M. mycoides cluster but unidentified by the set of primers used. However, it was identified as MmmLC by sequence analysis of the 16S rRNA genes.

For each of these two strains, colony immunostaining of a generation from a single cell showed phenotypic heterogeneity with respect to the expression of the “PG50” (Msp7) valence for Afssa strain 11670 (Fig. 3) and the expression of the “PG3” (Mmc) valence for Afssa strain 4149 (Fig. 4). On the contrary, no phenotypic heterogeneity was obtained using the rabbit hyper immune anti-California Kid (Mcc)
**Figure 4.** Colony immunostaining on the Afssa 4149 field strain after cloning. This strain was antigenically intermediary between Mmm LC and Mmc (MF dot), genetically belonging to the M. mycoides cluster, but unidentified by the set of primers used, and identified as Mmm LC by sequence analysis of the 16S rRNA genes. Immunostaining using a polyclonal antibody prepared on the reference strain Mmc PG3. (P) Positive colonies; (N) Negative colonies.

serum on Afssa strain 11670 and the rabbit hyper immune anti-Y goat (Mmm LC) serum on Afssa strain 4149.

**4. DISCUSSION**

With regards to routine diagnosis, the identification of mycoplasmas belonging to the M. mycoides cluster remains problematic using either serological techniques, such as MF dot, or genetic identification methods such as PCR. In the present study, performed on a large sample of field strains, genetic identification by PCR has not proven entirely satisfactory.

Amongst the 183 strains strictly belonging to the M. mycoides cluster (as identified by both antigenic MF dot and genetic PCR REAP methods), 95 strains could not be identified with the tested set of primers. Most of these 95 strains (n = 93) were antigenically intermediate, i.e. showing cross-reaction between Mcc and Msp7 or between Mmm LC and Mmc. We demonstrated that the specific primers to Mcc (MCCPL) and to Mmm LC/Mmc (MMMLC) have low intra-species sensitivity: 48% and 26% respectively. Target sequences of these primers, which were chosen on lppA genes, were probably not strictly conserved among these two subspecies.

Antigenic identification of mycoplasma species belonging to the M. mycoides cluster is hampered by the occurrence of field strains that were antigenically intermediate between some serotypes or subspecies. These intermediate profiles could not result from a mixture of species since all the strains were cloned before analysis.

Many field strains were found antigenically intermediary in the present study, either between the reference strains Mmm LC (Y Goat) and Mmc (PG3) (n = 96) or between the reference strains Mcc (California Kid) and Msp7 (PG50) (n = 65). It has been reported that Mmm LC and Mmc are antigenically very similar, as assessed by the numerical analysis of one-dimensional SDS-PAGE protein patterns [18]. Phylogenetic studies based on sequence analysis of 16S rRNA genes have shown that it is obviously impossible to genetically distinguish between the Mmm LC biotype and the subspecies Mmc (99.9% similarity) [26]. Therefore, Mmm LC and Mmc may represent two different phenotypes of a same and unique subspecies of the M. mycoides species [21]. The figures obtained by colony immunostaining on field strains Mmm LC/Mmc with a polyclonal sera anti-Mmc PG3, and illustrated by Figure 4, Afssa strain 4149, seem to corroborate this hypothesis. The mixture of positive and negative colonies demonstrated that two distinct phenotypes could simultaneously coexist within a same clonal lineage, one phenotype expressing the “PG3” valence and not the other. Moreover, sectored colonies (data not shown) were also noted on colony immunostaining. They are typical features encountered with surface antigens which undergo high-frequency phase variation; the immunostained sectors...
result from mutations that have induced ON/OFF switching of the target surface antigen during the colony growth [29]. Antigen variation is widely used by the mycoplasma in order to enhance colonization and to adapt to the host tissue environment at various stages of infection [5]. Thus the “PG3” valence may result from the expression of a variable surface antigen on strains genetically related to MmmLC. According to the relative proportion between both phenotypes in the cell population, a strain could be antigenically more or less intermediary between both MmmLC and Mmc phenotypes. This hypothesis would account for ambiguous results encountered in routine diagnosis. The same hypothesis may explain the high number of antigenically Mcci/Msp7 field strains. The results of colony immunostaining obtained on clonal lineage of field strains Mcci/Msp7 with a polyclonal sera anti-Msp7 strain PG50, and illustrated Figure 3, Afssa strain 11670, also show the coexistence of two phenotypes: one expressing the “PG50” valence (positive) and not the other (negative). Moreover, sectored colonies were observed, suggesting that the “PG50” valence could also be an antigen subjected to high frequency phase variations and expressed on the surface of strains genetically related to Mcci. Previous studies suggest that the antigenic valence “PG50” may be shared by different species of mycoplasma, including species outside of the M. mycoides cluster [27]. Nevertheless, unclassified M. species bovine serogroup 7 cannot be solely explained by a variably expressed antigen “PG50” which is shared by several different mycoplasma species. Among the field strains tested in this study, five strains were proven to be strictly identical to Msp7 strain PG50 by MF dot, by PCR and on the basis that these strains shared some strictly similar IS1296 and vmm Southern blot profiles. Most of the phylogenetic analysis [14, 25, 33], particularly based on the 16S rRNA sequences [26], place Msp7 strain PG50 as a sub-species of the M. capricolum species. However, other genetic particularities place Msp7 strain PG50 phylogenetically closer to the M. mycoides species. First, the subspecies of the M. mycoides species and Msp7 share highly conserved glycerol transport operons (gtsA, B and C) that are absent within the M. capricolum species [11]. Second, DNA sequence analysis of the lppA gene demonstrates that Msp7 may be clustered with the MmmSC type of the M. mycoides species [22]. All these observations illustrated the unclear phylogenetic and taxonomic position of the M. species bovine serogroup 7.

Identification of the MmmSC biotype is not as ambiguous. Target sequences of the SC3-NEST primers seem highly conserved within the biotype. However, amongst the 230 field isolates tested, 13 strains yielded reactions ranging from doubtful to very ambiguous. These doubtful strains were definitively confirmed as the non-MmmSC biotype by four other methods of genetic analysis (Tab. IV). They were antigenically and genetically diversified. In practice, these ambiguous reactions can severely reduce the reliability of the test, given the importance of finding the MmmSC biotype. Consequently, any identification of the MmmSC biotype by a single PCR test must be confirmed by another method.

Some discrepancies between the results of genetic identification by PCR and the results of antigenic identification by MF dot in this sample of 230 field strains were obvious. A number of problematic strains, along with strains that could not be identified with the various PCR tests, were further analyzed using sequencing of the genes coding for 16S rRNA (Tab. V). In all cases, sequence analysis confirmed the antigenic identification. Therefore, even if not entirely reliable, serological identification continues to be the most appropriate routine identification technique for strains from the M. mycoides cluster.

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REFERENCES


