Suppression-Subtractive Hybridization as a Strategy To Identify Taxon-Specific Sequences within the *Mycoplasma mycoides* Cluster: Design and Validation of an *M. capricolum* subsp. *capricolum*-Specific PCR Assay

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The phylogenetically related *Mycoplasma capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony are two small-ruminant pathogens involved in contagious agalactia. Their respective contributions to clinical outbreaks are not well documented, because they are difficult to differentiate with the current diagnostic techniques. In order to identify DNA sequences specific to one taxon or the other, a suppression-subtractive hybridization approach was developed. DNA fragments resulting from the reciprocal subtraction of the type strains were used as probes on a panel of *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony strains to assess their intrataxon specificity. Due to a high intrataxon polymorphism and important cross-reactions between taxa, a single DNA fragment was shown to be specific for *M. capricolum* subsp. *capricolum* and to be present in all *M. capricolum* subsp. *capricolum* field isolates tested in this study. A PCR assay targeting the corresponding gene (simpA51) was designed that resulted in a 560-bp amplification only in *M. capricolum* subsp. *capricolum* and in *M. capricolum* subsp. *capripneumoniae* (the etiological agent of contagious caprine pleuropneumonia). simpA51 was further improved to generate a multiplex PCR (multA51) that allows the differentiation of *M. capricolum* subsp. *capripneumoniae* from *M. capricolum* subsp. *capricolum*. Both the simpA51 and multA51 assays accurately identify *M. capricolum* subsp. *capricolum* among other mycoplasmas, including all members of the *M. mycoides* cluster. simpA51 and multA51 PCRs are proposed as sensitive and robust tools for the specific identification of *M. capricolum* subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae*.

The *Mycoplasma* genus is composed of wall-less bacteria with small genomes (0.58 to 1.35 Mb) and includes several species known to cause important diseases in humans and animals (24). Most of these species can be grown under laboratory conditions, although they require complex, undefined media and several days to several weeks of incubation because of their limited biosynthetic capacities (24). So far, mycoplasma identification and diagnosis have been based mainly on serological assays after cultivation, but recent ongoing efforts have been directed toward replacing these assays with more rapid and accurate molecular approaches based on the detection of specific DNA sequences.

For the veterinary field, members of the so-called *Mycoplasma mycoides* cluster are of particular concern, because they are important ruminant pathogens that are responsible for economic losses worldwide (1, 28). This cluster includes six taxa, which are phylogenetically closely related based on their 16S rRNA gene sequences (21); present similar serological patterns associated with important intertaxon cross-reactivity (12); and, consequently, are difficult to differentiate. Four taxa contain pathogens for small ruminants, with three, namely, *M. mycoides* subsp. *mycoides* biotype Large Colony, *M. mycoides*

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intrataxon sensitivity of only 48 and 26% for *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony, respectively (12).

Designing PCR assays that would accurately identify *M. mycoides* subsp. *mycoides* biotype Large Colony (or *M. mycoides* subsp. *capri*) and *M. capricolum* subsp. *capricolum* would be particularly valuable, as both mycoplasma species induce CA in goats; CA is a disease of economic importance with a worldwide distribution (1, 28). *M. mycoides* subsp. *mycoides* biotype Large Colony (or *M. mycoides* subsp. *capri*) and *M. capricolum* subsp. *capricolum* also can be isolated from asymptomatic animals (25), and in the absence of a reliable identification assay, the exact contribution of each taxon to CA has yet to be evaluated. Finally, *M. mycoides* subsp. *mycoides* biotype Large Colony (or *M. mycoides* subsp. *capri*) and *M. capricolum* subsp. *capricolum* both can occur in the same clinical specimen, but in culture the presence of *M. capricolum* subsp. *capricolum* often is masked by *M. mycoides* subsp. *mycoides* biotype Large Colony, which grows faster. Adequate, specific PCR assays would overcome this problem. Recently, the alignment of *M. mycoides* subsp. *mycoides* biotype Large Colony (strain GM12) genome draft sequence with the *M. capricolum* subsp. *capricolum* California Kid sequence (GenBank accession no. NC_007633) showed 91.5% nucleotide identity across 76% of the California Kid genome, underlying the important relatedness of the two taxa (10) and the difficulty in identifying DNA sequences that would be specific to each taxon while also being evenly distributed in field isolates.

In this study, DNA sequences that diverge between *M. mycoides* subsp. *mycoides* biotype Large Colony and *M. capricolum* subsp. *capricolum* strains were enriched using suppression subtractive hybridization (SSH), a method that previously has been successful in identifying sequences specific to two related mycoplasma species, *Mycoplasma bovis* and *Mycoplasma agalactiae* (14). Sequences specific to the type strain of *M. capricolum* subsp. *capricolum*, namely California Kid, and *M. mycoides* subsp. *mycoides* biotype Large Colony, namely Y-Goat, were further identified, and their distributions were assessed in a panel of field isolates belonging to the *M. mycoides* cluster. Based on these data, a PCR assay was developed that identifies *M. capricolum* subsp. *capricolum* among members of the *M. mycoides* cluster.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Escherichia coli* strains DH5α (Invitrogen Ltd., Glasgow, United Kingdom) and XL1-blue MRF (Strategene, La Jolla, CA) were used for DNA manipulations. They were both grown at 37°C in Luria-Bertani broth supplemented with ampicillin (100 μg/ml) and tetracycline (15 μg/ml) when required.

*M. capricolum* subsp. *capricolum* type strain California Kid (ATCC 27343) was purchased from the Pasteur Institute Collection (Paris, France). *M. mycoides* subsp. *mycoides* biotype Large Colony type strain Y-Goat was kindly provided by E. Vilei (University of Berne, Switzerland). These two strains were used as the driver and tester for SSH experiments. *Mycoplasma* field isolates were selected from the Agence Française de Sécurité Sanitaire des Aliments (AFSSA) collection (22). *Mycoplasmas* were grown in PPLO broth (Difco, Le Pont-De-Claix, France) at 37°C under 5% CO₂ as previously described (23). Field isolates were cloned by filtering the isolates on a 0.22-μm filter, growing the filtrates on 1% agar PPLO medium, and randomly selecting a single colony. This procedure was repeated twice.

The distribution of the DNA sequences identified in this study was assessed in three sets of mycoplasma isolates and clones. Set 1 is composed of (i) type strains (see below) that represent *Mycoplasma* species usually isolated from small ruminants in Europe and (ii) clones derived from field isolates of *M. mycoides* subsp. *mycoides* biotype Large Colony, *M. capricolum* subsp. *capricolum*, and *Mycoplasma* sp. bovine group 7 from various origins and clinical contexts (for details, see Table 2). These clones were identified by the method of dot immunobinding on membrane filtration (MF-dot) with specific rabbit antisera (23), by the analysis of a segment of their 16S rRNA (8, 21), fusc (13), and nboB (26) genes, and by the PCR assays performed as previously published (17, 16, 17, 20). The stability of the clones was tested by MF-dot and pulsed-field gel electrophoresis analyses after five successive subcultivation steps into liquid medium. The type strains of set 1 were PG1 (NCTC 10114) for *M. mycoides* subsp. *mycoides* biotype Small Colony, PG2 (NCTC 10123) for *M. agalactiae*, TS205 (ATCC 19852) for *Mycoplasma* sp. ovine/caprine group 11, KSI (ATCC 15718) for *Mycoplasma putrefaciens*, TS1726 (ATCC 51346) for *Mycoplasma auris*, TS1874 (ATCC 51347) for *Mycoplasma cottonei*, TS1973 (ATCC 51346) for *Mycoplasma yaitii*, PG3 (NCTC 10137) for *M. capricolum* subsp. *capri*, TS1399 (ATCC 27948) for *Mycoplasma adleri*, TS1581 (ATCC 25834) for *Mycoplasma conjunctivae*, TS2230 (ATCC 23838) for *Mycoplasma arginini*, and PG50 (NCTC 10114) for *Mycoplasma* sp. bovine group 7.

Set 2 included 66 clones derived from field isolates that either (i) belong to the *M. mycoides* cluster (15 *M. mycoides* subsp. *mycoides* biotype Large Colony/M. *mycoides* subsp. *capri* isolates, 8 *M. mycoides* subsp. *mycoides* biotype Small Colony isolates, 3 *Mycoplasma* sp. bovine group 7 isolates, and 10 *M. capricolum* subsp. *capricolum* isolates); (ii) are closely related to this cluster (five *M. putre- faciens* isolates and four *M. yeastii* isolates); or (iii) are from different phyloge- netic clades (eight *M. agalactiae* isolates, seven *M. bovis* isolates, and six *M. arginini* isolates). All clones of set 2 were identified by MF-dot and fusc sequence analyses, which were shown to be efficient for species identification within the *M. mycoides* cluster and less expensive than 16S rRNA gene analysis (which requires the assembly of five individual sequences).

Set 3 included 28 noncloned isolates of *M. capricolum* subsp. *capricolum* obtained from bulk cultures of clinical specimens (from udder, joints, respiratory tract, etc.) and were identified only by MF-dot analysis.

Additionally, eight *Mycoplasma* sp. bovine group 7 and three *M. capricolum* subsp. *capri* pneumoniae strains (including the F39 *M. capricolum* subsp. *capri* type strain), kindly provided by F. Thiisgård (Clinique du Vétérinaire, French Agricultural Research Centre Working for International Development, Montpellier, France), were used in the course of PCR assay validation.

**SSH and construction of subtracted libraries.** SSH was performed as previously described using the RN48 (5'-AGACCTCTCCAGCCTCAAGGCGAAGCATTGGATTAACGGGAG) and RN46 (5'-AGACCTCTCAAGGCGAAGCATTGGATTAACGGGAG) adapters (15).

Genomic DNA of *M. capricolum* subsp. *capricolum* and *Mycoplasma* species usually isolated from small ru-"
TABLE 1. Primers used for PCR assays

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5’-3’</th>
<th>Targeted species or gene fragment(s)</th>
<th>Target sequence</th>
<th>Size (bp)</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-REAP</td>
<td>GAAACGAAATATACCGCATGAG</td>
<td>M. mycoides cluster</td>
<td>Segment in the 16S rRNA genes of the operons rmaA and rmaB</td>
<td>785</td>
<td>20</td>
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<tr>
<td>R-REAP</td>
<td>CCACTTGTGCGGTGTCAGGCGCTC</td>
<td>M. capricolum subsp. capricolum</td>
<td>lppA</td>
<td>1,356</td>
<td>16</td>
</tr>
<tr>
<td>MCCPL1-L</td>
<td>AGACCAAATAAGCCATCCA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCCPL1-R</td>
<td>CTTCCACGGTTTGGAATG</td>
<td>M. capricolum sp. bovine group 7</td>
<td>Gene encoding lipoprotein P67</td>
<td>1,500</td>
<td>7</td>
</tr>
<tr>
<td>P67BG7-L</td>
<td>GTGAATCCGATATATGCTCTT</td>
<td>Mycoplasma sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMMLC2-L</td>
<td>TAAGTATTGAAATGGCGGCAATAGTTTGCATTAAATGATGAA</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>lppA</td>
<td>1,050</td>
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<tr>
<td>MMMLC1-R</td>
<td>CTCTCTATATTCCTCGATAAA</td>
<td>M. mycoides cluster</td>
<td></td>
<td>781</td>
<td>13</td>
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<tr>
<td>F-fusA</td>
<td>TGAATAATTTTAGATGTTGGAAGAAGAAC</td>
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<td></td>
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</tr>
<tr>
<td>R-fusA</td>
<td>GGTATTAAAATAGTCTACCGATATGAA</td>
<td></td>
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<td></td>
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<tr>
<td>CAMproB-L4</td>
<td>CCAATTATATGGATCAAAT</td>
<td>Mycoplasmas</td>
<td>rpoB, encoding β-subunit of the RNA polymerase</td>
<td>527</td>
<td>26</td>
</tr>
<tr>
<td>Rpob-R</td>
<td>GGTCACGTTGNACCAT</td>
<td>M. capricolum subsp. capricolum</td>
<td>3′ Region of MCAP0862</td>
<td>560</td>
<td>This study</td>
</tr>
<tr>
<td>Sha51-F</td>
<td>TAAATAAAGAAGCAAGGAAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mccp-spe-F</td>
<td>CAGAAAATCCTGCTCCTGAAAC</td>
<td>M. capricolum subsp. capricornueae</td>
<td>ArcD gene of the arginine deiminase pathway</td>
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<td>27</td>
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<tr>
<td>Mccp-spe-R</td>
<td>ATACATTTTATACCTTTCACAG</td>
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<td></td>
</tr>
<tr>
<td>J24</td>
<td>ACCGGACGCGATCTCCTCGAAGC</td>
<td>Cloned subtracted fragments</td>
<td>Subtracted sequences</td>
<td>Variable</td>
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<tr>
<td>N24</td>
<td>AGGCAACTGTGCTACTCAGGAGGAAATTAATTGCTCATTAAAT</td>
<td>MCAP0862 gene and homologs</td>
<td>3′ Region of MCAP0862 including the simpA51 PCR target</td>
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<td>This study</td>
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<td>Var51-A1-F</td>
<td>ACTTTTTTCTACATCATC</td>
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<tr>
<td>Var51-A1-R</td>
<td></td>
<td></td>
<td></td>
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</table>

94°C for 30 s, 48°C for 15 s, and 72°C for 15 s, followed by a final extension for 5 min at 72°C.

The detection limit of the simpA51 and multA51 PCRs was assessed directly on crude DNA extracts prepared from Mycoplasma liquid cultures of M. capricolum subsp. capricolum California Kid alone or mixed with 10-fold-concentrated M. mycoides subsp. mycoides biotype Large Colony Y-Goat. Briefly, 1.5 ml of pelleted cultures were incubated in 50 μl of proteinase K lysis buffer (0.1 M Tris-HCl, pH 8.5; 0.05% Tween 20; 0.25 mg/ml proteinase K) for 1 h at 37°C. Proteinase K then was inactivated for 10 min at 95°C. Serial 10-fold dilutions of this lysate were made. Each dilution was further diluted to 1/30, and 1 μl was used as the template in the PCR assays. The number of CFU in liquid broth was estimated by plating serial dilutions of the original culture onto 1% PPLO agar medium. The robustness of the multiplex PCR for diagnosis purposes was assessed by performing PCR on bulk bacterial extracts (pelleted cultures heated for 5 min at 100°C) or directly on colonies picked from plated liquid cultures incubated for 2 days.

DNA hybridization. For dot blotting, 400 ng of genomic DNA from subtracted strains was denaturated at 100°C and spotted onto a Hybond N+ membrane after electrophoresis. Membranes were hybridized with DNA probes corresponding to PCR products labeled with the enhanced chemiluminescence direct nucleic acid labeler. Hybridization signals were revealed using detection systems (GE Healthcare, Chalfont St. Giles, United Kingdom) according to the manufacturer’s instructions. Briefly, membranes were incubated overnight at 42°C with 10 ng/ml of heat-denatured probe in hybridization buffer (0.5 M NaCl and a 5% concentration of the blocking agent in the buffer provided by the supplier). Membranes then were washed three times for 10 min in washing buffer (6 M Urea, 0.4% sodium dodecyl sulfate, 0.5% SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and were washed twice for 5 min in 2× SSC. The detection of hybridized probes was performed with a 1-min incubation in detection reagents and exposure (5 min for dot blots and 30 min for Southern blots) to Hyperfilm ECL.

Sequence analyses. Nucleotide sequence comparisons with databanks were performed with BLASTN (without a filter) through the National Center for Biotechnology Information (NCBI) resource (http://www.ncbi.nlm.nih.gov). Alignments and phylogenetic trees were realized with SeaView and Phylo_Win programs at the PBIL website (http://phil.univ-lyon1.fr). The redundancy of subtracted libraries was checked using the CAP3 sequence assembly program (http://phil.univ-lyon1.fr/cap3.php). We checked that the A51 target sequence was present in a single copy in the M. capricolum subsp. capricolum California Kid genome by in silico analysis using the Molligen website (http://cbi.labri.fr/outilsmolligen/home.php). The genome sequence of California Kid also is available at http://www.ncbi.nlm.nih.gov (GenBank accession no. NC_007633).

RESULTS

Selection and characterization of field isolates belonging to the M. mycoides cluster. To assess the specificity and the distribution of M. capricolum subsp. capricolum and M. mycoides subsp. mycoides biotype Large Colony sequences identified in this study, clones derived from a representative panel of field goat isolates belonging to the M. mycoides cluster were selected on the basis of their reactivity with taxon-specific antisera using the previously described MF-dot assay (see Materials and Methods). The set of clones is described in Table 2 and includes (i) 10 isolates of M. capricolum subsp. capricolum of which 2 (no. 10276 and no. 14141) also gave positive results with Mycoplasma sp. bovine group 7-specific serum; (ii) 12 M. mycoides subsp. mycoides biotype Large Colony isolates, (iii) 4 Mycoplasma sp. bovine group 7 isolates, and (iv) one clone (no. 4234) that could not be clearly identified.

These 27 cloned isolates were further characterized by PCR assays described as specific (i) for the M. mycoides cluster (20) and (ii) for each of the Mycoplasma sp. bovine group 7, M. capricolum subsp. capricolum, and M. mycoides subsp. mycoides biotype Large Colony taxa (7, 16, 17). The PCR results confirmed that all isolates belonged to the M. mycoides cluster and that the discrimination between M. capricolum subsp. capricolum and M. mycoides subsp. mycoides biotype Large Colony was problematic, as only 6/10 M. capricolum subsp. capricolum clones and 4/12 M. mycoides subsp. mycoides biotype Large...
TABLE 2. Identification of cloned field isolates of the *M. mycoides* cluster

<table>
<thead>
<tr>
<th>Field isolate no.</th>
<th>Clone</th>
<th>PCR&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Identification by:</th>
<th>Phylogenetic analysis using:</th>
<th>Isolate source information&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gpM LC MCCPL P67</td>
<td>gpM</td>
<td>16S rRNA gene</td>
<td>ftsA</td>
</tr>
<tr>
<td>1</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d + -</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
</tr>
<tr>
<td>2</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d + +</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
</tr>
<tr>
<td>3</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d - +</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
</tr>
<tr>
<td>4</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d - -</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
</tr>
<tr>
<td>5</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d + -</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capricolum</td>
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<td>6</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d + -</td>
<td>M. capricolum subsp. capricolum</td>
<td>M. capricolum subsp. capriculum</td>
<td>M. capricolum subsp. capriculum</td>
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<td>7</td>
<td>M. capricolum subsp. capricolum</td>
<td>+ d + -</td>
<td>M. capricolum subsp. capriculum</td>
<td>M. capricolum subsp. capriculum</td>
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<td>M. capricolum subsp. capricolum</td>
<td>+ d - +</td>
<td>M. capricolum subsp. capriculum</td>
<td>M. capricolum subsp. capriculum</td>
<td>M. capricolum subsp. capriculum</td>
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<td>9</td>
<td>M. capricolum subsp. capriculum</td>
<td>+ d + +</td>
<td>M. capricolum subsp. capriculum</td>
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<td>10</td>
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<td>11</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>+ + - d</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<td>12</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>+ at - d</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>+ at - d</td>
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<td>14</td>
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<td>+ + + d</td>
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<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<tr>
<td>15</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>+ - - d</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<td>16</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>+ d - d</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<tr>
<td>18</td>
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<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<tr>
<td>19</td>
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<td>M. mycoides subsp. mycoides biotype Large Colony</td>
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<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
<td>M. mycoides subsp. mycoides biotype Large Colony</td>
</tr>
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</table>
MF-dot analyses were performed as described earlier (23). PCR results are expressed as doubtful (d), atypical (at), positive (p), and negative (n).

Phylogenetic analyses were based on sequencing segments of the 16S rRNA (8, 21), fusA (26), and rpoB (21) gene.

Origin means the country from which the isolates were collected, followed by the department number for French cases to underline the diversity of origin in the French territory. a, arthritis; m, mastitis; p, pneumopathy; d, u, unknown; and t, type strain.

MF-dot positive for Mycoplasma sp. bovine group 7 isolates gave the expected amplicon, but two isolates (no. 5475 and no. 10277) that were PCR positive for Mycoplasma subsp. mycoides biotype Large Colony and one isolate (no. 4234) that was predominantly identified by MF-dot analysis also were positive. Finally, the three species-specific PCRs also gave atypical amplifications (i.e., multiple PCR products or amplicons of different size than expected) with isolates not targeted by the primers (Table 2).

To resolve the discrepancies observed between MF-dot and PCR results, three housekeeping genes known or proposed to be good markers of the Mycoplasma cluster phylogeny were partially sequenced. These genes were (i) the 16S rRNA gene (8); (ii) rpoB, which encodes the DNA-directed β subunit of the RNA polymerase (26); and (iii) fusA, which encodes an elongation factor (13). Phylogenetic trees constructed with 16S rRNA gene sequences failed to discriminate Mycoplasma capricolum subsp. capricolum from Mycoplasma sp. bovine group 7. The rpoB- and fusA-based phylogenetic trees gave more stringent subclustering of these two taxa and were in agreement with the MF-dot results. Interestingly, the rpoB phylogenetic tree revealed four subgroups among the Mycoplasma subsp. mycoides biotype Large Colony isolates (see isolates 1 to 4 in Table 2). Finally, trees constructed with rpoB, fusA, and 16S rRNA gene concatenated sequences showed the highest bootstrap values and were the most accurate in assigning a clone to a subcluster. The three clones identified as Mycoplasma subsp. mycoides biotype Large Colony or atypical by MF-dot analysis but as Mycoplasma sp. bovine group 7 by PCR assays turned out to be Mycoplasma subsp. mycoides biotype Large Colony by phylogeny analysis and were considered as such (Table 2).

Identification of DNA fragments specific to Mycoplasma subsp. capricolum California Kid or Mycoplasma subsp. mycoides biotype Large Colony Y-Goat. DNA sequences present in Mycoplasma subsp. capricolum California Kid and absent from Mycoplasma subsp. mycoides biotype Large Colony Y-Goat were enriched by SSH (SSH-A and SSH-B) and cloned into E. coli. After detailed analyses, 80 and 79 recombinant clones obtained from SSH-A and SSH-B, respectively, were selected that had a single DNA insert ranging from 100 to 1,000 bp. PCR products corresponding to each of these cloned, subtracted DNA fragments then were labeled and used as probes on a dot blot nylon membrane carrying the denatured tester and driver DNA. Hybridization results revealed that 27/80 probes (from SSH-A) and 40/79 probes (from SSH-B) reacted specifically with Mycoplasma subsp. capricolum California Kid DNA or Mycoplasma subsp. mycoides biotype Large Colony Y-Goat DNA, respectively, while the remaining probes recognized both species (Fig. 1A). These results were confirmed by Southern blot analysis as illustrated in Fig. 1B. Southern blot hybridization data further indicated that most California Kid-specific sequences occur as single copies in the genome. When two hybridizing fragments were observed, they were further shown to correspond after sequencing (see below) to (i) the presence of an EcoRI site within the hybridizing fragment, (ii) the presence of two paralogs (MCAP_0442 and MCAP_0561) in the California kid genome, and (iii) a concat-
enation during the cloning of two sequences nonadjacent on the chromosome.

Sequence analysis and intrataxon distribution of SSH-derived DNA fragments. The 27 and 40 DNA fragments described above that specifically hybridized with \textit{M. capricolum} subsp. \textit{capricolum} California Kid or \textit{M. mycoides} subsp. \textit{mycoides} biotype Large Colony Y-Goat, respectively, were sequenced. Sequences with an internal Sau3A site suspected to correspond to a concatenation of nonadjacent chromosomal fragments were excluded from the study. In total, 23 and 35 unique sequences corresponding to SSH-A and SSH-B, respectively, were used to search nonredundant data-bases using BLASTN algorithms. BLAST results indicated that 16 DNA sequences cloned from California Kid and 18 DNA sequences cloned from Y-Goat presented no or little identity with other mycoplasma sequences available in the databases and were, therefore, tester specific. To assess whether these sequences are good target candidates for diagnosis, their specificity and distribution were tested using a panel of cloned clinical isolates and type strains representing mycoplasma species usually isolated from small ruminants (Table 2). This was performed by dot blot hybridization using each tester-specific sequence as a probe. Results showed that only one (A51) out of the 16 probes specific for the California Kid strain reacted exclusively with all \textit{M. capricolum} subsp. \textit{capricolum} strains (Fig. 2). Of the remaining, 10 recognized some \textit{M. capricolum} subsp. \textit{capricolum} field strains, but not all, and 5 reacted with all \textit{M. capricolum} subsp. \textit{capricolum} field strains but also with other field isolates belonging to different mycoplasma species (four probes also recognized \textit{Mycoplasma} sp. bovine group 7; one probe recognized \textit{M. mycoides} subsp. \textit{mycoides} biotype Small Colony type strain PG1) (data not shown). In contrast, only 1 out of 18 Y-Goat-specific sequences hybridized with all \textit{M. mycoides} subsp. \textit{mycoides} biotype Large Colony isolates; however, it also cross-reacted with all \textit{M. capricolum} subsp. \textit{capricolum} and \textit{Mycoplasma} sp. bovine group 7 field strains as well as with the \textit{M. cottewii} and \textit{M. mycoides} subsp. \textit{capri} type strains.

Design of the \textit{M. capricolum} subsp. \textit{capricolum} PCR assay based on the MCAP0862 gene. Sequence alignments revealed that the entire A51 probe (394 bp) was identical to the 3'-terminal region of the MCAP0862 gene of California Kid and showed no significant identity with any other sequences from GenBank. The MCAP0862 gene is 2,253 bp long and codes a putative membrane protein with one transmembrane segment in the N-terminal region and four leucine zipper motifs. The MCAP0862 product belongs to a cluster of orthologous groups of ATPases involved in DNA repair (L-COG0419), but its exact function has not been explored so far.

Two primers were designed to specifically amplify a 570-bp DNA fragment from MCAP0862 with one primer, A51-R, located within the A51 fragment (Fig. 3). Using the Molligen database, we confirmed that the target sequence was present as a single copy in \textit{M. capricolum} subsp. \textit{capricolum} California Kid and was absent from other sequenced mollicutes. The PCR assay was performed using clones from set 1 as the DNA
Mycoplasma capricolum subsp. capripneumoniae sequences matched those of the M. capricolum subsp. capricolum MCAP0862 gene with 90% nucleotide identity. We therefore shortened the A51-F and A51-R primers (21-nucleotide primers designated ShA51-F and ShA51-R) to combine our PCR assay (simpA51) with that designed by Woubit et al. (27). As illustrated in Fig. 4, the resulting multiplex PCR assay (multA51) yields a 560-bp amplicon with both M. capricolum subsp. capricolum and M. capricolum subsp. capripneumoniae strains with an additional fragment of 316 bp specific for M. capricolum subsp. capripneumoniae strains.

Validation of the simplex and multiplex PCRs as diagnostic assays. Both simpA51 and multA51 PCR assays, run as described in Table 1, were validated using strains from set 1 with some additional Mycoplasma sp. bovine group 7 and M. capricolum subsp. capripneumoniae strains as mentioned above. The specificity of both simpA51 and multA51 PCR assays was assessed on a second set of cloned field strains from different species belonging or related to the M. mycoides sp. bovine group 7 (dots 22 to 25) as listed in Table 2 and from type strains (dots t1 to t12) as listed in Materials and Methods. Dot t13 corresponds to M. capricolum subsp. capricolum California Kid, and dot t14 corresponds to M. mycoides subsp. mycoides biotype Large Colony Y-Goat.

FIG. 3. Localization of the A51 sequence within the M. capricolum subsp. capricolum California Kid MCAP0862 gene. The MCAP0862 gene context is shown in the upper part of the figure by open arrows. The solid line represents the MCAP0862 gene coding sequence in which the A51 probe is located (gray box). Black arrows indicate ShA51-R and ShA51-F primers, and gray ones indicate the varA51-F and varA51-R primers used for the analysis of polymorphisms. Numbers represent nucleotide positions in the California Kid genome. Mcc, M. capricolum subsp. capricolum.
the template. For both PCR assays, the amplification reaction was not affected by the presence of *M. mycoides* subsp. *mycoides* biotype Large Colony (data not shown), and a PCR product was obtained up to a dilution of 10^-6, which corresponded to a limit of detection of 4 CFU per reaction (Fig. 5B).

**DISCUSSION**

The small-ruminant pathogens *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony are two members of the *M. mycoides* cluster, and both are involved in CA. Because of the economic importance of CA, control strategies have been developed that are based mainly on the eradication of the infection by an accurate detection of the CA causative agents. This goal can be achieved by two recently developed PCRs, one specific for *M. agalactiae* (14) and the other one globally targeting the members of the *M. mycoides* cluster (28). However, the identification of an isolate to the taxon level is important in order to survey the individual prevalence of taxa and the emergence of new molecular types (18). So far, PCR assays developed to distinguish *M. capricolum* subsp. *capricolum* from *M. mycoides* subsp. *mycoides* biotype Large Colony in a culture-independent manner were shown to be poorly specific (12). In this study, we applied an SSH approach to detect molecular differences existing between *M. capricolum* subsp. *capricolum* and *M. mycoides* subsp. *mycoides* biotype Large Colony type strains, with the ultimate goal of identifying candidate sequences for the design of taxon-specific PCR assays. The high relatedness of the subtracted strains and their very low %G+C content (24% for California Kid and an estimated 24% for Y-Goat [3]) were limiting factors for efficient SSH, and indeed only 34% (27/80) and 50% (40/79) of the subtracted fragments were shown to be specific for *M. capricolum* subsp. *capricolum* California Kid (SSH-A) and *M. mycoides* subsp. *mycoides* biotype Large Colony Y-Goat (SSH-B), respectively. Although only a part of the subtracted fragments was analyzed here, several fragments were found to overlap or to locate in the same genes, suggesting that they were representative of the overall genomic differences existing between Y-Goat and California Kid.

Subtracted sequences that were specific to the *M. mycoides* subsp. *mycoides* biotype Large Colony type strain Y-Goat were not found in some *M. mycoides* subsp. *mycoides* biotype Large Colony field isolates or cross-reacted with other members of the *M. mycoides* cluster. This result indicates that fragments obtained by SSH represent sequences that are highly variable not only between taxa but also within one taxon, such as insertion sequences (IS). This is in agreement with recent data obtained from the *M. mycoides* subsp. *mycoides* biotype Large Colony GM12 genomic draft sequence, which shows 91.5% identity with 76% of the *M. capricolum* subsp. *capricolum* California Kid genome, while the remaining 24% of the genome is composed mainly of IS (10). Thus, *M. mycoides* subsp. *my-
subsp. mycoides

context, the genome description of the currently sequenced
the evolution of these organisms over the past 10 years. In this
in isolation and identification methods, they also may reflect
M. mycoides biotype Large Colony was isolated from the eyes as
M. capricolum

SSH-B-subtracted fragments tend to indicate that the recent
strains from France may have diverged from a common ances-
ty that was isolated in 1956 in Australia and that of current field
assay, simpA51, for the identification of M. capricolum subsp.
capricolum within the M. mycoides cluster. The validation of
the specificity of the simpA51 assay was addressed using strains
from the M. mycoides cluster with a greater emphasis on M.
mycoides subsp. mycoides biotype Large Colony, which is the
Mycoplasma taxon most frequently recovered from small-rumin-
ant clinical samples in France (25), and Mycoplasma sp.
bovine group 7, the closest phylogenetic relative of M.
capricolum subsp. capricolum, which often gives cross-serological
reactivity with M. capricolum subsp. capricolum in diagnoses
derived from MF-dot analyses (12). The simpA51 PCR assay,
performed with 133 representative strains and isolates of M.
mycoides (48 M. capricolum subsp. capricolum strains and 85
non-M. capricolum subsp. capricolum strains), gave no ambigu-
ous results. This contrasts with the previously developed PCR
assay that targeted the lppA gene (16), which previously was
shown to be of poor specificity on field isolates (12) and which,
in our hands, gave false-negative results 40% of the time (Ta-
ble 2). One drawback of the A51 PCR assay is that it does not
discriminate the two M. capricolum subspecies, namely M.
capricolum subsp. capricolum and M. capricolum subsp. capri-
pneumoniae. In the alignment of the specific sequence targeted
by A51 PCR from M. capricolum subsp. capricolum and M.
capricolum subsp. capripneumoniae type strains, 90% identity
was obtained, underlining the high genetic relatedness of the
two subspecies. We solved this problem by multiplexing the
A51 PCR with the PCR described by Woubit et al. (27) to
exclude any non-M. capricolum subsp. capricolum isolates. Ini-
ially we did not include M. capricolum subsp. capripneumoniae
strains in the set of strains used for the A51 PCR validation,
Since CCPP is regarded as an exotic pathology raging in some

FIG. 5. Analysis of the robustness of the multA51 PCR (A) and the
multA51 PCR amplifications obtained when bulk bacterial extracts or
capricolum strains were used as the template. (B) Shown are simpA51 and multA51 PCRs obtained on serial dilutions (from 10^{-2} to 10^{-6}) of crude
DNA extracts prepared from a liquid culture of M. capricolum subsp. capricolum California Kid. The indicated dilutions correspond to 4 × 10^{5}
CFU per reaction for 10^{-2} down to 4 CFU per reaction for 10^{-6}. Lane M, molecular mass standard (Bench Top 1-kb ladder; Promega); lane T-, negative control.

M. mycoides biotype Large Colony GM12, an isolate found in the
United States that is considered to be serologically and bio-
chemically equivalent to M. mycoides subsp. mycoides biotype
Large Colony Y-Goat (6), and M. capricolum subsp. capri-
colum California Kid are not only highly related but also differ
mainly in mobile, repeated elements, such as IS. In contrast,
the overall specific genetic content of the Y-Goat type strain
that was isolated in 1956 in Australia and that of current field
strains from France may have diverged from a common ances-
tor. Indeed, hybridization patterns obtained in this study with
SSH-B-subtracted fragments tend to indicate that the recent
M. mycoides subsp. mycoides biotype Large Colony field iso-
lates are not very similar to the Y-Goat type strain, since only
1 probe out of 18 reacted with all M. mycoides subsp. mycoides
biotype Large Colony field strains while 10 probes reacted with
Mycoplasma sp. bovine group 7 or M. capricolum subsp. capri-
colum. This observation is in agreement with the evolution of
the clinical signs observed over time. In 1983, Cottew proposed
a clear distinction of the body sites affected by
M. capricolum subsp. mycoides biotype Large Colony (respiratory tract) and
M. capricolum subsp. capricolum (joints and udder) (4), while
in 1996 DaMassa was less definite (5) and reported the isola-
tion of both M. mycoides subsp. mycoides biotype Large Colony
and M. capricolum subsp. capricolum from the mouth, udder,
joints, respiratory tract, and external ear; M. mycoides subsp.
mycoides biotype Large Colony was isolated from the eyes as
well. Although these differences might reflect an improvement
in isolation and identification methods, they also may reflect
the evolution of these organisms over the past 10 years. In this
context, the genome description of the currently sequenced M.
mycoides subsp. mycoides biotype Large Colony strain, a field
strain isolated in France in 1995 (and the subject of an ongoing
sequencing project by V. Barbe [Genoscope] and F. Thiaw-
court [CIRAD]), will be very informative.

Six of the subtracted sequences isolated following SSH-A
and specific for the California Kid type strain recognized all M.
capricolum subsp. capricolum field strains that were included in

set 1. This suggests that the California Kid type strain is more
representative of the M. capricolum subsp. capricolum field
isolates than is Y-Goat for M. mycoides subsp. mycoides bi-
type Large Colony field isolates. One fragment, A51, which
specifically reacted with all M. capricolum subsp. capricolum
strains used in this study, was further used to design a PCR
assay, simpA51, for the identification of M. capricolum subsp.
capricolum within the M. mycoides cluster. The validation of
the specificity of the simpA51 assay was addressed using strains
from the M. mycoides cluster with a greater emphasis on M.
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exclude any non-M. capricolum subsp. capricolum isolates. Ini-
ially we did not include M. capricolum subsp. capripneumoniae
strains in the set of strains used for the A51 PCR validation,
since CCPP is regarded as an exotic pathology raging in some
regions of Africa and the Middle East. However, a recent short communication showed that CCPP was getting closer to European frontiers, with recent outbreaks in Thrace, in the western part of Turkey (19). Therefore, the choice of using *simp*A51 or *multi*A51 PCR for diagnosis will be determined by the local epidemiology.

Very recently, we became aware that an ortholog to the *M. capricolum* subsp. *capricolum* MCAP0862 gene that contains the A51 sequence is present in an *M. mycoides* subsp. *mycoides* biotype Large Colony strain currently being sequenced (F. Thiaucourt, personal communication). With an overall identity between the two genes of 65%, the sequence divergence between the two orthologs still opens the way for the design and validation of taxon-specific PCR assays. This finding not only allows us to confirm the high specificity of the A51 PCR but also illustrates the efficiency of the SSH approach to amplify specific chromosomal fragments despite the high genetic relatedness of subtracted strains.

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