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Original article

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 (*Mmm*SC), *M. mycoides* subsp. *mycoides* Large Colony type (*Mmm*LC), *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: *Mmm*SC) and Contagious Caprine Pleuropneumonia (CCPP) (causative agent: *Mccp*). CBPP is the most important animal disease in Africa and recently reappeared in Europe,

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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, *Mmm*LC 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 phylogenic analysis of the 16S rRNA genes has revealed that all three species belong to the *M. mycoides* cluster, that *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 *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 (*Mmm*LC, *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 *Mccp* and, to some extent, with *Mmm*SC [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 *Mmm*LC are 99.9% similar, suggesting they should be considered as two phenotypes of the same species, distinct from *Mmm*SC [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);

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.

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

Table II. Sequence and annealing temperatures of the specific primers tested.

Primers designation	Primers sequences 5'-3'	Specificity	Annealing temperature	Number of cycles	Reference
F-REAP	-GAAACGAAAGATAATACCGCATGTAG-	*M. mycoides* cluster	59 °C	33	[24]
R-REAP	-CCACTTGTGCGGGTCCCCGTC-				
SC3NEST1-L	-ACAAAAAGAAGATATGGTGTTGG-	*M. mycoides* subsp. *mycoides* SC	53 °C	35	[19]
SC3NEST1-R	-ATCAGGTTTATCCATTGGTTGG-				
MCCPL1-L	-AGACCCAAATAAGCCATCCA-	*M. capricolum* subsp. *capricolum*	51 °C	33	[20]
MCCPL1-R	-CTTTCACCGCTTGTTGAATG-				
P67BG7-L	-GGTAATTCGAATAATGATCCT-	*M. species* bovine serogroup 7	46 °C	35	[13]
P67BG7-R	-TAAGTTTATTGAATTAAAGCG-				
MMMLC2-L	-CAATCCAGATCATAAAAAACCT-	*M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*	49 °C	30	[21]
MMMLC1-R	-CTCCTCATATTCCCCTAGAA-				
Ssf 2	-ACTGAGATACGGCCCAGAC-	*M. putrefaciens*	64 °C	25	[30]
Ssr 1	-ACTTGAGTCGCGGCGTCGA-				

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), *Mmm*LC and *Mmc* (MMMLC), *Msp7* (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

at 100 °C. The PCR reactions were carried out in a DNA thermal cycler (BioRad, Marnes la Coquette, France) in 50 µL reaction mix (0.2mM dNTP (Roche Diagnostic, Meylan, France); 10 mM Tris-HCl pH 8.3; 1.5 mM $MgCl_2$; 50 mM forward and reverse primers; 1.25 U *Taq*polymerase (Roche Diagnostic)) using 4 µL of the lysate as the template. The amplification was 25 to 35 cycles according to the primers and performed at the temperature defined for each species (Tab. II). The PCR amplification products were analyzed by gel electrophoresis on 1% agarose gels and visualized after staining with ethidium bromide on a U.V. transilluminator.

For each primer, the repeatability of the results was assessed by repeating the assays at least five times on the reference strains, 2 sets of 10 field-strains each (one positive set and one negative set), and all strains remaining doubtful or atypical after reclon-ing. Repeatability was estimated "good" when the intensity and profile of amplification were identically repeated within the assays. The PCR reactions were classified as positive (molecular weight and intensity amplicon identical to the reference strain), negative (no amplification), and doubtful (amplification inferior to the reference strain and/or electrophoretic profile of the amplicon different from the reference strain).

The conventionally defined ratios, positive predictive value (PPV = True Positive / True Positive + False Positive) and intraspecies sensitivity [34], were calculated from the PCR results obtained for each primers on the sample of 230 field strains simultaneously classified by a serological method (MF dot).

2.5. Additional tests for identification

2.5.1. Nested PCR for Mmm*SC*

Nested PCR analysis was performed as described by Miserez et al. [19] with the inner primers SC3VII: 5'-ATTAGGATTAGCTGGTGGAGGAAC-3' and SC3IV-S: 5'-TCTGGGTTATTCGAACCATTAT-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 *Mmm*SC 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 *Mmm*SC lacks one of the *Alu*I restriction sites. Thereby, *Alu*I restriction of the amplicon from *Mmm*SC 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 *Hind*III 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 *Mmm*LC 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 Pétri 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 *Mmm*SC 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 *Mmm*LC*/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. conjonctivae* (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 *Mmm*LC and *Mmc* indistinctly.

Table III. Results obtained on the 230 field isolates: (i) antigenic identification by MF dot; (ii) PCR assays with oligonucleotide primers REAP (*M. mycoides* cluster), SC3 NEST (*Mmm*SC), P67 BG7 (*Msp7*), MCCPL (*Mcc*), MMMLC (*Mmm*LC and *Mmc*), Ssf2, Ssr1 (*Mp*).

Identification by MF dot		Number of strains	Results of PCR assays with oligonucleotides primers																	
			REAP			SC3 NEST			P67 BG7			MCCPL			MMMLC			Ssf2-Ssr1		
			P	D	N	P	D	N	P	D	N	P	D	N	P	D	N	P	D	N
Unclassifiable strains[a]		5	1	1	3	0	1	4	0	0	5	0	0	5	0	0	5	0	0	5
M. mycoides cluster	Atypical[b]	3	2	1	0	0	2	1	0	0	3	0	1	2	0	0	3	0	0	3
	Mcc/Msp7[c]	65	65	0	0	0	2	63	5	0	60	31	0	34	0	0	65	3	0	62
	*Mmm*LC/*Mmc*[d]	96	93	2	1	0	5	91	4	0	92	0	3	93	25	1	70	3	0	93
	*Mmm*SC[e]	23	23	0	0	23	0	0	0	0	23	0	0	23	0	0	23	0	0	23
M. putrefaciens		38	3	29[f]	6	0	3	35	0	0	38	0	0	38	0	1	37	38	0	0
Total		230	187	33	10	23	13[g]	194	9	0	221	31	4	195	25	2	203	44	0	186

P: positive; D: doubtful; N: negative.
[a] Strains isolated from goats and bovines but not belonging to the *M. mycoides* cluster and serologically unclassifiable until now.
[b] Strains with an atypical cross-reacting scheme with the *M. mycoides* cluster polyclonal anti-sera used.
[c] Strains cross-reacting with the anti-*Mcc* (reference strain California Kid) and/or the anti-*Msp7* (reference strain PG50) polyclonal sera.
[d] Strains cross-reacting with the anti-*Mmm*LC (reference strain Y Goat) and/or the anti-*Mmc* (reference strain PG3) polyclonal sera.
[e] Strains identified as *Mmm*SC with polyclonal sera (anti-PG1) and monoclonal antibody 3F3 [4].
[f] Atypical amplification profile.
[g] Reactions range from doubtful to very ambiguous and vary in intensity with different assays.

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 *Mmm*LC/*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 Mmm*SC*

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 *Mmm*SC biotype. The 13 strains which

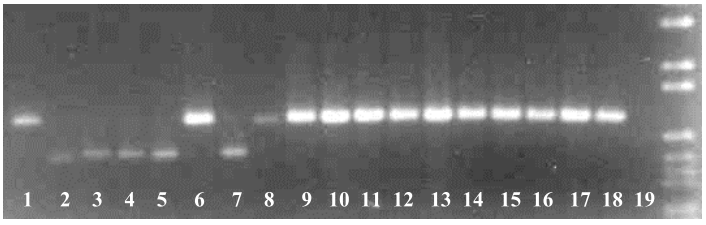

Figure 1. The results of PCR obtained on reference strains and field strains with REAP primers specific to the *M. mycoides* cluster. (i) Reference strains: Lane 7: KS1 *Mp*; Lane 8: HRC 581 *M. conjonctivae*; Lane 9: PG1 *Mmm*SC; Lane 10: PG50 *Msp7*; Lane 11: PG3 *Mmc*; Lane 12: Y Goat *Mmm*LC; Lane 13: California Kid *Mcc*. (ii) Field strains antigenically identified as *Mmm*LC: Lanes 1, 6, 14, 15, 17. (iii) Field strains antigenically identified as *Mp*: Lanes 2, 3, 4, 5. (iv) Field strains unclassifiable by MF dot: Lanes 16, 18. (v) Lane 19: negative sample.

yielded unclear to very ambiguous reactions were not antigenically of the *Mmm*SC 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 *Alu*I 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 *Mmm*SC biotype and were genetically and antigenically diverse (5 *Mmm*LC/*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, IS*1296* 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 *Mmm*LC/*Mmc*, had no *vmm*-like genes (like all strains of the LC type) and had distinct IS*1296* profiles from the PG50 reference strain. They were identified as *Mmm*LC by sequence analysis of the 16S rRNA genes (Tab. V, strains Afssa 4234, 5475, 10253, 10277).

Thus, the relative PPVof 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 misestimated. 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 *Mmm*LC/*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 *Mmm*SC biotype (SC3-NEST).

Afssa strains number	Antigenic identification by MF dot	Identification with primers REAP (*M. mycoides* cluster)	Identification with primers SC3-NEST	Identification by sequence analysis of the 16S rRNA genes	Identification with inner primers SC3 VI (*Mmm*SC)	Profile obtained with the IS *1296* probe	Profile obtained by PCR REAP followed by restriction with *Alu*I
4117	*Msp7*	positive	doubtful	nt	negative	nt	not *Mmm*SC
4119	atypical	doubtful	doubtful	*Msp11 / M. bovigenitalium*	negative	nt	nt
4120	unclassifiable	negative	doubtful	nt	negative	nt	nt
10252	*Mp*	positive	doubtful	nt	negative	nt	not *Mmm*SC
11164	*Mp*	doubtful	doubtful	nt	negative	not *Mmm*SC	nt
11184	*Mp*	doubtful	doubtful	nt	negative	not *Mmm*SC	nt
12586	*Mcc/Msp7*	positive	doubtful	nt	positive	nt	not *Mmm*SC
12587	atypical	positive	doubtful	nt	positive	nt	not *Mmm*SC
12645	*Mmm*LC	positive	doubtful	*Mmm*LC	positive	not *Mmm*SC	not *Mmm*SC
12649	*Mmm*LC	positive	doubtful	nt	negative	not *Mmm*SC	nt
12678	*Mmm*LC	positive	doubtful	*Mmm*LC	positive	not *Mmm*SC	not *Mmm*SC
12584	*Mmm*LC	positive	doubtful	nt	positive	nt	not *Mmm*SC
12595	*Mmm*LC/*Mmc*	positive	doubtful	*Mmm*LC	positive	not *Mmm*SC	not *Mmm*SC

nt: not tested.

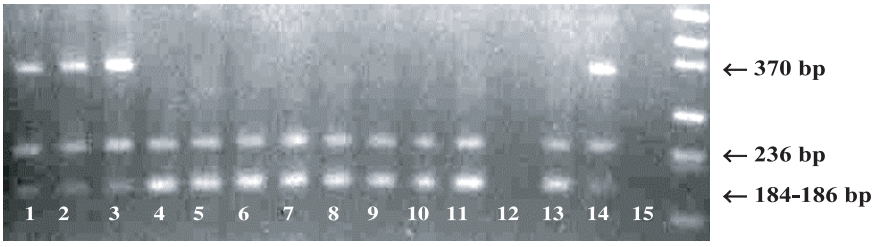

Figure 2. Comparison of the electrophoretic profiles of amplicons obtained by PCR-REAP after digestion with the *Alu*I endonuclease on reference strains of the *M. mycoides* cluster, on strains of the *Mmm*SC biotype and on field strains showing doubtful reactions with PCR SC3-NEST. (i) Reference strains of the *M. mycoides* cluster: Lane 4: PG50 *Msp7*; Lane 5: Y Goat *Mmm*LC; Lane 14: PG1 *Mmm*SC. (ii) Strains of the *Mmm*SC biotype: Lane 1: T_1SR; Lane 2: Afadé; Lane 3: Afssa 2091. (iii) Field strains antigenically other than the *Mmm*SC biotype and showing doubtful reactions with PCR SC3-NEST: Lanes 6, 7, 8: *Mmm*LC; Lane 9: *Mmm*LC/*Mmc*; Lane 10: atypical strain intermediate *Mmm*LC/*Msp7*; Lane 11: *Mcc/Msp*7; Lane 13: *Msp7*. (iv) Lane 12: *M. putrefaciens* (Cirad-COST-Tours 2-1972). (v) Lane 15: negative sample. (Double strand with fragments 81 bp and 98 bp not shown, common to all strains.)

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 Mmm*LC/Mmc*

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

Only 25 strains, among the 96 classified antigenically as *Mmm*LC/*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 *Mmm*LC/*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.

Afssa strains number	Identification with primers REAP (*M. mycoides* cluster)	Identification with the specific primers	Antigenic identification by MF dot	Identification by sequence analysis of the 16S rRNA genes
3266	positive	indeterminate	indeterminate	*M. yeatsii*
4234	positive	*Msp7*	*Mmm*LC/*Mmc*	*Mmm*LC
4348	positive	indeterminate	*Mmm*LC	*Mmm*LC
4369	positive	indeterminate	*Mcc/Msp7*	*Msp7*
4371	positive	indeterminate	*Mcc*	*Msp7* close to *Mcc*
4427	positive	indeterminate	*Mcc*	*Mcc* close to *Msp7*
5475	positive	*Msp7*	*Mmm*LC/*Mmc*	*Mmm*LC
5684	positive	indeterminate	*Mmm*LC	*Mmm*LC
10253	positive	*Msp7*	*Mmm*LC/*Mmc*	*Mmm*LC
10277	positive	*Msp7*	*Mmm*LC	*Mmm*LC
12583	positive	indeterminate	*Mmm*LC	*Mmm*LC
12590	positive	indeterminate	*Mcc*	*Msp7*
12596	positive	indeterminate	*Mmm*LC	*Mmm*LC
12607	positive	*Mp*	*Mmm*LC	*Mmm*LC
12648	positive	indeterminate	*Mmm*LC	*Mmm*LC
12652	positive	*Mcc*	*Msp7*	*Mmm*LC

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 *Mmm*LC and *Mmc*, genetically belonging to the *M. mycoides* cluster but unidentified by the set of primers used. However, it was identified as *Mmm*LC 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*)

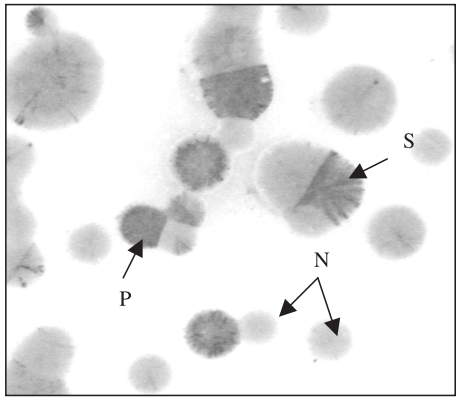

Figure 3. Colony immunostaining on the Afssa 11670 field strain after cloning. This strain is antigenically intermediary between *Mcc* and *Msp7* (MF dot) and genetically *Mcc* (PCR and sequence analysis of the 16S rRNA genes). Immunostaining using a polyclonal antibody prepared on the reference strain *Msp7* PG50. (P) Positive colonies; (N) Negative colonies; (S) Sectored colonies.

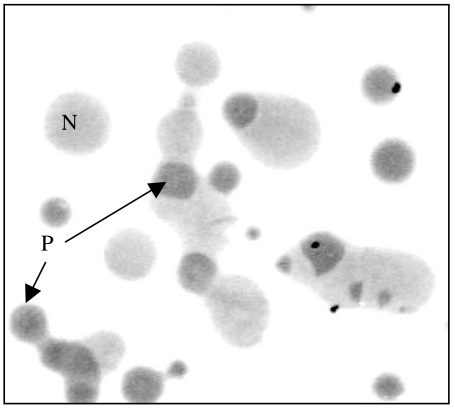

Figure 4. Colony immunostaining on the Afssa 4149 field strain after cloning. This strain was antigenically intermediary between *Mmm*LC and *Mmc* (MF dot), genetically belonging to the *M. mycoides* cluster, but unidentified by the set of primers used, and identified as *Mmm*LC by sequence analysis 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 intraspecies sensitivity: 48% and 26% respectively. Target sequences of these primers, which were chosen on *lppA* genes, were probably not strictly conserved among theses 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 *Mmm*LC. According to the relative proportion between both phenotypes in the cell population, a strain could be antigenically more or less intermediary between both *Mmm*LC and *Mmc* phenotypes. This hypothesis would account for ambiguous results encountered in routine diagnosis. The same hypothesis may explain the high number of antigenically *Mcc/Msp7* field strains. The results of colony immunostaining obtained on clonal lineage of field strains *Mcc/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 *Mcc*. 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 IS *1296* 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 *Mmm*SC 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 *Mmm*SC 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-*Mmm*SC 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 *Mmm*SC biotype. Consequently, any identification of the *Mmm*SC 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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