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5 # Further evidence to justify reassignment of *Mycoplasma mycoides*
6 # subspecies *mycoides* Large Colony type to *Mycoplasma mycoides*
7 # subspecies *capri*

8

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20 **Running title**: Taxonomy of *Mycoplasma mycoides*

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26 **Abstract**

27

28 Analysis, using the polymerase chain reaction (PCR), restriction enzyme

29 endonuclease analysis (REA), protein profile patterns, random amplification of

30 polymorphic DNA (RAPD) fingerprinting, 16S rRNA gene sequencing, and antisera

31 growth inhibition tests, of 22 strains of *Mycoplasma mycoides* subsp. *mycoides* Large

32 Colony type (MmmLC) and eight strains of *M. mycoides* subsp. *capri* (Mmc) is

33 presented, along with a summary of comparative data from the literature for over 100

34 strains, all of which supports the reclassification of the MmmLC and Mmc strains into

35 the single subspecies, *M. mycoides* subspecies *capri*.

36

37 **Keywords**: *Mycoplasma* taxonomy; *Mycoplasma mycoides* cluster; *Mycoplasma*

38 *mycoides* subspecies *capri*

39 ---

40

41 ## Introduction

42

43 While the animal pathogens now known as *Mycoplasma* have been studied for
44 more than a century [6,7], their affiliations and taxonomy did not begin to be resolved
45 until the 1950s [15,16]. Those early authors formalized the genus name *Mycoplasma*
46 Nowak (1928) [16], with *M. mycoides* as the type species [6,16,30]. The strains of
47 this species were classified into two subspecies, *M. mycoides* subsp. *mycoides*,
48 pathogenic to cattle, with strain PG1 as the representative strain, and *M. mycoides*
49 subsp. *capri*, causing infections in goats, with strain PG3 as the representative strain
50 [16]. Subsequently, *M. mycoides* subsp. *mycoides* (Mmm) was subdivided into two
51 morphotypes, one of which produced Large Colonies (MmmLC), and the other Small
52 Colonies (MmmSC), with strain PG1 being assigned as representative of MmmSC
53 [14]. Most strains of MmmLC and MmmSC were serologically indistinguishable
54 from each other by the growth inhibition test [2,44], but as well as their differing
55 growth characteristics, they were distinguished by their biochemical and physiological
56 properties, and by LC strains being goat pathogens, and SC strains causing disease in
57 cattle [13,14,44].
58 Many studies have shown that most strains of *M. mycoides* subsp. *mycoides*
59 (MmmLC) and *M. mycoides* subsp. *capri* (Mmc) are serologically distinct from each
60 other (see [2] for the earlier literature). Serological and metabolic studies of
61 numerous putative strains of each subspecies by Al-Aubaidi et al. [2] identified strain
62 PG3 as the neotype strain for Mmc, and proposed strain Y-goat as the representative
63 strain for MmmLC. Evidence has, however, accumulated for more than 30 years that
64 the serovars MmmLC and Mmc are actually very similar, perhaps taxonomically

65 identical [9,12,21,27,29,37,38]. This led increasingly to suggestions that the two
66 subspecies might be regarded as a single taxon [8,25,32,38,46], and to the formal
67 proposal that they should be amalgamated as strains of *Mycoplasma mycoides*
68 subspecies *capri* [28]. We provide new evidence to support this proposal, using
69 several taxonomic criteria, applied to 22 strains of MmmLC and eight strains of Mmc.
70 To date, the taxonomic evidence in the literature, and our new study, has been derived
71 from work on at least 112 strains (about 85 MmmLC and 27 Mmc), originating from
72 17 countries on several continents. We present new data on our 30 strains, 21 of
73 which have not previously been used in comparative studies, and summarize all the
74 key experimental evidence for the amalgamation of the two subspecies.
75

76 **Materials and methods**

77

78 *Mycoplasma* strains used in this study are listed in Table 1. All strains were
79 grown at 37°C in broth medium containing tryptose, yeast extract, glucose, glycerol,
80 heat-inactivated porcine serum, HEPES and fresh yeast extract [42]. *Mycoplasma*
81 DNA was extracted by the method of Bashiruddin [4]. The cluster-specific primers
82 MC323 and MC358, derived from the sequence of CAP-21 [5] were used for the
83 polymerase chain reaction on all the DNA samples.
84 Restriction endonuclease analysis (REA) of genomic DNA was used to assess any
85 differences between the strains. Digestion with endonucleases used 40 µl mixture
86 volumes containing 5-7 µg genomic DNA, with 10-40 units of the test endonuclease,
87 incubated at 37°C, 3 h. Enzymes tested were *Bam*HI, *Pst*I, *Bgl*I, *Alu*I, *Dra*, *Cla*I, *Sal*I,
88 *Sma*I, *Ava*I, *Vsp*I, *Eco*RI, *Dde*I, *Bsrs*I, *Bbu*I, *Bss*HII (all from Promega, Southampton,
89 UK), using the Web Cutter program (Max Heiman, Yale University). DNA fragments

90 were separated by electrophoresis in 1% (w/v) and 0.7% (w/v) agarose gels run for 18
91 h at 45V, respectively, then stained with ethidium bromide (0.4 mg ml^{-1}, 15 min), and
92 photographed under UV light. For each strain a control of undigested DNA was
93 subjected to electrophoresis to detect any extra chromosomal DNA: none was
94 detected.

95 RAPD (arbitrarily primed-PCR) fingerprinting, using the primer pair Mlip1 and
96 Mlip4, has been shown to assist in typing within the *M. mycoides* cluster [27,34]. The
97 methodology was essentially that of Rawadi et al. [34,35] using 50 µl reaction
98 volumes containing 400 ng *Mycoplasma* genomic DNA, 40 pmol of each
99 oligonucleotide primer, 200 nmol of each dNTP (Pharmacia ultra pure), and 2.5 U
100 Taq Gold polymerase (Applied Biosystems). Amplified products (20 µl) were
101 separated by electrophoresis at 110 V, 30 min, using 1% (w/v) agarose gels, and
102 bands visualized by UV fluorescence after staining with ethidium bromide.

103 16S rRNA gene sequencing used the method of Johansson et al. [22]. Sequences
104 were aligned and compared using the BioEdit programme package [19].

105 Serological differentiation by growth inhibition was based on the method of
106 Poveda and Nicholas [33], with antisera raised against Mmc $PG3^T$ and against two
107 separate strains of MmmLC (Y-$goat^R$ and F-30). Antisera (60 µl) were added to 6
108 mm wells in plates of agar medium, previously spread with dilutions of mid- to late-
109 exponential cultures. Diameters (mm) of zones of inhibition in the lawns of
110 mycoplasmas were measured after 24 h at 37°C.

111 Total cellular protein patterns were produced by SDS PAGE, using methods based
112 on Laemmli [23] and Costas et al. [12]. Electrophoresis was conducted in a Protean
113 double slab vertical electrophoresis cell (Bio-Rad, UK), run for 18 h at 40 V. Gels
114 were stained for 4 h with 0.1% (w/v) Coomassie brilliant blue in aqueous 10% (v/v)

115 acetic acid with 40% (v/v) methanol, and destained with 30% (v/v) methanol and 10
116 % glacial acetic acid (v/v) in distilled water for 12 h. Gels were scanned with a
117 Herolab E.A.S.Y. Enhanced Analysis System (Wiesloch, Germany).
118

119 Results and discussion

120

121 Numerous comparative criteria have been applied to more than 100 mycoplasma
122 strains by us and earlier workers (Table 2), which show that MmmLC and Mmc are in
123 fact essentially indistinguishable (Tables 2 and 3). Serological methods have been
124 widely used in the diagnosis of animals infected with members of the *Mycoplasma*
125 *mycoides* cluster, and is one approach that does enable some distinction of MmmLC
126 and Mmc strains (Table 3). Data presented cover the properties and analysis of their
127 DNA and proteins, as well as our work on their substrate utilization profiles [1,26,38].
128 New indicative data obtained by us apply to 30 strains, including 21 strains not
129 previously assessed (Table 1), and some tests previously applied to only a few strains
130 or to none at all.
131

132 PCR analysis and 16S RNA gene sequencing for the *M. mycoides* cluster

133

134 A single distinct and intense band of 1.5 kb was seen as expected after agarose gel
135 electrophoresis and ethidium bromide staining of PCR products from the 16S rRNA
136 gene from all 30 strains. Partial sequencing of the 16S rRNA gene products from the
137 strains showed >99% sequence identity among them [38] and full-length sequencing
138 (up to 1524 nucleotides) of the 16S rRNA gene from 17 of the strains (12 MmmLC
139 and five Mmc strains) showed all strains to be 99.9% identical to each other. Two

140 independent samples each of DNA from MmmLC strain Y-goat^{R} and Mmc PG3^{T}

141 were sequenced as internal controls to check the reproducibility of the method, and

142 were found to show 99.9% identity to GenBank reference sequences for strains of

143 both MmmLC (U26044, U26050) and Mmc (PG3^{T}; U26037). For MmmLC, one of

144 the two independently obtained sequences contained T at position 606 and C at

145 position 1447, as seen in the GenBank sequence for the *rrnB* gene from MmmLC Y-

146 goat^{R} (U26044). The other sequence had C and T at these positions, indicating it to be

147 for the *rrnA* gene (U26043), as reported by Pettersson et al. [32]. The base at

148 nucleotide positions 606 and 1447 in Mmc was C, as reported for the *rrn*A and *rrn*B

149 genes of Mmc [32]. These results confirmed that 16S rRNA gene sequencing is of

150 little use in distinguishing between strains of *M. mycoides*, as even the taxonomically

151 distinct *M. mycoides* subspecies *mycoides* Small Colony type (MmmSC) strains

152 showed 99.5% sequence identity to MmmLC and Mmc strains. Real-time PCR assays

153 were developed to discriminate between different subspecies within the *Mycoplasma*

154 *mycoides/capricolum* cluster [17]. These enabled the specific detection of MmmSC

155 but did not distinguish between strains of MmmLC and Mmc. The use of tRNA gene

156 fingerprinting [39], and DGGE fingerprinting of the V3 region of the 16S rRNA

157 genes [40] also showed a very close relationship between MmmLC and Mmc strains.

158

159 **Restriction enzyme analysis of 16S rRNA PCR gene products**

160

161 As expected from the sequencing results, all the MmmLC and Mmc strains gave

162 similar digestion patterns with six of the endonuclease enzymes tested (*Alu*I, *Cla*I,

163 *Hind*III, *Sau*3AI, *Rsa*I, *Dra*I), and thus did not differentiate the MmmLC and Mmc

164 strains from one another.

165

166 **Restriction endonuclease analysis (REA) of whole genomic DNA**

167

168 REA of the genomic DNA of the Mmm LC strains and Mmc strains with *Hind*III,
169 and *Pst*I showed DNA cleaved to produce a complex of 20-30 bands: patterns for
170 Mmc strains Pendik, BQT, G169, G105/A1, G108, and N108 were identical;
171 MmmLC strains 1141, FR1645, SP80, SP266 and Y-goat® formed one cluster with
172 60% similarity; strains Pendik, BQT, G169, G105/A1 and FR755 formed a cluster
173 with 65% similarity; and strains N108, G108, and JM formed another cluster showing
174 more than 85% similarity. Thus, this method did not allow discrimination between
175 the two subspecies. The profiles were highly reproducible when carried out in
176 duplicate with replicate and independent DNA extractions, and did not show any
177 changes after serial passaging *in vitro* for two of the MmmLC strains for 50, 60, 100
178 and 150 passages. No plasmids were detected on the agarose gel electrophoresis of
179 undigested DNA, showing that plasmid DNA did not contribute to the profiles.

180

181 **One-dimensional SDS-PAGE profiles of total cellular proteins**

182

183 All the strains tested (Table 2) showed very similar and highly reproducible
184 patterns of 15-25 polypeptide bands, but the patterns did not allow discrimination
185 between the MmmLC and Mmc strains (Table 2). All the strains formed cluster
186 groupings of 62-100% similarity, within which some pairs of MmmLC and Mmc
187 strains showed over 80% similarity, which exceeded the similarity between some
188 strains of each type individually. This is entirely consistent with the early
189 observations on other strains [12,36,37]. Serial passaging *in vitro* for two of the

190 MmmLC strains for about 150 generations did not produce any changes in the
191 patterns.
192
193 **Analysis of the MmmLC and Mmc strains using RAPD**
194
195 The RAPD technique using arbitrarily-primed PCR allows detection of specific
196 polymorphisms in the genomic fingerprints of related strains by amplification of
197 random segments of their genomic DNA, produced using random primer sets,
198 constructed without specific nucleotide sequence information [35]. RAPD using the
199 *M. mycoides* cluster-specific primers, Mlip1 and Mlip4 [34,35], produced diverse
200 genomic fingerprints showing high genomic polymorphism among the strains, but did
201 not differentiate between the subspecies. RAPD fingerprinting has previously been
202 shown to help distinguish between related bacterial strains better than multilocus
203 enzyme electrophoresis [47], and has proved useful for typing of different species of
204 mycoplasmas, including *M. pneumoniae* [45], *M. hyopneumoniae* [3], the *M.*
205 *mycoides* cluster [35], *M. gallisepticum* [18], and *M. bovis* [10]. It was, however,
206 clear that the high variation of genomic polymorphism within strains precluded
207 unequivocal separation of MmmLC and Mmc [35].
208
209 **Serological differentiation by growth inhibition tests**
210
211 As expected, the growth of most of 16 strains of MmmLC tested was not inhibited
212 by antiserum to Mmc, and five of six Mmc strains tested were not inhibited by either
213 of the MmmLC antisera (Table 3). MmmLC strains FR1645 and SP152 were
214 unaffected by any of the antisera; while MmmLC strain IT247 showed a 2 mm

215 inhibition zone with Mmc antiserum but no inhibition by either of the MmmLC
216 antisera. Mmc strain G169 was inhibited by both Y-goat^{R} and F-30 LC antisera (5
217 and 2.5 mm zones of inhibition), but was unaffected by the Mmc antiserum (Table 3).
218 Growth of MmmLC strain SP266 was depressed by both MmmLC and Mmc antisera,
219 suggesting it might be an intermediate strain. The affected MmmLC strains showed a
220 higher sensitivity to the Y-goat^{R} antiserum than to that for F-30. This diversity of
221 response has long been known, making the serological typing of a few MmmLC and
222 Mmc strains problematic [25].
223
224 **Disease profiles defining the "mycoides cluster"**
225
226 The *M. mycoides* cluster of mycoplasmas cause some serious diseases in
227 ruminants, the most severe of which are the notifiable contagious caprine
228 pleuropneumonia (CCPP), and contagious bovine pleuropneumonia (CBPP). CCPP
229 and CBPP are caused specifically by *M. capricolum* subsp. *capripneumoniae* and
230 MmmSC, respectively (for literature, see [28,31,46]). The most recently defined
231 distinct species in the cluster is *M. leachii* [28], the causative agent of mastitis and
232 polyarthritis in cattle [24,28]. This species, and *M. capricolum* subsp.
233 *capripneumoniae* and MmmSC, can be distinguished relatively unequivocally from
234 each other, and from MmmLC and Mmc, and each has a distinct disease profile.
235 MmmLC and Mmc cause disease almost exclusively in goats, with both producing
236 what has been described as the "MAKePS" syndrome by some workers, referring to
237 the mastitis, arthritis, keratoconjunctivitis, pneumonia and septicaemia seen in
238 affected animals [43]. The two subspecies cannot, however, be routinely

239 distinguished on the basis of host, pathology, virulence, or epidemiological impact,
240 thus further supporting the identity of these subspecies.

241 Our data all support the view that the MmmLC and Mmc strains of *Mycoplasma*
242 *mycoides* used by us and reported in other studies (Table 2) are representatives of a
243 single taxon, *M. mycoides* subspecies *capri*, only distinguishable serologically from
244 each other, with other strain differences being randomly distributed both within and
245 between the original MmmLC and Mmc designations. Many of these strain
246 differences are stable (e.g. REA and SDS-PAGE profiles, substrate oxidation kinetics;
247 Table 2; [38]), not being altered even after numerous generations in culture.

248

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251

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402 **Table 1.** Strains, and their sources, of *Mycoplasma mycoides* subsp. *mycoides* LC,
403 and *M. mycoides* subsp. *capri* used in this study[a]

404
405
406

Mycoplasma strains	Country of origin (and sources[b,c])
***Mycoplasma mycoides* subsp. *mycoides* LC**	
Y-goat® (NCTC 11706), 1141, 1164	Australia (1)
CH5, CH6	Chile (VLA)
FR755, FR1645	France (2)
SP80, SP152, SP266	Spain (VLA)
IT39se, IT247	Italy (3)
NZ67, NZ68	New Zealand (VLA)
PT994	Portugal (4)
GR50, GR51, GR52, GR55, GR59, GR60	Greece (VLA)
GM12	USA (VLA)
Mycoplasma mycoides* subsp. *capri	
JM	Australia (1)
Pendik, BQT, PG3^T (NCTC 10137)	Turkey (1)
N108	Nigeria (1)
G108	Kenya (1)
G105A1, G169	Brazil (1)

407–423

424 [a] Of the 22 strains of MmmLC, only Y-goat® seems previously to have been the subject
425 of direct comparison with Mmc strains. All the Mmc strains have previously been used
426 in some comparative studies (Table 2).

427 [b] 1, Dr D. Pitcher (deceased) and Dr R. Leach, Mycoplasma Research Facility, National
428 Collection of Type Cultures, CPHL, London, UK; 2, Dr M. Lambert, CNEVA,
429 Laboratoire de Pathologie des Petits Ruminants, France; 3, Dr J. Bashirudin, Instituto
430 Zooprofilattico Sperimentale, Teramo, Italy; 4, Dr J. Regalio, Laboratorio Nacional de
431 Veterinaria, Lisbon, Portugal.

432 [c] VLA – Strains from the collection of the Veterinary Laboratories Agency.

433

434 **Table 2.** Characteristics showing similarities between strains of *Mycoplasma mycoides* subspecies *mycoides* Large Colony type (MmmLC) and

435 *M. mycoides* subspecies *capri* (Mmc)

436

Line	Characteristic compared	Strains assessed		References
437–438		MmmLC	Mmc	
439				
440–441	PCR and 16S rRNA gene sequences	All 22 strains in Table 1	All 8 strains in Table 1	This study [19.30]
442–443		Y-goat, UM30847	PG3^{T} (NCTC 10137^{T})	[24]
444–446	Restriction endonuclease cleavage patterns of the 1.5 kb PCR product for 16S rRNA gene	All 22 strains in Table 1	All 8 strains in Table 1	This study
447–450	Restriction endonuclease cleavage patterns of the genomic DNA using *Hind*III and *Pst*I	Y-goat^{R}, 1141, FR755, FR1645 IT39, SP80, SP152, SP266, CH5, CH6, 1164	N108, Pendik, BQT, JM, G105/A1, G108, G169	This study
451–453	RAPD fingerprint analysis using Mlip1 and Mlip4	All 22 strains in Table 1	All 8 strains in Table 1	This study
454–455		Y-goat^{R}, GC 1177-2, 7730, Farcha	PG3^{T}, 88-117, L	[27]
456	16S-23S intergenic spacer region analyses	Y-goat^{R}	PG3^{T}	[13]

457				
458	Sequencing of the gene encoding the β-subunit	Y-goat^{R}, 152/93, LC8065, D2482/91,	PG3^{T}, N108, WK354/80,	[37]
459	of RNA polymerase (*rpoB*)	950010, D2083/91, CP271, D2503	213, 9139-11/91, capri L	
460				
461	Sequences for genes encoding concatenated	Y-goat^{R}, 9501-C1, 55507-1,	PG3^{T}, L, 2003-045-C2,	[20]
462	conserved proteins (*fusA, glpQ, gyrA, lepA, rpoB*)	Kombolcho, WK354	2002-054 (VP9L), N108	
463				
464	Coding sequences and restriction fragment	Y-goat^{R}, LC8065, D2503, D2482/91,	PG3^{T}, L, 9139-11/91,	[22]
465	analysis of lipoprotein LppA, and antigenic	D2083/91, B671/93, 266/94, 6P,	WK354/80, N108	
466	specificity of LppA	2/93, 152/93, 153/91, 80X3, 83/93,		
467		CP271, 9096-C9415, 8756-13, 8794-Inde		
468				
469	DNA-DNA hybridization	Y-goat^{R}	PG3^{T}	[8]
470				
471	DNA probe (CAP-21), sequencing,	Y-goat^{R}, KH1, Cov 2, LB2, 801,	PG3^{T}, BQT, YC, ZZ, N108	[33]
472	and Southern hybridization	M243/67, OSB42, EZG, F30		
473				
474	PAGE profiles of total cellular proteins	Y-goat^{R}, 1164, FR755, FR1645, CH5,	PG3^{T}, BQT, Pendik, G169,	This study
475		CH6, IT39, IT247, SP80, SP152, SP266,	G108, JM, N108	
476		PT994, NZ67, NZ68, G105/A1, GR50,		

477		GR60		
478				
479		Y-goat^R, $H_{22/1F}$, OSB42, KH1, ojo1,	PG3^T, 5907A, 5357L,	[9,13,18,28,29]
480		ojo2, 74/2488, Cov, F30 (M2055/75),	BQT, ZZ, 74/2907A	
481		74/2488, Cov 2, VR1/3172, LB2,	N108, YC, JM, Pendik,	
482		81.636.IC, GE.6A.79E, KH1,		
483		1217/77, GM12		
484				
485	Range and kinetics of substrates metabolized	All 22 strains in Table 1	All 8 strains in Table 1	[19,20]
486				
487		Y-goat^R, VR1, 74/2488, 81.636.1c,	PG3^T, N108, YC, ZZ,	[1]
488		GE.6A.79E, KH1, 78/441, 11041,	74.5907A, JM, BQT,	
489		11041, F30, ojo1, Cov 2, GM12, 977/79,	Pendik, G108/A2 clone(a),	
490		400/79, 755/80, 221/82, 1645/82,	G108/A2 clone(b), G108/A3,	
491		1729/82, 842/86	G105/A1, G169/Leite	
492				
493	Serological differentiation by growth	Y-goat^R, 1164, CH5, CH6, FR755	PG3^T, Pendik, BQT, G169,	This study
494	inhibition using antisera raised against	FR1645, SP80, P152, SSP206, IT39,	N108, G108A	
495	MmmLC and Mmc	IT247, NZ67, NZ68, PT994, GR50, GR60		
496				
497		Y-goat^R, OSB42, ojo1, Cov, Cov 2,	PG3^T, 74/2907A,	[18]
498		F30 (M2055/75),	BQT, ZZ, N108, YC, JM,	

499	74/2488, VR1/3172,	Pendik, G108/A2 (a) and (b)
500	81.636.IC, GE.6A.79E, KH1,	G108/A3, G169/Leite,
501	1217/77, GM12	G105/A1

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504

505 **Table 3.** Effects of immune sera on colony growth by *Mycoplasma mycoides*
506 subspecies *mycoides* LC (MmmLC) and *M. mycoides* subspecies *capri* (Mmc) strains.
507 Zones of inhibition are indicated in mm.
508
509

Strain tested	Antiserum to MmmLC strain Y-goat^R	Antiserum to MmmLC strain F-30	Antiserum to Mmc strain PG3^T
Mycoplasma mycoides subspecies *mycoides* LC			
Y-goat^R	5	3	0
1164	5	3.5	0
CH6	4	3	0
CH5	5	3	0
FR755	3	3	0
FR1645	0	0	0
SP80	2	0	0
SP266	4	2	2
SP152	0	0	0
IT39	3	2	0
IT247	0	0	2

NZ68	0	3	0
PT994	3	3	0
NZ67	5	3	0
GR60	5	3	0
GR50	4	3	0
Mycoplasma mycoides subspecies *capri*			
Pendik	NG*	0	2
PG3^{T}	0	0	2.5
BQT	NG	0	2.5
G169	5	2.5	0
N108	0	0	2.5
G108A	0	0	3

510

511 * NG, no growth. Data are the average of three or four tests on each strain.

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