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Characterisation of *Mycoplasma capricolum* P60 surface lipoprotein and its evaluation in a recombinant ELISA

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1 ABSTRACT

2 This paper reports the identification and characterization of a 60 kDa surface protein
3 antigen (P60) of *Mycoplasma capricolum* subspecies *capricolum* (*Mcc*), and describes its
4 diagnostic application. Genomic localization and presence in P60 of conserved functional
5 domains suggested a structural and functional relationship with the immunodominant antigen
6 P48 of *Mycoplasma agalactiae*, a basic membrane protein. A rP60-ELISA was developed,
7 and it resulted in a high specificity for *Mcc* infections after evaluation with 125 goat sera.
8 The comparison with an existent ELISA based on whole *Mcc* cell lysates showed that the two
9 assays have comparable sensitivities, but the rP60-ELISA has the significant advantage of a
10 greater specificity. Results indicate that P60 is a potential marker of *Mcc* infection, especially
11 useful in areas where the presence of *Mycoplasma capricolum* subspecies *capripneumoniae*
12 is also reported.

13
14 **KEYWORDS:** *Mycoplasma capricolum* subspecies *capricolum*; contagious agalactia;
15 ELISA; basic membrane proteins; P60.

17 INTRODUCTION

18 *Mycoplasma capricolum* subsp. *capricolum* (*Mcc*) (Leach *et al.*, 1993) is a highly
19 invasive pathogen belonging to the *Mycoplasma mycoides* cluster, (Cottew *et al.*, 1987;
20 Bradbury, 2001) together with the closely related subspecies *Mycoplasma capricolum* subsp.
21 *capripneumoniae*, the causative agent of contagious caprine pleuropneumonia (Bonnet *et al.*,
22 1993). *Mcc* is responsible for sporadic outbreaks of arthritis or polyarthritis, mastitis,
23 keratoconjunctivitis, pneumonia, and septicemia in goats (Cottew, 1985; Bolske *et al.*, 1988,
24 Damassa *et al.*, 1992), and more rarely in sheep (Jones *et al.*, 1983). *Mcc* infections are often
25 associated with high morbidity, and clinical signs may be rapidly followed by death (Cottew,

26 1985; Madanat *et al.*, 2001). Despite the low frequency of symptomatic infections, *Mcc* is
27 detected in geographically diverse situations (Perreau, 1979) and persists for long periods in
28 healthy animals (Cottew and Yeats, 1981). Identification of *Mcc* in carriers is therefore
29 needed for controlling the spread of this pathogen.

30 There is no widespread standard protocol for diagnosis of small ruminant
31 mycoplasmoses; clinical signs are not specific (Rosendal, 1994), and combined approaches
32 are often required for species identification (Lambert 1987). Also, members of the *M.*
33 *mycoides* cluster share genomic and antigenic features (Bonnet *et al.*, 1993; Le Grand *et al.*,
34 2004), complicating diagnosis (Rosendal, 1994; Woubit *et al.*, 2004). Although direct PCR-
35 based diagnostic methods for the *M. mycoides* cluster have been developed (Dedieu *et al.*,
36 1995; Woubit *et al.*, 2004), dedicated serological methods are still lacking, and the ones
37 which are currently available suffer from many drawbacks (Bergonier *et al.*, 1997). At
38 present, antibodies specific to *Mcc* are detected in blood or milk by the complement-fixation
39 test (CF) or by ELISA (Lambert *et al.*, 1998). These indirect methods rely on whole cell
40 preparations as antigens, and are therefore particularly vulnerable to potential cross-
41 reactivity, especially with *Mycoplasma capricolum* subspecies *capripneumoniae*. Therefore,
42 the need for a simple and sensitive indirect test with the advantage of a greater specificity
43 becomes apparent. Recently, our group developed two dedicated specific recombinant
44 ELISAs based on *M. agalactiae* (*Ma*) and *M. bovis* (*Mbo*) P48 proteins (Rosati *et al.*, 2000;
45 Robino *et al.*, 2005), two major surface antigens belonging to the basic membrane protein
46 family (Bmp). In this paper we report identification and characterisation of *Mycoplasma*
47 *capricolum* subspecies *capricolum* P60, a basic membrane protein (Bmp) sharing structural
48 and genetic similarities with the *Ma* and *Mbo* P48 genes. We also report evaluation of its
49 diagnostic potential in a recombinant ELISA (rP60-ELISA).

50

51 **MATERIALS AND METHODS**

52 **Bacterial strains and plasmids.** California kid^T, Y-goat^T, PG45^T, and PG2^T were used as
53 reference strains of *Mcc*, *M. mycoides* subsp. *mycoides* Large Colony (*MmmLC*), *Mbo*, and
54 *Ma*, respectively. Strains were obtained in 1988 from E. A. Freundt (Institute of Medical
55 Microbiology, University of Aarhus, Denmark). Three field isolates of *Mcc* were originally
56 obtained from geographically distinct outbreaks of goat arthritis and mastitis, and tested for
57 the presence of the *mcs4 a/b* genes, which are genetic markers of the *M. capricolum* species
58 (Ushida *et al.*, 2003). Two field isolates of *MmmLC* were also used. *Escherichia coli* strain
59 BL 21 was used as host for cloning and expression studies. Plasmids pCR2-TopoTA
60 (Invitrogen, Paisley, UK) and pGEX-2T (Pharmacia, Uppsala, Sweden), were used as cloning
61 and expression vectors, respectively (Sambrook *et al.*, 1989).

62
63 **Identification and sequencing of *Mcc p60*.** The presence of a gene related to *Ma p48* was
64 investigated in *Mcc* by nucleotide-nucleotide BLAST search using *Ma p48* as query sequence
65 (Altschul *et al.*, 1997), and by submitting the consensus sequences of the selected lipoprotein
66 associated motifs (SLAs, Calcutt *et al.*, 1999, Rosati *et al.*, 1999) SLA-1 and SLA-2 to
67 ScanProsite (EXPASY). Once identified, the *p60 Mcc* gene was amplified from the *Mcc*
68 strains CK and TO5812 using two primers obtained from the *Mcc* DNA sequence for
69 CONTIG MCABF (Bork *et al.*, 1995): Myc1CK
70 (TTATAAAGGAGAGTTGAAAATGAAAA), and Myc3CK
71 (TATCAGCCAACATCTGGAATTA). Full length *p60* genes obtained from the two strains
72 were cloned into pCR2TopoTA and double strand sequenced. The ABI PRISM Big Dye
73 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used for
74 sequencing, using universal M13 primers and sequence generated primers. At least two
75 positive clones were double-strand sequenced after each cloning event. The *Mcc p60*

76 nucleotide sequences generated during this study were deposited in the GenBank database
77 under accession numbers DQ988842 (strain CK^T), and DQ988843 (field isolate TO5812).

78

79 **Site directed PCR-mediated mutagenesis and cloning.** Standard overlap extension PCR
80 adapted to *Mycoplasma* (Robino *et al.*, 2005) was used to selectively mutate UGA codons
81 into UGG. Briefly, 6 mutagenic primers were used to generate three overlapping fragments
82 (A, B, C) in a first PCR run (Figure 1, 1). The three fragments were joined using primers
83 CKW6F and CKW9R in a second PCR run (Figure 1, 2). Fragment D, containing a frame
84 coding for a C-terminal-located region of the protein, was thus generated. Fragment D was
85 also amplified with the same primers bearing restriction sites for *Bam*HI (sense) and *Eco*RI
86 (antisense) at the 5'-termini to facilitate cloning in pGEX-2T. To generate the full length
87 product M (Figure 1, 4), fragment D amplified without restriction sites was joined to the
88 other 6 fragments generated (Figure 1, 3) using the outermost primers CKF and CKW10R,
89 carrying restriction sites for *Bam*HI and *Eco*RI. Both fragment D and fragment M were
90 digested with the appropriate restriction enzymes, purified, and cloned into the pGEX-2T
91 expression vector, in frame with the glutathione S-transferase (GST) gene. Two plasmid
92 preparations from two PCR-positive transformed clones were sequenced in order to confirm
93 the induced point mutations.

94

95 **Expression and purification of recombinant P60.** Early log phase bacterial cultures were
96 induced for 90 min with 1 mM IPTG (isopropyl-thio- β -D-galactopyranoside) under constant
97 agitation. Total cell lysates were obtained by sonication and proteins were solubilized using
98 1% sarkosyl and 2% triton X-100. The fusion proteins containing fragment D and M were
99 purified by affinity chromatography using glutathione sepharose 4B. Purity and yield of the

100 recombinant proteins were evaluated by SDS-PAGE and by the Bradford method (Bradford,
101 1976), respectively.

102

103 **SDS PAGE and sera used for western blotting.** Eight rabbit hyperimmune sera raised
104 against whole cell preparations of 8 mycoplasma species, namely *Mccp*, *M. ovipneumoniae*,
105 *Mcc*, *M. arginini*, *M. putrefaciens*, *M.m. capri*, *MmmLC*, and *Ma*, were kindly provided by E.
106 A. Freund. A rabbit hyperimmune antiserum (α -rP48) raised against *Ma* rP48 was obtained
107 as previously described (Robino *et al.*, 2005). A hyperimmune antiserum was obtained by
108 immunizing a rabbit with the purified recombinant fragment D of *Mcc* P60 expressed in *E.*
109 *coli*, according to standard protocols (Harlow and Lane, 1998). Protein preparations, SDS
110 PAGE, and western blotting were performed as described previously (Alberti *et al.*, 2006).

111

112 **Serum samples and ELISAs.** A panel of 125 goat sera was used. Twenty-eight sera were
113 collected during two outbreaks occurred in France and in Italy, in which involvement of *Mcc*
114 was confirmed by isolation (group A). Twenty-three sera were collected from 2 Italian flocks
115 and 1 Scottish flock in which mycoplasma infections were never clinically suspected or
116 diagnosed, and milk was negative for mycoplasma (group B). Twenty-six sera were collected
117 from 2 Italian confirmed outbreaks of *Ma* (group C), and were positive by *Ma* rP48 ELISA
118 (Rosati *et al.*, 2000). Eighteen sera were collected from 2 natural infections and from 1
119 experimental infection of *MmmLC*, recorded in Italy and France (group D), respectively.
120 Finally, 30 serum samples were collected from 15 *Mccp* naturally infected animals and from
121 15 *Mccp* experimentally infected animals (group E), and were kindly provided by John
122 March, Moredum Research Institute, Edinburg, UK. A high titre group A serum was chosen
123 as a positive control in all rP60-ELISA experiments. All group C, D, and E sera were
124 collected from *Mcc* free flocks.

125 ELISAs were carried out as previously described (Rosati *et al.*, 2000) with some
126 modifications. Briefly, microplates were coated overnight uncovered at 37 °C with 100
127 ng/well of the GST/*Mcc* rP60 fusion protein (odd columns) and 34 ng/well of the GST as
128 negative control (even columns). After three washes, plates were blocked with 2.5% bovine
129 casein for 1 h, and washed as before. Sera diluted 1/20 in PBS containing 1.25% bovine
130 casein were incubated at 37 °C for 1 h. After four washes, a peroxidase labeled anti-
131 goat/sheep IgG monoclonal antibody (Sigma, Saint Louis, Missouri, USA) was added, and
132 plates were incubated as above. After a final wash, reaction was developed with ABTS (2,2'-
133 azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; Chemicon, Temecula, CA, USA), and read
134 after 15 min at 405 nm. ELISA net absorbances were obtained for each serum sample by
135 subtracting the absorbance measured against GST from the absorbance measured against the
136 fusion protein. Cut-off was evaluated using a subset of well characterized sera in dose
137 titration experiments, and a positive reaction was defined as having a reactivity of >20% of
138 the positive control serum enclosed in each plate. Box plots of the percentage of reactivity for
139 each group tested were obtained using the R software Version 1.7.1 (Fleiss, 1981; Ihaka and
140 Gentleman, 1996). A subset of serum samples (n=73) from groups A, B, C, D and E was
141 tested with the *Mcc* whole cell ELISA (wc-ELISA) described by Lambert and co-workers
142 (Lambert *et al.*, 1998). Results were expressed as International Units/ml (UI/ml), as described
143 (Lambert *et al.*, 1998).
144 Significance of discordant results obtained by testing the same sera with wc-ELISA and rP60
145 ELISA was investigated by McNemar's χ^2 .

146

147 **RESULTS**

148 **Identification and characterization of *Mcc* P60.** The immunodominant antigen P48 of *Ma*
149 is a conserved surface membrane protein with a high immunogenicity, and forms the basis of

150 a consolidated diagnostic recombinant ELISA for ovine contagious agalactia. In order to
151 search for P48 immunologically related proteins in other pathogenic mycoplasma species,
152 including *Mcc*, the presence of crossreactive proteins was investigated by western blotting.
153 Specifically, 8 rabbit sera raised against 8 different mycoplasma species were challenged
154 with purified rP48 (Figure 2). A strong reactivity was observed for *Ma* (as expected) and *Mcc*
155 only, indirectly suggesting the presence in *Mcc* of a protein immunologically related to *Ma*
156 P48 (Figure 2). A lower level of reactivity was also observed with the *MmmLC* and *F38*
157 hyperimmune sera; these results remain to be investigated further.

158 A nucleotide-nucleotide BLASTn search was performed using *Ma p48* as query
159 sequence, but despite immunological crossreactivity, we did not observe significant
160 homologies with nucleotide sequences available in the GenBank for *Mcc*. *Ma p48* is located
161 immediately upstream of *p59*, the *ATP binding cassette* gene of a putative ABC transporter
162 operon (Rosati *et al.*, 1999; Rosati *et al.*, 2000). In view of the adjacent positions occupied by
163 the two genes in the *Ma* genome, the *p59* sequence was used to conduct a new BLASTn
164 search. This time BLASTn revealed a significant homology (453 bits, Expect = 1e-125)
165 between *Ma p59* and *Mcc* California Kid^T ATP-binding protein CcmA (accession number
166 ABC01656). Upstream of this gene, a nucleotide sequence coding for a putative lipoprotein
167 weighing about 20 kDa (accession no. Z33368, 7) was identified as an open reading frame
168 coding for a truncated Bmp containing exclusively the selective lipoprotein associated motif
169 SLA-1 (Calcutt *et al.*, 1999), but lacking the SLA-2 motif (Rosati *et al.*, 1999). This is in
170 agreement with the findings of Calcutt and co-workers (Calcutt *et al.*, 1999). The same *Mcc*
171 sequence was identified by submitting the consensus sequences of the selected lipoprotein
172 associated motifs SLA-1 and SLA-2 to ScanProsite. However, an additional analysis of
173 sequence Z33368 revealed that downstream of the stop codon truncating the ORF at
174 nucleotide position +601, producing a protein prematurely interrupted at position +201 with

175 respect to the methionine start codon, further amino acid homologies could be identified in
176 association with a SLA-2 motif.

177 Full-sequence analysis conducted on the *Mcc* CK type strain cultured in our
178 laboratories and in the field isolate TO5812 revealed the presence of an open reading frame
179 (orf) of 1644 bp, lacking the stop codon truncating the protein at amino acid +201. Moreover,
180 partial sequencing of *p60* in 3 *MmmLC* and in 2 additional *Mcc* strains confirmed the absence
181 of a stop codon at the same position. The 1644 bp orf identified in *Mcc* encodes for a basic
182 protein with a theoretical pI of 8.81 and a putative Mw of 60 kDa, as calculated with the
183 Compute pI/Mw tool (Expasy). The protein has a typical leader peptide of 20 residues, which
184 is putatively cleaved by a bacterial protease at the recognition site VISCS, upstream of the
185 cysteine residue representing the putative acylation site to which palmitate binds.

186 The levels of nucleotide and amino acid identity between *Mcc* CK^T P60 and TO5812
187 P60 were 97.9% and 97.5%, respectively. A low level of amino acid identity (about 15%)
188 was obtained when comparing *Mcc* P60 to *Ma* P48. Full length P60 is expressed both in the
189 *Mcc* CK type strain and in three field strains of *Mcc* obtained from three geographically
190 distinct outbreaks, as demonstrated by western blotting with the hyperimmune antiserum
191 directed against the recombinant fragment D (Figure 3, lanes 2, 3). The same hyperimmune
192 antiserum did not show any reactivity with *MmmLC* Y-goat^T and with *Mycoplasma mycoides*
193 subsp. *capri* (Figure 3, lanes 4, 5).

194

195 **Cloning and expression of *Mcc* P60.** Ten UGA codons were successfully mutated to UGG
196 by site-directed PCR-mediated mutagenesis. Sequence analysis confirmed the base changes
197 and that D and M were correctly inserted into the pGEX-2T vector in frame with the
198 glutathione S-transferase (GST) gene.

199

200 **Evaluation of cross-reactivity between *Ma* P48 and *Mcc* P60.** A hyperimmune antiserum
201 developed in rabbit against purified *Ma* rP48 was tested by western blotting for reactivity
202 against *Ma* rP48 and against total protein preparations of *Ma* PG2^T, *Mbo* PG45^T, *Mcc* CK^T, a
203 field isolate of *Mcc*, *MmmLC* Y-goat^T, a field isolate of *MmmLC*, and *Mycoplasma mycoides*
204 subspecies *capri*. As a result, anti-recombinant P48 antiserum reacted selectively with native
205 *Ma* P48 but not with whole *Mcc* protein extracts. However, low cross reactivity was observed
206 when the purified recombinant *Mcc* P60 protein was probed with anti-recombinant *Ma* P48
207 (data not shown).

208

209 **rP60-ELISA and wc-ELISA.** Sera collected from animals positive to *Mcc* (group A), from
210 animals negative to *Mycoplasma* (group B), and from animals positive to mycoplasmas other
211 than *Mcc* (groups C, D, and E), were tested by ELISA using *Mcc* rP60 as capture antigen
212 (rP60 ELISA). A boxplot of the absorbance values obtained is shown in Figure 4. With the
213 exception of 1 group E serum, only sera from *Mcc* infected animals (group A) produced a
214 positive reaction with rP60. A subset of serum samples (N= 73) was also tested with the wc-
215 ELISA, and results were compared to those obtained by rP60 ELISA (Table 1). All group A
216 sera of the subset tested positive by wc-ELISA and all but one were positive by rP60 ELISA.
217 Conversely, 13 group C and E sera positive by wc-ELISA were negative by rP60 ELISA,
218 whereas only one serum was positive by rP60 ELISA and was negative by wc-ELISA, and
219 the difference between these discordant results was statistically significant (13 vs 1,
220 McNemar's $\chi^2 = 10.3$, d.f. =1, $P < 0.01$). We therefore concluded that the probability of
221 obtaining a false positive by wc-ELISA was greater than the probability of obtaining a false
222 positive by rP60 ELISA. In conclusion, the two assays have a comparable sensitivity, but the
223 rP60 ELISA has the significant advantage of a greater specificity.

224

225 **DISCUSSION**

226 The characterization of mycoplasma surface proteins has long been a challenging
227 goal. In the last decade, several variable surface proteins (VSPs) involved in evasion of the
228 host immune response have been described in pathogenic mycoplasmas (Yogev *et al.*, 1991;
229 Nussbaum *et al.*, 2002). However, because of their intrinsic variable nature, these proteins are
230 unsuited to use as markers of infection or for development of related immunogenic products.
231 Among invariable antigens, the basic membrane proteins (Bmps) have shown a great
232 potential for the development of specific and sensitive serological tests. One of these is *Ma*
233 P48, which forms the basis for a recombinant ELISA, now validated and commercially
234 available, for detection of contagious agalactia in sheep (Rosati *et al.*, 2000).

235 Here we report the characterisation of *Mcc* P60, a protein sharing positional and
236 functional relationships with *Ma* P48, and demonstrate its application as a candidate antigen
237 in the development of a sensitive and specific serological test for detection of *Mcc* infections
238 in goats. Nucleotide and aminoacid sequence analyses revealed a striking similarity of P60 to
239 a protein described as P20 in *Mcc* CK^T by Bork and co-workers (1995). Interestingly, this
240 similarity extends to nucleotides following an in-frame TAA stop codon which prematurely
241 interrupts translation of the *p20* gene. This stop codon occurs at nucleotide position + 601,
242 leading to expression of a 20 kDa protein in *Mcc* CK^T (ATCC27343). Conversely, in this
243 study we detected an A to G transition at nucleotide position + 602, resulting in a TGA codon
244 instead of a stop codon in all the *Mycoplasma mycoides* cluster strains evaluated, including
245 *Mcc* CK^T ATCC27343. We speculate that the TGA codon observed at nucleotide position +
246 601 is conserved within the *Mycoplasma mycoides* cluster, and that the TAA stop codon
247 described in *Mcc* CK^T ATCC27343 could have arisen in the strain used by Bork and co-
248 workers (1995) during prolonged *in vitro* cultivation. Indeed, *Mcc* CK^T ATCC27343 cultured
249 in our laboratory encodes the full length version of the protein (P60).

250 In spite of the immunological crossreactivity seen by western blotting (Figure 2), a
251 low level of amino acid identity was observed between *Ma* P48 and *Mcc* P60 along the whole
252 sequence (about 15%). Analogy between the two proteins relies, however, in presence of two
253 consensus sequences for the Bmp functional domains SLA-1 and SLA-2, which are
254 conserved in this protein family. The elevated divergence observed in other protein regions
255 might reasonably be explained by the absence of tight functional constraints and by
256 immunological escape, given the surface localization. The hypothesis that the two proteins
257 might be functionally and structurally related is also supported by the finding that *p48* and
258 *p60* have the same genetic location in the genomes of *Mcc* and *Ma*, being both located within
259 a structurally conserved ABC transporter operon and upstream of a conserved protein
260 sequence. The presence of the SLA-1 and SLA-2 domains might be responsible for the
261 crossreactivity observed by western blotting when probing recombinant P60 with a rabbit
262 serum developed against recombinant P48. The reason remains unclear, however, why the
263 native *Mcc* P60 could not be detected within total cell lysates of *Mcc* probed by western
264 blotting with anti-recombinant P48 (Fig. 4b). It should be pointed out that this apparent lack
265 of reactivity could be due to low P60 abundance below the detection limits of this assay and
266 deserves further investigation.

267 P60 is conserved in size and is stably expressed in all the *Mcc* strains tested in this
268 work, as demonstrated by reactivity with a rabbit hyperimmune antiserum (Figure 3).
269 Moreover, experiments of colony blotting performed using the same hyperimmune antiserum
270 suggest the absence of clonal variability (data not shown). Comparison of the results obtained
271 by western blotting and ELISA with other mycoplasmas confirmed that P60 is specifically
272 recognized only by sera of goats infected by *Mcc* (Figure 4). The extremely low positivity
273 rate observed with sera from goats infected by other mycoplasmas (1/74) suggests the
274 suitability of rP60 as a specific marker of infection. In fact, the rP60-ELISA has a level of

275 sensitivity comparable to that of the wc-ELISA, but it has the significant advantage of a
276 higher specificity when challenged with sera from goats with infections caused by other
277 mycoplasmas (Figure 4), as demonstrated by the chi-square. This is especially relevant when
278 considering the lack of ELISA crossreactivity with the closely related *Mccp* subspecies,
279 which renders rP60-ELISA an additional specific and reliable tool for developing *Mcc*
280 diagnostic strategies in areas where both *Mcc* subspecies are present. Moreover, the
281 availability of a sensitive and highly specific serological test for detection of *Mcc* infections
282 in small ruminants could represent a practical and cost-effective approach for surveillance
283 and control programs, since *Mcc* infection is not always accompanied by visible symptoms.

284

285 **Conclusion**

286 We developed the first recombinant ELISA for diagnosis of *Mycoplasma capricolum*
287 subspecies *capricolum* based on the use of P60, a basic membrane protein structurally and
288 functionally related to the basic membrane proteins P48 of *M. agalactiae* and *M. bovis*,
289 respectively. This antigen could supplement the p48-based ELISA test already developed for
290 Contagious Agalactia, and might lead to the development of a multi-antigen test for diagnosis
291 and control of contagious agalactia syndromes associated with ovine and caprine
292 mycoplasmas.

293

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301

302 REFERENCES

303

304 Alberti, A., Addis, M.F., Chessa, B., Cubeddu, T., Profiti, M., Rosati, S., Ruiu, A., Pittau, M.,
305 2006. Molecular and antigenic characterization of a *Mycoplasma bovis* strain causing an
306 outbreak of infectious keratoconjunctivitis. J. Vet. Diagn. Invest. 18, 41-51.

307

308 Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z. Miller, W., Lipman, D.J.,
309 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search
310 programs. Nucleic Acids Res. 25, 3389-3402.

311

312 Bergonier, D., Berthelot, X., Poumarat, F., 1997. Contagious agalactia of small ruminants:
313 current knowledge concerning epidemiology, diagnosis and control. Rev. Sci. Tech. 16, 848-
314 873.

315

316 Bolske, G., Msami, H., Humleslo, N.E, Erno, H., Jonsson, L., 1988. *Mycoplasma capricolum*
317 in an outbreak of polyarthritis and pneumonia in goats. Acta Vet. Scand. 29, 331-338.

318

319 Bonnet, F., Saillard, C., Bové, J.M., Leach, R.H., Rose, D.L., Cottew, G.S., Tully, J.G., 1993.
320 DNA relatedness between field isolates of *Mycoplasma* F38 group, the agent of contagious
321 caprine pleuropneumonia, and strains of *Mycoplasma capricolum*. Int. J. Syst. Bacteriol. 43,
322 597-602.

323

- 324 Bork, P., Ouzounis, C., Casari, G., Schneider, R., Sander, C., Dolan, M., Gilbert, W.,
325 Gillevet, P.M., 1995. Exploring the *Mycoplasma capricolum* genome: a minimal cell reveals
326 its physiology. *Mol. Microbiol.* 16, 955-967.
- 327
- 328 Bradbury, J.M., 2001. International committee on systematic bacteriology, subcommittee on
329 the taxonomy of *Mollicutes*. *Int. J. Syst. Evol. Microbiol.* 51, 2227-2230.
- 330
- 331 Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram
332 Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* 72, 248-
333 254.
- 334
- 335 Calcutt, M.J., Kim, M.F., Karpas, A.B., Muhlradt, P.F., Wise, K.S., 1999. Differential
336 posttranslational processing confers intraspecies variation of a major surface lipoprotein and
337 a macrophage-activating lipopeptide of *Mycoplasma fermentans*. *Infect. Immun.* 67, 760-771.
- 338
- 339 Cottew, G.S., 1985. Infections with *Mollicutes* in sheep and goats. In: Gylstorff I. (ed),
340 Infektionen durch *Mycoplasmatales*, VEB Gustav Fischer Verlag, Jena, Germany, pp 368-
341 386.
- 342
- 343 Cottew, G.S., Yeats, F.R., 1981. Occurrence of mycoplasmas in clinically normal goats.
344 *Aust. Vet. J.* 57, 52-53.
- 345
- 346 Cottew, G.S., Bréard, A., DaMassa, A.J., Erno, H., Leach, R.H., Lefèvre, P.C., Rodwell,
347 A.W., Smith G.R., 1987. Taxonomy of the *Mycoplasma mycoides* cluster. *Israel J. Med. Sci.*
348 23, 632-635.

349

350 DaMassa, A.J., Wakenell, P.S., Brooks, D.L., 1992. Mycoplasmas of goats and sheep. *J. Vet.*
351 *Diagn. Invest.* 4, 101-113.

352

353 Dedieu, L., Mady, V., Lefèvre, P.C., 1995. Development of two PCRs for the identification
354 of mycoplasmas causing contagious agalactia. *FEMS Microbiol. Lett.*, 129, 243-250.

355

356 Fleiss, J. L., 1981. *Statistical Methods for Rates and Proportions*. New York: John Wiley &
357 Sons. (Second Edition).

358

359 Harlow, E., Lane, D., 1998. *Using antibodies: A laboratory manual*. Cold Spring Harbor
360 Laboratory Press, Cold Spring Harbor, New York.

361

362 Ihaka, R., Gentleman, R., 1996. R: A Language for Data Analysis and Graphics. *Journal of*
363 *Computational and Graphical Statistics* 5, 299-314.

364

365 Jones, G.E., Rae, A.G., Holmes, R.G., Lister S.A., Jones, J.M.W., Grater G.S., Richards N.,
366 1983. Isolation of exotic mycoplasmas from sheep in England. *Vet. Rec.* 113, 540.

367

368 Lambert, M., 1987. Contagious agalactia of sheep and goats. In: *Mycoplasmoses of*
369 *ruminants*. *Rev. Sci. Tech. OIE* 6, 699-711.

370

371 Lambert, M., Calamel, M., Dufour, P., Cabasse, E., Vitu, C., Pépin, M., 1998. Detection of
372 false-positive sera in contagious agalactia with a multiantigen ELISA and their elimination
373 with a protein G conjugate. *J. Vet. Diagn. Invest.* 10, 326-330.

374

375 Leach, R.H., Erno, H., MacOwan, K.J., 1993. Proposal for designation of F38-type caprine
376 mycoplasmas as *Mycoplasma capricolum* subsp. *capripneumoniae* subsp. nov. and
377 consequent obligatory relegation of strains currently classified as *M. capricolum* (Tully,
378 Barile, Edward, Theodore, and Erno 1974) to an additional new subsp., *M. capricolum* subsp.
379 *capricolum* subsp. nov. Int. J. Syst. Bacteriol. 43, 603-605.

380

381 Le Grand, D., Saras, E., Blond, D., Solsona, M., Poumarat, F., 2004. Assessment of PCR for
382 routine identification of species of the *Mycoplasma mycoides* cluster in ruminants. Vet. Res.
383 35, 635-649.

384

385 Madanat, A., Zendulková, D., Pospiil, Z., 2001. Contagious agalactia of sheep and goats. A
386 review. Acta Vet. Brno 70, 403-412.

387

388 Nussbaum, S., Lysnyansky, I., Sachse, K., Levison, S., Yogev, D., 2002. Extended
389 Repertoire of Genes Encoding Variable Surface Lipoproteins in *Mycoplasma bovis* Strains.
390 Infect. Immun. 70, 2220-2225.

391

392 Perreau, P., 1979. Les mycoplasmoses de la chèvre. Cah. Med. Vet. 48, 71-85.

393

394 Robino, P., Alberti, A., Pittau, M., Chessa B., Miciletta, M., Nebbia, P., Le Grand, D., Rosati,
395 S., 2005. Genetic and antigenic characterization of the surface lipoprotein P48 of
396 *Mycoplasma bovis*. Vet. Microbiol. 109, 201-209.

397

398 Rosati, S., Robino, P., Fadda, M., Pozzi, S., Mannelli, A., Pittau, M., 2000. Expression and
399 antigenic characterization of recombinant *Mycoplasma agalactiae* P48 major surface protein.
400 Vet. Microbiol. 71, 201-210.
401

402 Rosati, S., Pozzi, S., Robino, P., Montinaro, B., Conti, A., Fadda, M., Pittau, M., 1999. P48
403 major surface antigen of *Mycoplasma agalactiae* is homologous to a malp product of
404 *Mycoplasma fermentans* and belongs to a selected family of bacterial lipoproteins. Infect.
405 Immun. 67, 6213-6216.
406

407 Rosendal, S., 1994. Ovine and Caprine mycoplasmas. In: Whitford, H.W., Rosenbusch, R.F.,
408 Laueran, L.H. (Eds), Mycoplasmosis in Animals: Laboratory Diagnosis, Iowa State
409 University Press, Ames, Iowa, pp. 84-93.
410

411 Sambrook, J.E., Fritsch, F., Maniatis, T., 1989. Molecular cloning. 2nd ed. New York: Cold
412 Spring Harbor Laboratory Press.
413

414 Ushida, C., Yoshida, A., Miyakawa, Y., Ara, Y., Muto, A., 2003. Distribution of the MCS4
415 RNA genes in mycoplasmas belonging to the *Mycoplasma mycoides* cluster. Gene 314, 149-
416 155.
417

418 Woubit, S., Lorenzon, S., Peyraud, A., Manso-Silvan, L., Thiaucourt, F., 2004. A specific
419 PCR for the identification of *Mycoplasma capricolum* subsp. *capripneumoniae*, the causative
420 agent of contagious caprine pleuropneumonia (CCPP). Vet. Microbiol. 104, 125-132.
421

422 Yogev, D., Rosengarten, R., Watson-McKown, R., Wise, K.S., 1991. Molecular basis of
423 *Mycoplasma* surface antigenic variation: a novel set of divergent genes undergo spontaneous
424 mutation of periodic coding regions and 5' regulatory sequences. EMBO J. 10, 4069-4079.

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Table 1

Sera used for comparison of rP60-ELISA with wc-ELISA

Cluster	Group	Pathogen	Geographic origin	rP60-ELISA			wc-ELISA		
				N. of sera	Pos	Neg	N. of sera	Pos	Neg
1	A	<i>Mcc</i>	France/Italy	12	11	1	12	12	0
2	B	None	Scotland/Italy	14	0	14	14	0	14
3	C	<i>Ma</i>	Italy	15	0	15	15	5	10
	D	<i>MmmLC</i>	Italy	18	0	18	18	0	18
	E	<i>Mccp</i>	UK	14	1	13	14	8	6

Mcc, *Mycoplasma capricolum* subsp. *capricolum*; *Ma*, *Mycoplasma agalactiae*, *MmmLC*, *Mycoplasma mycoides* subsp. *mycoides* Large Colony; *Mccp*, *Mycoplasma capricolum* subsp. *capripneumoniae*. rP60 ELISA, recombinant *Mcc* P60-based ELISA; wc ELISA, *Mcc* whole-cell proteins-based ELISA.

Figure 1

Strategy used for introducing multiple distant point mutations. Eighteen primers (CKF, CKW1F/R, CKW2F/R, CKW3-4R, CKW4F, CKW5F/R, CKW6F/R, CKW7F/R, CKW8F/R, CKW9F/R, CKW10R) containing the desired mutations were used to generate seven overlapping fragments (E, F, G, H, I, D, and L). Purified fragments were joined in M using primer CKF and CKW10R in a second PCR run. Mutated nucleotides in primers and in PCR products are shown as vertical bars.

Figure 2

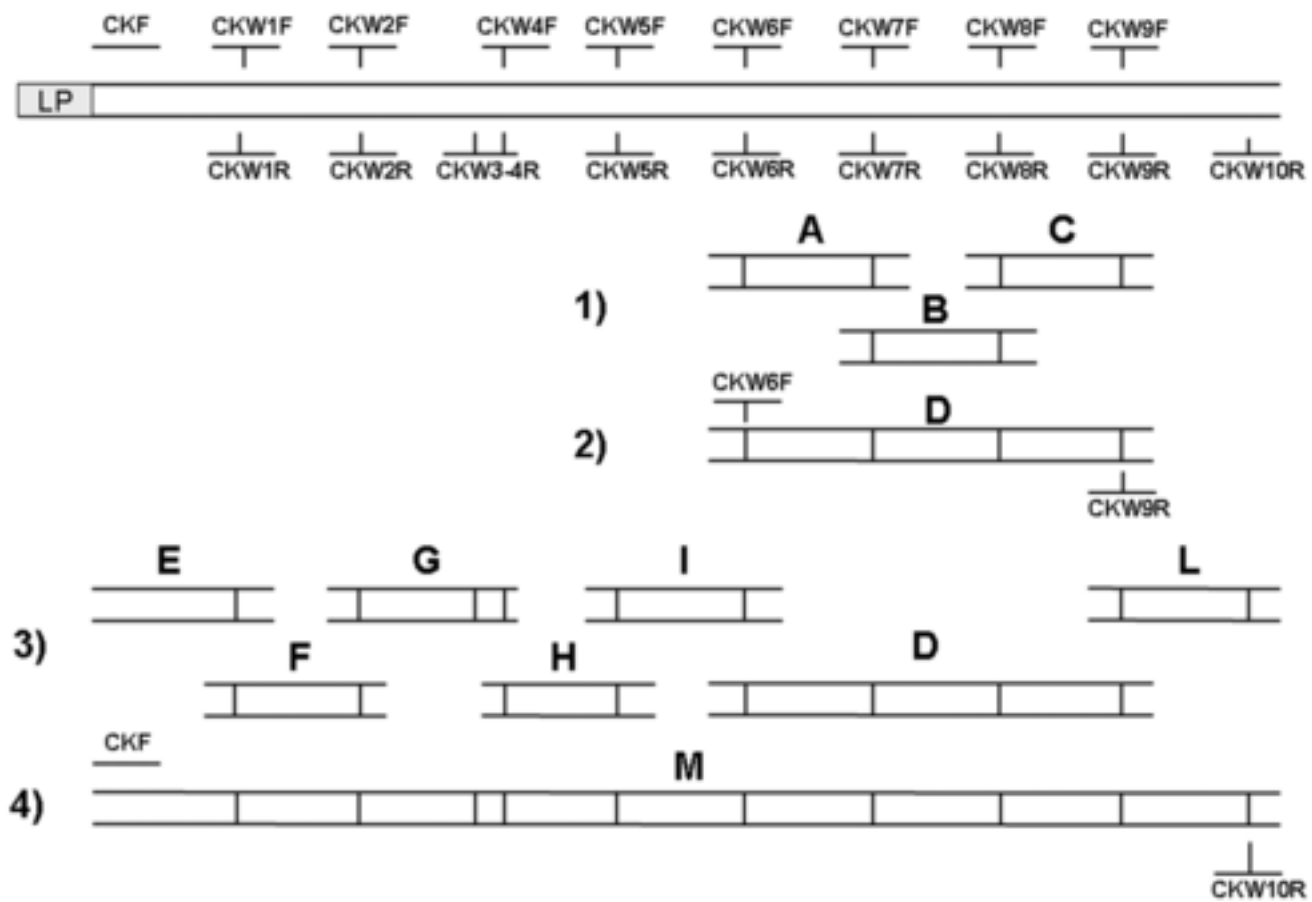
Western blotting showing reactivity of rP48 (P48) and GST-rP48 fusion protein (GSTP48) with 8 rabbit hyperimmune antisera raised against 8 different *Mycoplasma* species. M: molecular weight markers (weights are indicated on the left); 1, *M. capricolum* subspecies *capripneumoniae* F38; 2, *M. ovipneumoniae*; 3, *M. capricolum* subspecies *capricolum*; 4, *M. arginini*; 5, *M. putrefaciens*; 6, *M. mycoides* subspecies *capri*; 7, *M. mycoides* subspecies *mycoides* large colony; 8, purified GST-rP48 and rP48, respectively. Arrows indicate GST-rP48 and rP48. Strips were cut from a membrane obtained by blotting a single-well gel loaded with 4 µg of recombinant protein preparation.

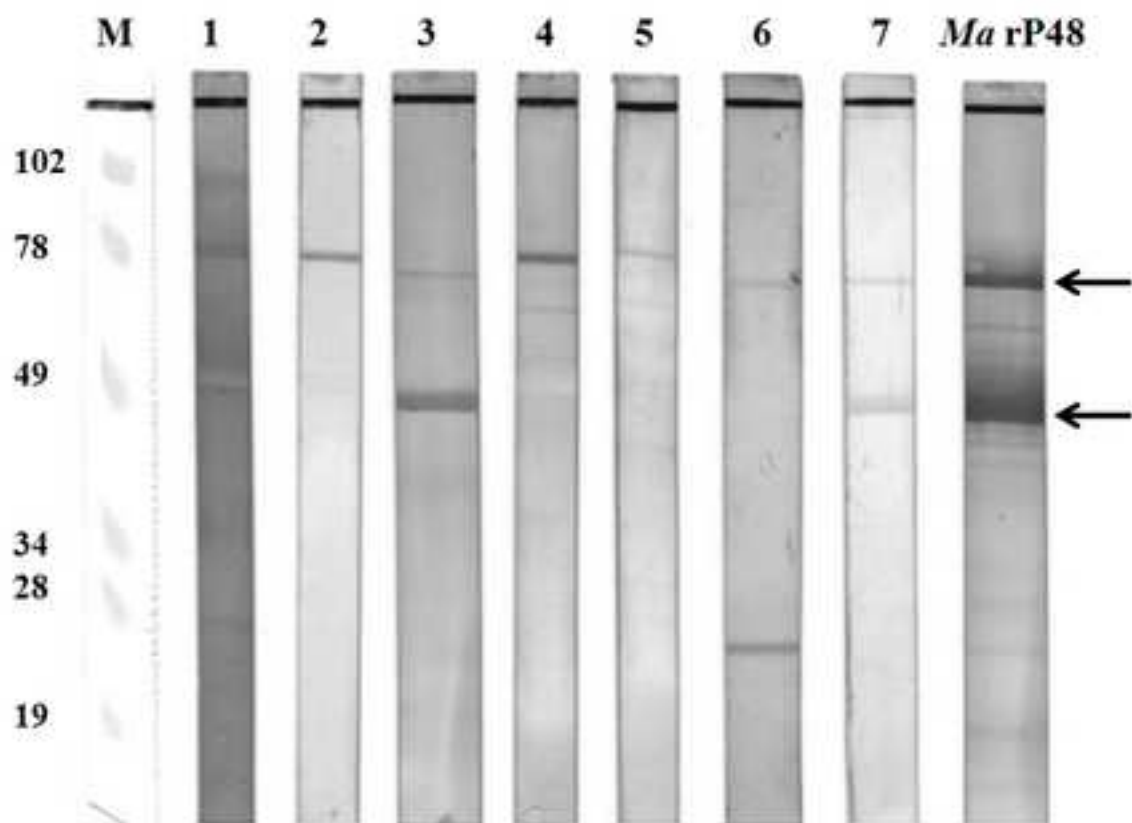
Figure 3

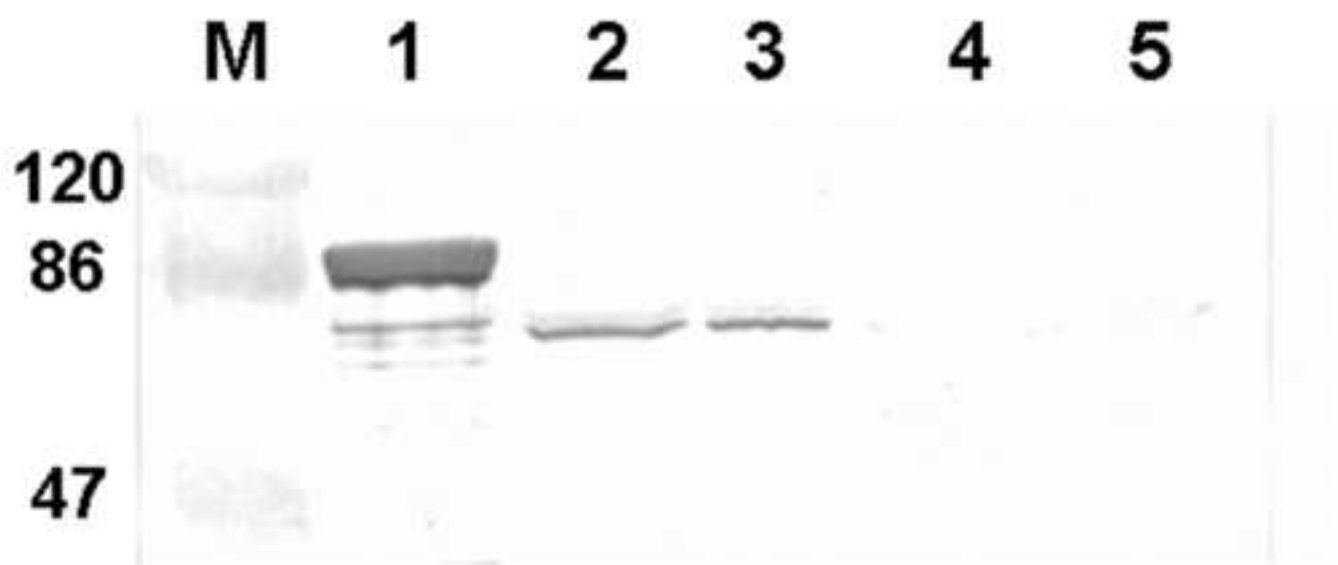
Western blotting performed to investigate presence and size of P60 in the *Mycoplasma mycoides* cluster using a rabbit antiserum raised against *M. capricolum* P60. 1, rP60 and rP60-GST fusion protein (0.5 µg); 2-5, total cell lysates of *Mcc* CK, a *Mcc* field isolate, *M. mycoides* subspecies *mycoides* large colony Y-goat^T, and *M. mycoides* subspecies *capri*, respectively; M, molecular weight markers. The amount of mycoplasma proteins loaded were the same in each lane (about 50 µg).

Figure 4

Boxplot of absorbance values obtained by rP60-ELISA: A-E: *Mcc* naturally infected goats; *Mycoplasma capricolum* subsp. *capricolum* (*Mcc*) seronegative goats; *M. agalactiae* positive sheep; *M. mycoides* subspecies *mycoides* large colony naturally infected goats, and *M. capricolum* subspecies *capripneumoniae* positive goats, respectively. Optical densities are expressed as percent of positivity respect to the positive control. Differences in optical density between the groups of sera were statistically significant ($P < 0,05$).







Script

