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1 **Phage display-based identification and potential diagnostic application of**
2 **novel antigens from *Mycoplasma mycoides* subsp. *mycoides* small colony type.**

3

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29 **Abstract**

30

31 Contagious Bovine Pleuropneumonia caused by *Mycoplasma mycoides* subsp.
32 *mycoides* small colony type is a respiratory disease of considerable economic
33 importance in sub-Saharan Africa; control of the disease in Africa is hampered by
34 diagnostic tests which are suited for herd-level but not for individual animal
35 diagnostics. In the work presented we identified 22 potential immunogenic
36 antigens of the Kenyan outbreak strain B237 by using phage display technology.
37 We determined the relative strength of immunogenicity, the discriminatory
38 capacity between bovine positive and negative sera, and the cross reactivity with
39 rabbit hyperimmune sera directed against 15 different mycoplasmal species. The
40 three best-performing antigens, a conserved hypothetical protein (MSC_0636), a
41 glycosyl transferase (MSC_0108), and an acyl carrier protein phosphodiesterase
42 (MSC_0029) were considered candidate diagnostic proteins. They were expressed
43 as GST-fusion proteins in *E. coli*, purified, and used in an ELISA as solid phase
44 antigens. The diagnostic potential of the recombinant antigens was tested using
45 the sera of ten experimentally infected animals and six control animals. This
46 prototype test resulted in 100 % diagnostic sensitivity and specificity. In
47 comparison, the complement fixation test and the competitive ELISA performed
48 with a diagnostic sensitivity of 70 % and 60 %, respectively.

49

50 **Keywords:** *Mycoplasma mycoides* subsp. *mycoides* small colony type; phage
51 display; diagnostic antigens

52 1. Introduction

53

54 Contagious Bovine Pleuropneumonia (CBPP) is a highly infectious respiratory
55 disease in cattle caused by *Mycoplasma mycoides* subsp. *mycoides* small colony
56 type (*MmmSC*) and solely transmitted by animal to animal contact. The disease is
57 of major economic importance in subsaharan Africa (FAO, 2003) with a constant
58 risk of reintroduction into other parts of the world. Diagnosis is primarily based
59 on serology currently done by complement fixation test (CFT), competitive
60 ELISA (cELISA) (Le Goff and Thiaucourt, 1998) and a lipoprotein Q-based
61 ELISA (Bruderer et al., 2002). Both ELISA tests closely correlate with the CFT
62 reported to have a diagnostic sensitivity of 64 to 70 % and a diagnostic specificity
63 of 98 % (Amanfu et al., 2000; Marobela-Raborokgwe et al., 2003). For a reliable
64 diagnosis of disease on the individual animal level the diagnostic sensitivity needs
65 to be improved without losing specificity. To achieve this goal, novel diagnostic
66 antigens need to be identified.

67 Recently it has been shown that this goal can be achieved effectively using a non-
68 lytic phage display approach based on phage M13 and designated as pHORF-
69 system (Kügler et al., 2008); it improves the panning and screening process
70 compared to the lytic lambda-based phage display system (Beghetto et al., 2009;
71 March et al., 2006; Kügler et al., 2008). Furthermore, the included open reading
72 frame selection and the packaging into “Hyperphage”, a helperphage not encoding
73 pIII protein on its genome, improve the subsequent panning process (Hust et al.,
74 2006; Kügler et al., 2008; Soltes et al., 2007; Rondot et al., 2001).

75 In the work presented, the phage M13-based pHORF system was used to identify
76 novel immunogenic epitopes of *MmmSC* which are potentially suitable not only
77 for herd-level but also for individual animal diagnostics.

78 **2. Materials and Methods**

79

80 *2.1 Bacterial strains, phages, growth conditions, plasmids and sera*

81

82 The strains, plasmids, primers and sera used in this work are listed in Table 1.

83 *Escherichia coli* strains were cultured in Luria-Bertani medium supplemented

84 with the appropriate antibiotics (ampicillin, 100 µg/l, kanamycin, 30 µg/l). The

85 *MmmSC* field isolate B237 was grown as stationary culture for three days in

86 modified PH medium (Kirchhoff and Rosengarten, 1984), supplemented with

87 20 % equine serum (WDT, Garbsen, Germany) at 37 °C in a 5 % CO₂ atmosphere.

88 The 10 bovine anti-*MmmSC* sera used in this study were derived from

89 experimentally infected cattle (Jores et al., 2008). A positive control serum was

90 obtained by pooling equal volumes of these sera. The six negative sera used were

91 obtained from uninfected cattle from a CBPP free region within Kenya. A

92 negative control serum was generated by pooling equal volumes of these sera. The

93 rabbit sera directed against different mycoplasmal species had been raised

94 previously against the respective whole cell bacterins.

95

96

97 *2.2 Construction of an MmmSC genomic phage display-library and enrichment of*

98 *open reading frames*

99

100 Site-specific mutation of the phagemid vector pHORF3 (Kügler et al., 2008) was

101 performed using primers oMHLacZPro_f, oMHORF3_pme_rX1 and

102 oMHORF3_pme_rXX2 (Table 1) such that the unique PmeI site used for insertion

103 of foreign DNA is shifted by 1 and 2 bp resulting in plasmids pHORF3X and

104 pHORF3XX, respectively (Fig. 1). Genomic DNA of the *MmmSC* African field
105 strain B237 was isolated as described previously (Meens et al., 2006) and digested
106 using five different blunt end-cutting restriction endonucleases (AluI, PsiI, SspI,
107 HpyCH4V, RsaI). DNA fragments with a size of 100-1000 bp were isolated after
108 separation in an agarose gel using the Qiagen PCR DNA and gel band purification
109 kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and
110 ligated into PmeI-restricted vectors pHORF3, pHORF3X and pHORF3XX (Fig.
111 1). The genomic *MmmSC* phage library was constructed as described previously
112 (Kügler et al., 2008).

113

114

115 *2.3 Selection of phage encoding immunogenic oligopeptides of MmmSC and* 116 *production of individual phage clones for screening*

117

118 The panning of phage presenting immunogenic peptides on the surface was
119 performed using the bovine positive control serum pre-absorbed to Hyperphage in
120 order to prevent unspecific reactivity with antibodies directed against phage-
121 surface epitopes. The preabsorbed serum was coupled to MaxiSorb® 96 well
122 microtitre plates (Nunc, Wiesbaden, Germany) via rabbit anti-bovine IgG (Sigma,
123 Deisenhofen, Germany), and four successive panning rounds were performed as
124 previously described (Kügler et al., 2008). Individual clones were picked from the
125 titration plates of the last panning round.

126 For production of individual phage clones, polypropylene 96-well U-bottom
127 plates (Greiner bio-one, Frickenhausen, Germany) containing 175 µl of 2 x TY-
128 GA medium (1 % Bacto-Yeast Extract, 1.6 % Bacto Tryptone, 0.05 % NaCl; 100
129 mM glucose, 100 µg/ml ampicillin) per well were inoculated with single clones

130 and incubated at 37 °C with constant shaking at 250 rpm overnight. Cultures were
131 expanded to 1.5 ml of 2 x TY-GA medium, infected with 5×10^9 cfu Hyperphage
132 per tube and incubated at 37 °C without shaking for 30 min, followed by 30 min
133 incubation with shaking at 250 rpm. Cells were centrifuged at 3,220 x g for
134 10 min and the supernatants were discarded. The bacterial pellets were
135 resuspended in 1.5 ml of 2 x TY medium containing ampicillin (100 µg/ml) and
136 kanamycin (30 µg/ml) and incubated at 30 °C at 250 rpm overnight for phage
137 production.

138 In order to confirm the antigenicity of individual phage clones PolySorb® 96 well
139 microtitre plates (Nunc) were coated overnight with 10 µl (1×10^{10} pfu/ml) of
140 individual phage in 100 µl of carbonate buffer (100mM NaHCO₃, 46 mM
141 Na₂CO₃, pH 9.6). The ELISA was developed using the positive control serum
142 (diluted 1:100), goat anti-bovine IgG conjugated with horseradish peroxidase
143 (HRP; Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany) as a
144 conjugate and 2,2-azino-di-[3-ethylbenzothiazoline sulfonate] (ABTS; Roche
145 Diagnostics, Mannheim, Germany) as a substrate. The staining reaction was
146 stopped by adding 100 µl of 50% (v/v) methanol. The absorbance at 405 nm and
147 scattered light at 492 nm were measured using a Sunrise® microtiter plate reader
148 (Tecan, Crailsheim, Germany).

149

150

151 *2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and*

152 *Western blot*

153

154 SDS-PAGE was performed following standard procedures using a Protean II

155 Minigel system (BioRad Inc, Munich, Germany). Western blotting of the SDS-
156 PAGE minigels was done using the Mini Trans-Blot[®] system (BioRad). The
157 bovine positive and negative control serum and a rabbit anti-GST serum were
158 used as primary antibodies; blots were developed using alkaline phosphatase-
159 conjugated goat anti-bovine or goat anti-rabbit IgG antibody (Jackson
160 ImmunoResearch Laboratories) and BCIP (5-bromo-4-chloro-3-indolyl
161 phosphate) and NBT (nitroblue tetrazolium) as substrate.

162

163

164 *2.5 Preparation of recombinant fusion proteins and ELISA set-up*

165

166 The *MmmSC* genes encoding immunogenic epitopes as assessed by ELISA of
167 individual phage clones were identified by database analysis. The respective parts
168 of the genes located between two TGA codons were amplified by PCR using
169 primer pairs with an EcoRI- (upstream primer) or NotI-restriction endonuclease
170 site (downstream primer) at their 5' ends (Table 1). The corresponding PCR
171 fragments were digested with EcoRI and NotI and ligated into pGEX-5x-2,
172 resulting in plasmids pMSC0029-500, pMSC0108-500 and pMSC0636-500.
173 Recombinant fusion proteins were prepared as inclusion bodies as described
174 previously (Gerlach et al., 1992) and dissolved in guanidine hydrochloride (4 M).
175 To remove small-sized contaminating material, the solution was washed twice in
176 guanidine hydrochloride (4M) using a 10 kDa cut-off (MicroCentricon 10 system,
177 Amicon, Beverly, Mass., U.S.A.). Using BSA as reference the concentration of the
178 purified protein (desalted by precipitation with trichloroacetic acid [TCA]) was
179 then determined by SDS PAGE and staining with Coomassie blue. The structure

180 and immunogenicity of the fusion proteins was confirmed by Western blot
181 analysis using positive and negative control sera as well as an anti-GST serum.
182 For use in an ELISA, fusion proteins in guanidine hydrochloride were pipetted in
183 a petri dish, diluted by adding the appropriate volume of carbonate coating buffer
184 with a 10 ml pipette, and used as solid phase antigens on a PolySorb 96 well
185 microtiter plate (Nunc) coating 100 µl volumes per well overnight at 4 °C. The
186 coating concentration was optimized by checkerboard titration of the antigen,
187 development of the ELISA using 2-fold serial dilutions of the positive and
188 negative control sera, goat-anti-bovine peroxidase, and ABTS, and analysis of the
189 log-log-transformed data. Prior to use in the ELISA sera were absorbed with
190 whole-cell lysate of *E. coli*-pGEX-5x-2 transformants for 1 h at room temperature
191 in order to remove antibodies directed against contaminating *E. coli* components
192 in the semi-purified antigen preparation.

193

194

195 **3. Results**

196

197 *3.1 Identification of immunogenic peptides*

198

199 After performing four panning rounds with the positive control serum a total of
200 147 clones (49 from each of the three libraries based on vectors with the cloning
201 site in three different reading frames with respect to the 3' pIII protein-encoding
202 ORF) were sequenced. One hundred and thirty four clones contained analyzable
203 sequence and showed an ORF consisting of a *Mmm*SC-derived genomic DNA
204 fragment in frame with both the *pelB* signal sequence and the PIII protein-
205 encoding ORF. Upon NCBI Blast analysis (<http://www.ncbi.nlm.nih.gov/blast/>

206 [Blast.cgi](#)) 22 different clones were identified with each clone present at least twice
207 in the random selection of sequenced clones (Table 2).

208

209

210 3.2 Selection of clones with diagnostic potential

211

212 The individual clones were amplified and the relative strength of serum reactivity
213 was determined by phage ELISA. Eight clones showing a reactivity of more than
214 30 OD% with respect to *MmmSC* whole cell lysate (Table 2) were considered to
215 be of potential diagnostic value and were subjected to further investigation. The
216 clones were individually amplified, and the discrimination between positive and
217 negative control serum was investigated by ELISA. For six of the eight clones
218 (MSC_0029, 0108, 0636, 0656, 1009 and 1062) at least a 3-fold difference in titre
219 was observed between the positive and the negative control serum (data not
220 shown). These six clones were tested with respect to their discriminatory potential
221 using the ten individual positive sera and the six individual negative sera
222 contained in the respective control sera. Four of the clones (MSC_029, 108, 636
223 and 1062) reacted with all individual positive sera; no overlap in OD%-values was
224 seen between positive and negative sera (data not shown).

225 These four clones were investigated for cross reactivity to other mycoplasmal
226 species using rabbit hyperimmune sera previously raised against 15 different
227 mycoplasmal species. All four clones (MSC_0029, MSC_0108, MSC_0636, and
228 MSC_1062) were highly positive with the hyperimmune serum directed against
229 *MmmSC*. Two clones showed clear cross-reactivity (i.e. more than 1.5-fold of the
230 average reactivity with the negative control serum) with the serum directed
231 against *M. capricolum* subsp. *capricolum*; for clone MSC_1062 an additional

232 cross-reactivity against *A. modicum* and *M. bovis* was observed (Table 3) and,
233 therefore, this clone was excluded from further analyses. The three remaining
234 clones (MSC_0029, MSC_0108 and MSC_0636) were expressed as GST fusion
235 proteins.

236

237

238 *3.3 Potential of recombinant GST-fusion proteins as diagnostic antigens*

239

240 All fusion proteins had a molecular mass corresponding to the calculated masses
241 of 32.5, 32.0, and 45.0 kDa, respectively. Approximately 90% of the fusion
242 protein preparations consisted of recombinant antigen (Fig. 2A). The proteins
243 reacted strongly positive with both the anti-GST and the positive control serum
244 but did not show any reaction with the negative control serum (Fig. 2B-D).

245 The purified fusion proteins, upon checkerboard titration performed to determine
246 the suitable coating concentration of 2.5 µg/ml, were used as solid-phase antigens.

247 In a first ELISA, using rabbit hyperimmune sera previously raised against
248 *MmmSC*, a 32-times higher titre of the positive versus the negative serum was
249 observed (data not shown) thereby showing suitability of the antigens in principal.

250 In a second ELISA, serial two-fold dilutions of the bovine positive and negative
251 control sera were used after absorption against a lysate of *E. coli* pGEX-5x-2

252 transformants. The slope of the regression curve of the log-log transformed data

253 obtained with the positive control serum was determined to be 0.72 thereby

254 confirming the suitability of the coating concentration; in addition, an at least

255 three-fold difference in titre and a 2.5-fold difference of OD%-values was

256 observed between positive and negative control serum thus confirming the

257 discriminatory power of the antigens.

258 Using these antigens and coating concentrations the ELISA was performed three
259 times in duplicate for 1:500 dilutions of the individual positive and negative sera.
260 The intra-assay variation for all three ELISAs was determined to be below
261 5 OD% and the inter-assay variation below 20 OD%. Applying a cut-off range of
262 50 OD% all three ELISAs resulted in an analytical sensitivity and specificity of
263 100 % as compared to a 70 % sensitivity for the complement fixation test and
264 60 % for the competitive ELISA commercially available (Fig. 3).

265

266

267 **4. Discussion**

268

269 The control of the continuous spread of CBPP in sub-Saharan Africa is severely
270 limited by the lack of sufficiently sensitive and specific diagnostic tests. In an
271 attempt to improve the diagnostic possibilities we set out to identify novel
272 *MmmSC*-derived antigens using a non-lytic M13-based phage display system
273 (Kügler et al., 2008). The construction of phage display libraries in three different
274 reading frames using five different restriction endonucleases with a 4mer
275 recognition sequence was considered to result in a reasonable coverage of the
276 *MmmSC* genome although the three vectors facilitate the generation of a
277 functional pIII fusion protein only for one of the three possible reading frames
278 (Fig. 1). Thus, in silico analysis of the *MmmSC* PG1 genome revealed that the
279 restriction enzyme digests resulted in a total of 9,178 restriction endonuclease
280 fragments of 100 to 1000 bp in length. In total these fragments cover 3,413,598 bp
281 which corresponds to 2.8 times the *MmmSC* genome size. The finding that each
282 peptide was present at least twice in the pool of 147 clones analyzed implied that
283 the number of panning rounds was sufficient to eliminate non-specific binders

284 (Eshaghi et al., 2005; Kügler et al., 2008). When comparing the open reading
285 frames (ORFs) identified with the results of a previous study by March et al.
286 (March et al., 2006) only ORF MSC_0266 encoding a pyruvate dehydrogenase
287 was identified in both studies. This is likely due to the different methodologies
288 used; thus, March et al. (March et al., 2006) used a lytic bacteriophage Lambda
289 ZAP Express vector with insert sizes of 6 to 8 kb and screened using rabbit IgG
290 antibodies.

291 Some of the initially identified antigens are intracellular proteins. This is in
292 accordance with many other studies, where intracellular proteins were found to be
293 immunogenic (Kügler et al., 2008; March et al., 2006; Delvecchio et al., 2006;
294 Cho et al., 2008; Kodzius et al., 2003). A possible explanation for this
295 phenomenon could be the release of intracellular proteins by cell lysis or by
296 programmed cell death (Lewis, 2000).

297 Four of 22 identified antigens were consistently recognized by all positive sera
298 and showed sufficient discrimination of all negative sera. Using rabbit
299 hyperimmune sera directed against 15 other bovine mycoplasmal species these
300 four antigens were tested for serological cross-reactivity which might limit
301 diagnostic specificity. The cross-reactivity of all four antigens with *M. capricolum*
302 subsp. *capricolum* appeared acceptable since sequence analysis of the 16S rRNA
303 (Pettersson et al., 1994, 1996) and of five conserved protein-coding sequences
304 (Manso-Silván et al., 2007) showed a high sequence similarity and, consequently,
305 a close phylogenetic relationship between *MmmSC*, *M. mycoides* subsp. *capri*
306 and *M. capricolum* subsp. *capricolum*, and the other members of the “mycoides-
307 cluster”. In addition, it has been reported that *M. capricolum* subsp. *capricolum*
308 can cause CBPP-like disease in cattle (Ajuwape et al., 2003). *Acholeplasma*
309 *modicum* and *M. bovis*, however, are not members of the “mycoides-cluster”;

310 since serological cross-reactivity particularly with the frequently occurring bovine
311 pathogen *M. bovis* is unacceptable for a diagnostic test, ORF MSC_1062 was
312 excluded from further analyses. Immunoblot analysis of the three remaining
313 antigen-GST fusion proteins showed a strong reaction particularly of antigens
314 MSC_0108 and MSC_0636 (Fig. 2) thereby confirming the initial panning results
315 where these two peptides had elicited the highest reactivity.

316 The testing of the cohort sera (16 infected and six non-infected) resulted in a
317 diagnostic sensitivity and specificity of 100 % each in comparison to a diagnostic
318 sensitivity of 70 % for the CFT and 60 % for the cELISA (Fig. 3). This result
319 implies that all three antigens are potentially suitable to improve the reliability of
320 current serological testing which is primarily hampered by low diagnostic
321 sensitivity (Marobela-Raborokgwe et al., 2003). However, the cohort sera used
322 were by no means representative; in addition, *MmmSC* is known to show a high
323 antigenic variability (Gonçalves et al., 1998; Persson et al., 2002) and different
324 breeds of cattle may show a distinct serological response to *MmmSC* infection.

325 Therefore, in order to support the potential suitability of these antigens for field
326 application, additional sera from experimentally infected cattle as well as field
327 sera from different geographical regions need to be investigated in the future.

328

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330

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333

334

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336

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428 **Figure captions:**

429

430 Fig. 1. Cloning vector pHORF3 and derivatives. (A) Schematic of the phage
431 display vector pHORF3. Abbreviations: Lac Pr.: promoter of the bacterial lac
432 operon; RBS: ribosome binding site; *pelB*: sequence encoding the signal peptide
433 of bacterial pectate lyase, mediating protein secretion into the periplasmic space;
434 ochre: ochre stop codon; amber: amber stop codon; tag: hexahistidine and c-myc
435 tags; terminator: sequence terminating transcription; bla: β -lactamase gene for
436 ampicillin resistance; ColE1: bacterial origin of DNA replication; M13 ori:
437 intergenic region of phage f1. (B) Comparison of the PmeI cloning site of
438 pHORF3, pHORF3X and pHORF3XX.

439

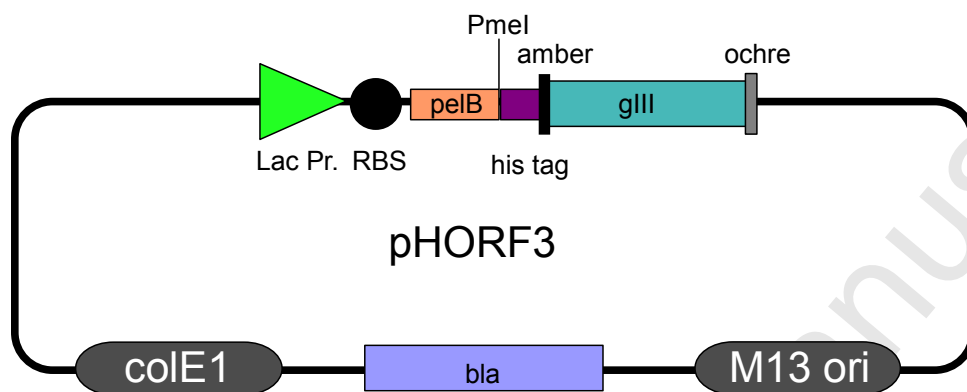
440 Fig. 2. Characterisation of GST fusion proteins. (A) SDS PAGE showing
441 aggregate preparations of recombinant fusion proteins of *E. coli* transformants
442 containing pMSC0029-500, pMSC0108-500, pMSC0636-500 and the cloning
443 vector pGEX-5x-2 (Lines 1 to 4). (B) Western blot using anti-GST antibody
444 (1:2000), (C) Western blot using the positive control serum obtained from CBPP-
445 infected animals (1:100) and (D) Western blot using the negative control serum
446 (1:100). All three clones pMSC0029-500, pMSC0108-500, pMSC0636-500 were
447 found to strongly react with the positive control serum and not with the negative
448 control serum. In addition, GST showed only minor reactivity with the positive
449 control serum.

450

451 Fig. 3. ELISA results using GST fusion proteins. Sera of ten individual CBPP-
452 infected and six uninfected animals were used in the commercial cELISA and in
453 ELISAs with the GST-fusion proteins as solid phase antigens. The horizontal line
454 indicates the cut-off given for the cELISA.. Solid symbols indicate a deviating
455 outcome of CFT test and ELISA for the respective sera. The OD%-values give the
456 arithmetic mean of three independent experiments.

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A



B

pHORF3:

3' pelB *PmeI* His Tag amber 5' gIII
 atg gcg gtt t|aa ac cat cat cac cat cat cat tag gga tcc aaa gat atc aga gct
 M A V * H H H H H H * G S K D D I R

pHORF3X:

3' pelB *PmeI* His Tag amber 5' gIII
 atg gcg gtt t|aa aca cat cat cac cat cat cat tag gga tcc aaa gat atc aga gct
 M A V * H H H H H H * G S K D D I R

pHORF3XX:

3' pelB *PmeI* His Tag amber 5' gIII
 atg gcg gtt t|aa aca a cat cat cac cat cat cat tag gga tcc aaa gat atc aga gct
 M A V * H H H H H H * G S K D D I R

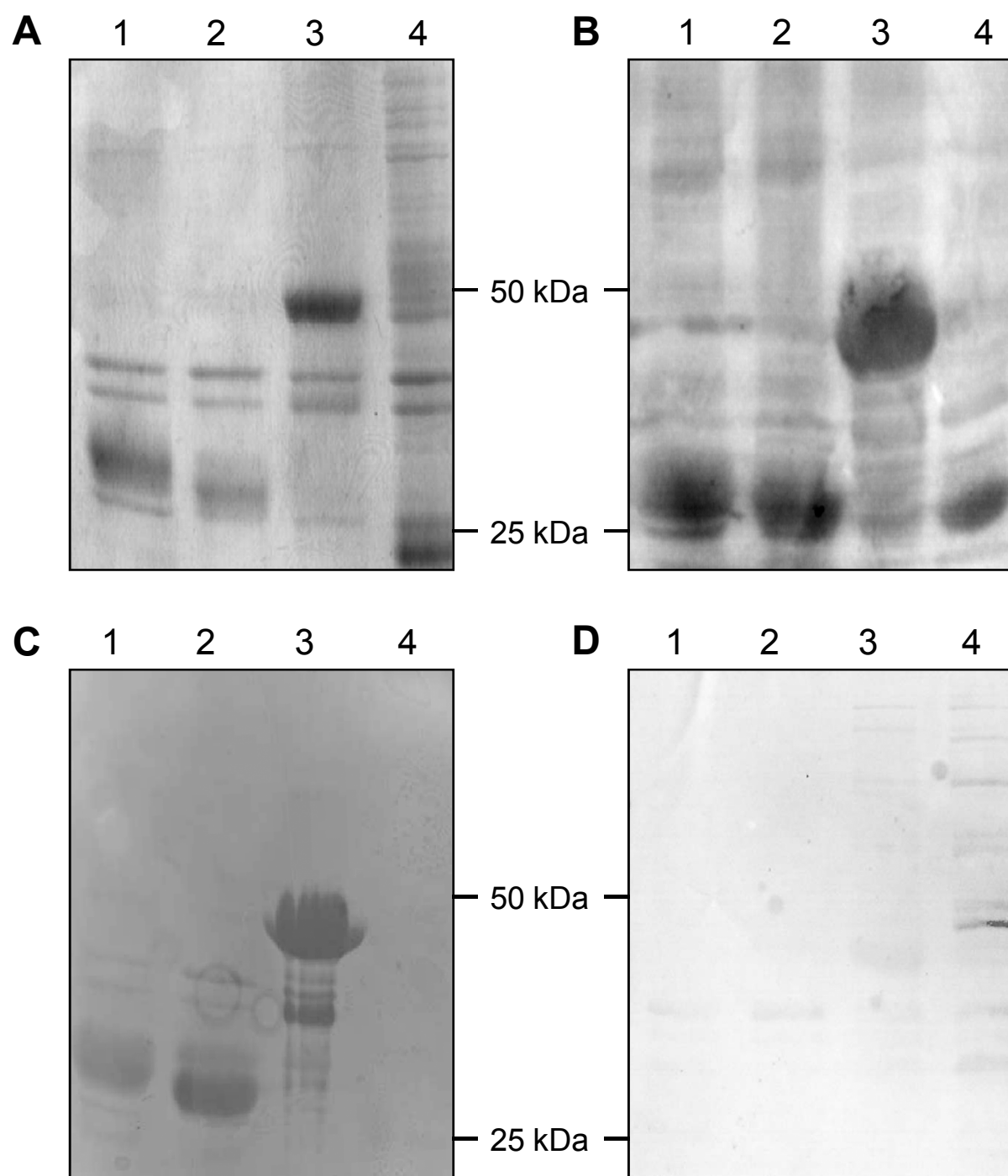


Fig. 2: Characterization of the GST fusion proteins.

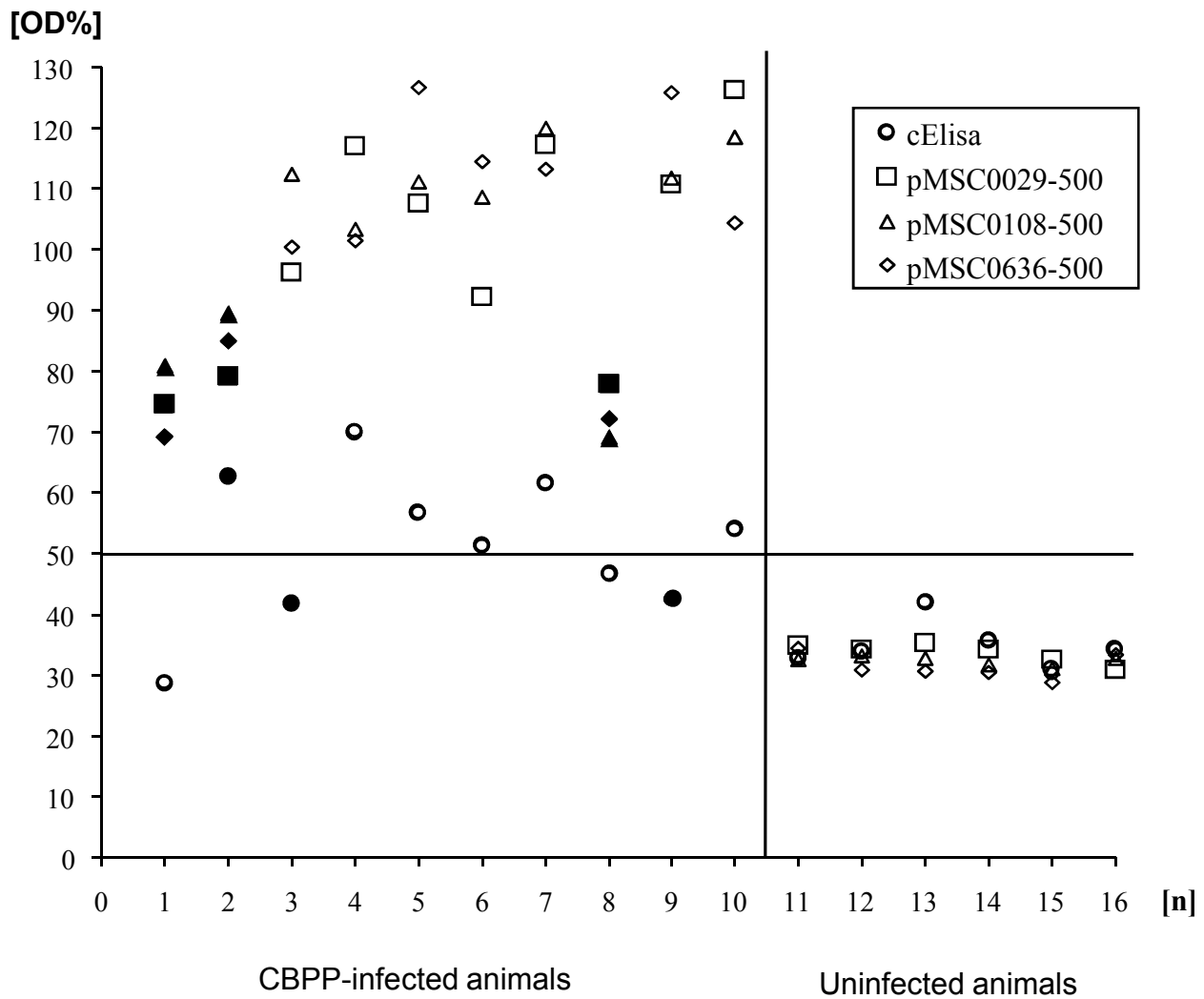


Fig. 3: ELISA results using GST fusion proteins.

Table 1

Bacterial strains, plasmids, primers and sera used in this work

Strains, plasmids, primers or sera	Characteristic(s)	Source or Reference
Strains		
<i>Mmm</i> SC	<i>Mmm</i> SC B237, isolated from an outbreak in Thika/Kenya in 1997	Jores et al., 2008
<i>E. coli</i> EC-TS40.6	K12 strain	AG Dübel
<i>E. coli</i> DH5 α F'	F' <i>endA1 hsdR17</i> ($r_K^- m_K^-$) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacZYA-argF</i>) <i>U169 deoR</i> [Φ 80 <i>dlacA(lacZ)</i> M15]	New England Biolabs, Frankfurt, Germany
M13K07	Helperphage carrying a kanamycin resistance determinant	Stratagene, Amsterdam, The Netherland
“Hyperphage”	Helperphage carrying a kanamycin resistance determinant and truncated gIII	Rondot et al., 2001
Plasmids		
pHORF3	pHORF3 used for insertion of foreign DNA	Kügler et al., 2008
pHORF3X	pHORF3 was modified such that the singular PmeI site used for insertion of foreign DNA is shifted by 1bp, allowing the in-frame fusion with gIII in an additional reading frame	This work
pHORF3XX	pHORF3 was modified such that the singular PmeI site used for insertion of foreign DNA is shifted by 2bp, allowing the in-frame fusion with gIII in an additional reading frame	This work
pGEX5x2	<i>E. coli</i> expression vector carrying an <i>bla</i> resistance determinant, for construction of GST fusion proteins	GE Healthcare, Munich, Germany
pMSC0029-500	plasmid encoding the GST-MSC_0029 fusion protein	This work
pMSC0108-500	plasmid encoding the GST-MSC_0108 fusion protein	This work
pMSC0636-500	plasmid encoding the GST-MSC_0636 fusion protein	This work

Table 1 - continued

Primers		
MHLacZPro_f	GGCTCGTATGTTGTGTGG; control for phagemids; binds with LacZ promoter	Kügler et al., 2008
MHgIII_r1	CTAAAGTTTTGTCGTCTTTCC; control for phagemids; binds with <i>pIII</i>	Kügler et al., 2008
oMHORF3_Pme_rX1	CTTTGGATCCCTAATGATGATGGTGATGA TG; primer for modification of pHORF3 by adding 1bp (pHORF3X)	This work
oMHORF3_Pme_rXX2	CTTTGGATCCCTAATGATGATGGTGATGA TGG; primer for modification of pHORF3 by adding 2bp (pHORF3XX)	This work
oMSC0029A	GATCGAATTCAATGGGGACAACACACTCA ATAT; forward primer to clone MSC_0029	This work
oMSC0029B	CGATGCGGCCGCTTAAAAAGTTCTAGCAG CTTCTAT; reverse primer to clone MSC_0029	This work
oMSC0108A	ATAATCGAATTCAATGGATTCAAGTGCT TATTCAATTCAA; forward primer to clone MSC_0108	This work
oMSC0108B	TCCGATGCGGCCGCTCATCAATTAGTTTG; reverse primer to clone MSC_0108	This work
oMSC0636A	GACGAATTCAAATGGTTTATTATGCAAGA AACAAATGAACCG; forward primer to clone MSC_0636	This work
oMSC0636B	ATATGCGGCCGCTCATGATTTTAATTGTTC TTTTAATTG; reverse primer to clone MSC_0636	This work
Sera		
Hyperimmune sera	hyperimmune sera raised in rabbits against different mycoplasmal species	FLI, Jena, Germany
CBPP negative sera	preimmune sera obtained from uninfected cattle	ILRI Nairobi Kenya
CBPP positive sera	sera from CBPP infection trials, ILRI Nairobi, Kenya	Jores et al., 2008

Table 2

Immunogenic peptides identified in *Mycoplasma mycoides* subsp. *mycoides* small colony type (*MmmSC*)

No	Fq ¹⁾	Genome position	Product from <i>MmmSC</i>	Insert length (bp)	Locus tag	OD% ²⁾
1	3	733268 - 733109	Conserved hypothetical	159	MSC_0636	56.7 %
2	2	127879 - 127937	Glycosyl transferase	59	MSC_0108	51.4 %
3	7	696995 - 697122	ATP dependent protease	128	MSC_0613	43.7 %
4	7	1153378 - 1153506	DNA directed RNA polymerase beta subunit	199	MSC_1009	39.5 %
5	8	32410 - 32351	Acyl carrier protein phosphodiesterase	60	MSC_0029	37.3 %
6	2	1205835 - 1205959	AAA family ATPase	124	MSC_1062	36.9 %
7	6	1032859 - 1032922	Amino acid permease	64	MSC_0908	34.3 %
8	5	750456 - 750516	Conserved hypothetical	60	MSC_0656	32.7 %
9	2	304438 - 304508	Pyruvate dehydrogenase	71	MSC_0266	30.0 %
10	5	218668 - 218814	Oligopeptide ABC transporter, component OppA	147	MSC_0184	30.0 %
11	8	159499 - 159643	Hypothetical transmembrane protein	144	MSC_0135	29.0 %
12	4	1160317 - 1160401	Glucose inhibited division protein A	85	MSC_1017	28.3 %
13	2	23668 - 23767	Mannitol-1-phosphate 5-dehydrogenase	99	MSC_0017	26.8 %
14	26	77529 - 77641	Transcriptional regulator; NifR3 family	184	MSC_0068	26.7 %
15	3	940581 - 940693	Conserved hypothetical	112	MSC_0826	25.2 %
16	19	1117031 - 1117172	UDP glucose 4-epimerase	141	MSC_0978	24.5 %
17	5	44289 - 44393	D-Lactate dehydrogenase	105	MSC_0034	24.5 %
18	4	701239 - 701317	Prolipoprotein	78	MSC_0617	23.8 %
19	6	1210760 - 1210841	Conserved hypothetical transmembrane protein	82	MSC_1066	22.3 %
20	4	698434 - 698492	Putative hydrolase of HAD family	69	MSC_0614	22.1 %
21	3	385965 - 386234	tRNA pseudouridine syntase B	269	MSC_0336	21.0 %
22	3	97781 - 98005	Prolipoprotein ; putative phosphate ABC transport	224	MSC_0079	21.0 %

¹⁾ Frequency of clones among the random sample of 134 clones sequenced.

²⁾ The optical density obtained with *MmmSC* whole cell lysate used as solid phase antigen served as positive control (100 %).

Table 3

ELISA cross-reactivity of four clones with rabbit sera directed against 16 different

Mycoplasma species

Serum raised against	culture collection number	OD% +/- standard deviation			
		MSC_029	MSC_0108	MSC_636	MSC_1062
<i>A. axanthum</i> S 743	NCTC 10138	28 ± 8.6	26 ± 4.1	35 ± 2.3	45 ± 1.8
<i>A. laidlawii</i> PG 8	NCTC 10116	39 ± 8.3	43 ± 0.2	42 ± 3.5	39 ± 1.7
<i>A. modicum</i> PG 49	NCTC 10134	32 ± 6.8	26 ± 0.6	32 ± 0.4	51 ± 1.2
<i>M. alkalescens</i> PG 51	ATCC 29103	17 ± 0.6	21 ± 2.1	43 ± 1.3	41 ± 0.5
<i>M. arginini</i> G 230	NCTC 10129	21 ± 1.6	21 ± 0.3	26 ± 1.6	32 ± 0.3
<i>M. bovirhinalium</i> PG 11	ATCC 19852	17 ± 0.3	17 ± 1.9	22 ± 1.1	25 ± 0.3
<i>M. bovirhinalis</i> PG 43	NCTC 10118	36 ± 4.8	40 ± 2.1	44 ± 14.9	44 ± 3.2
<i>M. bovis</i> Donetta PG 45	ATCC 25523	44 ± 13.7	39 ± 2.5	41 ± 5.6	49 ± 2.3
<i>M. bovoculi</i> M 165/69	NCTC 10141	44 ± 0.4	42 ± 8.8	44 ± 7.1	33 ± 2.9
<i>M. californicum</i> ST-6	ATCC 33461	21 ± 2.5	22 ± 0.9	32 ± 0.6	32 ± 2.2
<i>M. capricolum</i> subsp. <i>capricolum</i> california kid	NCTC 10154	45 ± 21.7	48 ± 0.5	45 ± 1.4	57 ± 4.9
<i>M. gallinarum</i> PG 16	ATCC 19708	19 ± 1.9	28 ± 2.4	45 ± 1.1	44 ± 0.3
<i>M. gatae</i> C 8	ATCC 23392	30 ± 4.2	21 ± 4.0	27 ± 1.5	31 ± 2.0
<i>M. leachii</i> PG 50	NCTC 10133	46 ± 6.0	27 ± 2.0	33 ± 4.3	45 ± 7.3
<i>M. mycoides</i> subsp. <i>capri</i> y-goat	NCTC 10706	30 ± 1.2	27 ± 2.3	30 ± 0.5	34 ± 3.4
MmmSC PG 1	NCTC 10114	100 ± 4.6	100 ± 12.1	100 ± 2.5	100 ± 8.9
Negative serum	-	27 ± 3.0	30 ± 2.6	29 ± 1.0	36 ± 0.7