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Use of *Mycobacterium avium* subsp. *paratuberculosis* specific coding sequences for serodiagnosis of bovine paratuberculosis

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1 **Use of *Mycobacterium avium* subsp. *paratuberculosis* specific coding**
2 **sequences for serodiagnosis of bovine paratuberculosis.**

3
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15 **Running title:** specific antigens for Johne's disease diagnosis

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27 **Abstract**

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29 In this study, the finished complete genome of *Mycobacterium avium* subsp.
30 *paratuberculosis* (Map) was screened for specific coding sequences that could be very
31 valuable in the design of a sensitive and specific Map detection serological assay. 87 Map
32 specific sequences were retained. Among these, three candidate antigens have been analysed
33 for their serodiagnostic potential. These antigens were selected on the basis of their putative
34 immunogenicity as predicted by *in silico* analysis. The antigens were cloned in *E. coli*,
35 expressed, and purified before testing in an antibody detection ELISA, test using a well
36 characterized panel of 18 and 48 sera from Map infected and uninfected cattle, respectively.
37 Two of these antigens, antigen 6 and MAP1637c, yielded in our conditions a sensitivity of 72
38 and 82%, respectively, for a specificity of 98%. It is particularly noticeable that, when probed
39 with the same serum panel, the most widely used European paratuberculosis commercial
40 seroassay (Pourquier test) yielded a sensitivity of 72% for a specificity of only 92%.

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42 **Keywords:** specific antigens, *Mycobacterium avium* subsp. *paratuberculosis*, serodiagnosis,
43 ELISA.

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51 **Introduction**

52 Bovine paratuberculosis is a chronic granulomatous enteritis caused by *Mycobacterium avium*
53 subsp. *paratuberculosis* (Map) and characterised by intermittent diarrhoea associated with
54 rapid weight loss. Variable prevalence has been reported (Cetinkaya et al., 1996; Giese and
55 Ahrens, 2000; Jakobsen et al., 2000; Kennedy and Benedictus, 2001; Streeter et al., 1995;
56 Wells and Wagner, 2000), ranging between 3.5% and 70% in developed countries, and annual
57 financial losses (Gonda et al., 2007) were evaluated to \$200 million for the US dairy industry
58 alone (Ott et al., 1999).

59 It is largely accepted that major improvements are needed in currently available
60 serodiagnostic tests to allow for an efficient control program to be set up (Collins et al., 2005;
61 Sweeney et al., 2006). Many laboratories have therefore chosen to improve the efficiency of
62 serological assays by replacing the current common “extract antigen” by “purified Map
63 specific subunit antigens”(Dupont et al., 2005; Huntley et al., 2005; Olsen et al., 2001;
64 Paustian et al., 2004; Willemsen et al., 2006). Different approaches have been used in the
65 search for such antigens. Among other things we can mention immunoproteomic approaches,
66 in which antigens are firstly selected on the basis of their antigenicity, and genomic
67 approaches, in which antigens are selected in the finished genome of Map on the basis of their
68 sequence specificity (Li et al., 2005). Recently, two large scale immunoproteomic analyses
69 have been published and reported very promising results obtained via such approach (Cho et
70 al., 2007; Cho et al., 2006; Leroy et al., 2007). To date, a number of antigens have also been
71 identified using the genomic approach, but data about their reactivity in ELISA using large
72 panels of sera, are still lacking (Bannantine et al., 2004a; Bannantine et al., 2008a; Bannantine
73 et al., 2004b).

74 Here, we present data on the bioinformatic screening of the Map genome in search for Map
75 specific genes. Among them, Map-specific coding sequences predicted to present the most

76 potent antigenicity, as determined *in silico*, were selected for cloning, expression and
77 serological analysis. Three candidate antigens were selected and probed in an ELISA test with
78 66 sera from Map- infected cattle and negative control cattle. Two of these candidate antigens
79 were strongly antigenic and might be included in a diagnostic assay based on purified
80 antigens.

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99 **Materials and methods**

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101 *Sera*

102 Sera used in this study have already been extensively described previously (Leroy et al.,
103 2007). Briefly, a panel of 48 sera from two *M. bovis* infected herds with no known cases of
104 paratuberculosis were used as negative controls. All animals included as negative controls
105 were tested in the *in vitro* interferon gamma assay using ESAT6 and CFP10 and post-mortem
106 by bacterial culture. Most had their *M. bovis*-sensitised status confirmed by the interferon
107 gamma test (41/48), or by post-mortem *M. bovis* isolation (20/48). These animals were 3 to 5
108 years old and were either low (5), medium (7) or high (6) faecal shedders of Map at time of
109 sampling. Three high faecal shedders showed clinical signs of JD and two out of 4 sacrificed
110 high shedders demonstrated extensive intestinal lesions. For all 18 positive controls, Map
111 infection was confirmed by faecal culture. Both positive and negative serum panels were also
112 tested with the Pourquier test as reference serological assay. All data about the sera and
113 results of Pourquier test are summarized in supplemental table SI

114

115 *Selection of candidate antigens*

116

117 Before the availability of the genome of Map (completed since February 2004
118 (*Mycobacterium avium* subsp. *paratuberculosis* str. k10/ Li,L., Bannantine,J., Zhang,Q.,
119 Amonsin,A., Alt,D. and Kapur,V. Direct Submission. Submitted (05-SEP-2003) Modified (30
120 -Jan -2004)) it was already feasible to blast against the available Map contigs on the National
121 Center for Biotechnology Information (NCBI) web site
122 (<http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi>). The genome of *Mycobacterium avium*
123 subsp. *avium* (Maa), that is the closest genome to Map, was used as query sequence for a

124 BLASTn against the Map contigs. As a result all the Map contigs were returned and
125 assembled in a unique silico-genome of 4 950 895 bases. In this case, borders of all joined
126 contigs were replaced by N.

127 Coding sequences (CDS) were detected by the program GLIMMER. This program tracks
128 down the stop codon of all genes and predicts a corresponding CDS. GLIMMER was used
129 because it was not stopped by the unknown (N) bases. The smallest sequence recognized by
130 GLIMMER is 90bp long. Other programs were used to predict the structure and the
131 localisation of the putative proteins. We used PsortII for Gram positives, TMHMM and
132 TMPred programs.

133 A last selection was carried out using three different T cell epitope prediction programs,
134 “Tsites” , “BIMAS “ and “SYFPEITHI”, in order to select the most promising antigenic
135 sequences, with respect to future vaccine development.

136

137 *Production of the candidate antigens*

138

139 Genes encoding candidate antigen proteins were amplified by PCR from *M. avium* subsp.
140 *paratuberculosis* ATCC 19698 genomic DNA (Rosseels et al., 2006) were initially cloned in
141 a V1J.ns-tPA vector (Merck Research Laboratories, PA, USA) (Roupie et al., 2008). Coding
142 sequences were subsequently subcloned by PCR amplification, (Expand High Fidelity PCR
143 System, Roche), agarose gel purification (QIAkit PCR kit, Qiagen), and ligation into a pQE-
144 80L (Qiagen) expression vector predigested with *Bam*HI / *Hind*III. After ligation (T4 DNA
145 ligase, Fermentas) and transformation into Top-10F' chemically competent *Escherichia coli*
146 cells (Invitrogen) for expression, positive clones were screened on LB-ampicillin medium
147 (100 µg/ml) and confirmed by restriction enzyme digestion. The integrity of cloned sequences
148 was checked by sequence analysis.

149 For antigen expression, *E. coli* positive clones were grown on LB-ampicillin medium at 37°C
150 under agitation until they reached an OD_{600nm} of 0.6 and antigen expression was induced by
151 addition of IPTG (1mM final concentration) for 4 hours for Ag6 and overnight for Ag5 and
152 Ag7.

153 Bacteria were lysed using lysozyme (r-lysozyme, Novagen) and bath sonicator (2 x 5 minutes
154 on ice) in extraction buffer (EB: 50 mM Tris-HCl pH 7.5, NaCl 500mM, Imidazole 10mM, β-
155 MSH 1mM) containing 8M urea and protease inhibitors (EDTA free miniprotease inhibitor
156 tabs, Roche). Extraction efficiency of the different supernatants was analysed by SDS PAGE.

157

158 *Purification of candidate antigens*

159

160 His-Select cartridges (Sigma) were used for candidate antigen purification following
161 manufacturer's instructions. Candidate antigens were eluted using an imidazole gradient of 10
162 to 300 mM in 100 ml. To evaluate their homogeneity, concentrated candidate antigens were
163 analysed in SDS-PAGE and all Coomassie Brilliant Blue stained bands were analysed by
164 mass spectrometry. Protein concentrations were measured using the Non-Interfering Protein
165 Assay kit (Novagen) following manufacturer's instructions.

166

167 *Protein characterization by mass spectrometry analysis*

168

169 The Coomassie stained proteins of interest were cut out, and destained and washed twice in
170 25 mM NH₄HCO₃ for 15 minutes under gentle agitation at room temperature, followed by
171 two 15-minute washes in 25 mM NH₄HCO₃, 50 % (v/v) CH₃CN. Cysteines were
172 carboxyamidomethylated by incubation of gel pieces in 25 mM NH₄HCO₃, 50mM DTE for
173 30 minutes at 56°C followed by 30 minutes in 25 mM NH₄HCO₃, 50mM iodoacetamide at

174 room temperature in the dark. After Speed vacuum dehydration, 10 μl of a 0.02 $\mu\text{g}/\mu\text{l}$ trypsin
175 solution in 25 mM NH_4HCO_3 (Promega Madison), were added, and samples were incubated
176 overnight at 37°C. Tryptic digestion was stopped by addition of 1 μl of 5 % (v/v) formic acid.
177 For MALDI-TOF analysis, 1 μl of each sample was mixed with 1 μl of matrix (5 mg/ml α -
178 cyano-4-hydroxycinnamic acid and 0.5 pmol/ μl rennin as internal standard, in 25 % (v/v)
179 ethanol, 25 % (v/v) acetonitrile, 0.05 % (v/v) TFA), then spotted onto a MALDI sample plate
180 and allowed to air dry. MALDI-TOF was performed using a M@ldiTM mass spectrometer
181 (Micromass, Manchester, UK) equipped with a 337 nm nitrogen laser. The instrument was
182 operated in the positive reflectron mode with 15 kV of source voltage, 2.5 kV of pulse voltage
183 and 2 kV of reflecting voltage. Proteins identification was realized using ProteinLynx global
184 server (Micromass) in an in-house Map genomic database, using mass tolerance of 100 ppm,
185 carbamidomethylation of Cysteine as fixed modification and oxidation of methionine as
186 variable modification.

187

188 *Antibody ELISA*

189

190 The commercial Pourquier ELISA was performed following the manufacturer's instructions.
191 Candidate antigens were tested in ELISA on 96 well plates (MaxiSorb, Nunc) coated
192 overnight at room temperature with 100 μl of candidate antigens diluted at 4 $\mu\text{g}/\text{ml}$ in 100
193 mM $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH 9.6 or formaldehyde 37% (v/v). After washing three times
194 in PBS containing 0.05% (v/v) Tween 20 (PBST), non specific interaction sites were blocked
195 by incubation with 150 μl of 2.5% caseine hydrolysate (w/v) in PBST. 100 μl of sera diluted in
196 PBST containing 1.25% caseine hydrolysate (w/v) (1:250) were then added and incubated 1
197 hour at 37°C. After washing three times with PBST, plates were incubated 1 hour at 37°C
198 with 100 μl of secondary antibody diluted in PBST containing 1.25% caseine hydrolysate

199 (w/v). Two different HRP conjugated secondary antibodies were used. The first one
200 purchased from Sigma-Aldrich and raised against bovine Ig was diluted 1:2000. The second
201 one is a monoclonal antibody specifically directed against type G bovine immunoglobulin.
202 This antibody developed by Letesson *et al.* (Letesson et al., 1985) was kindly provided by
203 Bio-X Diagnostic (Jemelle, Belgium) and diluted 1:50. After washing three times with PBST,
204 HRP activity immobilized on the plate was detected by reaction with tetramethylbenzidine for
205 5-15 minutes. Reaction was stopped by addition of an equal volume of 1N sulphuric acid and
206 evaluated by colorimetric measurement at 450 nm. ROC analysis was used to determine best
207 cut off value and associated sensitivity and specificity.

208

209 **Results**

210 *Selection of candidate antigens*

211

212 7137 CDS were predicted by GLIMMER and submitted to a BLAST 2.2.3. n (with an e value
213 $< 10^{-3}$) against the genome of *Mycobacterium avium* subsp. *avium* (Maa).

214 After the BLAST, 285 CDS appeared absent from Maa. Ten sequences were considered as
215 wrong because they overlapped two contigs. We translated the remaining 275 CDS into
216 protein sequences and used a BLAST 2.2.3. p / nr.: 101 sequences with a hit on proteins from
217 other mycobacteria were discarded. The ensuing selection of candidate antigens was based on
218 the analysis of the 174 remaining sequences: first, we discarded two sequences that were
219 overlapping each other, and second the sequences that were smaller than 120 bp were also
220 discarded. Finally we got 121 sequences: 27 with “No significant similarity found”, and 94
221 with one or more hits but without a BLAST mycobacterial hit.

222 19 sequences were discarded because already analysed in other publications ((Bannantine et
223 al., 2002; Bannantine et al., 2004b).

224 To continue this selection, the programs Psort II and TMHMM were used to find the
225 subcellular localisation of the protein. Proteins predicted to have more than 3 transmembrane
226 helices were also discarded.

227 Finally, 87 putative proteins specific for Map were identified by this *in silico* method (table
228 SII): 25 sequences belonged to the annotated proteins of Map; 22 sequences had a BLAST
229 with a result: “No significant similarity found”; and 40 putative proteins had a positive
230 BLASTp but without a hit for other mycobacteria.

231 The antigenicity-driven selection based on three online available T cell epitope prediction
232 programs (Tsites , BIMAS and SYFPEITHI) permitted finally to select 3 sequences as the
233 putatively most potent to be analysed for antigenicity in an antibody ELISA test (table I).
234 Sequence of Antigen 7 (coordinates: 1791069-1792508) corresponds to MAP1637c.
235 Sequence of antigen 6 (coordinates: complement 886138-886530) has not been annotated in
236 itself but it overlaps two sequences annotated on the complementary strand, MAP0864
237 (coordinates: 886023-886448) and MAP0865 (coordinates: 886451-887722). Sequence of
238 antigen 5 (2383504-2384184) has not been annotated.

239

240 *Production of candidate antigens*

241

242 The three antigens were successfully cloned into the pQE-80L expression vector (QIAGEN),
243 and expressed in *E. coli* as histidine-tagged proteins (data not shown). For an unknown
244 reason, overnight expression was not suitable for Ag6 and was replaced by a 4 hours
245 induction. It is possible that Ag6 presents some toxicity that would impair its overnight
246 expression. All three antigens were expressed in as inclusion bodies and required urea
247 extraction. Moreover, Ag5 was only partially extracted in urea and use of detergent was
248 necessary to obtain satisfactory yields.

249

250 *Purification of candidate antigens*

251 First, purified candidate antigens were analysed in mass spectrometry to confirm that the
252 produced proteins matched the selected specific nucleotide sequences. For antigen 5 and 6
253 peptide mass fingerprint of tryptic digest corresponded to the right sequence. For antigen 7,
254 on the other hand, in addition to a faster than expected migration in SDS-PAGE, no peptides
255 were identified in the N-terminal region of the purified protein. Sequencing of the cloned
256 insert reveals the loss of this particular region. Only corrected coordinates are presented in
257 table I.

258 IMAC strategy using an imidazole gradient rather than the common step procedure was
259 particularly efficient for antigen purification. Indeed, purified and concentrated antigens were
260 analysed by SDS-PAGE and all Coomassie stained bands were analysed in mass spectrometry
261 (Fig. 1). In this analysis, all proteins bands were identified as belonging to the candidate
262 antigens studied and resulting from polymerisation/degradation phenomena. Two weak
263 protein bands in Ag7 sample (indicated by # on figure 1) were not derived from this antigen
264 and were no *E. Coli* contaminants either. Purification yield were in the range of 0.3 to 0.6 mg
265 of candidate antigens/g of bacteria.

266

267 *Serological assay*

268

269 The three candidate antigens were tested individually for their ability to discriminate Map
270 infected animals from the negative controls originating from two *M. bovis* infected herds.
271 Most of the negative controls had their *M. bovis*-sensitised status confirmed by the ESAT6
272 and CFP10 based interferon gamma test or by post-mortem *M. bovis* isolation. Two different
273 coating conditions were tested as well as two different secondary antibodies. Whatever the

274 plate coating conditions, Ag5 gave the worst results. Indeed the discrimination that could be
275 made between the responses obtained with sera from Map infected animals and from the
276 negative controls was very poor (Se: 27.8% (9.70-53.48%, 95% CI); Sp: 97.92% (88.93-
277 99.95%, 95% CI)). Clearly, this antigen has no potential as diagnostic antigen since it failed
278 in discrimination of Map infected cattle (Fig. 2).

279 Results obtained with Ag6 and Ag7 using coating in formaldehyde were only slightly better,
280 resulting in sensitivities of 44.4% (21.5-67.4%, 95% CI) and 66.7% (44.9-88.4%, 95% CI),
281 respectively, at a given specificity of 97.9% (93.9-100%, 95% CI) (data not shown).
282 However, in contrast to what has been observed in previous studies (Leroy et al., 2007; Speer
283 et al., 2006), sensitivity was increased by using a carbonate buffer for plate coating. Indeed, as
284 shown in figure 2, sensitivities of 72.2% (46.52-90.31%, 95% CI) and 77.8% (52.36-93.59%,
285 95% CI) were obtained in four independent experiments carried out with Ag6 and Ag7
286 respectively, at a constant specificity of 97.9% for both (Fig. 2). For Ag7, the sensitivity could
287 be further increased by use of an IgG-specific monoclonal antibody from Bio-X Diagnostic.
288 Indeed, use of this secondary antibody permitted to enhance the sensitivity of the test to
289 83.3% for a specificity of 97.9% (data not shown). For Ag6, responses were not
290 systematically increased using this Mab.

291 All these results are particularly interesting when compared to results obtained with the
292 Pourquier test in a duplicate analysis (Table SI). Pourquier test when used to probe our panel
293 of sera yielded a sensitivity of 72.2% (46.52-90.31%, 95% CI) for a specificity of 91.7 (83.8-
294 99.5%, 95% CI) (Fig. 2). It thus seems that Ag6 and Ag7 performed better separately than the
295 Pourquier test in discriminating Map infected cattle from negative controls originating from a
296 *M. bovis* infected herds. Nevertheless, no statistical difference between both tests was
297 observed using a McNemar's test (data not shown).

298

299 **Discussion**

300 *Mycobacterium avium* subsp. *paratuberculosis* (Map) is the etiologic agent of bovine
301 paratuberculosis, also called Johne's disease. As no effective therapeutics presently exist to
302 combat Johne's disease, and because current vaccines interfere with control programs for
303 bovine tuberculosis, large scale testing and infected animal confinement is the only
304 reasonable way to limit disease prevalence in cattle (Harris and Barletta, 2001). However,
305 despite several years of development, currently available diagnostic tests still lack
306 performance to be used in large scale control programs. Among the different available
307 diagnostic tools (PCR, faecal culture...), serodiagnosis is the most promising. Indeed, ELISA
308 based serological tests are easy to perform, cheap to produce and do not require dedicated
309 facilities. In these current commercial ELISAs, antigens used for plate coating are different,
310 crude bacterial extracts requiring *M. phlei* preabsorption of sera in order to remove cross-
311 reacting antibodies. This pre-absorption step is not only time consuming but also affects test
312 sensitivity (McKenna et al., 2005; Sockett et al., 1992). This is the main reason why we have
313 chosen to focus our effort on the development of a serodiagnostic test based on one or a
314 handful of purified antigens rather than on a Map extract.

315 In this study, a genomic analysis of Map genome allowed us to identify *in silico* Map specific
316 candidate antigens. A similar approach has already been used by other authors using
317 approximately half of the *M. avium* subsp. *paratuberculosis* genome (Bannantine et al., 2002;
318 Bannantine et al., 2004b). On the contrary we used almost all the Map genome to perform our
319 research and actually nineteen sequences were found in common with these previous reports,
320 demonstrating the validity of the chosen selection criteria. As their serodiagnostic potential
321 has already been evaluated, these 19 sequences were discarded from our analysis.

322 Among the database of 87 Map specific sequences, potentially antigenic proteins were
323 identified using bioinformatic tools. These last steps led to the final selection of three Map
324 specific candidate antigens.

325 These three genes were cloned and produced as recombinant his-tagged proteins in *E. coli*.
326 Antibody ELISA based serological test was used to evaluate their ability to discriminate Map
327 infected cattle from negative controls. Ag5 clearly demonstrated insufficient antigenicity
328 since no clear increase in the signal obtained with sera of Map infected animals could be
329 recorded. Results obtained with the two other antigens were clearly more encouraging.
330 Indeed, ELISA based serological test using Ag6 and Ag7 as coating antigens permitted to
331 reach sensitivities of 72.2% and 83.3%, respectively. The Pourquier test, one of the mostly
332 used tests in Europe, yielded on the same panel of sera, a sensitivity of 72.2% and a Sp of
333 91.7%. Clearly, ELISA based serological test using Ag6 and Ag7 are at least as sensitive as
334 Pourquier test used in standard conditions and should now be evaluated with a larger panel of
335 sera.

336 Interestingly, the results obtained with Ag6 and Ag7 were better using coating with carbonate
337 than with formaldehyde. In a previous report the positive effect of formaldehyde was clearly
338 demonstrated (Leroy et al., 2007; Speer et al., 2006) and explained by a better fixation of the
339 antigen to the microwell plate through the fixative effect of formaldehyde. However, this
340 explanation is not supported by the observations with Ag6 and Ag7 reported here. It is
341 possible that formaldehyde plays a positive role in antigen detection by stabilization of some
342 epitopes by covalent cross-link formation. This cross-linking could be detrimental for
343 antibodies directed against certain proteins such as Ag6 and Ag7. In this study, Pourquier test
344 has been used according to the manufactory's protocols without evaluation of formaldehyde
345 treatment. A possible positive effect on sensitivity of the Pourquier test should be evaluated.

346 It is interesting to note that the sequence of antigen 6 overlaps the complementary strand of
347 MAP0864 and MAP0865 but on the reverse strand, protein sequences thus having no
348 similarities. These two sequences were also selected based on their specificity by Bannantine
349 et al (Bannantine et al., 2008b). MAP0865 was one of the most immunogenic antigens in the
350 developed protein array analysis. Moreover, in a very recent publication Bannantine et al.
351 (Bannantine et al., 2008a), showed that MAP0865 could be detected as early as 70 days post-
352 infection by sear from experimentally infected animals. Finally, these proteins also gave good
353 results in an ELISA-based test for the diagnosis of ovine paratuberculosis (Bannantine et al.,
354 2008c). It is astonishing that this particular nucleotide sequence leads to two completely
355 different proteins both appearing finally very immunogenic in independent studies. The
356 expressed sequence of antigen 6 has been verified by mass spectrometry and its peptide mass
357 fingerprint corresponds to the sequence deduced from the nucleotide sequence. Such a
358 precaution was also taken in study involving MAP0865 by sequencing cloned insert.

359 The control sera used in this analysis were all from animals from two *M. bovis* infected herds
360 with no known cases of Map infection. Most of the animals were positive in an ESAT6 and
361 CFP10 based interferon gamma test, confirming the within-herd sensitisation to *M. bovis*
362 (Aagaard et al., 2006) further demonstrated by its isolation in half of the cattle. This group of
363 negative control cattle thus represents very stringent discriminating conditions. Facing these
364 negative controls, specificity of the Map Pourquier test was only of 91.6%, *i.e.* four sera
365 among 48 were detected as false-positive. Specificity obtained with Ag6 and Ag7 as coating
366 antigens was 97.9% for both. It means that the strategy chosen here, *i.e. in silico* genomic
367 analysis for selection of Map specific genes has been efficient. Our results also suggest that
368 when tested with *M. bovis* sensitized animals, the specificity of the Pourquier test decreases
369 significantly in comparison to what is generally observed (>99%, (Christopher-Hennings et
370 al., 2003). It is possible that *M. bovis* cross reaction is not completely abolished by

371 preabsorption on *M. phlei* extract. The actual specificity of pre-absorbed tests is generally not
372 calculated using *M. bovis* sensitized animals.

373 Obviously, the use of a 'golden' antigen should be able to discriminate all infected animals
374 regardless of their clinical stage and with a very small false-positive rate. But it is now clear
375 that such an antigen probably does not exist and that the most efficient diagnostic test would
376 be based on a combination of purified antigens. Moreover, Bannantine and coworkers
377 (Bannantine et al., 2008a) have demonstrated, using an experimental infection model, that
378 efficiency of an antigen could vary and decrease over the course of the infection. Thus
379 antigens effective in detection of clinical cases could be inefficient for early diagnosis. In this
380 context, antigens presenting sensitivities of more than 70% in an individual test, such as Ag6
381 and Ag7, must be regarded as good candidates to formulate an antigen combination useful to
382 develop a high efficiency ELISA-based serological test.

383 Finally, the results obtained in this study clearly indicate that a genomic analysis in search for
384 specificity, combined with a bioinformatic analysis in search of potential immunogenicity is
385 efficient to select candidate antigens. This approach is undoubtedly complementary to the
386 immunoproteomic analysis we have already performed in the same context. Indeed, if the
387 immunoproteomic approach has been shown to be very efficient, not all proteins expressed *in*
388 *vivo* by Map are sampled in this analysis and thus some very potent antigens might be missed.
389 These antigens could be detected by the genomic *in silico* analysis. On the other hand,
390 immunoproteomic analysis has also shown us that some proteins even if they are not
391 completely Map specific, could lead to sufficient specificity to be included in a diagnostic
392 test.

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502 **Legends to figures**

503

504 Figure 1: SDS-PAGE analysis of purified candidate antigens. Purification efficiency was
505 very high thanks to the use of an imidazole gradient for elution instead of the generally used
506 step procedure. Contamination was monitored by mass spectrometry of all visible bands in the
507 sample. Except for two unidentified bands in Ag7 (#), all other protein bands belonged to the
508 studied proteins and resulted from degradation/polymerisation phenomena.

509

510 Figure 2: Comparison of results obtained using Pourquier and Ag5, Ag6 and Ag7 based
511 ELISA. For Ag5, no significant difference was found between signals obtained with control,
512 and MAP⁺ sera (Se: 27.8%; Sp: 97.92%). On the other hand, sensitivities of 72.2% (46.52-
513 90.31%, 95% CI) and 77.8 % (52.36-93.59%, 95% CI) were obtained in four independent
514 experiments realised with Ag6 and Ag7 respectively with a constant specificity of 97.9% for
515 both. These results clearly suggest that Ag6 and Ag7 could be valuable tools for development
516 of an ELISA based diagnostic test of Map infection in cattle.

Table I

	NCBI reference	Protein Name	Location	pCDS on NC_002944.2
Antigen 7	NP_960571.1	MAP1637c	complement:	1791402..1792508
Antigen 6	N.D.	N.D.	complement:	886138..886530
Antigen 5	N.D.	N.D.	complement:	2383504..2384184

Table I: coordinates of the three selected sequences on Map chromosome.

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