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15 **Abstract**

16 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a slow growing bacterium that can infect
17 ruminants and remain latent for years without development of any clinical signs or disease.
18 Diagnosis is often based on detection of MAP antibodies in milk or serum samples or culture of
19 bacteria from faeces; however, these diagnostic tools are often not applicable until years after
20 infection. Detection of MAP specific cell-mediated immune (CMI) responses can serve as an
21 alternative and be implemented in a diagnostic tool. CMI responses can be measured at an early
22 stage of infection, prior to development of antibodies and shedding of detectable amounts of MAP.
23 At present, available diagnostic assays are limited by the lack of MAP specific antigens included in
24 these assays resulting in poor specificity. The objective of this review is to provide a systematic
25 overview of diagnostic MAP antigen candidates described to date with special emphasis on antigen
26 candidates tested for CMI responses. Relevant information on 115 different MAP antigens was
27 systematically extracted from literature and summarized in 6 tables of CMI antigens, secreted
28 antigens, cell wall and membrane antigens, lipoprotein antigens, heat shock antigens and
29 hypothetical antigens. Strategies for evaluation of novel antigen candidates are discussed critically.
30 Relatively few of the described antigens were evaluated for their use in CMI based diagnostic
31 assays and so far, no obvious candidate has been identified for this application. Most of the novel
32 diagnostic candidates were evaluated in few animals and it is recommended that an appropriate
33 sample size is included for evaluation of antigen candidates in future studies.

34

35 *Key words: paratuberculosis, antigen, diagnostic antigens*

36

36 1. Introduction

37 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis in
38 ruminants. MAP is part of the large and diverse *Mycobacterium* family that is defined by their acid-
39 fast properties, mycolic acid-containing cell walls, and high genomic C+G content (61-71%). The
40 family currently counts more than 130 established and validated species and subspecies (Turenne et
41 al., 2007). Besides ruminants, MAP can infect a large range of host species including birds, rabbits,
42 foxes and badgers (Beard et al., 2001). Infection of dairy cattle leads to economic losses because of
43 reduced milk yield, premature culling and reduced slaughter value (Ott et al., 1999). Cattle are most
44 susceptible to infection as calves where they are infected while still in the uterus or by ingestion of
45 bacteria through milk, colostrum or faecal matter (Chiodini et al., 1984; Sweeney, 1996).

46 Development of a cell-mediated immune (CMI) response is essential to the host in defence against
47 intracellular MAP and is elicited at an early stage of infection before antibodies are detected. As the
48 disease progress from subclinical to clinical, the CMI response wanes and is replaced by a strong
49 humoral immune response characterised by antibody formation (Chiodini, 1996; Stabel, 2000).

50 Antibodies do not protect the animal against the intracellular pathogen and in the final stages of
51 infection suppression of the immune system and a state of anergy may occur (Waters et al., 1999).

52 Diagnosis of MAP infected animals is frequently based on detection of antibodies in milk or serum,
53 or by cultivation of bacteria from faeces, but these diagnostic methods are usually not applicable
54 until years after infection at an advanced stage of disease (Nielsen and Toft, 2006). Contrary, CMI
55 based diagnostics are applicable at an early stage of infection, prior to antibody formation and
56 bacterial shedding in faeces. Hence, by using various diagnostic test strategies it is possible to
57 detect infection with MAP at different stages of disease. The main limitation of available diagnostic
58 tools is the lack of MAP specificity of included antigens resulting in poor specificity.

59 The aim of the current review was to provide an overview of MAP antigen candidates that has been
60 used for diagnostic application to date. The emphasis will be on diagnostics measuring CMI,
61 although humoral based approaches and different types of antigen will also be discussed.

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63 **2. Literature review**

64 A review of MAP antigen candidates was carried out by searching the databases Web of Science
65 and Medline through PubMed. The search terms were paratuberculosis; Johne's or Johnes
66 combined with antigen and generated 1169 hits including duplicate records. The number of
67 publications was reduced to 96 by exclusion of duplicate records and exclusion of studies, where
68 the abstract indicated that the study did not test antigens for MAP diagnosis or did not characterize
69 MAP antigens. From available publications the following information was extracted and tabulated:
70 a) Antigen name, locus and gene name, if available; b) size in kDa; c) characteristic; d) whether the
71 antigen candidate was purified from MAP or recombinant; e) immunogenicity of antigen candidate
72 and type of assay, i.e. in vitro CMI, skin test, enzyme linked immunosorbent assay (ELISA); f)
73 animal species and g) species specificity. These publications describe 115 different antigen
74 candidates originating from MAP, antigen candidates from mycobacteria other than MAP used for
75 MAP diagnosis, antigen candidates of different specificity and antigen discovery and
76 characterisation by various approaches.

77

77 3. Immune-based test platforms for MAP diagnostics

78 The true state of infection can often only be established through culture of multiple tissues.
79 However, bacterial growth is slow with test results being available only after months of incubation,
80 and up to 100 tissues may be required to establish the infection status of an animal (Whitlock et al.,
81 1996). Further, shedding of MAP at detectable levels in faeces is irregular (Nielsen and Toft, 2008).
82 Therefore immune-based diagnostic tests are relevant alternatives to faecal culture and several tests
83 measuring either specific CMI or antibodies have been developed.

84

85 3.1. *Host immune response to MAP*

86 MAP infected macrophages present antigens associated with major histocompatibility complex
87 class I and II on the cell surface to CD8⁺ and CD4⁺ T cells. As a result CD4⁺ T helper type 1 cells
88 (T_H1) are activated and produces a range of cytokines, including interferon- γ (IFN- γ). The T_H1
89 cytokine signals through a network of receptors and starts a cascade of reactions characterising the
90 CMI response (Coussens, 2001). As the infection progress, the CMI response wanes and an
91 antibody-mediated CD4⁺ T helper type 2 (T_H2) response becomes predominant. Antibody
92 production provides little, if any, protection against MAP and the infection expands without the
93 important T_H1 cell subset, which is considered to be necessary for protective immunity (Toman et
94 al., 2003). Therefore it appears that IFN- γ producing T_H1 cells recognizing peptide antigens are the
95 major mediators of specific immunity early in the infection. Hence, specific T cell responses can be
96 exploited for early detection of infection whereas assays of humoral responses may be relevant for
97 detecting the later stages of MAP infection (Stabel, 2000).

98

98 3.2. *CMI based diagnostics*

99 The intradermal skin test measures the delayed-type hyper-sensitivity response 72 hours after
100 intradermal injection of purified protein derivative (PPD) (Kalis et al., 2003; Antognoli et al.,
101 2007). PPDs are crude undefined extracts of mycobacterium antigens of different origin such as:
102 MAP (PPDj or Johnin), *M. avium* subsp. *avium* (MAA) (PPDa) or *M. bovis* (PPDb) (Gilot and
103 Cocito, 1993; Semret et al., 2006). For MAP diagnostics the situation is complicated by the lack of
104 standardization of the PPD used. For many years the alleged reference strain 18 was used for
105 preparation of antigens including PPDj, although it was later shown to be a laboratory adapted
106 strain of *M. avium* that is not a representation of field strains of MAP isolated from cattle (Chiodini,
107 1993). Lack of standardized PPDs is of concern, because the genomic variability is considerably
108 high in the organisms used for preparation of different PPDs (Semret et al., 2006). A standardized
109 production of PPDs, including a detailed production protocol and the use of the same strains, would
110 enable comparison of results from various laboratories.

111 Development of a positive skin test against PPDj is considered an indication of MAP infection.
112 However, the current skin test protocol based upon PPDs not only cross-reacts with environmental
113 mycobacteria but also with currently used MAP and *M. bovis* vaccines (Köhler et al., 2001).
114 Another drawback of the skin test is that each individual animal needs to be restrained twice within
115 a period of 72 h, first for the application of the test, and later for the reading of the results. As a
116 consequence, the skin test has to some extent been replaced by an *in vitro* CMI assay measuring
117 secreted IFN- γ in response to an antigen (Wood et al., 1989).

118 The IFN- γ assay requires only one intervention for blood collection. Whole blood samples are
119 cultured with MAP antigens in a proliferation assay, and released IFN- γ is measured in the
120 supernatant by ELISA (Wood et al., 1989). Similar to the skin test, the current IFN- γ MAP assay is
121 based on PPD as antigen for stimulation and will therefore inherently have the same specificity

122 problems. Furthermore, large scale use of the IFN- γ assay for routine diagnostics is limited by the
123 short timeframe of less than 24 hours allowed from blood sampling at the farm to stimulation with
124 antigens in the laboratory (Jungersen et al., 2005). Finally, fluctuating IFN- γ responses to PPDs in
125 calves younger than 15 months puts another limitation of the usefulness of the IFN- γ assay in its
126 current form (Jungersen et al., 2002; Huda et al., 2003). We have previously investigated if the IFN-
127 γ response to PPDj could be used as an early indicator of later antibody results in milk samples of
128 cattle with different faecal culture status (Mikkelsen et al., 2009). Samples were collected during a
129 period three years of 975 cows from 18 Danish MAP infected dairy herds. The study showed that a
130 small but significant association exists between IFN- γ test results obtained prior to first calving and
131 milk antibody ELISA results at different ages after calving. In addition, we observed that only some
132 of the IFN- γ positive animals developed a positive antibody response against MAP, which may
133 indicate that CMI responses can control or eradicate MAP in many animals.

134

135 3.3. *Serology based diagnostics*

136 Antibodies to MAP in serum can be detected by different methods such as: complement fixation
137 (Morris and Stevens, 1977), agar gel immuno-diffusion (AGID) (Sherman et al., 1990) and ELISA
138 (Reichel et al., 1999). AGID can reach a specificity of 100% but it is less sensitive than ELISA
139 which is capable of detecting small amounts of antibodies and therefore show the highest sensitivity
140 of the serological tests for MAP (Harris and Barletta, 2001). Specificity of ELISA tests has been
141 improved by removing cross-reacting antibodies by absorbing sera with *Mycobacterium phlei*.
142 Using this method, studies have reported ELISA specificity of 98.8% (Cox et al., 1991) and 99.7%
143 (Reichel et al., 1999). However, this absorption step compromises assay sensitivity. Sensitivity of
144 serological tests is highest for animals with clinical symptoms as antibodies develop in the later
145 stages of infection (Sweeney et al., 1995; Nielsen and Toft, 2008). Consequently, the main

146 limitation of these antibody based assays is that they cannot be used for diagnosis of animals at an
147 early stage of infection. The major advantages of antibody based diagnostic assays are that it is
148 relatively easy to implement into a format that is useful for large scale screening, and can be used in
149 the field without any expensive equipment or highly trained personnel.

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150 **4. *Mycobacterium avium* subsp. *paratuberculosis* antigen candidates**

151 4.1. *The ideal diagnostic antigen*

152 To improve on CMI and serology based diagnostic assays it will be important to identify well-
153 defined antigens contributing to high test sensitivity and specificity. In general, there is a lack of
154 well-defined and standardized antigens for use in diagnostic assays even though a number of
155 antigens have been described in the literature. The ideal characteristics of a new diagnostic MAP
156 antigen are that it is antigenic, unique to MAP, recognized by MAP infected animals in the early
157 subclinical stages of infection and that it remains detectable throughout the course of the disease.
158 Thus far, no such antigen has been discovered for MAP infections. In practice, it is likely that a
159 diagnostic test for MAP will require a cocktail of antigens due to the range of heterogeneous MHC
160 molecules of out-bred animals (Kathaperumal et al., 2009) and due to differentiated antigen
161 expression patterns of MAP bacteria (Radosevich et al., 2007). The same antigens will most likely
162 not be useful for both CMI and serology assays.

163

164 4.2. *Antigen candidates for CMI diagnostics*

165 Specificity and sensitivity of CMI based assays are poor primarily because of the applied antigens.
166 Most studies have been conducted using PPDs (Köhler et al., 2001). However, a number of studies
167 have described novel well-defined MAP antigens for measuring CMI responses. Table 1
168 summarizes these antigen candidates based on publications from 1989 through 2009. The antigen
169 candidates include secreted antigens, cell wall and membrane antigens, lipoproteins, heat shock
170 proteins and hypothetical proteins. The earliest studies used native proteins purified from MAP:
171 Avi-3 (Abe et al., 1989), A36-complex (Gilot et al., 1992), P30 (Burrells et al., 1995), alkyl
172 hydroperoxid reductase C (AhpC) and alkyl hydroperoxid reductase D (AhpD) (Olsen et al., 2000),
173 MPP14 (Olsen and Storset, 2001; Olsen et al., 2005) and MPB70 (Olsen et al., 2005). All of these

174 antigens were described to recall CMI responses in *in vitro* assays but they all lacked MAP
175 specificity because of orthologues in other mycobacteria species. Two exceptions that were specific
176 to MAP were antigens AphC and AphD, as demonstrated by immunoblot reaction of monospecific
177 rabbit antiserum against the two antigens with 9 MAP strain lysates but not with lysates from 20
178 other mycobacterial species, except *M. goodnae* that produced weak cross-reactive bands (Olsen et
179 al., 2000). The diagnostic potential of AphC and AphD were evaluated in four goats experimentally
180 infected with MAP. T cells from all four goats produced IFN- γ to *in vitro* stimulation with either
181 antigen, whereas a non-infected control animal did not respond. AhpC and AhpD are the most
182 promising CMI antigens among the natively purified MAP antigens as they appear to be species
183 specific with a high sensitivity.

184 In recent years recombinant antigen candidates have replaced purified antigen candidates in the
185 development of diagnostic assays. The advantages of recombinant antigens over purified antigens
186 are higher product yield, high homology of antigen preparation and faster production. Several
187 recombinant antigen candidates have been evaluated for detection of CMI against MAP. In some
188 studies the species specificity was validated by comparison of the recombinant antigen sequence
189 with available sequences of other mycobacteria (Koets et al., 1999; Nagata et al., 2005; Rosseels et
190 al., 2006). Only the 67kDA heat shock protein (HspX) was described to be species specific
191 (Bannantine and Stabel, 2000). The remaining recombinant antigen candidates were either highly
192 homologous to proteins in other mycobacteria species or the homology was not reported.
193 Unfortunately, the HspX protein did not produce a CMI recall response when tested in cell culture
194 from 10 infected cows (Bannantine and Stabel, 2000). Apart from HspX and heat shock protein 65
195 (Hsp65) (Koets et al., 1999; Nagabhushanam et al., 2001), which induced only a low CMI response
196 in both mice (Nagabhushanam et al., 2001) and cattle (Koets et al., 1999) all the described
197 recombinant antigen candidates in Table 1 were reported to produce a CMI response.

198 To study recombinant heat shock protein 65 (Hsp65) and 70 (Hsp70) blood samples were collected
199 from 179 cows and distributed to groups of: cows naturally infected and vaccinated against MAP
200 (n=30), naturally infected and not vaccinated (n=79), clinical cases (n=11) and non-infected animals
201 (n=59) all from dairy farms (Koets et al., 1999). Each group was further divided into groups of
202 faecal culture positive or negative cows. From blood, peripheral blood mononuclear cells were
203 purified to evaluate proliferative responses following stimulation with Hsp65 or Hsp70 compared to
204 PPDj. Proliferative responses to Hsp65 were generally low in all groups of cows whereas responses
205 to Hsp70 varied between groups. Highest proliferative responses was observed for the group of
206 faecal culture positive cows that were not vaccinated (88% positive), followed by faecal culture
207 negative cows that were vaccinated (93.3% positive), 54 faecal negative cows that were non-
208 vaccinated (74.1% positive) and 11 clinical cases of faecal culture positive cows (72.2% positive).
209 In the control group, 30.5% produced a positive response to Hsp70 and 16.9 % produced a positive
210 response to Hsp65, which indicate low specificity of both antigens.

211 Another large study including 54 young clinical healthy cattle was conducted by Olsen et al. (2005)
212 evaluating IFN- γ responses to recombinant ESAT-6, PPDb, purified antigen MPP14 and purified
213 antigen MPB70. The study investigated the role of unspecific IFN- γ production by natural killer
214 (NK) cells in young animals and concluded that NK cells were responsible for a large proportion of
215 the IFN- γ producing cells in peripheral blood mononuclear cells from uninfected calves. A third
216 study including a decent number of animals was the investigation of recombinant Ag85A, Ag85B
217 and Ag85C. This study included 18 healthy control cows, 16 low shedders and 4 medium shedders
218 and resulted in highest responses by cows from the group of medium shedders and lowest responses
219 in the healthy control cows (Shin et al., 2005). The remaining studies in Table 1 included a much
220 lower number of animals. Validation of immunogenicity of antigen candidates requires an
221 appropriate sample size otherwise the results cannot be applied to herd conditions with genetically

222 heterogeneous animals demonstrating different stages of MAP infection. Further, some of the listed
223 antigens in Table 1 remain to be validated in bovine species, as they have only been validated in
224 other species such as mice, rabbits or guinea pigs.

225

226 4.3. *Types of characterized MAP antigen candidates*

227 The majority of characterised or described MAP antigen candidates were not tested as CMI
228 diagnostic antigens but either tested for immunogenicity in humoral assays or no records of immune
229 reactivity were published. These antigen candidates are grouped and listed as: secreted antigens
230 (Table 2), cell wall and membrane antigens (Table 3), lipoprotein antigens (Table 4), heat shock
231 antigens (Table 5) and hypothetical antigens (Table 6). Due to the limited number of animals
232 included in most studies it is impossible to validate the influence of variance of host immune
233 response between animals, changes in MAP protein expression profiles during infection progress
234 and influence of disease stage.

235

236 4.3.1. *Secreted antigen candidates*

237 Secreted antigens (Table 2) are expected to be highly immunogenic or immunodominant due to
238 their presence in the extracellular environment where they are more likely to encounter sensitized
239 immune cells. This was confirmed in a study comparing secreted antigens from supernatants and
240 antigens of intracellular origin (Cho and Collins, 2006). Serum from infected cattle was used for
241 immunoblot analysis which showed that infected sera reacted more strongly to the secreted antigens
242 compared to the antigens of intracellular origin. The majority of the antigen candidates were
243 evaluated with relatively few positive and negative control sera. However, five recombinant
244 antigens, MAP2411, ClpP (MAP2281c), Ppa (MAP0435c), MAP0593c and GreA (MAP1027c),
245 were tested for immunogenicity with sera from 41 sheep with a known MAP infection and 41 non-

246 infected control sheep (Gumber et al., 2009b). Two of these antigens, MAP0593c and ClpP reacted
247 against 58.5% and 46.3% test positive sera and 12.1% and 4.9% of the negative control sera.
248 MAP2411 could not distinguish between MAP positive or negative serum samples. The specificity
249 of these antigen candidates towards MAP was not reported and hence it is difficult to evaluate their
250 usefulness. An earlier study tested recombinant antigens of Ag85A, Ag85B, Ag85C and SOD in an
251 ELISA with sera from 60 MAP shedding cows and 22 non-shedding cows (Shin et al., 2004).
252 Antigens of the Ag85 complex and SOD showed high reactivity against sera from the MAP
253 shedding cows and little reactivity against sera from the non-shedding cows. Conserved proteins
254 such as antigens of the Ag85 complex (Dheenadhayalan et al., 2002) are found in all mycobacteria
255 species. At the protein level the three Ag85 components of MAP share 99% sequence identity with
256 MAA (Rosseels et al., 2006). For vaccine design the use of conserved proteins may provide cross
257 protection towards other infections as a positive side-effect. However, for diagnostic purposes this
258 cross reactivity is undesirable as high specificity and sensitivity is essential. The specificity for
259 MAP was not reported for a large part of the antigen candidates listed in Table 2.

260

261 4.3.2. *Cell wall and membrane antigens*

262 Antigen candidates originating from cell wall or membrane are listed in Table 3. Similar to secreted
263 antigens, cell wall and membrane antigens are expected to be highly immunogenic due to their
264 accessibility to components of the immune system. Several of the reported studies have
265 characterised and tested a 34kDa membrane protein antigen candidate (p34) (Gilot et al., 1992;
266 Ostrowski et al., 2003) or parts of the protein (Dekesel et al., 1993; Vannuffel et al., 1994; Malamo
267 et al., 2006) that belongs to the major antigen complex A36. The p34 protein was reported to elicit a
268 predominant humoral immune response (Dekesel et al., 1992) and to be MAP specific (Gilot et al.,
269 1993). In general, the characterised antigen candidates were evaluated with sera of few animals.

270 However, the polypeptide a362, which represent the carboxyl termini of p34, was evaluated by
271 ELISA with sera from 208 MAP positive and 175 MAP negative cattle (Vannuffel et al., 1994). The
272 peptide reacted with 40.4% of the positive samples and 5% of the negative samples and hence
273 showed low sensitivity but high specificity. The majority of the listed antigen candidates were
274 reported to cross react with other mycobacteria species (Table 3).

275 A comparative study of different mycobacteria species genomes showed that the major differences
276 are in the gene products constituting the cell wall and in particular genes encoding the PE and PPE
277 proteins (Marri et al., 2006), which are unique to mycobacteria. PE and PPE proteins are named
278 after a proline-glutamic acid (PE) motif and a proline-proline-glutamic acid (PPE) motif found in
279 conserved domains near the N termini in these proteins. Their function remains unknown but
280 members of the family have been linked to virulence (Li et al., 2005b). A recent genomic
281 comparison study concluded that several PPE proteins were unique to MAP compared to other
282 members of the *Mycobacterium avium* complex (Mackenzie et al., 2009). These proteins may
283 therefore be useful antigen candidates in MAP specific diagnostic assays. Two PPE proteins
284 expressed on the MAP cell wall are listed in Table 3, MAP1506 and MAP3420c (Newton et al.,
285 2009). Recombinant MAP3420c showed positive immunoblot reaction with sera from four MAP
286 faecal culture positive cows and did not react with sera from four faecal culture negative cows.
287 Three of the faecal culture positive cows were confirmed tissue culture positive, whereas one faecal
288 culture negative cow were tested tissue culture positive. Sera from the eight cows were also tested
289 by three commercial ELISAs, but only one, two and four of the cows tested positive in each ELISA.
290 The two PPE proteins could be promising diagnostic antigen candidates, however orthologous
291 genes were found in *M. avium* subsp. *hominissuis* and MAA genomes (Newton et al., 2009) and if
292 they are expressed in these species it would jeopardize the specificity.

293

294 *4.3.3. Lipoprotein antigens*

295 Lipids (Table 4) comprise a large part of the mycobacterial cell wall and, similar to cell wall
296 antigens, many of these structures have demonstrated high seroreactivity as a consequence of their
297 accessibility for antibodies and immune cells (Biet et al., 2008). In an early study lipoarabinomanan
298 (LAM) was purified, analysed for chemical properties, and shown to react with serum from a MAP
299 infected cow (Sugden et al., 1987). LAM is a highly immunodominant component of the MAP cell
300 wall and has been widely used in serological assays for MAP diagnosis (Jark et al., 1997; Reichel et
301 al., 1999). Antigen candidates L5P (Biet et al., 2008), Para-LP-01 (Eckstein et al., 2006) and
302 lipopeptide PstA (Wu et al., 2009) were reported to be absent in MAA. The diagnostic value of
303 these antigen candidates remains to be thoroughly validated with appropriate sample sizes.

304

305 *4.3.4. Heat shock protein antigens*

306 Hsp's (Table 5) are highly conserved molecules produced by both eukaryotic and prokaryotic cells.
307 Expression of Hsp's is upregulated during cellular stress, such as heat stress in response to infection
308 and inflammation (Wu et al., 2007). In addition to their chaperone function, these proteins have
309 demonstrated to play an important role in activation and modulation of dendritic cells (Langelaar et
310 al., 2002). Due to their conserved nature Hsp's are likely to be immunogenic but are not ideal
311 antigens for diagnostic purposes as cross reactivity with other bacteria are expected.

312 Five different Hsp antigen candidates are listed in Table 5: GroES, Hsp65K, Hsp70, MAP3840 and
313 MAP3841. All five antigens were tested with relatively few sera, and as expected, none of them
314 were reported to be MAP specific. One study focused on the interaction of Hsp70 and bovine
315 antigen presenting cells investigating the use of recombinant Hsp70 as a tool to chaperone antigens
316 into the cell to mediate a cytotoxic T-cell response initiated by presentation of antigens by MHC
317 class I antigen presenting pathway (Langelaar et al., 2002). The results indicate that a Hsp70

318 receptor is present on bovine dendritic cells and macrophages and that Hsp70 may be useful as an
319 adjuvant in vaccines, if not as the vaccine antigen itself (Koets et al., 2006).

320

321 *4.3.5. Hypothetical proteins*

322 Table 6 includes characterised hypothetical protein antigen candidates of unknown function or other
323 antigen candidates of MAP. The majority of the listed antigen candidates are recombinant proteins.

324 Several of these were reported from studies conducting large proteome analysis of MAP

325 (Bannantine et al., 2004; Bannantine et al., 2008; Hughes et al., 2008) and were validated with sera

326 from few animals.

327

328 **5. Future studies**

329 In the pre-genomic era, the primary technique for discovery and characterization of novel MAP
330 antigen candidates was immunoproteomics. Screening of expression libraries was employed in
331 parallel but to a much lesser degree. With the completion of the MAP genome (Li et al., 2005a)
332 proteomic approaches has opened up for more targeted strategies that will be exploited further in
333 the future. Immunoproteomic approaches are based upon selection of antigen candidates by a
334 combination of antigenicity and protein expression profiles (White et al., 1994). Proteins are
335 resolved by two-dimensional (2D) gel-electrophoresis and interesting spots are identified by mass
336 spectrometry followed by cloning, recombinant expression and ultimately testing for antigenicity
337 with serology or CMI based assays (Bannantine and Paustian, 2006; Gumber and Whittington,
338 2009). Progress in proteomic techniques has resulted in high-resolution 2D electrophoresis
339 proteomic maps comprising more than 1500 spots and combined with mass spectroscopy in the
340 identification of several hundred expressed proteins (Leroy et al., 2007).

341 Comparison of the MAP genome against other mycobacteria genomes, including the highly
342 homologous MAA genome, allowed the identification of coding sequences present only in MAP
343 (Bannantine et al., 2002). Comparative studies revealed that less than 40 coding sequences are
344 uniquely present in the MAP genome (Bannantine et al., 2002; Paustian et al., 2004). This relatively
345 small number of unique coding sequences reflects the genetic similarity among members of the
346 mycobacteria (Bannantine et al., 2003).

347 Isolation and mass spectrometric identification of MHC binding peptides is a new approach that
348 will allow the selection of a limited number of highly relevant proteins based upon the MAP
349 genome sequence being publicly available. As an alternative, the genome could be screened *in*
350 *silico* in a non-biased way for epitopes that will be recognized by the bovine immune system

351 (Lundegaard et al., 2007). This approach relies upon computer algorithms to identify T cell epitopes
352 that bind the MHC II most strongly and promiscuously. In order for a protein to be recognized
353 strongly in a diagnostic test over the entire course of infection it has to be constitutively and
354 strongly expressed. Two recent studies demonstrated that protein expression of MAP differ from
355 control conditions during starvation, hypoxia (Gumber et al., 2009a) and heat stress (Gumber and
356 Whittington, 2009) in MAP strains originating from cattle and sheep. These results suggest that
357 MAP expresses a diverse range of antigens throughout the course of infection during which the
358 environmental conditions for the MAP bacteria change.

359 Regardless of which strategy we choose to follow there is currently no reliable way to predict
360 diagnostic antigens and we are left with the task of screening each selected antigen for its potential,
361 but in contrast to screening of the entire proteome it will be a doable task.

362

362 **6. Summary**

363 A number of MAP antigen candidates of different characteristic and species specificity have been
364 identified and tested for immunogenicity to evaluate their diagnostic potential. Few of these
365 antigens have been tested for CMI reactions that allow for early detection of MAP infected animals.
366 Until now, no obvious antigen candidates for use in a CMI based diagnostic assay have been
367 identified. However, with several mycobacteria genome sequences available along with new tools
368 for *in silico* analysis and genomic techniques, specific and immunogenic antigens for
369 paratuberculosis diagnosis should be expected soon. It is recommended that appropriate sample size
370 is included for validation of future antigen candidates for diagnostic purposes. In addition, the use
371 of a selected antigen cocktail for diagnostic application should be considered, to ensure that all
372 infected animals are detected independent of disease status and MHC make-up.

373

374 **References**

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Table 1. Antigen candidates tested for cell mediated immunity against *Mycobacterium avium* subsp. *paratuberculosis*.

Antigen	Locus	Gene	Size (kDa)	Characteristic	Antigen	Test assay	MAP specific	Reference
Secreted								
MPP14	-	-	14	Protein from MAA complex	Purified	IFN- γ test, resp. of 14 calves during two months. pos: 5/7 pos and 0/7 neg	Specific for MAA	Olsen et al., 2005
MPP14	-	-	14	Protein of MAIS complex	Purified	IFN- γ test, pos: 6/8 MAP inf, 1/7 control 23 weeks p.i.	No	Olsen and Storset, 2001
MPT-TP	-	-	16.7	Hypothetical thiol peroxidase	Recombinant	IFN- γ response of pooled mice spleen cells from 24-60 mice	Not reported	Mullerad et al., 2003
SOD	-	-	28	Superoxide dismutase	Recombinant	IFN- γ signif. higher of 20 FC pos vs. 18 FC neg cows	81-93% homologous to <i>M. TB</i> and <i>M. leprae</i> , <i>M. habana</i>	Mullerad et al., 2002a
P30	-	-	30	Protein	Purified	IFN- γ test, pos: 2/2 MAP inf., 0/2 uninf. sheep	Homologous to other mycobacteria	Burrells et al., 1995
Ag85A	MAP0216	<i>fbpA</i>	30-32	Protein, T-cell epitope	Recombinant	IFN- γ test, pos: 5 inf. mice (Ag85A) and 5 inf. calves (Ag85 complex)	Identical sequence in <i>M. bovis</i>	Rosseels et al., 2006
Ag85A	MAP0216	<i>fbpA</i>	30-32	Protein, T-cell epitope	Recombinant	IFN- γ signif. higher of 20 FC pos vs. 18 FC neg cows	Identical sequence in <i>M. bovis</i>	Shin et al., 2005
Ag85B	MAP1609c	<i>fbpB</i>	30-32	Protein, T-cell epitope	Recombinant	IFN- γ test, pos: 5 inf. mice (Ag85B) and 5 inf. calves (Ag85 complex)	Identical sequence in <i>M. bovis</i>	Rosseels et al., 2006
Ag85B	MAP1609c	<i>fbpB</i>	30-32	Protein, T-cell epitope	Recombinant	IFN- γ signif. higher of 20 FC pos vs. 18 FC neg cows	Identical sequence in <i>M. bovis</i>	Shin et al., 2005
Ag85C	MAP3531c	<i>fbpC2</i>	30-32	Protein, T-cell epitope	Recombinant	IFN- γ test, pos: 5 inf. mice (Ag85C, low resp.) and 5 inf. calves (Ag85 complex)	Identical sequence in <i>M. bovis</i>	Rosseels et al., 2006
Ag85C	MAP3531c	<i>fbpC2</i>	30-32	Protein, T-cell epitope	Recombinant	IFN- γ signif. higher of 20 FC pos vs. 18 FC neg cows	Identical sequence in <i>M. bovis</i>	Shin et al., 2005
ESAT-6	-	-	-	Early secretory antigenic target 6 of M.TB complex	Recombinant	IFN- γ test, resp. of 14 calves during two months, pos: 7/7 pos and 1/7 neg	No	Olsen et al., 2005
MPB70	-	-	-	Protein from M.TB complex	Purified	IFN- γ test, resp. of 14 calves during two months, pos: 0/7 pos and 0/7 neg	No	Olsen et al., 2005
SOD	-	-	-	Superoxide dismutase	Recombinant	IFN- γ signif. higher of SOD imm. mice vs. control mice. Pooled samples of 24-60 mice	No	Shin et al., 2005
Cell wall and membrane								
Map39	Map3184	-	39	Possible T-cell Ag, homologous to PPE protein family. Hypothetical protein	Recombinant	IFN- γ test, pos*: 5/5 pos and 0/5 neg	Homologous to MAA	Nagata et al., 2005
Map41	Map1518	-	41	Possible T-cell Ag, homologous to PPE protein family. Hypothetical protein	Recombinant	IFN- γ test, pos*: 5/5 pos and 0/5 neg	Homologous to MAA	Nagata et al., 2005
A36-complex	-	-	-	TMA family, part of PPD	Purified	Skin test: reaction to A36 by sensitized rabbits	94% homology to MAA	Gilot et al., 1992
Lipoproteins								
MAP0261c	MAP0261c	-	19	Homologue protein of <i>M. TB</i> is immunodominant in mice and humans	Recombinant	IFN- γ test: low resp, but signif. difference between 21 naturally inf and 9 neg control cows	Homologues in <i>M. bovis</i> , MAA and <i>M. intracellulare</i>	Huntley et al., 2005
MAP1138c	MAP1138c	<i>lprG</i>	22	Exported lipoprotein (LppX/LprAFG family)	Purified/recombinant	Proliferation assay, no signif. difference between MAP FC pos (n=12) and FC neg (n=14) cows. Serum antibody levels: signif. difference between groups	67% homology with <i>M. bovis</i>	Santema et al., 2009
P22	MAP1138c	<i>lprG</i>	22	Exported lipoprotein (LppX/LprAFG family)	Recombinant	IFN- γ test, pos: 8/9 MAP vaccinated and 0/5 control sheep	75% homology with <i>M. leprae</i> and M.TB	Dupont et al., 2005
P22	MAP1138c	<i>lprG</i>	22	Exported lipoprotein (LppX/LprAFG family)	Recombinant	IFN- γ test, pos: 3/5 and 2/5 P22-imm sheep and	75% homology with <i>M. leprae</i>	Rigden et al., 2006

					0/6 and 0/6 control at 13 and 29 weeks p.i.	and M.TB		
Heat shock proteins								
Hsp65	-	-	65	From <i>M. bovis</i> BCG	Recombinant	Positive PBMC proliferation: 48% of FC pos, 20.4% of FC neg, 16.9% of control (FC neg), and 60% of MAP vaccinated cows	Highly conserved	Koets et al., 1999
Hsp65	-	-	65	From MAA	Recombinant	ELISPOT, IFN- γ pos: 0/5 MAP infected mice	Highly homologous to other mycobacteria	Nagabhushanam et al., 2001
HspX/MBP	-	<i>hspX</i>	67	May be involved in adhesion or heat shock. Protein is not secreted.	Purified/recombinant	INF- γ test, pos: 0/10 inf. and 0/3 control cattle. Serum antibody positive: 4/24 inf. and 0/3 control cows	Specific for MAP	Bannantine and Stabel, 2000
Hsp70	-	-	70	From <i>M. bovis</i> BCG	Recombinant	Positive PBMC proliferation: 88% of FC pos, 74.1% of FC neg, 30.5% of control (FC neg), and 93.3% of MAP vaccinated cows	Highly conserved	Koets et al., 1999
Hypothetical proteins								
Map10	-	-	10	Possible T-cell Ag. Part of fusion protein of β -galactosidase	Recombinant	IFN- γ test, pos*: 4/5 pos and 0/5 neg	Not reported	Nagata et al., 2005
AhpD	MAP1588c	<i>ahpD</i>	19	Alkyl hydroperoxid reductase D	Purified	IFN- γ test, pos: 3/3 inf. and 0/1 control goats. Serum antibody pos: 0/4 inf. and 0/1 control goats	Specific compared to 20 different mycobacteria strains	Olsen et al., 2000a; Olsen et al., 2001
Ag85B	MAP1609c	<i>fbpB</i>	34.9	Mycolyl transferase, involved in cell wall assembly	Recombinant	IFN- γ response of pooled mice spleen cells from 24-60 mice	No	Mullerad et al., 2002b
P35	-	-	35	Associated with invasion of bovine intestinal epithelial cells	Recombinant	IFN- γ test, pos*: 3/3 pos and 0/3 neg	Not reported	Basagoudanavar et al., 2006
35kDa	-	-	35	Associated with invasion of bovine intestinal epithelial cells	Recombinant	Proliferation assay, IFN- γ signif. higher in 20 FC pos vs. 18 FC neg cows	Not reported	Shin et al., 2005
AhpC	MAP1589c	<i>ahpC</i>	45	Alkyl hydroperoxid reductase C	Purified	IFN- γ test, pos: 3/3 inf. and 0/1 control goats. Serum antibody pos: 4/4 inf. and 0/1 control goats. ELISA pos: 13/56 pos, 0/10 neg cattle	Specific compared to 20 different mycobacteria strains	Olsen et al., 2000a
Apa	MAP1569	<i>modD</i>	50-60	Alanine and Proline rich Ag or Fibronectin Attachment Protein	Recombinant	IFN- γ test, pos: 6/45 FC pos and 0/45 FC neg. cows. Not significant.	98% homologous to MAA	Gioffre et al., 2009
74F	MAP3527 and MAP1519	<i>pepA</i>	74	Polyprotein of MAP3527 (a peptidase) and MAP1519 (a hypothetical protein)	Recombinant	Proliferation of pooled spleen cells of 24 mice. IFN- γ signif. higher of MAP74F immunised vs. control mice	Homologous to M. TB	Chen et al., 2008
MAP1718c	MAP1718c	-	-	-	Purified/recombinant	Proliferation assay, no signif. difference between MAP FC pos (n=12) and FC neg (n=14) cows	99% homologous to MAA	Santema et al., 2009
MAP3515c	MAP3515c	-	-	-	Purified/recombinant	Proliferation assay, no signif. difference between MAP FC pos (n=12) and FC neg (n=14) cows	No	Santema et al., 2009

Abbreviations: Ag: antigen; BCG: Bacillus Calmette-Guérin; ELISPOT: enzyme linked immunosorbent spot; FC: faecal culture; IFN- γ : interferon gamma; imm.: immunised; inf.: infected; MAA: *Mycobacterium avium* subsp. *avium*; MAIS: *M. avium-intracellulare-scrofulaceum*; MAP: *Mycobacterium avium* subsp. *paratuberculosis*; M.TB: *Mycobacterium tuberculosis*; neg: negative; TMA: thermostable macromolecular antigen; PBMC: peripheral blood mononuclear cells; p.i.: post infection; pos: positive; PPD: purified protein derivative; PPE: proline-proline-glutamate; resp.: response; signif.: significant.

* Number of positive and negative animals extracted from available data

Table 2. Characterized secreted antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*

Antigen	Locus	Gene	Size (kDa)	Characteristic	Antigen	Test assay	MAP specific	Reference
MAP2609	MAP2609	-	9	Conserved hypothetical protein	Recombinant	Immunoblot pos: 11/11 pos	Homologous to <i>M. TB</i> and <i>M. bovis</i>	Willemsen et al., 2006
MAP0593c	MAP0593c	-	14.7	Hypothetical protein. Degradation of proteins, peptides, and glycopeptides	Recombinant	ELISA pos: 24/41 pos and 5/41 neg	Not reported	Gumber et al., 2009
14kDa	-	-	14	Putative signal peptide	Purified	Immunoblot pos ELISA pos: 13/30 pos; 1/10 neg	Homologous in all species of MAC	Olsen et al., 2000b; Olsen et al., 2001
MAP1693c	MAP1693c	-	15	Peptidyl prolyl-cis-trans isomerase	Purified /recombinant	ELISA pos*: 3/7 pos and 0/3 neg	Absent in <i>M. bovis</i>	Leroy et al., 2007
MAP2942c	MAP2942c	<i>mpt53</i>	15	Ag mpt53	Recombinant	Immunoblot pos: 11/11 pos	Homologous to <i>M. TB</i> and <i>M. bovis</i>	Willemsen et al., 2006
MAP1693c	MAP1693c	-	15.3	Hypothetical peptidyl-propyl cis-trans isomerase	Purified/ recombinant	Immunoblot and ELISA pos	Not reported	Cho et al., 2006; Cho et al., 2007
MAP2411	MAP2411	-	15.5	Hypothetical protein. Biosynthesis of cofactors, prosthetic groups, and carriers: Pyridoxine	Recombinant	ELISA, no difference between infected (n=41) and control sera (n= 41)	Not reported	Gumber et al., 2009
MAP2168c	MAP2168c	-	15.95	Hypothetical protein	Purified/ recombinant	Immunoblot and ELISA pos	Not reported	Cho et al., 2006; Cho et al., 2007
GreA	MAP1027c	<i>greA</i>	17.8	Transcription elongation factor GreA	Recombinant	ELISA pos: 10/41 pos and 2/41 neg	Not reported	Gumber et al., 2009
Ppa	MAP0435c	<i>ppa</i>	18.5	Inorganic pyrophosphatase involved in metabolism	Recombinant	ELISA pos: 14/41 pos and 2/41 neg	Not reported	Gumber et al., 2009
ClpP	MAP2281c	<i>clpP</i>	21.6	ATP-dependent Clp protease proteolytic subunit. Degradation of proteins, peptides, and glycopeptides	Recombinant	ELISA pos: 19/41 pos and 2/41 neg	Not reported	Gumber et al., 2009
SOD	MAP0187c	<i>soda</i>	23	Superoxide dismutase	Purified	Not tested	Not reported	Liu et al., 2001
MAP3199	MAP3199	-	26	Hypothetical protein. Microtubule-associated	Purified /recombinant	ELISA pos*: 0/7 pos and 0/3 neg	Absent in <i>M. bovis</i>	Leroy et al., 2007
Antigen-A	-	-	31	Extracted from culture filtrate and sonicate	Purified	ELISA pos: 18/22 pos at sp=90, AGID pos 4/22 pos	Similar to BCG85 complex Ag's of <i>M. bovis</i>	Sugden et al., 1991
PepA	MAP3527	<i>pepA</i>	31.8	Serine protease	Purified/ recombinant	Immunoblot and ELISA pos	Not reported	Cho et al., 2006; Cho et al., 2007
32kDa	-	-	32	Fibronectin binding properties	Recombinant	Not tested	Not reported	ElZaatari et al., 1994
ModD	MAP1569	<i>modD</i>	32,3	Associated with MHC II and fibronectin attachment	Purified/ recombinant	Immunoblot and ELISA pos	Homologous to <i>M. TB</i> and <i>M. bovis</i>	Cho et al., 2006; Cho et al., 2007
MAP1272c	MAP1272c	-	33.3	Putative invasin, NlpC/P60 superfamily	Recombinant	Immunoblot, ELISA pos: 7/7 pos, 0/2 neg	Homologous to MMP-1 of MAA and <i>M. leprae</i>	Li et al., 2007
Map0210c	Map0210c	<i>pirG</i>	34	-	Recombinant	Immunoblot pos in 10/11	Homologous to <i>M. TB</i> and <i>M. bovis</i>	Willemsen et al., 2006
Ag85C	MAP3531c	<i>fbpC2</i>	34.1	Putative mycolyl transferase	Purified/ recombinant	Immunoblot and ELISA pos	Highly conserved	Cho et al., 2006; Cho et al., 2007
MAP4308c	MAP4308c	-	36	Possible L-lactate-2-monooxygenase	Purified /recombinant	ELISA pos*: 4/7 pos and 0/3 neg	Absent in <i>M. bovis</i>	Leroy et al., 2007
44,3kDa	-	-	44.3	Soluble protein	Purified	Not tested	Not reported	Mutharia et al., 1997
Antigen-D	-	-	400	Peroxidase, extracted from culture filtrate + sonicate	Purified	ELISA pos: 22/22 pos at sp=90, AGID pos: 18/22 pos	Not reported	Sugden et al., 1991
Antigen-D	-	-	400	Proxidase	Purified	Not tested	Not reported	Brooks et al., 1991
Ag85A	MAP0216	<i>fbpA</i>	-	T-cell epitope	Purified	Not tested	Homologous to MAA	Dheenadhayalan et al., 2002

Ag85A	MAP0216	<i>fbpA</i>	-	T-cell epitope	Recombinant	ELISA pos ¹ : 40/60 pos, 4/22 neg	Homologous to MAA	Shin et al., 2004
Ag85B	MAP1609c	<i>fbpB</i>	-	T-cell epitope	Purified	Not tested	Homologous to MAA	Dheenadhayalan et al., 2002
Ag85B	MAP1609c	<i>fbpB</i>	-	T-cell epitope	Recombinant	ELISA pos ¹ : 43/60 pos, 3/22 neg	Homologous to MAA	Shin et al., 2004
Ag85C	MAP3531c	<i>fbpC2</i>	-	T-cell epitope	Purified	Not tested	Homologous to MAA	Dheenadhayalan et al., 2002
Ag85C	MAP3531c	<i>fbpC2</i>	-	T-cell epitope	Recombinant	ELISA pos ¹ : 41/60 pos, 4/18 neg	Homologous to MAA	Shin et al., 2004
MAP0586c	MAP0586c	-	-	Hypothetical protein. Possible transglycosylase SLT domain	Purified /recombinant	ELISA pos*: 0/7 pos and 0/3 neg	Absent in <i>M. bovis</i>	Leroy et al., 2007
MAP2677c	MAP2677c	-	-	Hypothetical protein, glyoxylase	Purified /recombinant	ELISA pos*: 4/7 pos and 0/3 neg	Absent in <i>M. bovis</i>	Leroy et al., 2007
SOD	-	-	-	Superoxide dismutase. Induce B-cell response	recombinant	ELISA pos ¹ : 49/60 pos, 2/22 neg	Not reported	Shin et al., 2004

Abbreviations: as in Table 1; MAC: *Mycobacterium avium* complex; MHC II: Major Histocompatibility Complex class II.

* Number of positive and negative animals extracted from available data.

¹ Based on available data on number of animals, specificity and sensitivity.

Table 3. Characterized cell wall and membrane antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*

Antigen	Locus	Gene	Size (kDa)	Characteristic	Antigen	Test assay	MAP specific	Reference
MAP1087	MAP1087	-	15.4	Putative surface protein. Peptide/nickel transport system permease	Recombinant	Immunoblot pos: 2/2 MAP infected cattle, 0/2 prior to infection	Homologous to MAA and <i>M. smegmatis</i>	Bannantine et al., 2008a
MAP1087	MAP1087	-	15.4	Putative surface protein. Peptide/nickel transport system permease	Recombinant	Immunoblot: high spot intensity in 6/6 infected cattle	Cross-reaction with <i>M. bovis</i> infected sera	Bannantine et al., 2008b
34kDa-C	-	-	18	Cell wall Ag, carboxyl termini of 34kDa protein,	Purified/ recombinant	ELISA pos: 18/18 pos, 0/50 neg	Not reported	Malamo et al., 2006
MAP3968	MAP3968	-	30	Hypothetical protein, putative heparin binding hemagglutinin protein	Recombinant	ELISA pos: 9/23 pos, 4/46 neg	92% homology to MAA	Sechi et al., 2006
a362 P34	-	-	34	B-cell epitope (part of A36, TMA complex)	Recombinant	ELISA pos: 25/25 pos, 0/7 neg	Homologous to <i>M. leprae</i> , but two species specific epitopes	Dekesel et al., 1993
P34	-	-	34	B-cell epitope (part of A36, TMA complex)	Purified	Not tested	Specific based on EMBL data bank	Gilot et al., 1993
P34-p5	-	-	34	B-cell epitope peptide A	Recombinant	ELISA pos: 6/10 pos, 0/10 neg	Cross-reaction with <i>M. bovis</i> infected sera (0/6)	Ostrowski et al., 2003
P34-p8	-	-	34	B-cell epitope peptide B	Recombinant	ELISA pos: 8/10 pos, 0/10 neg	Cross-reaction with <i>M. bovis</i> infected sera (0/6)	Ostrowski et al., 2003
34kDa	-	-	34	B-cell epitope (part of A36, TMA complex)	Purified	Immunoblot: high spot intensity in 3/3 infected cattle	Low cross-reaction with MAA, <i>M. bovis</i> and <i>M. phlei</i>	Dekesel et al., 1992
MAP2121c	MAP2121c	-	35	Major membrane protein I, putative role in invasion of epithelial cells	Recombinant	Immunoblot: high spot intensity in pos rabbit and mouse, low in 5/5 pos cattle	Homologous to <i>M. leprae</i>	Bannantine et al., 2008d
P35	-	-	35	-	Recombinant	Immunoblot reaction with anti-MAP rabbit serum and skin reaction in guinea pigs	Cross-reaction with MAA in skin test	Basagoudanavar et al., 2004
MMP	-	-	35	T-cell Ag (homologous to major membrane protein, MMP)	-	Not tested	Homologous to <i>M. leprae</i> and MAA	Banasure et al., 2001
MMP	-	-	35	Major membrane protein. Role in invasion of epithelial cells.	Recombinant	Not tested	Homologous to <i>M. leprae</i> and MAA	Bannantine et al., 2003
TMA	-	-	100	Cytoplasmic protein of TMA complex	Purified	Not tested, comparison of TMA from MAP, MAA and <i>M. bovis</i>	Homologues in MAA and <i>M. bovis</i>	Bruneteau et al., 1992
a362	-	-	-	Ag containing B-cell epitope. Polypeptide of carboxyl termini of 34kDa protein	Recombinant	ELISA pos ¹ : 84/208 pos, 9/175 neg, 2/38 <i>M. bovis</i> pos	Specific compared to MAA, <i>M. bovis</i> and <i>M. phlei</i> . Low cross-reaction with <i>M. bovis</i>	Vannuffel et al., 1994
MAP1204	MAP1204	-	-	Hypothetical protein, putative invasin	Recombinant	Immunoblot: high spot intensity in 6/6 infected cattle	Cross-reaction with <i>M. bovis</i> cow sera	Bannantine et al., 2008b
MAP1506	MAP1506	-	-	Hypothetical protein, PPE protein family	Purified peptide	Not tested	Homologous in <i>M. avium</i> subsp. <i>hominissuis</i> and MAA	Newton et al., 2009
MAP3817c	MAP3817c	-	-	Putative membrane protein	Recombinant	Immunoblot: high spot intensity in 5/5 pos cattle	Sequence unique to MAP	Bannantine et al., 2008d
MAP3420c	MAP3420c	-	-	Hypothetical protein, PPE protein family	Purified/ recombinant	Immunoblot pos*: 4/4 FC pos, 0/4 FC neg; 3/4 tissue pos, 1/3 tissue neg. ELISA pos: 1 pos in all 3 ELISA/4 FC pos.	Homologous in <i>M. avium</i> subsp. <i>hominissuis</i> and MAA	Newton et al., 2009
rcP34	-	-	-	Carboxyl termini of 34kDa protein	Recombinant	Production of monoclonal antibodies	Common epitopes of MAP, MAA and <i>M. intracellulare</i>	Malamo et al., 2007

Abbreviations: as in Table 1

*Number of positive and negative animals extracted from available data.

¹Based on available data on number of animals, specificity and sensitivity.

Table 4. Characterized lipoprotein antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*

Antigen	Locus	Gene	Size (kDa)	Characteristic	Antigen	Test assay	MAP specific	Reference
Lpp34	-	-	20.8	Putative lipoprotein	Recombinant	Immunoblot pos: 48 pos, 3 neg and 7 doubtful of 23 pos, and 35 neg cattle	Homologous to MAA and <i>E. coli</i> , but absent in <i>M. TB</i> complex	Gioffre et al., 2006
L5P	-	-	-	Lipopentapeptide, a non-ribosomal synthase	Purified	ELISA pos: 2/2 pos goats, 3/3 pos cattle, 0/3 experimental infected mouse/cattle	Not present in MAA or <i>M. smegmatis</i> .	Biet et al., 2008
LAM	-	-	-	Lipoarabinomannan	Purified	AGID pos: 1/1 pos	Not reported	Sugden et al., 1987
Para-LP-01	-	-	-	Cell wall lipopeptide, likely major component of outer part of cell envelope	Purified	ELISA pos*: 5/6 pos, 0/3 neg	Not present in MAA	Eckstein et al., 2006
Polar GPL-1	-	-	-	Glycopeptidolipid, belongs to polar mycoside C glycopeptidolipid family	Purified	ELISA pos: 1(high)/9 pos, 0(?) neg	Not reported	Camphausen et al., 1985
PstA	-	<i>pstA</i>	-	Cell wall lipopeptide, maybe involved in GLP-biosynthesis and biofilm formation	Recombinant	Peptides tested in immunoblot	Present in MAA	Wu et al., 2009

Abbreviations: as in Table 1.

*Number of positive and negative animals extracted from available data

Table 5. Characterized heat shock antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*

Antigen	Locus	Gene	Size (kDa)	Characteristic	Antigen	Test assay	MAP specific	Reference
GroES	MAP4264	<i>GroES</i>	10	-	Recombinant	Not tested	Identical to MAA and homologous to other mycobacteria	Cobb and Frothingham, 1999
MAP3841	MAP3841	<i>grpE</i>	23.7	-	Recombinant	ELISA pos*: 4/6 pos, Dot blot neg, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
Hsp65K	-	-	65	Member of GroEL family	Recombinant	Immunoblot reaction with rabbit anti-MAP serum	Homologous to other mycobacteria	EiZaatari et al., 1994
Hsp65K	-	-	65	Member of GroEL family	Recombinant	Immunoblot pos*: 8/35 pos cattle, 2/2 pos sheep, 1/2 pos goats, 0/10 neg cattle	Homologous to <i>M.TB</i> , <i>M. leprae</i> , MAA	EiZaatari et al., 1995
Hsp70	-	-	70	Interaction with bovine antigen presenting cells	Recombinant	Not tested. Investigation of mechanisms	Not reported	Langelaar et al., 2002
MAP3840	MAP3840	<i>dnaK</i>	70	Molecular chaperone, protein folding and stabilization	Recombinant	Immunoblot: high spot intensity in pos rabbit and mouse, high in 3/5 pos cattle, low 2/5 pos cattle	Not reported	Bannantine et al., 2007; Bannantine et al., 2008d

Abbreviations: as in Table 1.

*Number of positive and negative animals extracted from available data

Table 6. Characterized hypothetical proteins, antigen candidates of unknown function or other antigen candidates of *Mycobacterium avium* subsp. *paratuberculosis*

Antigen	Locus	Gene	Size (kDa)	Characteristic	Antigen	Test assay	MAP specific	Reference
MAP3155c	MAP3155c	-	9	-	Recombinant	Immunoblot: high spot intensity in 5/5 pos cattle, high in pos rabbit and low in pos mouse	Not reported	Bannantine et al., 2008d
Ag6	Overlap MAP0864 and MAP0865	-	16	T-cell epitope, putative protein	Recombinant	ELISA pos*: 8/18 pos, 1/48 neg	Specific compared to MAA. Low cross-reaction with <i>M. bovis</i> (1/48)	Leroy et al., 2009
MAP3857	MAP3857	<i>umpA</i>	18.7	UmpA (orotate phosphoribosyltransferase), biosynthesis	Recombinant	ELISA pos* in 0/6 pos, Dot blot pos, Western blot neg	Higher expression in MAP>MAA	Hughes et al., 2008
21K	-	-	21	Unknown function	Recombinant	Not tested	Not reported	EIzaatari et al., 1994
MAP2685	MAP2685	-	21.3	Hypothetical conserved protein	Recombinant	ELISA pos*: in 6/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
Ag5	-	-	22.5	T-cell epitope, putative protein	Recombinant	ELISA pos*: 5/18 pos, 1/48 neg	Specific compared to MAA. Low cross-reaction with <i>M. bovis</i> (1/48)	Leroy et al., 2009
MAP1564c	MAP1564c	-	23.1	Short chain dehydrogenase	Recombinant	ELISA pos*: 6/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP3627	MAP3627	-	23.1	Hypothetical protein involved in metabolism	Recombinant	ELISA pos*: 6/6 pos, Dot blot neg, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP0268c	MAP0268c	-	23.8	Hypothetical conserved protein	Recombinant	ELISA pos*: 0/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP3491	MAP3491	-	25.2	Hypothetical hydrolase	Recombinant	ELISA pos*: 6/6 pos, Dot blot pos, Western blot neg	Higher expression in MAP>MAA	Hughes et al., 2008
MAP1204	MAP1204	-	25.4	Putative exported p60 protein, virulence factor	Recombinant	Immunoblot pos: 2/2 infected cattle, 0/2 prior infection	Present in MAA and <i>M. smegmatis</i>	Bannantine et al., 2008a
MAP1297	MAP1297	<i>hisA</i>	25.4	HisA (phosphoribosyl isomerase)	Recombinant	ELISA pos*: 0/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP2878c	MAP2878c	<i>dapB</i>	25.6	DapB (dihydrodipicolinate reductase), amino acid biosynthesis	Recombinant	ELISA pos*: 5/6 pos, Dot blot pos, Western blot neg	Higher expression in MAP>MAA	Hughes et al., 2008
MAP0865	MAP0865	-	30.9	Hypothetical protein	Recombinant	ELISA*: 3/3 sheep anti-MAP sera recognised antigen	Unique to MAP	Bannantine et al., 2008c
MAP1365	MAP1365	<i>ardF</i>	33.6	ArgF (ornithine carbamoyltransferase)	Recombinant	ELISA pos*: 5/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
34kDa	-	-	34	Putative serine protease	Purified	Immunoblot pos: 8/14 pos sheep, 2/2 pos goats, 3/3 deer	Homologous to a number of bacteria	Cameron et al., 1994
34.5kDa	-	-	34.5	Cytoplasmic protein	Purified	Not tested	Cross-reaction with tested mycobacteria	Mutharia et al., 1997
MAP0334	MAP0334	-	34.5	-	Recombinant	ELISA pos*: 6/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
P35	-	-	35	-	Recombinant	Immunoblot pos: 31/36 pos sheep/goats/cows, 0/3 <i>M. bovis</i> infected, 0/3 inoculated cows, 0/15 healthy controls	Cross-reaction with anti- <i>M. bovis</i> and anti-MAA serum	EIzaatari et al., 1997
35kDa	-	-	35	-	Recombinant	ELISA pos ¹ : 56/60 pos, 3/22 neg	Not reported	Shin et al., 2004
MAP1012c	MAP1012c	-	37.5	-	Recombinant	ELISA pos*: 6/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
Ag7	MAP1637c	-	40	T-cell epitope. Biosynthesis of cofactors, prosthetic groups, and carriers	Recombinant	ELISA pos*: 12/18 pos, 1/48 neg	Specific compared to MAA. Low cross-reaction with <i>M. bovis</i> (1/48)	Leroy et al., 2009

MAP3932c	MAP3932c	<i>moaA3</i>	41.4	MoaA3 (molybdopterin biosynthesis protein MoeA). Biosynthesis of cofactors, prosthetic groups and carriers	Recombinant	ELISA pos*: 0/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP3175c	MAP3175c	<i>prfB</i>	41.5	Peptide chain release factor 2, protein synthesis	Recombinant	ELISA pos*: 0/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
42kDa	-	-	42	Cytoplasmic protein	Purified	Not tested	Higher expression in MAP>MAA	White et al., 1994
MAP4147	MAP4147	-	42.2	Hypothetical protein involved in energy metabolism, electron transport	Recombinant	ELISA pos*: 0/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP3457	MAP3457	<i>metC</i>	47.6	MetC(o-acetylhomoserine aminocarboxypropyltransferase) involved in metabolism	Recombinant	ELISA pos*: 0/6 pos, Dot blot neg, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP1293	MAP1293	<i>hisD</i>	49.4	HisD (histidinol dehydrogenase)	Recombinant	ELISA pos*: 0/6 pos, Dot blot pos, Western blot pos	Higher expression in MAP>MAA	Hughes et al., 2008
MAP0860	MAP0860	-	-	-	Recombinant	Immunoblot pos: 0/1 pos mouse, 0/2 pos rabbit and 0/2 pos cattle	Unique to MAP	Paustian et al., 2004
MAP0860c	MAP0860c	-	-	-	Recombinant	Immunoblot: high spot intensity in 5/5 pos cattle, high in pos rabbit and low in pos mouse	Unique to MAP	Bannantine et al., 2008d
MAP0862	MAP0862	-	-	-	Recombinant	Immunoblot pos: 1/1 pos mouse, 2/2 pos rabbit and 2/2 pos cattle	Unique to MAP	Paustian et al., 2004
MAP0862	MAP0862	-	-	-	Recombinant	ELISA pos: 9/11 pos. ELISA*: 3/3 sheep anti-MAP sera recognised antigen	Unique to MAP	Bannantine et al., 2008c
MAP1643	MAP1643	<i>aceAB</i>	-	Putative isocitrate lyase	Recombinant	Not tested	Not reported	Bannantine et al., 2007
MAP1730c	MAP1730c	-	-	-	Recombinant	Immunoblot: high spot intensity in 6/6 infected cattle	Cross reaction with <i>M. bovis</i> cow sera	Bannantine et al., 2008b
MAP2154c	MAP2154c	-	-	-	Recombinant	Immunoblot pos: 1/1 pos mouse, 1/2 pos rabbit and 0/2 pos cattle	Unique to MAP	Paustian et al., 2004
MAP2182c	MAP2182c	-	-	Hypothetical conserved protein	Recombinant	Immunoblot: high spot intensity in 4/5 pos cattle, high in pos mouse and rabbit	Highly conserved protein	Bannantine et al., 2008d
MAP2963c	MAP2963c	-	-	Hypothetical conserved protein containing heme binding domain	Recombinant	Immunoblot pos: 1/1 pos mouse, 2/2 pos rabbit and 2/2 pos cattle	Unique to MAP	Paustian et al., 2004
MAP3732c	MAP3732c	-	-	-	Recombinant	Immunoblot pos: 1/1 pos mouse, 2/2 pos rabbit and 2/2 pos cattle	Unique to MAP	Paustian et al., 2004
MAP-antigen	-	-	-	Protoplasmic antigen	Purified	ELISA pos: 50/60 pos, 5/44 neg cattle	Cross-reaction with several bacteria	Abbas et al., 1983
MBP-gene56	-	-	-	Protein of unknown function produced by MAP (selected in Bannantine et al., 2002)	Recombinant	Immunoblot pos: 5/6 pos, 0/4 neg cattle	Specific compared to 9 mycobacteria species	Bannantine et al., 2004
MBP-gene135	-	-	-	Protein of unknown function produced by MAP (selected in Bannantine et al., 2002)	Recombinant	Immunoblot pos: 4/6 pos 1/4 neg cattle	Specific compared to 9 mycobacteria species	Bannantine et al., 2004
MBP-gene218	-	-	-	Protein of unknown function produced by MAP (selected in Bannantine et al., 2002)	Recombinant	Immunoblot pos: 5/6 pos, 0/4 neg cattle	Specific compared to 9 mycobacteria species	Bannantine et al., 2004
MBP-gene241	-	-	-	Protein of unknown function produced by MAP (selected in Bannantine et al., 2002)	Recombinant	Immunoblot pos: 6/6 pos, 1/4 neg cattle	Specific compared to 9 mycobacteria species	Bannantine et al., 2004
MBP-gene254	-	-	-	Protein of unknown function produced by MAP (selected in Bannantine et al., 2002)	Recombinant	Immunoblot pos: 5/6 pos, 4/6 neg cattle	Specific compared to 9 mycobacteria species	Bannantine et al., 2004

Abbreviation: as in Table 1.

*Number of positive and negative animals extracted from available data.