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► To cite this version:

Rebeka Lucijana Berčič, Brigita Slavec, Miha Lavrič, Mojca Narat, Andrej Bidovec, et al.. Identification of major immunogenic proteins of isolates. *Veterinary Microbiology*, 2007, 127 (1-2), pp.147. 10.1016/j.vetmic.2007.07.020 . hal-00532296

HAL Id: hal-00532296

<https://hal.science/hal-00532296>

Submitted on 4 Nov 2010

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Accepted Manuscript

Title: Identification of major immunogenic proteins of *Mycoplasma synoviae* isolates

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PII: S0378-1135(07)00364-1
DOI: doi:10.1016/j.vetmic.2007.07.020
Reference: VETMIC 3770

To appear in: *VETMIC*

Received date: 27-4-2007
Revised date: 19-7-2007
Accepted date: 20-7-2007

Please cite this article as: Berčič, R.L., Slavec, B., Lavrič, M., Narat, M., Bidovec, A., Dovč, P., Benčina, D., Identification of major immunogenic proteins of *Mycoplasma synoviae* isolates, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.07.020

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Identification of major immunogenic proteins of *Mycoplasma synoviae* isolates**

2

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23

24 Abstract

25

26 *Mycoplasma synoviae* isolates differ in patterns of immunogenic proteins,
27 but most of them have not been identified yet. The main aim of this study was
28 their identification in two closely related *M. synoviae* isolates, ULB 02/P4 and
29 ULB 02/OV6, recovered recently from chickens in Slovenia. N-terminal
30 sequencing identified seventeen *M. synoviae* proteins. Amongst them were 14
31 major, highly expressed but previously unidentified proteins, including enzymes,
32 chaperones and putative lipoproteins. ULB 02/P4 proteins with increasing
33 molecular weight (Mw) in the region above the lipoprotein MSPB (~ 40 kDa)
34 were: elongation factor EF-Tu, enolase, NADH oxidase, haemagglutinin MSPA,
35 ATP syntase beta chain, trigger factor, pyruvate kinase and chaperone DnaK.
36 Enolase (~ 47 kDa) seemed to be immunogenic for chickens infected with *M.*
37 *synoviae*, whereas EF-Tu, which might cross-react with antibodies to the P1
38 adhesin of *Mycoplasma pneumoniae*, was not. ULB 02/OV6 synthesized several
39 immunogenic proteins and those with Mw of approximately 70, 78, 82, 90, 110
40 and 160 kDa, cross-reacted with antibodies to *Mycoplasma gallisepticum*. They
41 remain to be identified, because besides putative lipoproteins, protein bands of
42 78, 82, 85 and 110 kDa contained also dehydrogenase PdhD, elongation factor
43 EF-G, enzyme PtsG and putative neuraminidase, respectively.

44

45 Key words: *Mycoplasma synoviae*, proteome, major proteins

46

47 1.Introduction

48

49 *Mycoplasma synoviae* and *Mycoplasma gallisepticum* are major poultry
50 pathogens. Their strains differ in their infectivity, tissue tropism and
51 pathogenicity, but factors associated with their pathogenicity are largely
52 undetermined (Ley, 2003; Kleven,2003).

53 Recent comparison of the *M. gallisepticum* genome (Papazisi et al., 2003)
54 with the *M. synoviae* genome revealed that a number of their genes have been
55 transferred horizontally (Vasconcelos et al., 2005). However, with the exception
56 of the *vlhA* genes encoding haemagglutinins there is no data about the
57 expression of genes shared by both *Mycoplasma spp.* (Benčina, 2002).

58 In most *M. synoviae* strains their VlhA haemagglutinins are cleaved into
59 the lipoprotein MSPB and the C-terminal part, a putative haemagglutinin
60 MSPA, both of them can differ in lengths and antigenic determinants
61 (Noormohammadi et al., 1997; Narat et al., 1998; Benčina et al., 1999; 2001).

62 It has been reported that antibodies to the P1 adhesin of the human
63 pathogen *Mycoplasma pneumoniae* detected a protein of 45 kDa in all *M.*
64 *synoviae* strains and that this *M. synoviae* protein may be associated with the
65 pathogenicity of *M. synoviae* (Lockaby et al., 1999). In our previous study all *M.*
66 *synoviae* strains examined synthesized about 43- kDa elongation factor EF-Tu
67 and unidentified protein (about 45-kDa protein) recognized by monoclonal

68 antibody (mAb) 5 (Benčina et al., 1999). In addition, Avakian et al. (1992) have
69 reported that protein profiles of *M. synoviae* strains differ, particularly in the
70 molecular size range of 38-61 kDa. Amongst them may be MSPB and MSPA,
71 (Noormohammadi et al., 1997; Benčina et al., 2001) but other proteins have not
72 been characterized or identified.

73 The main aim of this study was to identify selected major proteins of two
74 *M. synoviae* isolates by N-terminal sequencing. In ULB 02/P4, we focused on
75 major proteins positioned in its protein profile between MSPB (~ 40 kDa) and
76 DnaK (~ 65 kDa), whereas in ULB 02/OV6 its major immunogenic proteins
77 with molecular weights from 78 kDa to about 160 kDa were sequenced.

78

79 2. Materials and methods

80

81 2.1 *Mycoplasma synoviae* strains

82 The *M. synoviae* type strain WVU 1853 and closely related isolates of *M.*
83 *synoviae* from the chicken broiler breeder flocks in Slovenia (breed RO) were
84 cultured in modified Frey's media (Benčina et al., 2001, 2005b; Lavrič et al.,
85 2007). They were, ULB 02/P4 isolated from the choanal cleft (Benčina et al.,
86 2005b, Lavrič et al., 2007) and ULB 02/T6 and ULB 02/OV6, isolated from the
87 trachea and oviduct of another hen of the same flock.

88

89 2.2 Antibodies

90 Chicken and rabbit antibodies as well as monoclonal antibodies (mAbs)
91 recognizing distinct *M. synoviae* proteins were used (Table 1).

92

93 *2.3 Electrophoresis and immunoblotting*

94 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-
95 PAGE), isoelectric focusing (IEF) and immunoblotting were performed with the
96 PhastSystem (Pharmacia, Sweden) or in larger gels as described in detail
97 elsewhere (Benčina et al., 1994, 1999, 2001, 2005b; Narat et al., 1998; Lavrič et
98 al., 2007).

99

100 *2.4 N-terminal sequencing of proteins and their identification*

101 Proteins were separated by SDS-PAGE and transferred to the Immobilon-
102 P (Millipore) membrane. Preparation of selected proteins of ULB02/P4 and
103 ULB02/OV6 for N-terminal sequencing was done as described earlier (Benčina
104 et al., 1999; Benčina et al., 2005a).

105 The determined N-terminal aa sequences were compared with sequences
106 encoded by the *M. synoviae* strain 53 genome (Vasconcelos et al., 2005), as well
107 as with sequences from public databases (NCBI, SwissProt, BCM Search
108 Launcher). The predicted molecular weights (Mw) and isoelectric points (pI) of
109 proteins annotated from the *M. synoviae* genome were considered, but their
110 values were corrected, if the determined N-terminal sequences required such
111 corrections, using ExPASy proteomic tools (<http://www.expasy.org/tools/>).

112

113 **3. Results**

114

115 *3.1 M. synoviae proteins recognized by specific antibodies*

116 Pattern of proteins recognized by chicken antibodies in the *M. synoviae*
117 type strain WVU 1853 was considerably different from that recognized in its
118 isolate ULB02/P4 (Fig.1 and Fig.1 shown in Benčina et al., 2005b). ULB02/P4
119 synthesized a shorter MSPB (~ 40 kDa) than WVU1853 (~ 45 kDa MSPB), and
120 proteins of about 55, 85, 90, 103 and 110 kDa which reacted more intensively in
121 ULB02/P4 in comparison with WVU1853 (Fig.1). On the other hand, amounts
122 of the elongation factor EF-Tu and of about 47 kDa protein detected by specific
123 mAb R and mAb5 were similar in both strains. (Supplement. Fig. 1, and data not
124 shown).

125 A protein detected by mAb5 was expressed on the surface of native
126 colonies of all *M. synoviae* strains examined by the indirect immunoperoxidase
127 assay (Benčina et al., 1994, 1999). Chickens infected with *M. synoviae* had
128 serum antibodies that blocked binding of mAb5 to its colonies, indicating that
129 the protein (~ 47 kDa) recognized by mAb5 is immunogenic (Narat et al.,1998).
130 In contrast, rabbit antibodies to the P1 adhesin of *M. pneumoniae* did not react
131 with *M. synoviae* colonies, although they in immunoblots reacted in all strains
132 of *M. synoviae* with about 43 kDa protein (see Lockaby et al., 1999). Further
133 analyses revealed that this protein is EF-Tu (see Figs. 2 and Supp. Fig.1).

134 Indeed, antibodies to P1 reacted with the same protein as did mAb R (~ 43 kDa
135 EF-Tu), and both detected also ~ 37 kDa EF-Tu fragment.

136

137 3.2 Identification of ULB02/P4 major proteins by N-terminal sequencing

138 In the ULB02/P4 protein profile there were six major protein bands
139 located between MSPB (~ 40 kDa) and DnaK (~ 65 kDa) whose positions were
140 determined by mAb 125 and mAb 3, respectively (Fig. 2). Five major proteins
141 bands from this region were submitted for N-terminal sequencing (Fig. 2 and
142 Table 2). The N-terminal aa sequence of a 43-kDa protein was identical to the
143 EF-Tu protein sequence (Table 2). A major protein (~ 47 kDa) above EF-Tu was
144 recognized by mAb5. The N-terminal sequence of this protein
145 (AIKKIHAREVLDSRG) was identical to the sequence of the *M. synoviae*
146 enolase (residues 3-17) encoded by the *eno* gene (MS53_0009 in the genome of
147 strain 53).

148 Sequencing of the protein band with an estimated Mw of approximately
149 50 kDa revealed three different aa sequences (Table 2). The first sequence,
150 MENKKIIVVGANHAG was identical to that of the NADH oxidase Nox
151 encoded by the *nox* gene (MS53_0522). The sequence of a protein comigrating
152 with Nox was, (M)GEAAEDSEXSETQL (Table 2). It is identical in all residues
153 determined to a putative MSPA sequence encoded by the expressed *vlhA* gene of
154 ULB02/P4 (corresponds to aa residues 337-350 in the mature VlhA protein).
155 The third sequence shared the highest sequence identity with two ATP synthase

156 beta chains, ATP D-3 and ATP D-2 (genes MS53_0464 and MS53_0160).
157 Sequencing of an approximately 55-kDa protein band revealed two distinct aa
158 sequences (Fig. 2 and Table 2). The first sequence was identical to that of a
159 putative trigger factor (Tig). The second sequence was identical in all residues
160 determined to the N-terminal sequence of the pyruvate kinase (Pyk) encoded by
161 the *pyk* gene (Table 2).

162 Approximately 65 kDa protein recognized by mAb 3 to DnaK revealed
163 the N- terminal sequence of DnaK (Table 2).

164

165 *3.3. Variable expression of immunogenic proteins in closely related M. synoviae*
166 *isolates cultures*

167 Initial analyses indicated that certain *M. synoviae* proteins can undergo
168 variation in expression and/or size variation. To address this topic in more detail
169 closely related cultures of *M. synoviae* were analyzed (Fig. S2 in Supplementary
170 Material). Culture isolated from the oviduct, ULB 02/OV6, abundantly
171 synthesized proteins of approximately 85, 110 and 160 kDa (designated P85,
172 P110 and P160), that were absent in a culture IP2K derived from the *M.*
173 *synoviae* (ULB 02/T6) isolate from the trachea of the same chicken (Fig. 2 in
174 Supplement).

175 Those proteins of ULB 02/OV6 (i.e. P85, P110, P160) were immunogenic
176 for chickens infected with different *M. synoviae* strains and reacted also with
177 rabbit antibodies to *M. synoviae* (Fig. 3). Those antibodies reacted with several

178 proteins positioned above DnaK, including P70, LP 78, P 82, P 110 and P160
179 (Fig. 3). In addition, a number of ULB 02/OV6 proteins also reacted with
180 antibodies to *M. gallisepticum* (Fig. 3). Some of them have similar Mw as those
181 which reacted with antibodies to *M. synoviae*.

182

183 3.4 N-terminal sequencing of ULB 02/OV6 immunogenic proteins

184 ULB02/OV6 proteins LP78, P82, P85, P90, P110 and P160 were
185 submitted for N-terminal sequencing (Table 2). Although individual protein
186 bands were excised carefully, sequence analysis revealed the presence of more
187 than one protein per band. Approximately 78-kDa protein band, contained a
188 sequence corresponding to that of LP78 (encoded by MS53_0334), as well as
189 the sequence MYTFKFADI (Table 2). This sequence is identical to that of
190 dihydrolipoamide dehydrogenase PdhD encoded by the gene *pdhD*. Protein of
191 about 82 kDa (P82) also contained two aa sequences. The first, ARDYDLKDY
192 was identical to the elongation factor EF-G sequence encoded by the *fusA* gene
193 (Table 2). The second sequence probably belonged to a putative lipoprotein
194 encoded by the gene MS53_0517 (Table 2). Sequencing of a «P85» protein
195 band also yielded two aa sequences. The first sequence, XGAPQEEKK
196 corresponded to a putative lipoprotein LP 85 (Table 2). The second sequence
197 had the highest aa sequence identity with a protein of the PTS system encoded
198 by the *ptsG* gene (MS53_0147).

199 Sequencing of P90 failed to identify an appropriate *M. synoviae* protein
200 (Table 2). A protein band designated P110 yielded two distinct aa sequences.
201 The first sequence probably belonged to a putative lipoprotein encoded by the
202 gene MS53_0349 (Table 2). The second sequence, MKKXK, is similar to the
203 sequence of a putative neuraminidase (sialidase) NanH encoded by the gene
204 MS53_0199. The determined aa sequence of P160 was too short and incomplete
205 for efficient retrieval of a relevant protein (Table 2).

206

207 **4. Discussion**

208

209 The *M. synoviae* genome has been sequenced (Vasconcelos et al., 2005)
210 whereas its proteome was not examined. We identified 10 previously
211 unidentified major *M. synoviae* proteins involved in metabolism and protein
212 processing, as well as three putative lipoproteins.

213 Nox seems to be a major *M. synoviae* protein and this observation is
214 consistent with its high NADH oxidase activity (Taylor et al., 1996). It should
215 be noted that the N-terminal sequence of Nox was MENKKIIVVGAN.., (rather
216 than MIVVGAN), and that its gene should start with a typical translation start
217 codon ATG, rather than with ATT codon which is 15 nt downstream of ATG.

218 Enolase (phosphopyruvate hydratase) is also a major *M. synoviae* protein.
219 In the protein profile of ULB02/P4 and related strains, enolase is well separated
220 from other major proteins (Figs.2 and Fig. S1 in Supplementary Material). In

221 certain other strains, like WVU1853, their MSPB proteins have Mw similar to
222 that of enolase. It seems that infection of chickens with *M. synoviae* induces
223 antibodies to its enolase. Antibodies to bacterial enolase are involved in variety
224 of autoimmune diseases, because they can cross-react with the host enolase
225 (Pancholi, 2001). *M. synoviae* is well known to cause autoimmune processes
226 (Kleven, 2003). Since its enolase shares approximately 53% sequence identity
227 with the chicken enolase, it seems possible that antibodies raised against the *M.*
228 *synoviae* enolase may be involved in autoimmune processes caused by *M.*
229 *synoviae*. However, further investigations are required to address this topic.

230 Two other enzymes involved in the sugar metabolism, PyK and PdhD,
231 also belong to major *M. synoviae* proteins. It is not a surprise that Pyk and
232 trigger factor, Tig, were in the same protein band, because their calculated Mw
233 are very similar (Table 2). It is likely that they can be easily separated by IEF,
234 because the pI of Pyk (pI > 8) is quite different from that of Tig (pI ~ 5). Both
235 chaperones, Tig and DnaK seem to be abundant in *M. synoviae*. In the absence
236 of chaperonines GroEL and GroES (Vasconcelos et al., 2005), the role of DnaK
237 and possibly, of Tig should be important for protein folding.

238 Along with an abundant synthesis of EF-Tu, this study showed also the
239 presence of another elongation factor, EF-G. However, it is not clear if EF-G
240 that comigrated in SDS-PAGE with a lipoprotein LP 78 was a protein that cross-
241 reacted with rabbit antibodies to *M. gallisepticum* (Fig. 3). This seems possible,
242 because EF-G C-terminal region share a high sequence identity with a short

243 form of EF-G of *M. gallisepticum*. Notably, *M. synoviae* duplicated
244 corresponding part of the *fusA* gene (genes MS53_0038 and MS53_0039) is in
245 the region that has been transmitted horizontally (Vasconcelos et al., 2005).

246 Besides Nox, approximately 50-kDa band of ULB02/P4 contained also
247 sequences indicating the presence of the cleaved VlhA (C-terminal part MSPA)
248 and ATP synthase beta chain that has three copies in the *M. synoviae* genome
249 (genes *atp D*, *atp D-2* and *atp D-3*). The region that includes *atp D-3* has also
250 been transferred between *M. synoviae* and *M. gallisepticum* (Vasconcelos et al.,
251 2005).

252 The sequence of a putative MSPA of ULB02/P4 (Table 2) indicates the
253 VlhA cleavage site downstream of such site proposed for the *M. synoviae* strain
254 ULB925 (Benčina et al., 1999). Because recombination of the *vlhA* gene
255 generate very diverse VlhA sequences (Noormohammadi et al., 2000; Benčina
256 et al., 2001) the VlhA may cleave at different sites.

257 It has been reported that *M. synoviae* protein of ~ 45 kDa reacted with
258 antibodies to the P1 adhesin of *M. pneumoniae* (Lockaby et al., 1999). In our
259 study, antibodies to P1 recognized in *M. synoviae* EF-Tu protein. It should be
260 noted that the genome of *M. synoviae* does not contain sequences related to that
261 of the gene encoding the P1 adhesin (Vasconcelos et al., 2005).

262 The genome of *M. synoviae* encodes about 40 putative lipoproteins.
263 (Vasconcelos et al., 2005) In many *Mycoplasma* species lipoproteins induce
264 strong antibody response, whereas their phase-variable expression mediates

265 immune evasion (Citti et al., 2005). At least two lipoproteins of *M. synoviae*,
266 VlhA and LP78, are immunogenic for poultry (Noormohammadi et al., 1997;
267 Narat et al., 1998; Markham et al., 2004).

268 In this study, besides VlhA (MSPB and MSPA) and LP78, also two
269 putative lipoproteins (LP85 and LP110) could be identified. It seems that LP85
270 could be expressed variably (Suppl. Fig. 2) in and might cross-react with
271 antibodies to *M. gallisepticum* (Fig. 3 and Suppl. Fig. 2). Shared epitopes may
272 be associated with *M. gallisepticum* proteins sharing aa sequences with LP85
273 (e.g. protein encoded by the gene MGA_0579 and MGA_0565). Moreover,
274 those *M. gallisepticum* genes share much higher sequence identity with another
275 *M. synoviae* gene (MS53_0463).

276 Variable expression and possibly, cross-reactivity with antibodies to *M.*
277 *gallisepticum*, also might be attributed to a putative LP110 (Fig. 3 and Suppl.
278 Fig. 2). *M. synoviae* has proteins that may bind IgG (via Fc part) of chickens and
279 some other birds and mammals (Lauerman and Reynolds-Vaughn, 1990). We
280 observed that ULB02/P4 and ULB02/OV6 proteins of about 85 and 90 kDa
281 proteins bound chicken and goat IgG, but it is not clear if they were among
282 proteins submitted for sequencing and shown in Table 2. It would be necessary
283 to affinity-purify *M. synoviae* proteins that bind chicken IgG (using IgG as a
284 ligand) and to identify them by sequencing.

285 Our data indicates that *M. gallisepticum* and *M. synoviae* cells have
286 neuraminidase activity and cleave sialic acid from specific substrates, but further

287 research is required to prove that about 110-kDa protein of *M. synoviae* is its
288 functional neuraminidase.

289 Whereas certain *M. synoviae* proteins could not be identified, some
290 proteins in the protein profile did not belong to *M. synoviae* but rather to porcine
291 IgG from the serum used in the growth medium (Table 2, footnote a). This
292 confirmed problems associated with “contamination” of mycoplasma antigens
293 with immunoglobulins from the growth medium (Benčina, 2002).

294

295 **5. Conclusions**

296

297 In this study we identified a number of major proteins from the *M.*
298 *synoviae* proteome. They belong to enzymes of the sugar metabolism,
299 chaperons, elongation factors and possibly, to neuraminidase NanH. Several
300 putative lipoproteins were identified. They seemed to be immunogenic variably
301 expressed proteins, probably sharing epitopes with *M. gallisepticum* proteins.

302

303 **Acknowledgements**

304 This research was supported by a grant from the Ministry of Education and
305 Science of the Republic Slovenia. We thank Dr L. Stipkovits, Dr D.H. Ley, Dr
306 B. Heinrich and Dr G. Czifra for mAbs and Dr D. C. Krause for the antibodies
307 to P1 adhesin. We thank Dr I. Križaj for the sequence analyses of *M. synoviae*
308 proteins. Technical help of I. Beretič and S. Salmič is acknowledged.

309

310 **References**

311

312 Avakian, A.P., Ley, D.H., Kleven, S.H., 1992. Comparison of *Mycoplasma*
313 *synoviae* isolates by immunoblotting. Avian Pathol. 21, 633–642.

314 Benčina, D., Kleven, S.H., Elfaki, M.G., Snoj, A., Dovč, P., Dorrer, D., Russ, I.,
315 1994. Variable expression of epitopes on the surface of *Mycoplasma*
316 *gallisepticum* demonstrated with monoclonal antibodies. Avian Pathol. 23,
317 19–36.

318 Benčina, D., 2002. Haemagglutinins of pathogenic avian mycoplasmas. Avian
319 Pathol. 31, 535–547.

320 Benčina, D., Narat, M., Dovč, P., Drobnič-Valič, M., Habe, F., Kleven, S.H.,
321 1999. The characterization of *Mycoplasma synoviae* EF-Tu protein and
322 proteins involved in hemadherence and their N-terminal amino acid
323 sequences. FEMS Microbiol. Lett. 173, 85–94.

324 Benčina, D., Drobnič-Valič, M., Horvat, S., Narat, M., Kleven, S.H., Dovč, P.,
325 2001. Molecular basis of the length variation in the N-terminal part of
326 *Mycoplasma synoviae* hemagglutinin. FEMS Microbiol. Lett. 203, 115–123.

327 Benčina, D., Slavec, B., Narat, M., 2005a. Antibody response to GroEL varies in
328 patients with acute *Mycoplasma pneumoniae* infection. FEMS Immunol.
329 Med. Microbiol. 34, 399–406.

- 330 Benčina, D., Narat, M., Bidovec, A., Zorman-Rojs, O., 2005b. Transfer of
331 maternal immunoglobulins and antibodies to *Mycoplasma gallisepticum* and
332 *Mycoplasma synoviae* to the allantoic and amniotic fluid of chicken embryos.
333 Avian Pathol. 34, 463–472.
- 334 Citti, C., Browning, G.F., Rosengarten, R., 2005. Phenotypic diversity and cell
335 invasion in host subversion by pathogenic mycoplasmas. In: Blanchard, A.,
336 Browning, G. (Eds.), Mycoplasmas, molecular biology, pathogenicity and
337 strategies for control. Horizon Bioscience, Norfolk, pp. 439–483.
- 338 Kleven, S.H., 2003. *Mycoplasma synoviae* infection. In: Saif, Y.M., Barnes,
339 H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. (Eds.),
340 Diseases of Poultry. 11th ed. Iowa State University Press, Ames, pp. 756–
341 766.
- 342 Laureman, L.H., Reynolds-Vaughn, R.A., 1991. Immunoglobulin G Fc
343 receptors of *Mycoplasma synoviae*. Avian Dis. 35, 135–138.
- 344 Lavrič, M., Benčina, D., Kothlow, S., Kaspers, B., Narat, M., 2007.
345 *Mycoplasma synoviae* lipoprotein MSPB, the N-terminal part of VlhA
346 haemagglutinin, induces secretion of nitric oxide, IL-6 and IL-1beta in
347 chicken macrophages. Vet. Microbiol. 121, 278–87.
- 348 Lockaby, S.B., Hoerr, F.J., Laureman, L.H., Smith, B.F., Samoylov, A.M.,
349 Toivio-Kinnucan, M.A., Kleven, S.H., 1999. Factors associated with
350 virulence of *Mycoplasma synoviae*. Avian Dis. 43, 251–261.

- 351 Markham, P.F., Zohari, S., Kanci, A., Bradbury, J.M., Hellman, U., Czifra, G.,
352 2004. The non-variable 78 kDa *Mycoplasma synoviae* membrane protein has
353 a diagnostic potential. In: 15th Congress of the International Organization for
354 Mycoplasmology, Athens, Georgia, July 11-16, 2004, Program, pp. 86.
- 355 Narat, M., Benčina, D., Kleven, S.H., Habe, F., 1998. Hemagglutination-
356 positive phenotype of *Mycoplasma synoviae* induces experimental infectious
357 synovitis in chickens with a higher frequency than the hemagglutination-
358 negative phenotype. *Infect. Immun.* 66, 6004–6009.
- 359 Noormohammadi, A.H., Markham, P.F., Whithear, K.G., Walker, I.D.,
360 Gurevich, V.A., Ley, D.H., Browning, G.F., 1997. *Mycoplasma synoviae* has
361 two distinct phase-variable major membrane antigens, one of which is a
362 putative hemagglutinin. *Infect. Immun.* 65, 2542–2547.
- 363 Noormohammadi, A.H., Markham, P.F., Duffy, M.F., Whithear, K.G.,
364 Browning, G.F., 1998. Multigene families encoding the major
365 hemagglutinins in phylogenetically distinct mycoplasmas. *Infect. Immun.* 66,
366 3470–3475.
- 367 Noormohammadi, A.H., Markham, P.F., Kanci, A., Whithear, K.G., Browning,
368 G.F., 2000. A novel mechanism for control of antigenic variation in the
369 haemagglutinin gene family of *Mycoplasma synoviae*. *Mol. Microbiol.* 35,
370 911–923.
- 371 Papazisi, L., Gorton, T.S., Kutish, G., Markham, P.F., Browning, G.F., Nguyen,
372 D.K., Swartzell, S., Madan, A., Maharais, G., Geary, S.J., 2003. The

- 373 complete genome: sequence of the avian pathogen *Mycoplasma*
374 *gallisepticum* strain R low. Microbiology. 149, 2307–2316.
- 375 Pancholi, V., 2001. Multifunctional alpha-enolase: its role in diseases. Cell.
376 Mol. Life Sci. 58, 902–920.
- 377 Taylor, R.R., Mohan, K., Miles, R.J., 1996. Diversity of energy-yielding
378 substrates and metabolism in avian mycoplasmas. Vet. Microbiol. 51, 291–
379 304.
- 380 Vasconcelos, A.T.R., Ferreira, H.B., Bizarro, C.V., Bonatto, S.L., Carvalho,
381 M.O., Pinto, P.M., Almeida, D.F., Almeida, L.G.P., Alves-Filho, R.A.L.,
382 Assunção, E.N., Azevedo, V.A.C., Bogo, M.R., Brigido, M.M., Brocchi, M.,
383 Burity, H.A., Camargo, A.A., Camargo, S.S., Carepo, M.S., Carraro, D.M.,
384 de Mattos Cascardo, J.C., Castro, L.A., Cavalcanti, G., Chemale, G.R.,
385 Collevatti, Cunha, C.W., Dallagiovanna, B., Dambrós, P., Dellagostin, O.A.,
386 Falcão, C., Fantinatti-Garboggini, F., Felipe, M.S.S., Fiorentin, L., Franco,
387 G.R., Freitas, N.S.A., Frías, D., Grangeiro, T.B., Grisard, E.C., Guimarães,
388 C.T., Hungria, M., Jardim, S.N., Krieger, M.A., Laurino, J.P., Lima, L.F.A.,
389 Lopes, M.I., Loreto, É.L.S., Madeira, H.M.F., Manfio, G.P., Maranhão, A.Q.,
390 Martinkovics, C.T., Medeiros, S.R.B., Moreira, M.A.M., Neiva, M.,
391 Ramalho-Neto, C.E., Nicolás, M.F., Oliviera, S.C., Paixão, R.F.C., Pedrosa,
392 F.O., Pena, S.D.J., Pereira, M., Pereira-Ferrari, L., Piffer, I., Pinoto, L.S.,
393 Potrich, D.P., Salim, A.C.M., Santos, F.R., Schmitt, R., Schneider, M.P.C.,
394 Schrank, A., Schrank, I.S., Schuck, A.F., Seunanz, H.N., Silva, D.W., Silva,

395 R., Silva, S.C., Soares, C.M.A., Souza, K.L. R., Souza, R.C., Staats, C.C.,
396 Steffens, M.B.R., Teixeira, S.M.R., Urmenyi, T.P., Vainstein, M.H.,
397 Zuccherato, L.W., Simpson, A.J.G., Zaha, A., 2005. Swine and poultry
398 pathogens: the complete genome sequences of two strains of *Mycoplasma*
399 *hyopneumoniae* and a strain of *Mycoplasma synoviae*. J. Bacteriol. 187,
400 5568–5577.

401 **Figure 1. Immunoblot of *M. synoviae* strains WVU 1853 and ULB 02/P4 proteins.**

402 Reactions of serum IgG antibodies of a chicken infected naturally with *M. synoviae*
403 ULB 9122 (Benčina et al., 2005b). Several proteins of ULB 02/P4 (lane P4) showed
404 more intense reaction e.g. P85 and P110 in comparison to WVU 1853 (lane WVU).
405 Reactions of MSPB of ULB 02/P4 (~40 kDa) and WVU 1853 (~45 kDa) are similary
406 intense. The position of Mw markers is indicated at the left side. Note, the same *M.*
407 *synoviae* antigens were used in our previous study (see Fig. 1 in the publication by
408 Benčina et al., 2005b).

409

410 **Figure 2. Comparison of protein profiles of *M. synoviae* isolates ULB 02/T6 and ULB**
411 **02/P4.**

412 Proteins of *M. synoviae* isolates ULB 02/T6 (lane T6) and ULB 02/P4 were
413 transferred to the membrane (lane P4) and stained with coomassie blue. Only proteins
414 in the region from MSPB (~40 kDa) to DnaK (~ 65-67 kDa) are presented. ULB
415 02/P4 proteins (indicated in lane P4) were submitted for N-terminal sequencing (see
416 Table 2).

417

418 **Figure 3. Reactions of ULB 02/OV6 proteins with rabbit antibodies to *M. synoviae* (MS)**
419 **or to *M. gallisepticum* (MG).**

420 ULB 02/OV6 proteins from the same SDS-PAGE run (a parallel lane on the same
421 membrane) were stained by coomassie blue, excised and submitted for N-terminal
422 sequencing. Relevant proteins, LP 78, P 82, P 85, P 90, P 110 and P 160 are indicated
423 at the left side (see Table 3). Estimated Mw of proteins reacting with antibodies to MG
424 are 70, 75, 82, 90, 110 and 160 kDa.

425

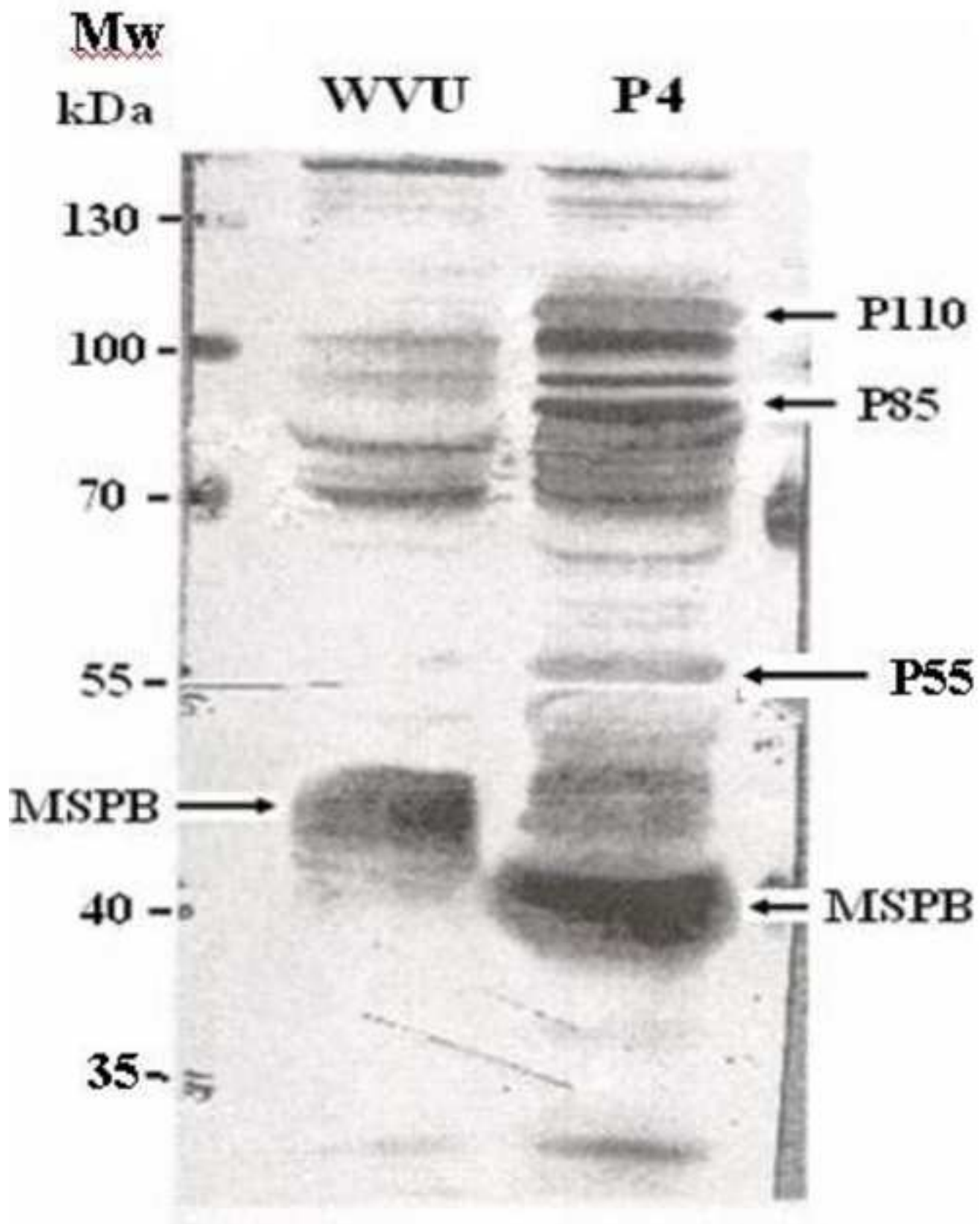
426 **Supplementary Figure S1. Immunoblot of proteins of *M. synoviae* ULB 02/P4.**

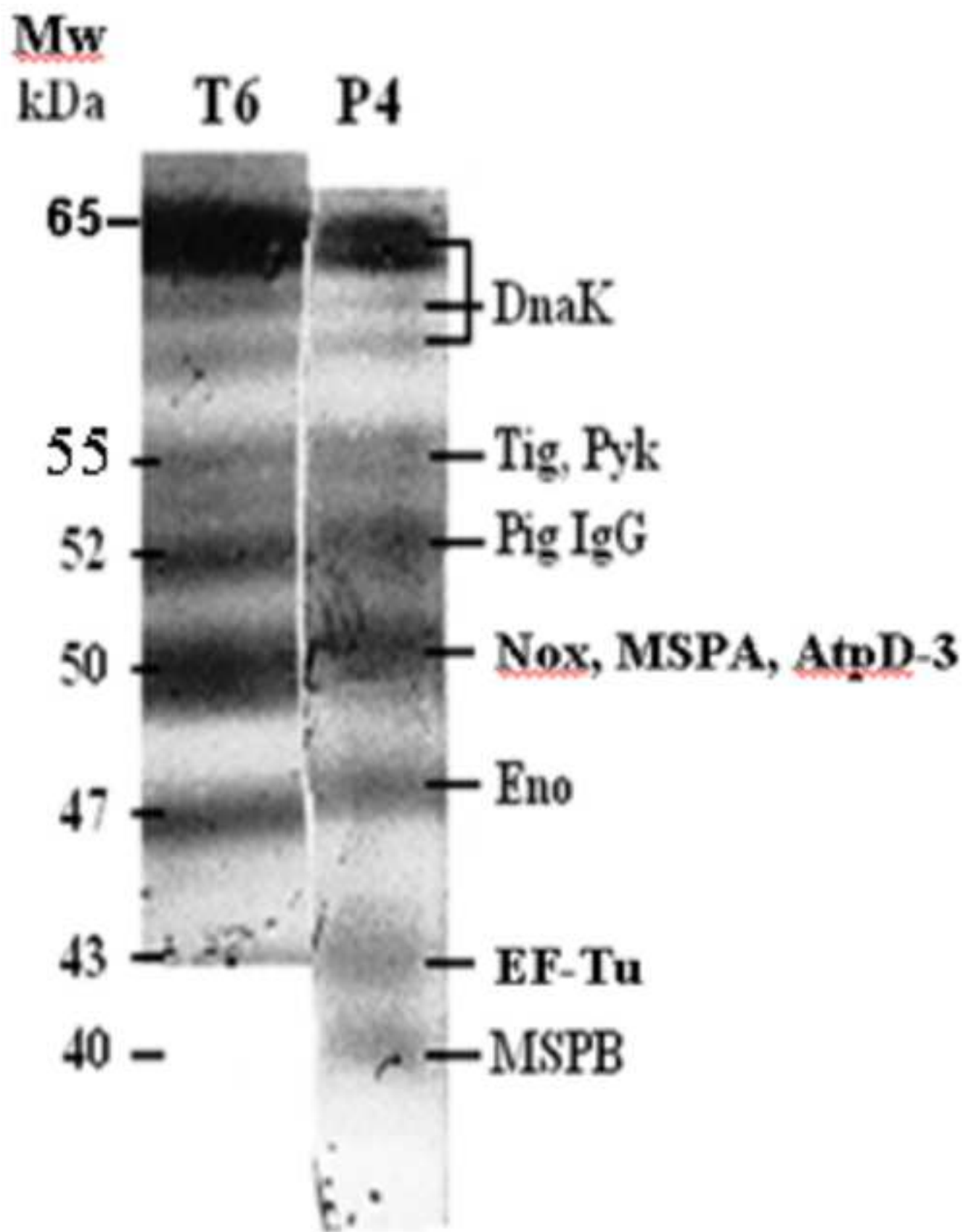
427 Blot A: proteins were transferred to the membrane and stained with coomassie blue.
428 Following destaining, the upper part of the membrane was probed with rabbit
429 antibodies to *M. synoviae* (ULB 925) to identify immunogenic proteins, e. g. P70, LP
430 78 (defined by mAb 1H6), P85 and P 110 (see arrows). Blot B: following destaining,
431 this part of the membrane was immunostained using mAb 5 to show the position of a
432 surface-exposed membrane protein of approximately 47 kDa, i.e. of enolase (Eno).
433 The position of EF-Tu was determined by mAbR (see its position in A).

434

435 **Supplementary Figure S2. Synthesis of proteins in closely related *M. synoviae* cultures.**

436 Proteins of ULB 02/OV6 (lane OV6), IP 2K, IT 2A and ULB 02/T6 (lane T6) were
437 separated by SDS-PAGE and stained with coomassie blue. ULB 02/OV6 and ULB
438 02/T6 were isolated from the oviduct (OV6) and trachea (T6) of the same chicken.
439 Cloned cultures IP2K (from the choanal cleft) and IT2A (from the trachea) derived
440 from cultures isolated from an experimentally infected chicken approximately 2
441 months after infection with ULB 02/T6. Mw markers are the left side of the gel (from
442 55kDa to 170 kDa). Proteins that are more abundant in ULB 02/OV6 in comparison
443 with IP2K (P160, P110, P85) are indicated by arrowheads. Note that ULB 02/OV6
444 proteins of approximately 78, 82, 85, 90, 110 and 160 kDa were chosen for N-terminal
445 sequencing (see Fig. 3 and Table 2).





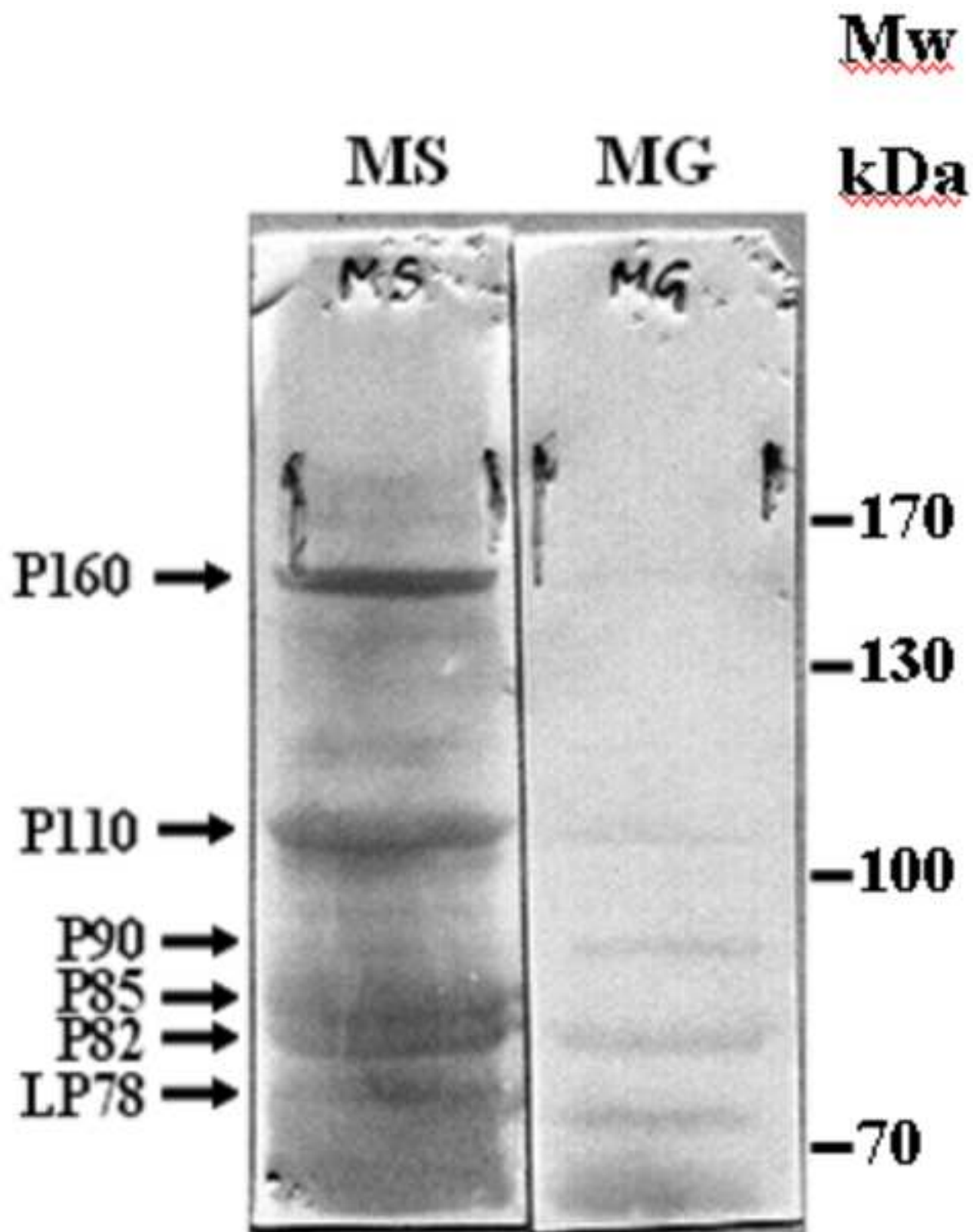


Table 1: Antibodies used in this study

<i>Antibodies/designation^a</i>	<i>Specificity</i>	Reference
RAb to <i>M. synoviae</i> (ULB925)	MS antigens ^a	Narat et al., 1998; Benčina et al. 2005b
RAb to <i>M. gallisepticum</i> (S6,R)	MG antigens ^a	Benčina et al., 1994
Ra1b to P1 adhesin	M.pneumoniae, P1 ^b	Lockaby et al., 1999
CAB to <i>M. synoviae</i> (ULB9122)	MS antigens ^a	Benčina et al., 2005b
CAB to <i>M. gallisepticum</i> (S6)	MG antigens ^a	Benčina et al., 1994
mAb 125 (<i>M. synoviae</i>)	MSPB, tMSPB	Benčina et al., 2001
mAb 5 (<i>M. synoviae</i>)	enolase	Benčina et al., 1999
mAb R (<i>M. orale</i>)	EF-Tu ^c	Benčina et al., 1999
mAb 3 (<i>M. hyopneumoniae</i>)	DnaK ^d	Benčina et al., 2005a
mAb 1H6 (<i>M. synoviae</i>)	Lipoprotein LP78 ^e	Markham et al., 2004

^a- Those antibodies reacted with a number of proteins in immunoblots (RAb- rabbit antibodies; CAB- chicken antibodies, mAb- monoclonal antibodies)

^b- Reacted with ~ 45 kDa protein of five *M. synoviae* strains

^c- Reacted with EF-Tu of many avian *Mycoplasma* species from the *Hominis* group

^d- Reacted with DnaK of all *Mycoplasma* species (>35 species) examined, so far

^e- mAb conjugated with horseradish peroxidase (Diagnostic kit SVANOVIR™, Svanova Biotech AB, Uppsala, Sweden)

Table 2. Identification of *M. synoviae* proteins by N-terminal sequencing^a

Protein ^b	N-terminal amino acid sequence ^c	Protein	Identification ^d		Theoretical ^e		
			Residues	Gene	ORF MS (AE017245)	Mw	pI
P 42	A K L D F D R S K E	EF-Tu	2-11	<i>tufA</i>	0667	43	5.6
P 47	A I K K I H A R E V L D S R G	enolase	3-17	<i>eno</i>	0009	49	6.6
P 50-1	<u>M</u> E N K K I I V V G A N H A G	NADH oxidase	1-15	<i>nox</i>	0522	50	6.4
P 50-2	M G E A A E D S E X S E T Q L	MSPA	336-350 f	<i>vlhA</i>	0232		
P 50-3	A K I T <u>K</u> <u>I</u> X <u>S</u> D V V	ATP D-3	3-13	<i>atpD-3</i>	0464	50	5.4
P 55-1	S D F K F T N T E T E L V V S	trigger factor	2-16	<i>tig</i>	0603	54	5.3
P 55-2	M Y K E T D K R T K L V A X I	pyruvate kinase	1-15	<i>pyk</i>	0648	53	8.1
P 65	A K E I V L X I D L G	DnaK	2-12	<i>dnaK</i>	0351	65	5.2
P 78-1	M Y T F K F A D I G	PdhD	1-10	<i>pdhD</i>	0275	66	5.9
P78-2	X G D K T D G G G	LP 78	2-9 f		0334	80	8
P 82-1	A R D Y D L K D Y	EF-G	2-10	<i>fusA</i>	0047	77	5.3
P 82-2	X G A P Q E E K	LP 85	2-9		0517	85	5.7
P 85-1	S I A K L G S F F R	PtsG	2-11	<i>ptsG</i>	0147	92	9.4
P 85-2	X G A P Q E E K K	LP85	2-9 f		0517	85	5.7
P 90	M E K Q	?ABC transporter	1-4		0391	80	9
P 110-1	X G R S D S E	LP 110	2-7 f		0349	110	6
P 110-2	M K K X K	?NanH	1-5	<i>nanH</i>	0199	108	9
P 160	M K N X N	?cons. hypoth.	1-5		0371	169	9

^a ULB 02/P4 proteins (P 42- P 65) and ULB 02/T6 proteins (P 78-1 – P 160). Two proteins positioned among proteins of ULB 02/P4 belonged to the heavy chain of swine immunoglobulin G i.e. P 52 and P 85. Their sequences were: EEKLVESG (P52) and EEKLVESGGGL (P85).

^b Numbers indicate molecular weight of proteins e.g. P 42 means a proteins of 42 kDa.

^c Determined amino acid residues; X denotes residue which could not be determined, whereas residues which are underlined were shared with a comigrating protein.

^d Identification according to the annotated genome of *M. synoviae* (Vasconcelos et al. 2005) (GenBank acc. no. AE017245)

^e Molecular weights (Mw) and isoelectric points (pI) calculated from predicted aa sequence of an appropriate gene, but were corrected according to the N terminal sequence determined in this study.

^f The numbering of aa residues according to putative mature lipoprotein sequence with cleaved signal peptide upstream of the cysteine residue (presumably x in sequences of LP 78 (Markham et al., 2004), and putative LP 85 and LP 110. Note, ORF MS 0232 is a *vlhA* pseudogen.

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