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Serodiagnosis of *Mycobacterium avium* infections in pigs

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31 **Abstract**

32 The aim of this study is the development and evaluation of a serodiagnostic assay for *Mycobacterium*
33 *avium* (MA). After screening MA lipid fractions in an ELISA format, a polar lipid fraction was selected as
34 antigen because of its superior recognition by serum antibodies in experimentally infected pigs. The
35 resulting MA-ELISA was evaluated as an alternative for detection of MA infection by traditional
36 pathological examination of pig lymph nodes for granulomatous lesions by meat inspectors. By comparing
37 with bacteriological examination, the MA-ELISA showed significantly better sensitivity (69%) as compared
38 to pathological examination (31%) in experimentally infected pigs. The MA-ELISA also appeared
39 significantly more specific in a set of serum samples from MA negative pigs: only 1 out of these 153
40 serum samples reacted positive, whereas 99 (65%) of these had displayed false positive results by
41 detection of lymph nodes lesions that appeared not to be associated with MA (Komijn et al., 2007).
42 The MA-ELISA was subsequently evaluated using serum samples from two farms with pigs known to be
43 infected with MA. Bacteriological examination of the sub-maxillary and mesenteric lymph nodes showed
44 that 56% (103/184) and 35% (41/117) of the pigs, respectively were positive for MA in these farms. In the
45 first farm, 16% (29/184) of the pigs tested positive in MA-ELISA and 31% (57/184) by pathological
46 examination. On the contrary, in the second farm, more pigs tested positive 17% (15/117) in MA-ELISA
47 with 8% (9/117) positivity by pathological examination. Taking the results on both farms together, the
48 sensitivity of the MA-ELISA was 14% and the specificity 83%, whereas the sensitivity of the pathological
49 examination was 31% and the specificity 86%.

50 For practical reasons use of a serological test as the MA-ELISA may be preferred over pathological or
51 bacteriological examination. Our studies in experimentally infected and negative "field" sera indicate that
52 the MA-ELISA is significantly more specific and more sensitive than detection by classical pathological
53 examination. However, the studies in two MA infected farms show a variable picture with pathological
54 examination overall performing better. Study in a wider range of "positive" farms will be needed to provide
55 a more comprehensive view of the quality of both tests for detection of MA in infected farms. At the same
56 time further optimization of MA-ELISA with use of lipid antigens from a broader range of serotypes may
57 improve its performance in the face of infections with different MA serotypes.

58

59 1. Introduction

60 *Mycobacterium avium* (MA) is an important pathogen in both animals and humans which belongs to the
61 *Mycobacterium avium* complex (MAC). To date, 28 MAC serotypes have been identified from which the
62 serotypes 1-6, 8-11 and 21 belong to *Mycobacterium avium* subsp. *avium* (MAA). The serotypes 1, 2 and
63 3 are isolated from birds and have been designated as the MAA “bird” serotypes whereas the serotypes
64 4-6, 8-11 and 21 are identified as the “non-bird” serotypes (Dvorska et al., 2004). The “non-bird”
65 serotypes are isolated from humans and pigs and for these reasons Mijs et al., (2002) proposed to name
66 these serotypes *M. avium* subsp. *hominissuis* (MAH). These “non-bird” serotypes are also found in the
67 environment, especially in soil (Dvorska et al., 2004).

68 MA is a potential zoonotic pathogen as it can produce disease in immunocompromised individuals with
69 acquired immunodeficiency syndrome and pulmonary disease in immunocompetent adults (Biet et al.,
70 2005; Wagner and Young, 2004). An infection with MA in pigs develops mostly subclinically leading to
71 granulomatous lesions in the lymph nodes. Most of the lesions are formed in the sub-maxillary and
72 mesenteric lymph nodes and disseminated lesions are occasionally observed in the liver, spleen and
73 lungs (Thoen, 1992).

74 Sources of MA infections in humans are still uncertain. Results of high resolution genotyping methods
75 showed that humans and pigs can be infected with the same MAC strains (Komijn et al., 1999). The
76 authors discussed the possibilities that humans and pigs share common sources of infection or that
77 contaminated meat originating from pigs is a potential source for infection in susceptible humans. To
78 exclude pigs as a reservoir for infection in man, regulations in European Union legislation (2004/854/EC)
79 are prescribed for special handling of pig carcasses in slaughter houses in which evidence of an MA
80 infection is observed.

81 Diagnosis of MA infections in pigs is based on detection of granulomatous lesions in lymph nodes of pigs
82 by palpation and incision at slaughter (Brown and Neuman, 1979; Komijn et al., 2007). Because MA has
83 been isolated from lymph nodes without lesions, and, on the other hand, non mycobacterial species such
84 as *Rhodococcus equi* have been frequently isolated from granulomatous lesions, sensitivity and
85 specificity of the current detection method have been questioned (Brown and Neuman, 1979; Dvorska et
86 al., 1999; Komijn et al., 2007; Offermann et al., 1999). In this study, we describe a serodiagnostic test that
87 may provide a more reliable tool for detecting MA infected pig herds.

88 Based on earlier observations that indicate that MA-derived lipid preparations contain dominant antigens
89 of MA, we focused on the applicability of antigenic lipid fractions for the development of an antibody

90 based immunodiagnostic test for detection of MA infections in 26-weeks old slaughter pigs (Brennan et
91 al., 1981; Inderlied et al., 1993; Kitada et al., 2002, 2005). Using serum samples of pigs that were
92 experimentally infected with MA, lipid fractions of MA were evaluated for applicability, and a polar lipid
93 fraction was selected for developing an ELISA for MA. The ELISA was validated using serum samples
94 from pigs, experimentally and naturally infected with MA. Bacteriological examination of the lymph nodes
95 for MA was used as reference to indicate the true infection status and the outcome of the ELISA was
96 compared to the traditional method of detecting lesions in lymph nodes (Komijn et al., 2007).

97

98

99 **2. Materials and Methods**

100 *2.1 Bacterial strain*

101 In this study MAH serotype 4 strain 17404 was used. This strain was isolated in the course of a study to
102 the prevalence of MA in Dutch pig herds and it was selected as a representative of one of the most
103 frequently isolated serotypes in the Netherlands (Komijn et al., 1999).

104

105 *2.2 Experimental infection with MA*

106 Thirty-two pigs, three weeks of age and crossbreeds of Yorkshire and Dutch landrace were obtained from
107 a high health status herd of the Animal Sciences Group of Wageningen UR (Lelystad, The Netherlands).

108 Pigs were allotted in four groups of eight pigs each and housed in separate boxes at the animal facilities
109 of Central Veterinary Institute of Wageningen UR (Lelystad, The Netherlands).

110 For preparation of the inoculum for the experimental infection, MAH strain 17404 was cultured in
111 Middlebrook 7H9 medium enriched with OADC (Becton Dickinson, Breda, The Netherlands) at 35°C to an

112 Optical Density (OD) of 2 at 600 nm. The cells were harvested by centrifugation at 3000 x g for 30

113 minutes and washed twice in isotonic saline. Until use, aliquots of one ml were stored in Tryptone Soya

114 Broth medium (Oxoid B.V., Badhoevedorp, The Netherlands) in the presence of 20% glycerol at – 70 °C.

115 Viability of the inoculum was determined by subculture on Coletsos Osein (Bio-Rad, Veenendaal, The

116 Netherlands) and Dubos Tween albumin medium (Becton Dickinson, Breda, The Netherlands). Purity of

117 the inoculum was determined from smear specimens stained by Ziehl-Neelsen. Prior to administration to

118 the pigs the stored MAH bacteria were revived by culturing one ml of the stored inoculum in four ml

119 Middlebrook 7H10 supplemented with OADC for two days at 37 °C.

120 To study the influence of an early and late infection with MA on bacteriological, serological and
121 pathological status we infected pigs at different ages. Three of the four groups were infected at 2½, 4½
122 and 18 weeks of age, respectively. To increase the probability of infection, pigs in the fourth group were
123 inoculated with MAH at three time points (Table 1). A syringe was used to deposit the inoculum in the
124 caudal area of the pharynx. To promote the penetration of the bacteria, the pharynx epithelial tissue was
125 scarified with a cotton-wool swab before inoculation.

126 Until the end of the experiment at 24 weeks of age, pigs were monitored once a week for clinical signs of
127 disease. Furthermore, blood serum samples were collected weekly to monitor the immune response.
128 White blood cells were counted, using a semi-cell bloodcounter (Sysmex, model F 800, Charles Goffin
129 Medical Systems). The number of neutrophils was calculated after a differential count of Giemsa-stained
130 blood smears. Three days before the end of the experiment, all pigs underwent an intradermal tuberculin
131 test in the ear with 0.1 ml Avian Tuberculin PPD (25.000 I.U., ASG, Lelystad, The Netherlands). The
132 presence at the injection site of erythema and induration was scored 24, 48 and 72 hours after injection.
133 After reading, pigs were euthanized. Organs were examined pathologically for deviations and lymph
134 nodes were examined pathologically for granulomatous lesions and bacteriologically for MA. For
135 bacteriological examinations, the surface of the lymph node specimens was decontaminated for 6 to 8
136 seconds in boiling water and cut aseptically into small pieces of 1 cm³. The specimens were sealed in
137 polyethylene bags and squeezed in a Stomacher (Laméris Laboratorium B.V., Breukelen, The
138 Netherlands). This material was treated for 10 min at room temperature with 3 volume parts of 6% H₂SO₄
139 and subsequently centrifugated for 10 min at 1000 g. The supernatant was discarded and the sediment
140 was washed twice with an excess of phosphate-buffered saline (PBS), 136.89 mM NaCl, 2.68 mM KCl,
141 8.1 mM Na₂HPO₄, 2.79 KH₂PO₄ and resuspended in 0.5 ml PBS. This suspension was inoculated on
142 Middlebrook 7H10 plates enriched with OADC, on Coletsos Osein and on Dubos Tween albumin
143 medium, incubated for eight weeks at 35 °C and observed every week for growth. To identify colonies,
144 Ziehl-Neelsen stain was performed for acid-fast bacilli and PCRs for the presence of IS1245 and IS901,
145 which are characteristic for MA and MAA respectively (Kunze et al., 1992; van Soolingen et al., 1998).

146

147 *2.3 Collection of pig serum samples in the field*

148 Field serum samples of pigs with a different MA health status were collected. Serum samples negative for
149 infections with MA (n=153) were collected in the course of a previous study for the prevalence of MA
150 infections in the Netherlands. The design and results of this study were described by Komijn et al. (2007).

151 In order to obtain positive field serum samples in the period October 2006 till June 2008 two herds (A and
152 B) were selected where recently cases of MAH infections were detected in slaughter pigs. Both farms
153 were farrow-to-finish farms. From farm A four batches of pigs were sampled in the slaughterhouse
154 (n=184) and from farm B two batches (n=117). Blood serum samples and the sub-maxillary and
155 mesenteric lymph nodes were collected at slaughter. In the laboratory, serum samples were stored at -20
156 °C and the lymph nodes were examined pathologically for granulomatous lesions and bacteriologically for
157 MA as described above.

158

159 *2.4 Isolation of lipid fractions from MAH*

160 In order to harvest lipids as antigen for the serodiagnostic ELISA assay, MAH strain 17404 was
161 inoculated in Middlebrook 7H9 supplemented with OADC (Becton Dickinson, Breda, The Netherlands).
162 The growth of MA was monitored by measuring weekly the OD at 600 nm in a spectrophotometer. The
163 incubation was stopped at an OD of 1 and the MA cells were pelleted by centrifugation (15 min 5000g).
164 Lipids were extracted from the MA cells according to a protocol of Dobson et al., (1985). Briefly, the
165 apolar lipids were extracted from MA cells by mixing these cells with methanol and petroleum ether. The
166 mixture was centrifuged, the upper layer with apolar lipids was harvested and stored. The lower layer was
167 treated with petroleum ether to extract remaining apolar lipids. After centrifugation, the upper layer
168 containing a second yield of apolar lipids was harvested and combined with the already stored apolar
169 fraction. The combined fractions of apolar lipids were dried under a gentle flow of nitrogen. The polar
170 lipids were further extracted by adding a mixture of chloroform and methanol to the lower layer. The
171 mixture was mixed during one hour and centrifuged. Supernatant was mixed with chloroform for 5
172 minutes and centrifuged. The lower layer containing the polar lipids was dried under a gentle flow of
173 nitrogen.

174

175 *2.5 Selection of lipid fraction for ELISA*

176 Polar and apolar lipid fractions of MAH strain 17404 were tested in indirect ELISA assays for selection of
177 the most promising fraction for use. Each well of polysorb microtiterplates (Nunc, Roskilde, Denmark) was
178 coated for 16 hours at 37°C with 1 µg polar or apolar lipids suspended in 50mM NaHCO₃, PH 9.6.
179 Endpoint serum samples of eight pigs, 21½ weeks after an experimental infection with MAH strain 17404
180 (see above and Table 1 group 1) were tested in a twofold serial dilutions from 1:40 to 1:81920 in PBS
181 containing 0.5% Tween 80. As conjugate monoclonal antibody IgL 27.2.1 was used. This monoclonal

182 antibody recognizes the kappa chains of pig immunoglobulins (Sinkora et al., 2001). As substrate TMB (3,
183 3', 5, 5'-tetramethylbenzidine) was used. Development of the reaction was stopped by adding 0.5 M
184 H₂SO₄. The OD was determined at 450 nm in an ELISA reader. Titers were expressed as the reciprocal
185 of the ²log of the highest dilution showing an absorbance of more than 50% of the positive control.

186

187 *2.6 Execution of the ELISA*

188 For evaluation of the ELISA basically the test was performed as described above. As positive control,
189 endpoint serum samples of a pig, 21½ weeks after an experimental infection with MAH strain 17404 was
190 used (see above and in Table 1). As negative control, a serum sample was used from a pig negative in
191 bacteriological examination for MA in its sub-maxillary and mesenteric lymph nodes (Komijn et al., 2007).
192 In each microtiterplate these positive and negative control serum samples were tested in twofold in a
193 dilution of 1:200. Test results were calculated as percentage positivity (PP). To calculate the PP, the OD
194 of the test serum samples was calculated as a percentage of the difference in OD of the negative and
195 positive control serum samples. This results in a uniform and continuous scale from 0-100% calculated
196 according to the following formula: $PP = \{OD(\text{test sample}) - OD(\text{negative control})\} / \{OD(\text{positive}$
197 $\text{control}) - OD(\text{negative control})\}$.

198

199 *2.7 Statistical analysis*

200 The cut-off value of the MA-ELISA was determined as the mean plus three-fold standard deviation of the
201 results on a set of serum samples (n=153) negative in bacteriological examination for MA (Komijn et al.,
202 2007). Sensitivity and specificity with 95% confidence intervals (CI) from the MA-ELISA and from the
203 pathological examination of the sub-maxillary lymph nodes were calculated considering the results of
204 bacteriological examination of lymph nodes for MA as a gold standard. McNemar's test was used as a
205 significance test for differences in sensitivity and specificity of both tests. *P* values below 0.05 were
206 considered statistically significant.

207

208

209 **3. Results**

210 *3.1 Development of MA-ELISA*

211 We started the development of the MA-ELISA by selection of the most promising lipid fraction for use in
212 an ELISA. For this purpose we used serum samples of eight pigs, collected 21½ weeks after an

213 experimental infection with MAH. All these serum samples displayed a higher titer against the polar lipid
214 fraction (range 290-1577) as compared to the apolar lipid fraction (range 20-250). Therefore the polar
215 lipid fraction was selected for further use in an MA-ELISA.

216 Checkerboard titrations using a concentration range of the same set of serum samples and of the polar
217 lipid fraction from MAH strain 17404 resulted in the selection of an optimal concentration of 2.4 µg/well of
218 polar lipid and a dilution of 1:200 of pig serum samples for the MA-ELISA.

219 The development of the MA-ELISA was finalized with the determination of a cut-off value for negative and
220 positive test results. For this purpose we tested serum samples of pigs (n=153) free of an MA infection
221 (Komijn et al., 2007) in the MA-ELISA. The PP in 80 (52.3%) of these serum samples, was lower than 0%
222 (Table 2). The highest titer detected in these 153 serum samples was 16 PP. The mean and standard
223 deviation of the ELISA for these serum samples was 0.26 PP and 3.45 PP, respectively. The mean plus
224 three standard deviations for the assay: 10.6 PP, was used as the cut-off value for the MA-ELISA.

225

226 *3.2 Experimental infection with MAH*

227 To evaluate the serodiagnostic ELISA test as an alternative for an inspection of lymph nodes by eye at
228 slaughter we infected pigs experimentally with MAH strain 17404 and examined the correlation between
229 the presence of MAH bacteria, the formation of lesions in lymph nodes of pigs and the antibody response.
230 Four groups of eight pigs were infected, one group at 2½ weeks of age, one group at 4½ weeks of age,
231 one at 18 weeks of age, and one group at all three time points (2½, 4½ and 18 weeks of age). The
232 experiment was terminated when all pigs were 24 weeks old. During the experimental phase the pigs
233 showed no clinical disease symptoms and their leucocyte counts were normal (results not shown). In
234 tuberculin skin tests three days before necropsy 31 out of 32 pigs reacted positive; the skin of the ear at
235 the site of injection showed redness and focal swelling. Size of reactions 72 hours after injection varied
236 between 10 and 20 mm. Pigs were subsequently euthanized and from the results of the bacteriological
237 examination all 32 pigs appeared to be infected with MAH. Irrespective of the age at which pigs were
238 infected, MAH bacteria were detected in one or more of the lymph nodes, most frequently in the sub-
239 maxillary and mesenteric lymph nodes (Table 3). Corresponding to the results of the bacteriological
240 examination of the lymph nodes, pathological examination showed that most frequently the sub-maxillary
241 and mesenteric lymph nodes were affected. However, bacteriological examination gave more positive
242 results as compared to pathological examination. Sixteen (50%) pigs had granulomatous lesions in one or
243 more of the lymph nodes and 10 (31%) of the pigs in the sub-maxillary lymph nodes (Table 3). Results of

244 the MA-ELISA on longitudinally serum samples obtained during the experimental phase showed that
245 titers (in PP) were low at the time of infection and started to increase 6 to 8 weeks after infection. At 10-12
246 weeks after infection the mean PP of groups of pigs infected at an age of 2½ and 4½ weeks was
247 maximal. In serum samples of pigs infected at three time points (2½, 4½ and 18 weeks of age) titers
248 increased further and at necropsy, 21½ weeks after infection these titers were at a maximum (Figure 1).
249 At that timepoint 69% of the pigs (22/32) were serologically positive (Table 4). Taken together, the
250 sensitivity of the pathological examination as performed at slaughter was 31% and of the serological
251 examination 69%. According to McNemar's test this difference in sensitivity was significant ($p = 0.01$).
252 We determined whether the length of the infection period influenced the formation of granulomatous
253 lesions and the anti MA antibody response. In groups of pigs infected at 2½ and 4½ weeks of age, 7 out
254 of 8 pigs showed granulomatous lesions in their lymph nodes at necropsy when pigs were 24 weeks of
255 age. No lesions were detected in lymph nodes of pigs infected at 18 weeks of age when they were
256 slaughtered 6 weeks later (Table 3). The results of the MA-ELISA showed that most (20/24) of the pigs
257 infected at 2½ and 4½ weeks of age had detectable anti-MA antibody responses at an age of 24 weeks
258 whereas 2 out of 8 pigs infected at 18 weeks of age had a positive antibody titer (Table 4). In conclusion,
259 these results indicated that lesions and antibody responses can generally be detected 19½ weeks after
260 experimental infection, but are relatively inconsistent after 6 weeks of infection.

261

262 3.3 Natural infections with MA

263 In order to evaluate the serodiagnostic ELISA test for use under field conditions we collected a
264 considerable number of serum samples from pigs from two farms (A and B) with a recent history of MAH
265 infections and performed bacteriological, serological and pathological examinations of particular lymph
266 nodes. Results of bacteriological examination of the sub-maxillary and mesenteric lymph nodes showed
267 that 56% (103/184) of the pigs on farm A were infected with MAH and 35% (41/117) of the pigs on farm B
268 (Table 5). Granulomatous lesions in the sub-maxillary lymph nodes were detected by pathological
269 examination in 31% (57/184) of the pigs originating from farm A and in 8% (9/117) of the pigs from farm
270 B. Using the MA-ELISA, on farm A, 16% (29/184) of the pigs tested serologically positive and on farm B,
271 17% (15/117) of the pigs. These results indicate that, compared to the results of the pathological and
272 serological examination, the bacteriological examination for MA is the most sensitive diagnostic method
273 for detection of MA infections in these two herds. Furthermore, that on farm A the pathological

274 examination had more positive results than the serological examination, whereas on farm B the
275 serological examination showed more positive results.

276 Sensitivity and specificity of the pathological and serological examination were calculated on the samples
277 of pigs from these two farms using the results of the bacteriological examination as a reference. For the
278 MA-ELISA, sensitivity was 14% (9% - 21% CI) and the specificity 83% (77% – 89% CI). For the
279 pathological examination of the sub-maxillary lymph nodes, sensitivity was 31% (23% - 39% CI) and the
280 specificity 86% (79% - 91% CI). According to McNemar's test pathological examination of the sub-
281 maxillary lymph nodes for granulomatous lesions had a significantly ($p = 0.005$) higher sensitivity than the
282 serological examination by the MA-ELISA whereas statistically no difference was found in specificity of
283 both tests.

284

285

286 4. Discussion

287 Diagnosis of MA infection in pigs is traditionally based on the detection of granulomatous lesions in lymph
288 nodes seen by eye at slaughter. Since sensitivity and specificity of this approach is questioned (Brown
289 and Neuman, 1979; Komijn et al., 2007; Offermann et al., 1999) we developed as an alternative a
290 serodiagnostic ELISA assay on the basis of a polar lipid fraction from MA. We evaluated the MA-ELISA
291 and found in samples from experimentally infected pigs a significantly higher sensitivity for the MA-ELISA
292 (69%) as compared to pathological examination (31%). The MA-ELISA seemed also more specific since
293 on a selected set of sera from MA negative pigs (Komijn et al., 2007) only one out of 153 pigs was
294 positive in the MA-ELISA whereas 99 (65%) pigs had granulomatous lesions. This shows that the MA-
295 ELISA was highly specific even when a high number of serum samples originated from pigs with lesions.
296 Results of the evaluation of the MA-ELISA test on serum samples from pigs originating from two MA
297 infected herds showed a variable picture. On samples of these pigs the sensitivity of the pathological
298 examination (31%) was significantly higher than the MA-ELISA (14%). However, more positive results
299 were obtained with the MA-ELISA on the first farm whereas on the second farm more pigs showed
300 lesions in the sub-maxillary lymph nodes. A possible reason for the variation in results on the two MA
301 infected farms may be related to antigenic variation between the MAH strain used for the preparation of
302 polar lipid fraction for the MA-ELISA and the MAH strains circulating on both farms. Study in a wider
303 range on MA infected farms will be needed to provide a more comprehensive view of the quality of both
304 tests for detection of MA in infected farms. At the same time further optimization of MA-ELISA with use of

305 lipid antigens from a broader range of serotypes may improve its performance in the face of infections
306 with different MA serotypes.

307 Currently, diagnosis of MA infections in pigs is based on detection of granulomatous lesions in lymph
308 nodes of pigs by incision and visual inspection at slaughter (Brown and Neuman, 1979; Komijn et al.,
309 2007). In accordance with results of others we frequently detected MA bacteria whereas lesions were
310 absent (Brown and Neuman, 1979; Offermann et al., 1999). Although the sensitivity we calculated on
311 naturally and experimentally infected pigs was low (31%), it cannot be excluded that this sensitivity is still
312 overestimated as we performed pathological examinations of the lymph nodes by multiple incisions and
313 under optimal conditions in the facilities of the Central Veterinary Institute of Wageningen UR. Lesions in
314 lymph nodes vary in size from pinpoint to pea size and especially mild lesions can easily be missed at
315 slaughter due to the rapid line speed.

316 The specificity of the pathological examination of sub-maxillary lymph nodes on the two MA infected
317 farms in this study was calculated to be 86%. Results of an earlier study of us on selected lymph node
318 samples from nine pig herds in the Netherlands had given evidence about a low specificity since we
319 found that granulomatous lesions in lymph nodes of slaughter pigs were not associated with the presence
320 of MA bacteria but with *R. equi* (Komijn et al., 2007). The MA infection level of the two for this study
321 selected herds was high but low in the nine herds in our earlier study. Therefore it is most likely that in
322 highly MA infected herds the pathological examination of lymph nodes is better correlated with the
323 presence of MA bacteria than in populations where MA infections are absent or low. In the Netherlands
324 the prevalence of MA infections is low (Komijn et al., 2007), for this reason the specificity as calculated in
325 this study is probably lower in the real field situation. Brown and Neuman (1979) calculated a specificity of
326 53%, which confirms the idea that specificity could be lower than the 86% we found on the two MA
327 infected herds.

328 We developed the MA-ELISA with the aim to detect MA infected pigs at slaughter when they are 26
329 weeks of age. Results of the experimental infection with MA showed that titers started to increase 6-8
330 weeks after infection and stayed detectable till the end of the experiment, 21½ weeks after infection.

331 There is no clear evidence at which age pigs are most at risk for an infection with MA. Infections in herds
332 are reported when peat or compost are provided as a source of iron and minerals to piglets soon after
333 birth (Engel et al., 1978; Matlova et al., 2005) but infections later in on lifetime cannot be excluded. MA
334 bacteria are present in bedding materials, sawdust and drinking water in the stables (Matlova et al., 2003;
335 Thoen, 1992), to this materials are pigs constantly exposed during their whole lifetime. Taking the results

336 of the MA-ELISA into account, serodiagnosis offers possibilities to detect MA infections during the whole
337 lifetime of the pig, except when pigs are infected in the latest weeks before slaughtering.

338 It was unexpected that in the experimental infection pigs repeatedly infected with MAH had a lower
339 number of lesions compared to pigs once infected (Table 3). No differences were seen between groups
340 regarding tuberculin reactions and isolation of MAH bacteria, but the development of immune response
341 differed between the group of repeatedly infected pigs and the groups of once infected pigs. Whereas
342 from the other groups the mean anti MA antibody titer was maximal at 10-12 weeks after infection, in the
343 repeatedly infected group the mean anti MA antibody titer still further increased. Apparently, the repeated
344 MA infection leads to this different immune response associated with a lack of lesion formation.

345 For immunodiagnosis of human MA infections lipid antigens derived from MA are successfully used
346 (Enomoto et al., 1998; Kitada et al., 2008, 2002, 2005), here we show that lipid fractions are also suitable
347 for immunodiagnosis of MA infections in pigs. The polar lipid fraction showed a higher reactivity with sera
348 from experimentally infected pigs than the apolar lipid fraction, for this reason the polar lipid fraction was
349 chosen as antigen in the MA-ELISA. For detection of MA infections in humans, purified glycopeptidolipids
350 (GPL) are used as antigen in ELISA tests with sufficient sensitivity and specificity (Kitada et al., 2008).
351 GPLs are immunodominant antigens from MAC and are well-characterized polar lipids (Brennan et al.,
352 1981; Inderlied et al., 1993). In preliminary experiments we found that GPLs are indeed present in the
353 polar lipid fraction we used for the MA-ELISA assay (results not shown). Although there are no reports on
354 the immunogenicity of GPLs in MA infections in pigs it can be speculated that in our ELISA test, GPLs
355 contribute to the difference between reactivity of the polar and apolar lipid fraction of MAH.

356 It can be concluded that sensitivity and specificity of the use of granulomatous lesions in lymph nodes as
357 read out criterion for a potential MA infection performs poorly. A more sensitive and objective test for
358 routine diagnosis of MA infections in slaughter pigs is needed. The MA serodiagnostic test could meet
359 these requirements and may be a valuable tool for screening herds to prevent and control MA infections
360 in pigs.

361

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433 **Figure legend**

434 Three groups of eight pigs each were experimentally infected with *M. avium* subsp. *hominissuis* strain
435 17404 at 2½ (◆), 4½ (■) and 18 (▲) weeks of age, the fourth group was infected three times at 2½, 4½
436 and 18 (X) weeks of age. All pigs were necropsied at 24 weeks of age. Longitudinal antibody responses
437 were determined in ELISA tests against the polar lipid fraction of MAH strain 17404. Mean Percentage
438 Positivity and Standard Deviation was calculated for each group and for each timepoint.

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Table 1Design experimental infection of pigs with *Mycobacterium avium* subsp. *hominissuis*

Group	No. of pigs	Age (weeks)	
		Infected	Necropsied
1	8	2½	24
2	8	4½	24
3	8	18	24
4	8	2½, 4½, 18 ^a	24

^a Pigs were three times infected.

Table 2

Distribution of PP obtained in the MA-ELISA assay on serum samples from pigs (n=153) negative for infections with *Mycobacterium avium* (MA)^a.

Percentage Positivity	Number (%)
< 0%	80 (52.3)
0 - 5%	60 (39.2)
5 - 10%	10 (6.5)
10 – 15%	2 (1.3)
15 – 20%	1 (0.7)
> 25%	0 (0)
Total	153 (100)

^a Serum samples were collected in the course of a study for the prevalence of MA infections in the Netherlands. The design and the outcome of the study was previously described by Komijn et al. (2007).

Table 3Post-mortem results recorded in pigs after experimental infection with *Mycobacterium avium* subsp. *hominissuis*

Group ^a	No. of pigs with lesions at														Total no. of pigs	
	Tonsil		Md ^b		Mes ^c		Ing ^d		Trach-br (le) ^e		Trach-br (ri) ^f		Retro-phar ^g		with lesions	
	Pt ^h	Bac ⁱ	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac	Pt	Bac
1	1	2	3	5	6	8	0	0	2	2	2	3	3	7	7	8
2	0	1	7	8	7	7	0	2	0	0	2	1	4	6	7	8
3	0	8	0	8	0	5	0	0	0	0	0	0	0	6	0	8
4	0	3	0	6	2	7	0	0	0	1	0	0	0	3	2	8
Total	1	14	10	27	15	27	0	2	2	3	4	4	7	22	16	32

^a Experimental design is described in Table 1A^b Lnn mandibularis^c Lnn mesenterialis^d Lnn inguinalis^e Lnn trachea-bronchialis^f Lnn retro-pharyngeal^g Lnn retro-pharyngeal^h Pathological lesionsⁱ Isolation of *M. avium* subsp. *hominissuis* at necropsy

Table 4

Results of the MA-ELISA assay on endpoint serum samples of pigs experimentally infected with *Mycobacterium avium* subsp. *hominissuis*.

Group	No. of pigs	Weeks after infection	Number of pigs	
			Negative	Positive
1	8	21.5	0	8
2	8	19.5	3	5
3	8	6	6	2
4	8	21.5, 19.5, 6 ^a	1	7
Total	32		10	22

^aPigs were three times infected.

Table 5

Results of bacteriological, pathological and serological examinations of pigs from two *Mycobacterium avium* infected herds for detection of *M. avium* infections.

Examination	Number of pigs (%) tested		
	Negative	Positive	Total (n)
<i>Farm A</i>			
Bacteriological ¹	81 (44)	103 (56)	184
Pathological ²	127 (69)	57 (31)	184
Serological ³	155 (84)	29 (16)	184
<i>Farm B</i>			
Bacteriological	76 (65)	41 (35)	117
Pathological	108 (92)	9 (8)	117
Serological	100 (85)	17 (15)	117

¹ Positive when *M. avium* bacteria were detected by bacteriological examination on sub-maxillary lymph nodes and mesenteric lymph nodes.

² Positive when granulomatous lesions were seen in the sub-maxillary lymph nodes.

³ As detected by the MA-ELISA with a cut-off value of 10.6 PP.

