

1 Experimental infection of SPF pigs with *Actinobacillus pleuropneumoniae* serotype 9 alone or in
2 association with *Mycoplasma hyopneumoniae*.

3

4 Corinne Marois^{1*}, Marcelo Gottschalk², Hervé Morvan³, Christelle Fablet⁴, François Madec⁴, Marylène
5 Kobisch¹

6

7 ¹ Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Unité de Mycoplasmologie-
8 Bactériologie, BP53, 22440 Ploufragan, France

9 ² Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de Médecine Vétérinaire,
10 Université de Montréal, Saint-Hyacinthe, Québec J2S 7C6

11 ³ Laboratoire de Développement et d'Analyses des Côtes d'Armor, BP54, 22440 Ploufragan, France

12 ⁴ Agence Française de Sécurité Sanitaire des Aliments (AFSSA), Unité d'Epidémiologie et Bien-Etre
13 Porcins, BP 53, 22440 Ploufragan, France

14 *Corresponding author: Tel: +33-2-96-01-01-72; Fax: +33-2-96-01-62-73

15 Email address: c.marois@ploufragan.afssa.fr

16

17

18 **Abstract**

19

20 The purpose of this study was to compare in SPF pigs, the pathogenicity of an *A. pleuropneumoniae*
21 serotype 9 strain 21 (isolated from the palatine tonsils of a healthy gilt on a French nucleus pig farm,
22 with no clinical signs or lung lesions but a highly positive reaction to *A. pleuropneumoniae* serotype 9
23 antibodies) with a pathogenic *A. pleuropneumoniae* strain 4915 serotype 9 (isolated in France from an
24 outbreak of porcine pleuropneumonia). The pathogenicity of one *M. hyopneumoniae* strain alone or
25 associated with *A. pleuropneumoniae* strain 21 was also compared. Eight groups of 7 pigs were
26 infected (at 6 or 10 weeks of age) and a control group was kept non-infected. Results showed that
27 sensitivity to *A. pleuropneumoniae* was related to the age of the pig (6 weeks vs 10 weeks) whatever
28 the strain. Surviving pigs infected at 6 weeks of age developed severe clinical signs, lung lesions

29 typical of *A. pleuropneumoniae* and they seroconverted. In contrast, symptoms and lung lesions were
30 almost non-existent in pigs infected with strain 21 at 10 weeks of age, but a seroconversion was
31 observed with very high ELISA titres. These results were in accordance with those observed in the
32 nucleus pig farm. Infection with *M. hyopneumoniae* alone induced typical mycoplasmal symptoms,
33 pneumonia and seroconversion. Symptoms and lung lesions were the most noticeable in pigs infected
34 with *M. hyopneumoniae* at 6 weeks of age and with *A. pleuropneumoniae* 4 weeks later. Our results
35 show that the presence of *A. pleuropneumoniae* serotype 9 in a pig herd may be clinically unnoticed
36 and that *M. hyopneumoniae* may potentiate *A. pleuropneumoniae* infection.

37

38 **Keywords:** *Actinobacillus pleuropneumoniae* serotype 9; *Mycoplasma hyopneumoniae*; dual infection;
39 SPF pigs; experimental infection

40

41

42 1. Introduction

43

44 Porcine respiratory disease complex (PRDC) is a major concern in the pig production throughout the
45 world and is due to a combination of multiple bacterial and viral agents. Two pathogenic bacteria,
46 *Mycoplasma hyopneumoniae* (the primary agent of swine enzootic pneumonia) (Ross, 1999; Thacker,
47 2006) and *Actinobacillus pleuropneumoniae* (the etiologic agent of swine pleuropneumonia), alone or
48 associated, can induce severe respiratory disorders in pigs (Kobisch et al., 1993; Gottschalk and
49 Taylor, 2006). An acute *A. pleuropneumoniae* infection can induce severe clinical signs and lung
50 lesions. The infection may become chronic or subclinical without previous signs of the disease,
51 outbreaks may suddenly appear or subclinical infections may remain silent. *A. pleuropneumoniae* is
52 able to persist in pig tissues, particularly in tonsillar crypts and in sequestered necrotic lungs. Thus,
53 the early identification of subclinically infected pig herds is necessary to control carrier pigs and
54 prevent *A. pleuropneumoniae* transmission between herds, especially from nucleus pig farms to
55 multipliers (Gottschalk and Taylor, 2006). The virulence of *A. pleuropneumoniae* is known to be
56 variable: biotype I has been divided into 13 serotypes and biotype II into 2 serotypes, for a total of 15

57 serotypes (Gottschalk and Taylor, 2006). Serotype 2 and serotype 9, two virulent serotypes, are
58 prevalent in outbreaks in European countries and particularly in France (Gottschalk et al., 2005). *A.*
59 *pleuropneumoniae* produces four RTX toxins (ApxI-ApxIV) associated with virulence. All serotypes of
60 *A. pleuropneumoniae* encode for at least two RTX toxins (Frey et al., 1993; Frey et al., 1995; Schaller
61 et al., 1999; Kuhnert et al., 2003). It is relatively easy to isolate *A. pleuropneumoniae* from pneumonic
62 lesions in freshly dead animals but bacteriological detection is more difficult in chronic infections or in
63 healthy carrier pigs. The presence of *A. pleuropneumoniae* in nasal cavities and tonsils can be
64 revealed by specific PCR tests (Savoie et al., 2000; Fittipaldi et al., 2003). Serological monitoring has
65 been used widely to control *A. pleuropneumoniae* infection in pig farms. The most commonly used are
66 ELISA tests with LC-LPS as antigens (Gottschalk et al., 1994; Dubreuil et al., 2000).

67 Previously, we described an experimental infection of 10-week-old SPF piglets, with a pathogenic *A.*
68 *pleuropneumoniae* strain (4915, belonging to serotype 9), isolated in France from an outbreak of
69 porcine pleuropneumonia. The clinical signs were acute: hyperthermia, respiratory distress and death
70 in some cases. The severe lung lesions included fibrinous pleurisy and lung haemorrhages in the
71 acute stage, pleural adhesions and focal pulmonary necrosis in the chronic stage. The surviving pigs
72 became seropositive in less than 6 days (Jobert et al., 2000).

73 The objectives of the present study were: (i) to compare the pathogenicity in SPF pigs, of
74 *A. pleuropneumoniae* strain 4915, with that of *A. pleuropneumoniae* strain 21 (serotype 9), isolated in
75 France from the palatine tonsils of a healthy gilt from a nucleus pig farm. This farm presented no
76 clinical signs or lung lesions related to *A. pleuropneumoniae* but a relatively highly positive prevalence
77 for *A. pleuropneumoniae* serotype 9 antibodies at the end of the finishing period and (ii) to investigate
78 the interactions of this *A. pleuropneumoniae* strain with *M. hyopneumoniae*, under experimental
79 conditions.

80

81

82 **2. Materials and methods**

83

84 **2.1. Bacterial strains and culture conditions**

85

86 *A. pleuropneumoniae* strains (4915 and 21) were grown on PPLO medium supplemented with 10
87 µg/mL nicotinamide adenine dinucleotide, 1 mg/mL glucose, 5% decomplexed horse serum
88 (PPLOsup) for 6 h at 37°C (Jobert et al., 2000). The titre of the *A. pleuropneumoniae* cultures was
89 expressed in colony forming units (CFU/mL). The two strains were identified by biochemical tests and
90 PCR (Savoie et al., 2000). Serotyping by coagglutination test with type-specific hyperimmune serum
91 showed that they belonged to serotype 9 (Gottschalk and Taylor, 2006). These *A. pleuropneumoniae*
92 strains were also positive for their capacity to produce two RTX toxins (ApxI and ApxII) as detected by
93 PCR (Frey et al., 1995). *M. hyopneumoniae* (strain 116) was isolated from an outbreak of enzootic
94 pneumonia in France and was cultivated in Friis broth medium (FBM) at 37 °C, as previously
95 described (Marois et al., 2007). The titre of the *M. hyopneumoniae* cultures was expressed as colour
96 changing units per millilitre (CCU/mL).

97

98 2.2. Experimental design

99

100 2.2.1. Experimental infections

101 Sixty-three SPF pigs (hysterectomy derived piglets) were obtained from the experimental pig herd of
102 the French Food Safety Agency of Ploufragan. Animal experiments were performed in accordance
103 with current legislation and ethical and welfare recommendations (Agreement B-22-745-1). Very strict
104 biosecurity measures were implemented in order to avoid undesirable contaminations of the pigs: air
105 filtration system and airlocks for each unit, unit-specific clothes and compulsory showering before and
106 after visiting the pigs (Cariolet et al., 1994). All animals were confirmed to be serologically negative to
107 *A. pleuropneumoniae* and *M. hyopneumoniae*.

108 Groups of seven pigs were randomly allocated to nine separate rooms (Table 1). At six weeks of age,
109 pigs in group 2 were inoculated intranasally (1 mL per nostril), each pig received 1.4×10^8 CFU of *A.*
110 *pleuropneumoniae* strain 4915 . Pigs in groups 3, 7 and 9 were experimentally infected, in the same
111 conditions, with 1.3×10^8 CFU of *A. pleuropneumoniae* strain 21 for each pig. Pigs in groups 5 and 8
112 were infected with the latter strain, at 10 weeks of age. In addition, pigs in groups 4, 8 and 9 were

113 experimentally infected with *M. hyopneumoniae* at six weeks of age and those in groups 6 and 7 four
114 weeks later (Table 1). They were inoculated intratracheally (5 mL per day), as previously described
115 (Kobisch and Ross, 1996). Each pig was infected, on 2 consecutive days, with 5×10^9 CCU by
116 tracheal intubation. The pigs in group 1 were controls (uninfected pigs).

117

118 2.2.2. Clinical monitoring

119 Daily clinical examinations consisted of taking rectal temperature (normally 39.5°C in an SPF pig but
120 noted as hyperthermia when $\geq 40.5^\circ\text{C}$) and looking for symptoms such as dyspnea, coughing (from
121 daily counts of the number of coughs for 15 min), cyanosis, nasal discharge or foaming. Body weight
122 was recorded once a week.

123

124 2.2.3. Samples and analysis

125 Blood samples were taken once a week from live pigs for serological analysis (D0 to D63). Sera were
126 stored at -20°C and tested with a blocking ELISA (DAKO ELISA, Kitvia, Labarthe-Inard, France) to
127 detect *M. hyopneumoniae* antibodies. The percentage of inhibition for each serum was calculated with
128 the following formula: Percent inhibition = $100 - [100 \times (\text{sample mean OD} \div \text{buffer control mean OD})]$. A
129 sample was classified as positive if the percent of inhibition was $> 50\%$. Sera were also analysed to
130 detect *A. pleuropneumoniae* antibodies, by an ELISA technique using long chain purified
131 polysaccharides (LC-LPS) specific for serogroup 1-9-11 (Swinecheck App, Biovet, AES Laboratoire,
132 Combourg). The positive threshold was fixed at an optical density (OD) of 0.55.

133 If mortality occurred or after euthanasia, maximum D30-D65 after *M. hyopneumoniae* or *A.*
134 *pleuropneumoniae* infection, the pigs were necropsied and their thoracic organs were thoroughly
135 examined (Table 2). Pneumonia and pleurisy were scored as previously described by Madec and
136 Derrien (1981). The maximum total scores possible for each lung were 28 for pneumonia and 4 for
137 pleurisy.

138 Swabs were collected from trachea, palatine tonsils and lungs, from all the pigs. The swabs were
139 placed in 2 mL of Buffered Pepton Water Broth (samples). *A. pleuropneumoniae* was cultured from 50
140 μL of each sample placed on PPLOsup agar and incubated overnight at 37°C in $5\% \text{CO}_2$. *A.*

141 *pleuropneumoniae* like colonies were identified by PCR (Savoye et al., 2000). *M. hyopneumoniae* was
142 cultured from samples by diluting 100 µL of each sample in 900 µL of FBM supplemented with
143 bacitracin (150 µg/mL), amphotericin B (2.5 µg/mL), ampicillin (100 µg/mL) and colistin (7.5 µg/mL) to
144 avoid contamination by non-specific bacteria and optimise *M. hyopneumoniae* recovery. Each sample
145 was grown in FBM, 10-fold diluted up to 10⁻³ and incubated at 37°C until the culture developed an acid
146 colour change or up to 30 days. When a colour change of the FBM was observed, *M. hyopneumoniae*
147 cultures were confirmed by a specific PCR (test described by Verdin et al., 2000 and modified by
148 Marois et al., 2007).

149 Finally, microscopic lung examinations were conducted on each pig. Lung samples (a piece of the
150 right middle lobe) were fixed in 10% buffered formalin: paraffin-embedded sections were cut at 5 µm,
151 stained by a trichrome coloration (hematoxylin, eosin and safran) and examined by light microscopy.

152

153 2.3. Statistical analysis

154

155 The data obtained from the experimental study (average daily weight gains, macroscopic and
156 microscopic lesions and serological results) for each group were compared simultaneously by
157 Kruskal-Wallis test and in 2 by 2 tables by Kolmogorov-Smirnov test. Differences in the number of
158 pigs (with clinical signs, macroscopic or microscopic lesions and with positive bacteriological or
159 serological analyses) between groups were assessed by Fisher exact test ($n \leq 5$) or chi-square test (n
160 > 5) of independence in 2 by 2 tables. These tests were carried out with Systat 9.0 program for
161 Windows (Systat Software GmbH, Erkrath, Germany). Differences were considered significant when P
162 ≤ 0.05 .

163

164

165 3. Results

166

167 3.1. Clinical signs and macroscopic lesions

168

169 As shown in Table 1, clinical signs and pulmonary lesions varied with the group of pigs. The necropsy
170 plan is presented in Table 2.

171 No clinical signs or lesions were observed in non-infected animals (group 1). The average daily weight
172 gain (ADG) of these pigs was 932 g.

173

174 3.1.1. Positive controls of the experiment: our standardized experimental models

175 Pigs infected at 6 weeks of age with *A. pleuropneumoniae* strain 4915 (group 2), developed
176 hyperthermia (41-41.4°C), dyspnea and anorexia. All pigs with respiratory distress died two days after
177 infection, so the ADG could not be evaluated. Macroscopic lung lesions, typical of *A. pleuropneumoniae*
178 infection (pneumonia, pleurisy, fibrinous pleuropneumonia and haemorrhage mostly located in the
179 cranial, middle and caudal lobes) and hypertrophy of the tracheo-bronchial lymph nodes were
180 observed in 7 pigs.

181 All pigs infected at either 6 or 10 weeks of age with *M. hyopneumoniae* strain 116 (groups 4 and 6)
182 developed coughing with a significantly higher frequency in group 4 ($P \leq 0.05$). Typical mycoplasmal
183 pneumonia, mostly located in the apical and cardiac lobes, was noted and no significant differences
184 were observed between the two groups. The ADG of both groups, compared with that of group 1, was
185 significantly affected, particularly that of group 4 ($P \leq 0.05$).

186

187 3.1.2. Experimental infections with *A. pleuropneumoniae* strain 21

188 Pigs infected with *A. pleuropneumoniae* alone, at 6 weeks or at 10 weeks of age (groups 3 and 5)

189 The results differed considerably between these two groups. The symptoms and lung lesions
190 observed in pigs of group 3 were typical of *A. pleuropneumoniae* infection and three pigs died (24-48h
191 after infection). Lung lesions were located in the diaphragmatic lobes and in some cases, in the apical
192 and cardiac lobes. With the exception of pleurisy scores and mortality rates, no significant differences
193 were apparent between groups 2 and 3 from the overall results.

194 No clinical signs or lung lesions were present in group 5, with the exception of tracheo-bronchial lymph
195 nodes hypertrophy in 4 pigs and hyperthermia for a single day (40.7°C), in only one pig, 6 days after

196 infection. The ADG in groups 3 and 5 were similar. But, a significant difference ($P \leq 0.05$) was noted
197 between the ADG of the pigs of these two groups and those of the negative control group (group 1).

198

199 Pigs infected with *A. pleuropneumoniae* in association with *M. hyopneumoniae* (groups 7, 8 and 9)

200 Group 7: pigs infected with *A. pleuropneumoniae* at 6 weeks of age and with *M. hyopneumoniae* at 10
201 weeks of age. One pig died two days after *A. pleuropneumoniae* infection. Hyperthermia and lung
202 lesions were similar to those of group 3 but the coughing score was higher in group 7. Lung lesions
203 were located in the apical and cardiac lobes (*M. hyopneumoniae* infection) as well as in the
204 diaphragmatic lobes (*A. pleuropneumoniae* infection). Pulmonary necrosis was observed in four pigs.
205 The ADG of pigs in group 7 were significantly different from those of group 1 ($P \leq 0.05$) but the ADG
206 values for groups 3, 6 and 7 were similar.

207 Group 8: pigs infected with *M. hyopneumoniae* at 6 weeks of age and with *A. pleuropneumoniae* at 10
208 weeks of age. Four pigs died 2 or 3 days after *A. pleuropneumoniae* infection and a fibrinous and
209 haemorrhagic pleuropneumoniae was apparent. Clinical signs and lung lesions were typical of both *M.*
210 *hyopneumoniae* and *A. pleuropneumoniae* infections. Coughing score and pneumonia mean score
211 were significantly higher in group 8 than in group 7 ($P \leq 0.05$). The comparison with group 5 gave
212 similar results. The ADG of the survivors was significantly affected in group 8, in comparison with the
213 negative control group and with pigs in groups 5 and 7.

214 Group 9: pigs simultaneously infected with *A. pleuropneumoniae* and *M. hyopneumoniae* at 6 weeks
215 of age. One pig died one day after the double infection. Clinical signs and lung lesions were similar to
216 those observed in groups 3 and 7, but significantly different from those observed in group 8 ($P \leq 0.05$).
217 The ADG was affected, in comparison with the negative control group and with group 7 ($P \leq 0.05$), but
218 no differences were noted with pigs of groups 3 and 8.

219

220 3.2 Microscopic lung lesions

221 Results are presented in Table 3 and Figure 1. Pigs infected with *M. hyopneumoniae* alone or in
222 association with *A. pleuropneumoniae*: infiltrating lymphocytes were observed in the peribronchiolar,
223 peribronchial and perivascular areas at the beginning of *M. hyopneumoniae* infection. This was

224 followed by interstitial pneumonia, lymphoid nodules associated with the airways and collapse of the
225 alveoli. These two phases were noted in pigs infected with *M. hyopneumoniae*, independently of the
226 day of necropsy. With the exception of two pigs that died, either firstly or simultaneously infected with
227 *A. pleuropneumoniae* (groups 7 and 9), all other pigs developed typical mycoplasmal lesions.
228 Microscopic examination did not reveal any differences between the lungs of pigs infected with *M.*
229 *hyopneumoniae* alone (groups 4 and 6) or with both bacterial species (groups 7, 8 and 9).
230 Pigs infected with *A. pleuropneumoniae*, alone or in association with *M. hyopneumoniae*: the
231 histopathologic changes of the lungs, in the early phase of *A. pleuropneumoniae* infection, were
232 characterised by haemorrhage, vascular thrombosis, oedema, necrosis and the presence of fibrinous
233 exudate. Acute lung lesions were apparent in similar numbers of pigs in groups 2, 3 and 8 but
234 significant differences ($P \leq 0.05$) were observed between group 2 and groups 5, 7 and 9.
235 In the chronic phase, marked fibrosis around areas of necrosis and fibrinous pleuritis were observed.
236 In group 5, two pigs without any macroscopic lung lesions, showed very mild and superficial
237 interlobular fibrosis. One pig in group 9, with macroscopic pneumonia, did not show any microscopic
238 lesions in the section observed. Similar numbers of pigs were affected in groups 3, 5, 7, 8 and 9.
239 Finally, no lesions were observed in the negative control group.

240

241 3.3. *M. hyopneumoniae* and *A. pleuropneumoniae* detection

242

243 *M. hyopneumoniae* and *A. pleuropneumoniae* were not recovered from any non-infected pigs (Table
244 4).

245 *M. hyopneumoniae* was re-isolated and identified by PCR, in all infected pigs (groups 4, 6, 7, 8 and 9)
246 and no significant differences were observed between these groups. In group 7, one pig (that died two
247 days after *A. pleuropneumoniae* infection) could not be experimentally infected with *M.*
248 *hyopneumoniae*. The most appropriate sites for the detection of *M. hyopneumoniae* appeared to be
249 the trachea and lungs rather than the tonsils.

250 *A. pleuropneumoniae* was recovered from all infected pigs (groups 2, 3, 5, 7, 8 and 9). Sampling the
251 lungs and trachea was most effective in the early phase of infection, whereas swabbing the tonsils

252 was more appropriate in the chronic phase. *A. pleuropneumoniae* was not isolated from the lungs of
253 pigs in group 5.

254

255 3.4. Serological results

256

257 Serological results are shown in Figure 2. All non-infected pigs were seronegative throughout the
258 experiment. In all pigs infected with *M. hyopneumoniae*, seroconversion occurred 2-3 weeks after
259 infection (Figure 2A). At the end of the experiment, the ELISA titres in the different groups of pigs were
260 similar (mean results:100 percent inhibition). All surviving pigs infected with *A. pleuropneumoniae*
261 were seropositive three or four weeks after infection (groups 3, 7 and 9) or two weeks after infection
262 (groups 5 and 8). After 42 days, the ELISA titres (mean OD almost 1.0) for the different groups of pigs
263 were similar (Figure 2B).

264

265

266 4. Discussion

267

268 In our experimental conditions, SPF pigs intranasally inoculated, at six weeks of age, with 1.4×10^8
269 CFU of *A. pleuropneumoniae* strain 4915 (serotype 9, isolated from an outbreak of porcine
270 pleuropneumoniae), developed severe clinical signs as well as lung lesions typical of the acute phase
271 of infection. All pigs died 24 to 48 h after experimental infection and *A. pleuropneumoniae* was
272 recovered from their respiratory tract. These results are in accordance with those of our previous study
273 showing that SPF pigs experimentally infected, at 10 weeks of age, with the same *A.*
274 *pleuropneumoniae* strain, developed acute clinical signs and severe lung lesions (Jobert al., 2000).
275 Nevertheless, in this first experiment with *A. pleuropneumoniae* strain 4915, the pigs received
276 ampicillin to avoid death. Thus, surviving pigs showed lung lesions corresponding to the chronic phase
277 of infection. According to our results in the present study, the sensitivity of pigs to *A.*
278 *pleuropneumoniae* infection is probably age-related: six weeks vs ten weeks. Thus, the results
279 obtained by Jobert et al. (2000) are in agreement with those obtained in the present study with *A.*

280 *pleuropneumoniae* strain 21 (also a serotype 9 producing ApxI and ApxII toxins): SPF pigs infected at
281 6 weeks of age with 1.3×10^8 CFU of *A. pleuropneumoniae* showed very severe clinical signs and lung
282 lesions, analogous to those induced with *A. pleuropneumoniae* strain 4915. On the other hand, lung
283 lesions and symptoms were almost non-existent in the group of pigs infected at 10 weeks of age.
284 Nevertheless, the ADG of the pigs was affected. In group 3, surviving pigs were seropositive three to
285 four weeks after infection and all the pigs of group 5 seroconverted after two weeks. In both cases, the
286 ELISA titres were similar and very high (mean OD almost 1) at the end of the experiment. These
287 results are in accordance with those observed in the nucleus pig farm from which *A.*
288 *pleuropneumoniae* strain 21 had been isolated. The young sows of this pig farm became
289 contaminated, were *A. pleuropneumoniae* positive in the palatine tonsils and clearly seroconverted
290 without any manifestation of *A. pleuropneumoniae* infection. This might be explained by the excellent
291 housing conditions and hygienic environment of this particular breeding herd.

292 Checks for respiratory disorders in breeding herds are made during routine farm visits by veterinarians
293 in the context of clinical surveillance (Gottschalk and Taylor, 2006). Serological testing of a
294 representative number of animals from the herd and detection of *A. pleuropneumoniae* by
295 bacteriological isolation or PCR, are very useful for epidemiological investigations. Positive serological
296 results must be taken seriously even if clinical signs are absent from the herd. Asymptomatic carrier
297 pigs are a major source for introduction of *A. pleuropneumoniae* into an uninfected herd (Chiers et al.,
298 2002a; Chiers et al., 2002b). Some authors indicated that asymptomatic pigs carrying bacteria in the
299 tonsils do not generally develop measurable antibody titres and the lower respiratory tract appears to
300 be involved to obtain a humoral response (Chiers et al., 2002a; Chiers et al., 2002b; Maas et al.,
301 2006). However, it has been clearly demonstrated that clinically healthy animals colonized at tonsils
302 can develop a strong serological reaction (Gottschalk and Taylor, 2006). Krejci et al. (2005) also
303 showed that the presence of *A. pleuropneumoniae* in the tonsils could induce immunity of the airway
304 mucosa and, in some cases, prevent bacterial colonisation of the lower part of the respiratory tract.
305 According to Haesebrouck et al. (1997), the presence of antibodies in the serum of pigs does not
306 provide complete protection against *A. pleuropneumoniae* infection. However, these antibodies may
307 prevent severe forms of the disease (Crujisen et al., 1992; Krejci et al., 2005).

308 Pigs infected with *M. hyopneumoniae* alone developed coughing, lung lesions and showed
309 seroconversion and *M. hyopneumoniae* was re-isolated whatever the time of infection (6 weeks vs 10
310 weeks). The ADG of the pigs was affected and antibodies were present in all the pigs three weeks
311 after infection. These results are similar to those described in previous studies (Kobisch and Ross,
312 1996; Fano et al., 2005; Marois et al., 2007).

313 Under the conditions of this study, when pigs were firstly infected with *A. pleuropneumoniae* or
314 simultaneously with *M. hyopneumoniae* and *A. pleuropneumoniae*, at six weeks of age, clinical signs
315 and lung lesions were severe and corresponded to the pathogenicity of the two bacterial strains
316 combined. However, pigs experimentally infected with *M. hyopneumoniae* at six weeks of age and four
317 weeks later with *A. pleuropneumoniae* (strain 21) were particularly affected. Very severe clinical signs
318 (hyperthermia, death and coughing) and lung lesions, corresponding to the double infection, were
319 observed in these animals. The ADG was the lowest of all the experimental groups (650g vs 932g in
320 the negative control group). *M. hyopneumoniae*, associated with enzootic pneumonia, is also
321 considered to play a primary role in PRDC (Thacker, 2006). *M. hyopneumoniae* is able to induce
322 damage in the respiratory tract and to predispose pigs to other respiratory pathogens, especially
323 bacteria and viruses (Yagihashi et al., 1984; Ross, 1999; Maes et al., 2008). According to Thacker
324 (2006), *M. hyopneumoniae* and *A. pleuropneumoniae* co-infection is a common cause of PRDC. *M.*
325 *hyopneumoniae* may potentiate the severity of *A. pleuropneumoniae*-induced lesions in co-infected
326 pigs (Ciprián et al., 1994). These observations are in accordance with the results of the present study
327 showing changes in pigs experimentally infected with *M. hyopneumoniae* and *A. pleuropneumoniae*.
328 The breeding herd, from which *A. pleuropneumoniae* strain 21 was isolated, was not infected with *M.*
329 *hyopneumoniae* (routine checks conducted by serology). *A. pleuropneumoniae* was probably not
330 associated with other respiratory pathogens in this farm with a high health status. *A.*
331 *pleuropneumoniae* was only detected in the palatine tonsils of gilts. Routine checks by veterinarians
332 are very useful in pig farms, particularly in breeding herds. Positive serological results, especially
333 concerning *A. pleuropneumoniae* serotype 9, a major respiratory pathogen of pigs, should be
334 interpreted with care.

335

336

337 **Acknowledgements**

338 The authors would like to thank the Regional Council of Brittany, the "Comité Régional Porcin" and
339 Boehringer Ingelheim Animal Health France, Fort Dodge Animal Health, Intervet S.A., Pfizer Animal
340 Health and Schering-Plough Animal Health for their financial support. They also thank Roland Cariolet,
341 Laëtitia Le Devendec, Véronique Tocqueville, Bernard Beaurepaire, Gérard Bénévent, Pierre
342 Ecobichon and Jean-Claude Rault for their technical assistance.

343

344

345 **References**

346 Cariolet, R., Marie, P., Moreau, G., Robert, H., 1994. Rappel des différentes méthodes d'obtention de
347 porcelets assainis : conditions de maintien du statut sanitaire et valorisation de ces animaux, Journées
348 de la Recherche Porcine en France 26, 1-12.

349 Chiers, K., Donné, E., Van Overbeke, I., Ducatelle, R., Haesebrouck, F., 2002a. *Actinobacillus*
350 *pleuropneumoniae* infections in closed swine herds: infection patterns and serological profiles. Vet.
351 Microbiol. 85, 343-352.

352 Chiers, K., Donné, E., Van Overbeke, I., Ducatelle, R., Haesebrouck, F., 2002b. Evaluation of
353 serology, bacteriological isolation and polymerase chain reaction for the detection of pigs carrying
354 *Actinobacillus pleuropneumoniae* in the upper respiratory tract after experimental infection. Vet.
355 Microbiol. 88, 385-392.

356 Ciprián, A., Cruz, T.A., de la Garza, M., 1994. *Mycoplasma hyopneumoniae*: interaction with other
357 agents in pigs, and evaluation of immunogens. Arch. Med. Res. Summer 25, 235-239.

358 Cruijssen, T.L., Van Leengoed, L.A., Dekker-Nooren, T.C., Schoevers, E.J., Verheijden, J.H., 1992.
359 Phagocytosis and killing of *Actinobacillus pleuropneumoniae* by alveolar macrophages and
360 polymorphonuclear leukocytes isolated from pigs. Infect. Immun. 60, 4867-4871.

361 Dubreuil, J.D., Jacques, M., Mittal, K.R., Gottschalk, M., 2000. *Actinobacillus pleuropneumoniae*
362 surface polysaccharides: their role in diagnosis and immunogenicity. Anim. Health. Res. Rev. 1, 73-
363 93.

364 Fano, E., Pijoan, C., Dee, S., 2005. Dynamics and persistence of *Mycoplasma hyopneumoniae*
365 infection in pigs. Can. J. Vet. Res. 69, 223-228.

366 Fittipaldi, N., Broes, A., Harel, J., Kobisch, M., Gottschalk, M., 2003. Evaluation and field validation of
367 PCR tests for detection of *Actinobacillus pleuropneumoniae* in subclinically infected pigs. J. Clin.
368 Microbiol. 41, 5085-5093.

369 Frey, J., 1995. Virulence in *Actinobacillus pleuropneumoniae* and RTX toxins. Trends Microbiol. 3:257-
370 261.

371 Frey, J., Bosse, J.T., Chang, Y.F., Cullen, J.M., Fenwick, B., Gerlach, G.F., Gygi, D., Haesebrouck, F.,
372 Inzana, T.J., Jansen, R., Kamp, E.M., Macdonald, J., Macinnes, J.I., Mittal, K.R., Nicolet, J., Rycroft,
373 A.N., Segers, R.P.A.M., Smits, M.A., Stenbaek, E., Stuck, D.K., Van Den Bosch, J.F., Wilson, P.J.,
374 Young, R., 1993. *Actinobacillus pleuropneumoniae* RTX toxins: uniform designation of haemolysins,
375 cytolysins, pleurotoxin and their genes. J. Gen. Microbiol. 139, 1723-1728.

376 Gottschalk, M., Altman, E., Charland, N., De Lasalle, F., Dubreuil, J.D., 1994. Evaluation of a saline
377 boiled extract, capsular polysaccharides and long-chain lipopolysaccharides of *Actinobacillus*
378 *pleuropneumoniae* serotype 1 as antigens for the serodiagnosis of swine pleuropneumonia. Vet.
379 Microbiol. 42, 91-104.

380 Gottschalk, M., Morvan, H., Broes, A., Desrosiers, R., Kobisch, M., 2005. Actualités sur la
381 pleuropneumonie porcine. Journées de la Recherche Porcine 37, 341-346.

382 Gottschalk, M., Taylor, T., 2006. *Actinobacillus pleuropneumoniae*. In: Straw, B.E., Zimmerman, J.J.,
383 D'Allaire, S., Taylor, D.J. (Eds.), Diseases of Swine, 9th ed. Blackwell Publishing Ltd. Oxford, UK, pp.
384 563-576.

385 Haesebrouck, F., Chiers, K., Van Overbeke, I., Ducatelle, R., 1997. *Actinobacillus pleuropneumoniae*
386 infections in pigs: the role of virulence factors in pathogenesis and protection. *Vet. Microbiol.* 58, 239-
387 249.

388 Jobert, J.L., Savoye, C., Cariolet, R., Kobisch, M., Madec, F., 2000. Experimental aerosol
389 transmission of *Actinobacillus pleuropneumoniae* to pigs. *Can. J. Vet. Res.* 64, 21-26.

390 Kobisch, M., Labbé, A., Morvan, P., Le Moine, M.M., Beaurepaire, B., Cariolet, R., Pansart, J.F., 1993.
391 Pathologie pulmonaire du porc : un modèle expérimental associant *Mycoplasma hyopneumoniae* et
392 *Actinobacillus pleuropneumoniae*. *Journées de la Recherche Porcine* 25, 339-344.

393 Kobisch, M., Ross, R.F., 1996. Experimental infections of swine. In: Tully, J.G., Razin, S. (Eds.),
394 Molecular and Diagnostic Procedures in Mycoplasmaology, vol II, Academic Press, San Diego,
395 California, pp. 371-376.

396 Krejci, J., Nechvatalova, K., Kudlackova, H., Faldyna, M., Kucerova, Z., Toman, M., 2005. Systemic
397 and local antibody responses after experimental infection with *Actinobacillus pleuropneumoniae* in
398 piglets with passive or active immunity. *J. Vet. Med. B Infect. Dis. Vet. Public Health.* 52, 190-196.

399 Kuhnert, P., Berthoud, H., Christensen, H., Bisgaard, M, Frey, J. 2003. Phylogenetic relationship of
400 equine *Actinobacillus* species and distribution of RTX toxin genes among clusters. *Vet. Res.* 34, 353-
401 359.

402 Maas, A., Jacobsen, I.D., Meens, J., Gerlach, G.F., 2006. Use of an *Actinobacillus pleuropneumoniae*
403 multiple mutant as a vaccine that allows differentiation of vaccinated and infected animals. *Infect.*
404 *Immun.* 74, 4124-4132.

405 Madec, F., Derrien, H., 1981. Fréquence, intensité et localisation des lésions pulmonaires chez le porc
406 charcutier, *Journées de la Recherche Porcine en France* 13, 231-236.

407 Maes, D., Segales, J., Meyns, T., Sibila, M., Pieters, M., Haesebrouck, F., 2008. Control of
408 *Mycoplasma hyopneumoniae* infections in pigs. *Vet. Microbiol.* 126, 297-309.

409 Marois, C., Le Carrou, J., Kobisch, M., Gautier-Bouchardon, A.V., 2007. Isolation of *Mycoplasma*
410 *hyopneumoniae* from different sampling sites in experimentally infected and contact SPF piglets. *Vet.*
411 *Microbiol.* 120, 96-104.

412 Ross, R.F., 1999. Mycoplasmal Diseases. In: Straw, B.E., D’Allaire, S., Mengeling, W.L., Taylor, D.J.
413 (Eds.), *Diseases of Swine*, 8th ed. Iowa State University Press, Ames, Iowa, pp. 495-501.

414 Savoye, C., Jobert, J.L., Berthelot-Hérault, F., Keribin, A.M., Cariolet, R., Morvan, H., Madec, F.,
415 Kobisch, M., 2000. A PCR assay used to study aerosol transmission of *Actinobacillus*
416 *pleuropneumoniae* from samples of live pigs under experimental conditions. *Vet. Microbiol.* 73, 337-
417 347.

418 Schaller, A., Kuhn, R., Kuhnert, P., Nicolet, J., Anderson, T.J., MacInnes, J.I., Segers, R.P.A.M., Frey,
419 J., 1999. Characterization of *apxIVA*, a new RTX determinant of *Actinobacillus pleuropneumoniae*.
420 *Microbiol.* 145, 2105–2116.

421 Thacker, E.L., 2006. Mycoplasmal Diseases. In: Straw, B.E., Zimmerman, J.J., D’Allaire, S., Taylor,
422 D.J. (Eds.), *Diseases of Swine*, 9th ed. Blackwell Publishing Ltd. Oxford, UK, pp. 701-717.

423 Verdin, E., Saillard, C., Labbe, A., Bove, J.M., Kobisch, M., 2000. A nested PCR assay for the
424 detection of *Mycoplasma hyopneumoniae* in tracheobronchiolar washings from pigs. *Vet. Microbiol.*
425 76, 31-40.

426 Yagihashi, T., Nunoya, T., Mitui, T., Tajima, M., 1984. Effect of *Mycoplasma hyopneumoniae* infection
427 on the development of *Haemophilus pleuropneumoniae* pneumonia in pigs. *Nippon Juigaku Zasshi.*
428 46, 705-713.

429

429 Table 1: Experimental design, clinical and pathological observations

430 ^a D: Day

431 ^b UI: uninfected pigs with *A. pleuropneumoniae* (App) or *M. hyopneumoniae* (Mhp)

432 ^c No. of dead pigs

433 ^d Mortality observed on D1 to D2

434 ^e Pigs surviving after infection

435 ^f No. of pigs with hyperthermia (body temperature $\geq 40.5^{\circ}\text{C}$)

436 ^g No. of pigs with coughing during the trial (Mean of coughing per day and per pig during the
437 trial) (D0-D59)

438 ^h No. of pigs with pneumonia

439 ⁱ Maximum total score possible for each complete lung was 28

440 ^j No. of pigs with pleurisy

441 ^k Maximum total score possible for each complete lung was 4

442 ^l No. of pigs with fibrinous and haemorrhagic pleuro-pneumonia

443 ^m No. of pigs with pulmonary necrosis

444 ⁿ No. of pigs with hypertrophy of tracheo-bronchial lymph node (TBLN)

445 ^o Average Daily Gain (g) of pigs per group during the trial (D0 to D49)

446

447 Table 2: Planning of necropsies

448

449 Table 3: Microscopic lung lesions

450 ^a Necropsy days

451 ^b No. of pigs with microscopic lung lesions / No. of necropsied pigs

452 ^c Typical mycoplasmal lesions

453 ^d Typical lung lesions corresponding to the early phase of *A. pleuropneumoniae* infection

454 ^e Typical lung lesions corresponding to the chronic phase of *A. pleuropneumoniae* infection

455

456

457 Table 4: Bacteriological results (at time of necropsy)

458 ^a TO, Tonsil

459 ^b T, Tracheal

460 ^c L, Lung

461

462 Figure 1: Microscopic examinations in the lungs of four pigs (LN: Lymphoid Nodules, N:
463 Necrosis, H: Haemorrhage, FE: Fibrinous Exudates, FN: Fibrosis around areas of Necrosis,
464 FP: Fibrinous Pleuritis)

465 (A) Lung of a control pig (group 1) (x50)

466 (B) Lung of a pig infected with *M. hyopneumoniae* (group 4) (x25)

467 (C) Lung of a pig infected with *M. hyopneumoniae* simultaneously with *A. pleuropneumoniae*
468 (group 9). Early phase of infection (x50-Figure C1 and x100-Figure C2)

469 (D) Lung of a pig infected with *M. hyopneumoniae* (6 weeks of age) and with *A.*
470 *pleuropneumoniae* (10 weeks of age) (group 7). Chronic phase of infection (x50-Figure D1
471 and x25-Figure D2).

472

473 Figure 2 : Serological response from uninfected pigs and pigs experimentally infected with *M.*
474 *hyopneumoniae* or *A. pleuropneumoniae* or both. Figure 2A: antibodies to *M.*
475 *hyopneumoniae*, group 1: uninfected pigs, groups 4, 6, 7, 8 and 9: infected with *M.*
476 *hyopneumoniae*. Figure 2B: antibodies to *A. pleuropneumoniae*, group 1: uninfected pigs,
477 groups 3, 5, 7, 8 and 9: infected with *A. pleuropneumoniae* (surviving pigs).

478 Table 1: Experimental design, clinical and pathological observations

	Groups of pigs (n=7)								
	1	2	3	4	5	6	7	8	9
Challenge at 6 weeks of age (D0 ^a)	UI ^b	App 4915	App 21	Mhp 116	-	-	App 21	Mhp 116	App 21 Mhp 116
Challenge at 10 weeks of age (D28 ^a)	UI	-	-	-	App 21	Mhp 116	Mhp 116	App 21	-
Mortality (D) ^c	0	7 (D1-D2 ^d)	3 (D1-D2)	0	0	0	1 (D2)	4 (D30-D31)	1 (D1)
Hyperthermia ^{e,f} (D)	0	4 (D1-D2)	5 (D1-D4)	0	1 (D34)	0	6 (D1-D6)	6 (D29-D36)	4 (D1)
Beginning of coughing	-	-	D7	D8	-	D32	D1	D9	D4
Coughing (Mean± SD) ^{e,g}	0	0	4 (0.05±0.21)	7 (0.56±0.95)	0	7 (0.28±0.68)	6 (0.32±0.63)	7 (0.67±0.99)	6 (0.86±1.10)
Pneumonia ^h (Mean score ± SD ⁱ)	0	7 (22.3±3)	5 (11.3±12.9)	7 (5.7±6.8)	0	7 (6.1±3.8)	7 (5.7±9.5)	7 (16±10)	7 (7.9 ±6)
Pleurisy ^j (Mean score ± SD ^k)	0	7 (4±0)	7 (2.9±1.2)	0	0	0	5 (1.9±1.7)	7 (3±1.2)	5 (2.1±1.9)
Fibrinous and haemorrhagic pleuro-pneumonia ^l	0	7	3	0	0	0	1	4	1
Pulmonary necrosis ^m	0	0	1	0	0	0	4	1	2
Hypertrophy of TBLN ⁿ	0	7	5	0	4	0	4	2	5
ADG (± SD) ^{e,o}	932 (±175)	-	816 (±257)	780 (±178)	791 (±222)	879 (±194)	798 (±262)	650 (±305)	737 (±246)

480 ^a D: Day

481 ^b UI: uninfected pigs with *A. pleuropneumoniae* (App) or *M. hyopneumoniae* (Mhp)

482 ^c No. of dead pigs

483 ^d Mortality observed on D1 to D2

484 ^e Pigs surviving after infection

485 ^f No. of pigs with hyperthermia (body temperature ≥40.5°C)

486 ^g No. of pigs with coughing during the trial (Mean of coughing per day and per pig during the trial) (D0-D59)

487 ^h No. of pigs with pneumonia

488 ⁱ Maximum total score possible for each complete lung was 28

489 ^j No. of pigs with pleurisy

490 ^k Maximum total score possible for each complete lung was 4

491 ^l No. of pigs with fibrinous and haemorrhagic pleuro-pneumonia

492 ^m No. of pigs with pulmonary necrosis

493 ⁿ No. of pigs with hypertrophy of tracheo-bronchial lymph node (TBLN)

494 ^o Average Daily Gain (g) of pigs per group during the trial (D0 to D49)

495 Table 2: Planning of necropsies

No. of pigs necropsied at:	Groups of pigs (n=7)								
	1	2	3	4	5	6	7	8	9
D1 and D2	0	7	3	0	0	0	1	0	1
D30 to D35	0	-	0	3	0	0	0	4	2
D48 and D49	3	-	0	4	0	0	0	3	4
D57 and D58	0	-	1	-	3	3	2	-	-
D63 to D65	4	-	3	-	4	4	4	-	-

496

497

498 Table 3: Microscopic lung lesions

	Groups of pigs (n=7)								
	1	2	3	4	5	6	7	8	9
Challenge at 6 weeks of age (Day 0)	UI	App 4915	App 21	Mhp 116	-	-	App 21	Mhp 116	App 21 Mhp 116
Challenge at 10 weeks of age (Day 28)	UI	-	-	-	App 21	Mhp 116	Mhp 116	App 21	-
Mycoplasmal lesions ^c	D1 and D2 ^a	0/7 ^b	0/3				0/1		0/1
	D30 to D35			3/3				4/4	2/2
	D48 and D49	0/3		4/4				3/3	4/4
	D57 and D58			0/1		0/3	3/3	2/2	
	D63 to D65	0/4		0/3		0/4	4/4	4/4	
	<i>Total</i>	0/7	0/7	0/7	7/7	0/7	7/7	6/7	7/7
Early phase of App infection ^d	D1 and D2		7/7	3/3			1/1		1/1
	D30 to D35				0/3			4/4	0/2
	D48 and D49	0/3		0/4				0/3	0/4
	D57 and D58			0/1		0/3	0/3	0/2	
	D63 to D65	0/4		0/3		0/4	0/4	0/4	
	<i>Total</i>	0/7	7/7	3/7	0/7	0/7	0/7	1/7	4/7
Chronic phase of App infection ^e	D1 and D2		0/7	0/3			0/1		0/1
	D30 to D35				0/3			0/4	1/2
	D48 and D49	0/3		0/4				3/3	4/4
	D57 and D58			1/1		1/3	0/3	2/2	
	D63 to D65	0/4		3/3		1/4	0/4	3/4	
	<i>Total</i>	0/7	0/7	4/7	0/7	2/7	0/7	5/7	3/7

499 ^a Necropsy days

500 ^b No. of pigs with microscopic lung lesions / No. of necropsied pigs

501 ^c Typical mycoplasmal lesions

502 ^d Typical lung lesions corresponding to the early phase of *A. pleuropneumoniae* infection

503 ^e Typical lung lesions corresponding to the chronic phase of *A. pleuropneumoniae* infection

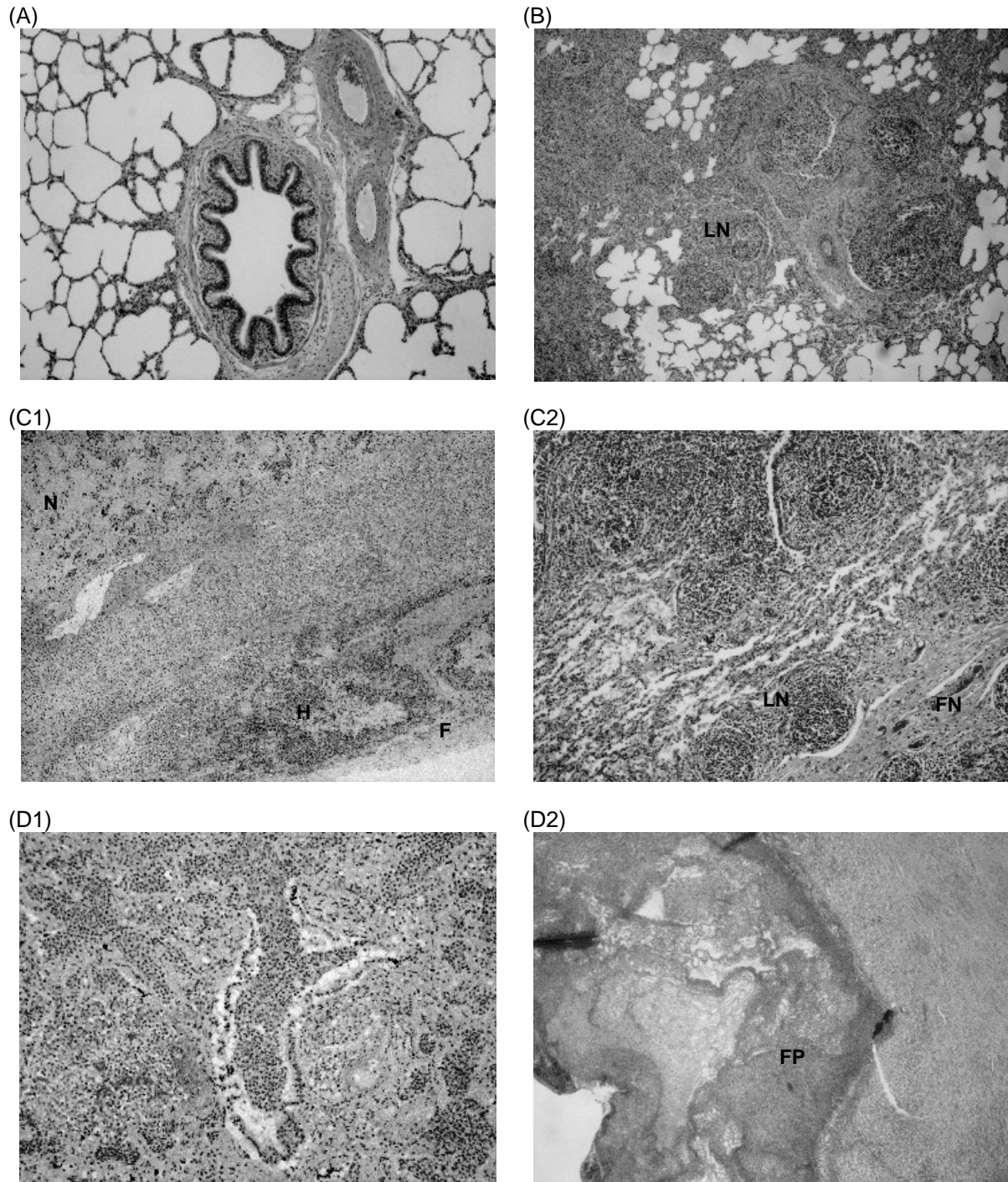
504

505

505 Table 4: Bacteriological results (at time of necropsy)

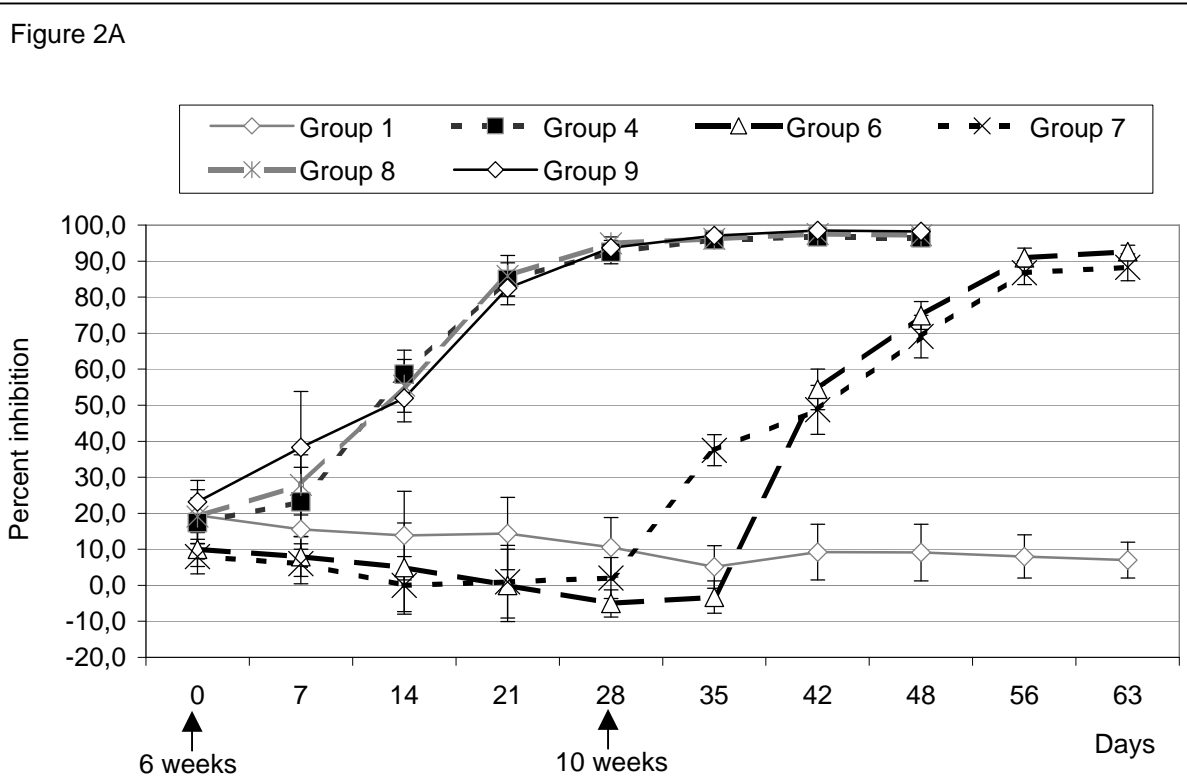
Samples	Groups of pigs (n=7)									
	1	2	3	4	5	6	7	8	9	
Challenge at 6 weeks of age (Day 0)	UI	App 4915	App 21	Mhp 116	-	-	App 21	Mhp 116	App 21 Mhp 116	
Challenge at 10 weeks of age (Day 28)	UI	-	-	-	App 21	Mhp 116	Mhp 116	App 21	-	
Number of positive Mhp cultures	TO ^a	0	0	0	4	0	3	5	4	4
	T ^b	0	0	0	7	0	7	6	7	7
	L ^c	0	0	0	7	0	7	6	7	7
Number of positive App cultures	TO	0	5	7	0	7	0	7	7	7
	T	0	7	4	0	4	0	6	6	7
	L	0	7	3	0	0	0	3	5	3

506 ^a TO, Tonsil
507 ^b T, Tracheal
508 ^c L, Lung
509
510

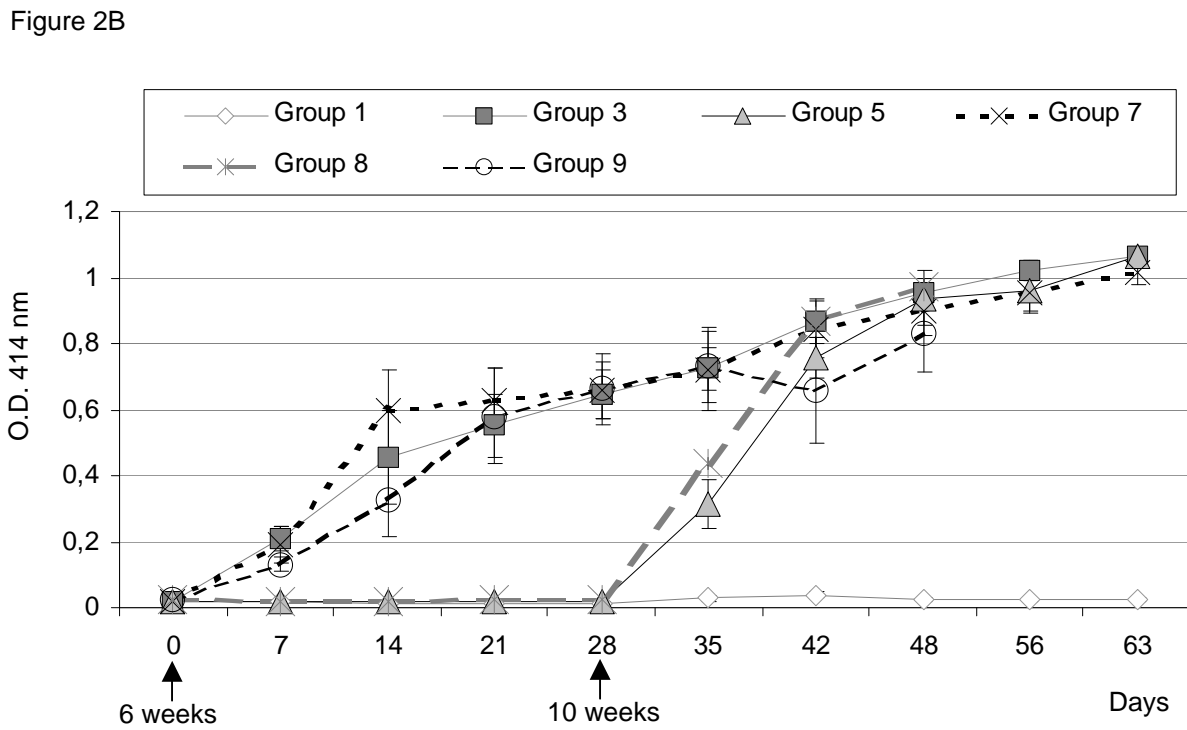


512 Figure 1: Microscopic examinations in the lungs of four pigs (LN: Lymphoid Nodules, N: Necrosis, H:
 513 Haemorrhage, FE: Fibrinous Exudates, FN: Fibrosis around areas of Necrosis, FP: Fibrinous Pleuritis)
 514 (A) Lung of a control pig (group 1) (x50)
 515 (B) Lung of a pig infected with *M. hyopneumoniae* (group 4) (x25)
 516 (C) Lung of a pig infected with *M. hyopneumoniae* simultaneously with *A. pleuropneumoniae* (group
 517 9). Early phase of infection (x50-Figure C1 and x100-Figure C2)
 518 (D) Lung of a pig infected with *M. hyopneumoniae* (6 weeks of age) and with *A. pleuropneumoniae* (10
 519 weeks of age) (group 7). Chronic phase of infection (x50-Figure D1 and x25-Figure D2).

520



521
522



523
524

525 Figure 2 : Serological response from uninfected pigs and pigs experimentally infected with *M.*
 526 *hyopneumoniae* or *A. pleuropneumoniae* or both. Figure 2A: antibodies to *M. hyopneumoniae*, group
 527 1: uninfected pigs, groups 4, 6, 7, 8 and 9: infected with *M. hyopneumoniae*. Figure 2B: antibodies to
 528 *A. pleuropneumoniae*, group 1: uninfected pigs, groups 3, 5, 7, 8 and 9: infected with *A.*
 529 *pleuropneumoniae* (surviving pigs).