

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

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

3

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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

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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  $1.4 \times 10^8$  CFU of *A.*  
110 *pleuropneumoniae* strain 4915 . Pigs in groups 3, 7 and 9 were experimentally infected, in the same  
111 conditions, with  $1.3 \times 10^8$  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  $5 \times 10^9$  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  $-20^\circ\text{C}$  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  $\mu\text{L}$  of each sample placed on PPLOsup agar and incubated overnight at  $37^\circ\text{C}$  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<sup>-3</sup> 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  $\leq 0.05$ .

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  $1.4 \times 10^8$   
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  $1.3 \times 10^8$  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

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343

344

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429

429 Table 1: Experimental design, clinical and pathological observations

430 <sup>a</sup> D: Day

431 <sup>b</sup> UI: uninfected pigs with *A. pleuropneumoniae* (App) or *M. hyopneumoniae* (Mhp)

432 <sup>c</sup> No. of dead pigs

433 <sup>d</sup> Mortality observed on D1 to D2

434 <sup>e</sup> Pigs surviving after infection

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

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

438 <sup>h</sup> No. of pigs with pneumonia

439 <sup>i</sup> Maximum total score possible for each complete lung was 28

440 <sup>j</sup> No. of pigs with pleurisy

441 <sup>k</sup> Maximum total score possible for each complete lung was 4

442 <sup>l</sup> No. of pigs with fibrinous and haemorrhagic pleuro-pneumonia

443 <sup>m</sup> No. of pigs with pulmonary necrosis

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

445 <sup>o</sup> 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 <sup>a</sup> Necropsy days

451 <sup>b</sup> No. of pigs with microscopic lung lesions / No. of necropsied pigs

452 <sup>c</sup> Typical mycoplasmal lesions

453 <sup>d</sup> Typical lung lesions corresponding to the early phase of *A. pleuropneumoniae* infection

454 <sup>e</sup> Typical lung lesions corresponding to the chronic phase of *A. pleuropneumoniae* infection

455

456

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

458 <sup>a</sup> TO, Tonsil

459 <sup>b</sup> T, Tracheal

460 <sup>c</sup> 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 <sup>a</sup> )	UI <sup>b</sup>	App 4915	App 21	Mhp 116	-	-	App 21	Mhp 116	App 21 Mhp 116
Challenge at 10 weeks of age (D28 <sup>a</sup> )	UI	-	-	-	App 21	Mhp 116	Mhp 116	App 21	-
Mortality (D) <sup>c</sup>	0	7 (D1-D2 <sup>d</sup> )	3 (D1-D2)	0	0	0	1 (D2)	4 (D30-D31)	1 (D1)
Hyperthermia <sup>e,f</sup> (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) <sup>e,g</sup>	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 <sup>h</sup> (Mean score ± SD <sup>i</sup> )	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 <sup>j</sup> (Mean score ± SD <sup>k</sup> )	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 <sup>l</sup>	0	7	3	0	0	0	1	4	1
Pulmonary necrosis <sup>m</sup>	0	0	1	0	0	0	4	1	2
Hypertrophy of TBLN <sup>n</sup>	0	7	5	0	4	0	4	2	5
ADG (± SD) <sup>e,o</sup>	932 (±175)	-	816 (±257)	780 (±178)	791 (±222)	879 (±194)	798 (±262)	650 (±305)	737 (±246)

480 <sup>a</sup> D: Day

481 <sup>b</sup> UI: uninfected pigs with *A. pleuropneumoniae* (App) or *M. hyopneumoniae* (Mhp)

482 <sup>c</sup> No. of dead pigs

483 <sup>d</sup> Mortality observed on D1 to D2

484 <sup>e</sup> Pigs surviving after infection

485 <sup>f</sup> No. of pigs with hyperthermia (body temperature ≥40.5°C)

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

487 <sup>h</sup> No. of pigs with pneumonia

488 <sup>i</sup> Maximum total score possible for each complete lung was 28

489 <sup>j</sup> No. of pigs with pleurisy

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

494 <sup>o</sup> 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	-	-

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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 <sup>c</sup>	D1 and D2 <sup>a</sup>		0/7 <sup>b</sup>	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 <sup>d</sup>	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 <sup>e</sup>	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 <sup>a</sup> Necropsy days

500 <sup>b</sup> No. of pigs with microscopic lung lesions / No. of necropsied pigs

501 <sup>c</sup> Typical mycoplasmal lesions

502 <sup>d</sup> Typical lung lesions corresponding to the early phase of *A. pleuropneumoniae* infection

503 <sup>e</sup> Typical lung lesions corresponding to the chronic phase of *A. pleuropneumoniae* infection

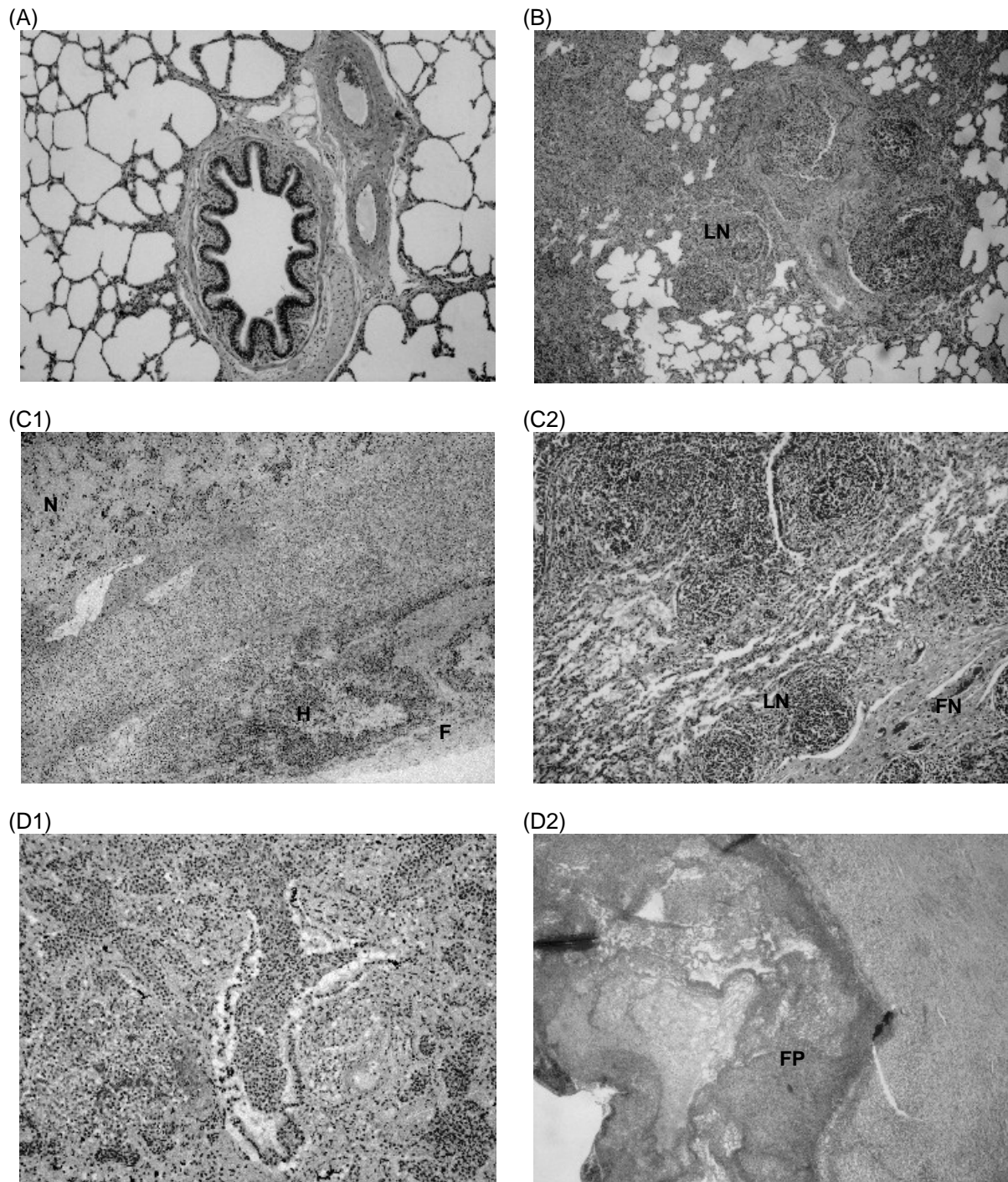
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505 Table 4: Bacteriological results (at time of necropsy)

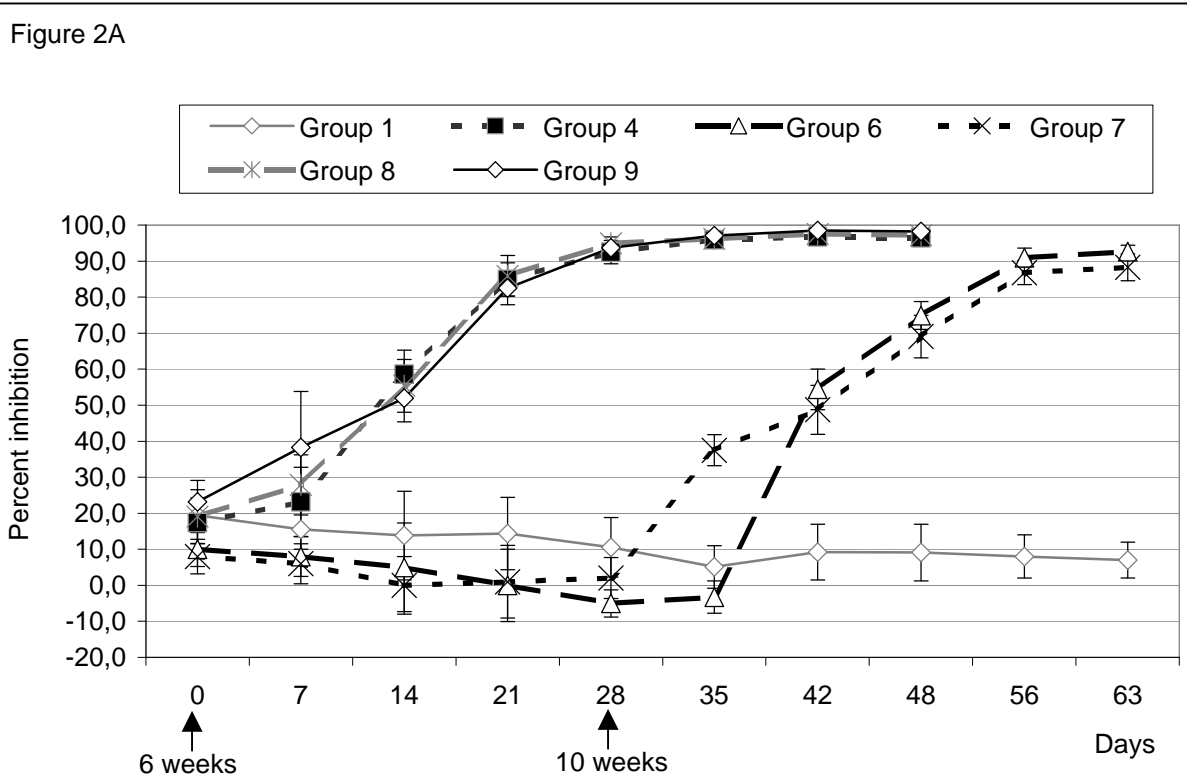
Samples	Groups of pigs (n=7)									
	1	2	3	4	5	6	7	8	9	
Challenge at 6 weeks of age (Day 0)	UI	App 4915	App 21	Mhp 116	-	-	App 21	Mhp 116	App 21 Mhp 116	
Challenge at 10 weeks of age (Day 28)	UI	-	-	-	App 21	Mhp 116	Mhp 116	App 21	-	
Number of positive Mhp cultures	TO <sup>a</sup>	0	0	0	4	0	3	5	4	4
	T <sup>b</sup>	0	0	0	7	0	7	6	7	7
	L <sup>c</sup>	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 <sup>a</sup> TO, Tonsil  
507 <sup>b</sup> T, Tracheal  
508 <sup>c</sup> L, Lung  
509  
510

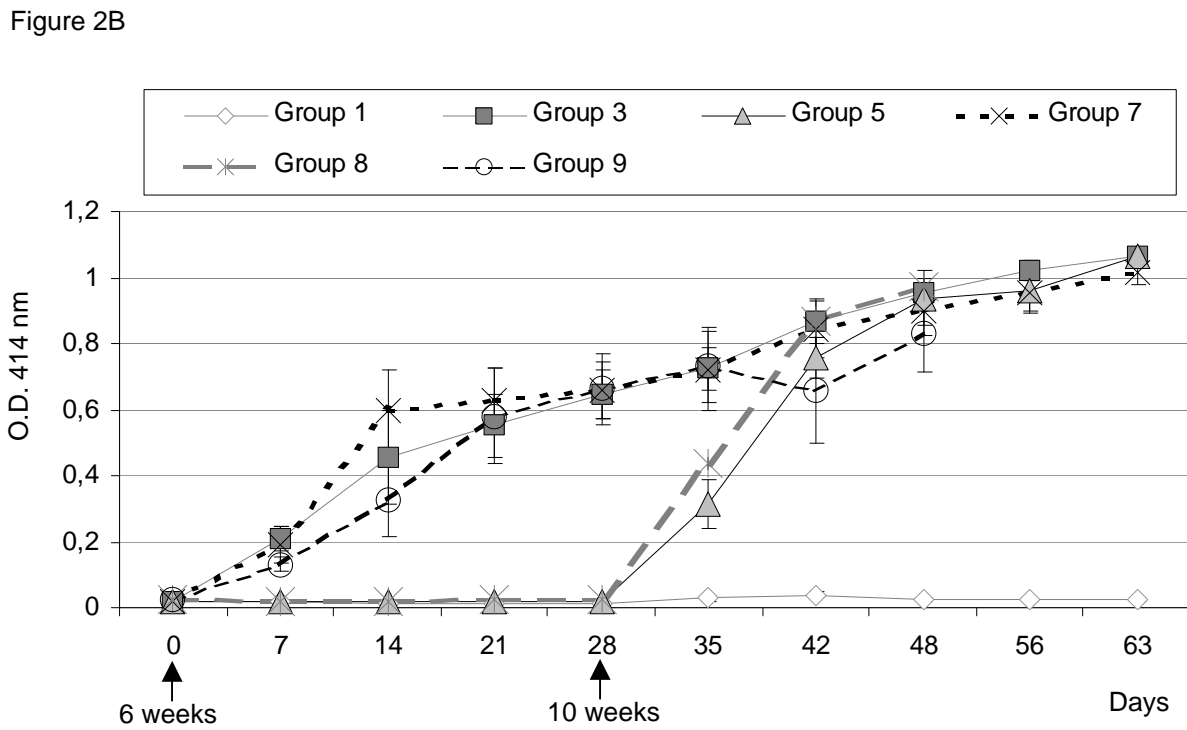


512 Figure 1: Microscopic examinations in the lungs of four pigs (LN: Lymphoid Nodules, N: Necrosis, H:  
 513 Haemorrhage, FE: Fibrinous Exudates, FN: Fibrosis around areas of Necrosis, FP: Fibrinous Pleuritis)  
 514 (A) Lung of a control pig (group 1) (x50)  
 515 (B) Lung of a pig infected with *M. 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).

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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).