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

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Abstract

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

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Bacterial strains and culture conditions

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

2.2. Experimental design

2.2.1. Experimental infections

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

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

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

2.2.2. Clinical monitoring

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

2.2.3. Samples and analysis

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

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

Swabs were collected from trachea, palatine tonsils and lungs, from all the pigs. The swabs were placed in 2 mL of Buffered Pepton Water Broth (samples). *A. pleuropneumoniae* was cultured from 50 μL of each sample placed on PPLOsup agar and incubated overnight at 37°C in 5% CO_2. *A.*

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

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

2.3. Statistical analysis

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

3. Results

3.1. Clinical signs and macroscopic lesions

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

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

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

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

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

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

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

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

No clinical signs or lung lesions were present in group 5, with the exception of tracheo-bronchial lymph 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. pleuropneumonia* 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

followed by interstitial pneumonia, lymphoid nodules associated with the airways and collapse of the alveoli. These two phases were noted in pigs infected with *M. hyopneumoniae*, independently of the day of necropsy. With the exception of two pigs that died, either firstly or simultaneously infected with *A. pleuropneumoniae* (groups 7 and 9), all other pigs developed typical mycoplasmal lesions. Microscopic examination did not reveal any differences between the lungs of pigs infected with *M. hyopneumoniae* alone (groups 4 and 6) or with both bacterial species (groups 7, 8 and 9).

Pigs infected with *A. pleuropneumoniae,* alone or in association with *M. hyopneumoniae*: the histopathologic changes of the lungs, in the early phase of *A. pleuropneumoniae* infection, were characterised by haemorrhage, vascular thrombosis, oedema, necrosis and the presence of fibrinous exudate. Acute lung lesions were apparent in similar numbers of pigs in groups 2, 3 and 8 but significant differences ($P \leq 0.05$) were observed between group 2 and groups 5, 7 and 9.

In the chronic phase, marked fibrosis around areas of necrosis and fibrinous pleuritis were observed. In group 5, two pigs without any macroscopic lung lesions, showed very mild and superficial interlobular fibrosis. One pig in group 9, with macroscopic pneumonia, did not show any microscopic lesions in the section observed. Similar numbers of pigs were affected in groups 3, 5, 7, 8 and 9.

Finally, no lesions were observed in the negative control group.

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

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

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

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

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

3.4. Serological results

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

4. Discussion

In our experimental conditions, SPF pigs intranasally inoculated, at six weeks of age, with $1.4x10^8$ CFU of *A. pleuropneumoniae* strain 4915 (serotype 9, isolated from an outbreak of porcine pleuropneumoniae), developed severe clinical signs as well as lung lesions typical of the acute phase of infection. All pigs died 24 to 48 h after experimental infection and *A. pleuropneumoniae* was recovered from their respiratory tract. These results are in accordance with those of our previous study showing that SPF pigs experimentally infected, at 10 weeks of age, with the same *A. pleuropneumoniae* strain, developed acute clinical signs and severe lung lesions (Jobert al., 2000). Nevertheless, in this first experiment with *A. pleuropneumoniae* strain 4915, the pigs received ampicillin to avoid death. Thus, surviving pigs showed lung lesions corresponding to the chronic phase of infection. According to our results in the present study, the sensitivity of pigs to *A. pleuropneumoniae* infection is probably age-related: six weeks *vs* ten weeks. Thus, the results obtained by Jobert et al. (2000) are in agreement with those obtained in the present study with *A.*

pleuropneumoniae strain 21 (also a serotype 9 producing ApxI and ApxII toxins): SPF pigs infected at 6 weeks of age with $1.3x10^8$ CFU of *A. pleuropneumoniae* showed very severe clinical signs and lung lesions, analogous to those induced with *A. pleuropneumoniae* strain 4915. On the other hand, lung lesions and symptoms were almost non-existent in the group of pigs infected at 10 weeks of age. Nevertheless, the ADG of the pigs was affected. In group 3, surviving pigs were seropositive three to four weeks after infection and all the pigs of group 5 seroconverted after two weeks. In both cases, the ELISA titres were similar and very high (mean OD almost 1) at the end of the experiment. These results are in accordance with those observed in the nucleus pig farm from which *A. pleuropneumoniae* strain 21 had been isolated. The young sows of this pig farm became contaminated, were *A. pleuropneumoniae* positive in the palatine tonsils and clearly seroconverted without any manifestation of *A. pleuropneumoniae* infection. This might be explained by the excellent housing conditions and hygienic environment of this particular breeding herd.

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

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

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

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Table 1: Experimental design, clinical and pathological observations

[a] D: Day

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

[c] No. of dead pigs

[d] Mortality observed on D1 to D2

[e] Pigs surviving after infection

[f] No. of pigs with hyperthermia (body temperature ≥40.5°C)

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

[h] No. of pigs with pneumonia

[i] Maximum total score possible for each complete lung was 28

[j] No. of pigs with pleurisy

[k] Maximum total score possible for each complete lung was 4

[l] No. of pigs with fibrinous and haemorraghic pleuro-pneumonia

[m] No. of pigs with pulmonary necrosis

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

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

Table 2: Planning of necropsies

Table 3: Microscopic lung lesions

[a] Necropsy days

[b] No. of pigs with microscopic lung lesions / No. of necropsied pigs

[c] Typical mycoplasmal lesions

[d] Typical lung lesions corresponding to the early phase of *A. pleuropneumoniae* infection

[e] Typical lung lesions corresponding to the chronic phase of *A. pleuropneumoniae* infection

Table 4: Bacteriological results (at time of necropsy)

[a] TO, Tonsil

[b] T, Tracheal

[c] L, Lung

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

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

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

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

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

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

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 haemorraghic 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)

[a] D: Day
[b] UI: uninfected pigs with *A. pleuropneumoniae* (App) or *M. hyopneumoniae* (Mhp)
[c] No. of dead pigs
[d] Mortality observed on D1 to D2
[e] Pigs surviving after infection
[f] No. of pigs with hyperthermia (body temperature ≥40.5°C)
[g] No. of pigs with coughing during the trial (Mean of coughing per day and per pig during the trial) (D0-D59)
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[k] Maximum total score possible for each complete lung was 4
[l] No. of pigs with fibrinous and haemorraghic pleuro-pneumonia
[m] No. of pigs with pulmonary necrosis
[n] No. of pigs with hypertrophy of tracheo-bronchial lymph node (TBLN)
[o] Average Daily Gain (g) of pigs per group during the trial (D0 to D49)

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	-	-

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		
	Total	*0/7*	*0/7*	*0/7*	*7/7*	*0/7*	*7/7*	*6/7*	*7/7*	*6/7*
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		
	Total	*0/7*	*7/7*	*3/7*	*0/7*	*0/7*	*0/7*	*1/7*	*4/7*	*1/7*
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		
	Total	*0/7*	*0/7*	*4/7*	*0/7*	*2/7*	*0/7*	*5/7*	*3/7*	*5/7*

[a] Necropsy days
[b] No. of pigs with microscopic lung lesions / No. of necropsied pigs
[c] Typical mycoplasmal lesions
[d] Typical lung lesions corresponding to the early phase of *A. pleuropneumoniae* infection
[e] Typical lung lesions corresponding to the chronic phase of *A. pleuropneumoniae* infection

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

		Groups of pigs (n=7)								
Samples		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
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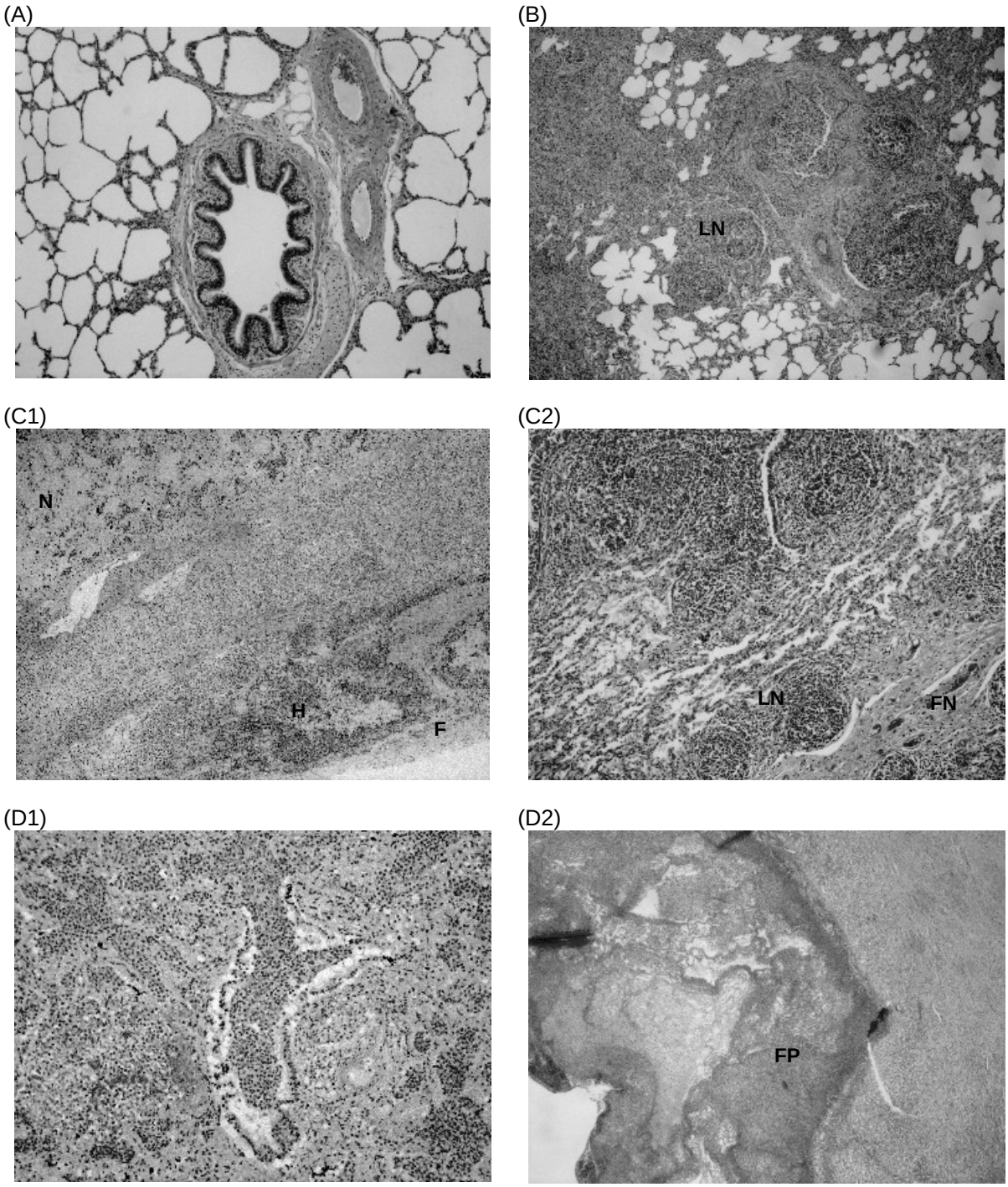

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519 weeks of age) (group 7). Chronic phase of infection (x50-Figure D1 and x25-Figure D2).

Figure 2A

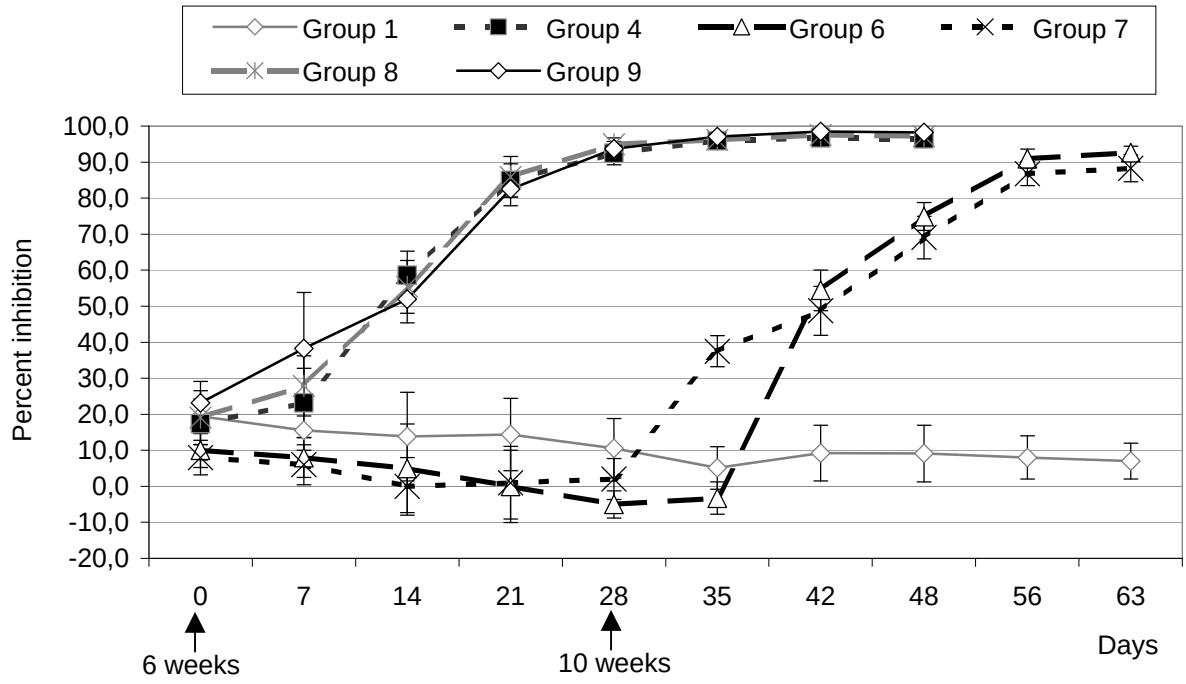
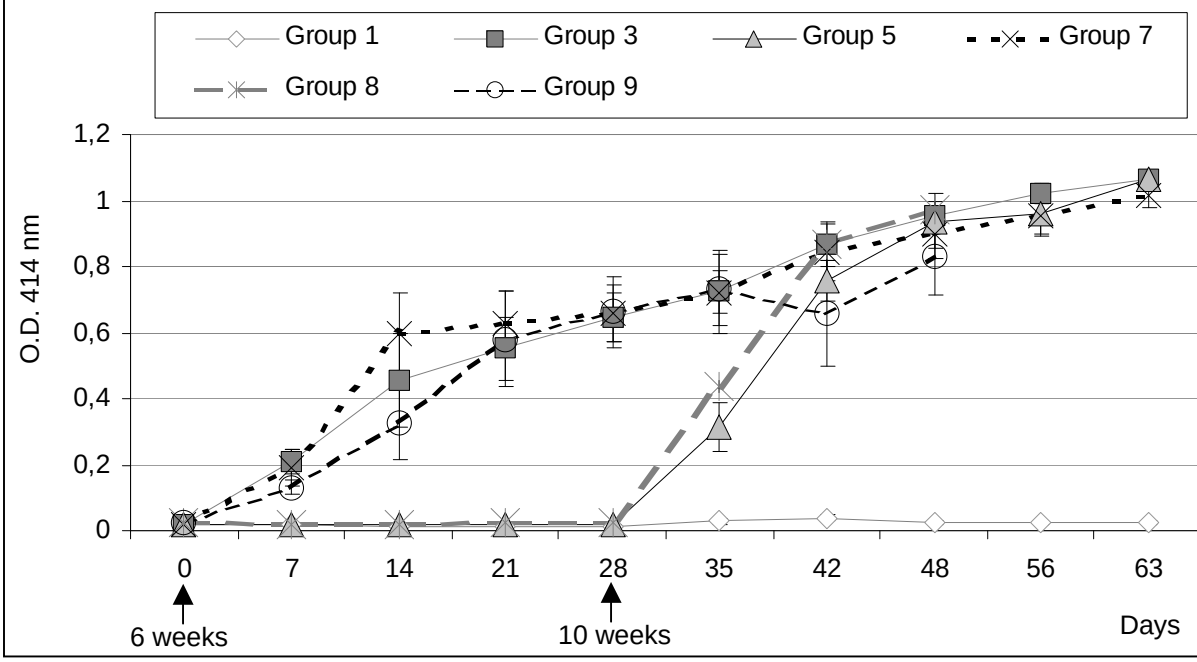


Figure 2B



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