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Pathogenicity study of *Mycoplasma hyorhinis* and *M. flocculare* in specific-pathogen-free pigs pre-infected with *M. hyopneumoniae*

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ABSTRACT

Mycoplasma (*M.*) *hyopneumoniae* is the initiator agent of the porcine respiratory disease complex (PRDC) and the etiological agent of enzootic pneumonia. *M. hyorhinis* and *M. flocculare* are also found in extensive gross pneumonia-like lesions, but their role is not known. We investigated the pathogenicity of *M. hyorhinis* and *M. flocculare* in specific-pathogen-free pigs pre-infected or not with *M. hyopneumoniae*. Mono-inoculated pigs with *M. flocculare* showed no clinical signs, hematological changes or macroscopic lesions upon necropsy. Mono-inoculated pigs with *M. hyorhinis* showed, overall seven days after inoculation, an increase in mean temperature with increases in white blood cell (monocyte) counts and in concentrations of pig major acute phase protein, whereas the average daily weight gain (ADWG) decreased compared with non-infected animals. *M. hyorhinis* was detected in serous membranes (polyserositis) but not in bronchi. Co-infected pigs with *M. hyopneumoniae* and *M. hyorhinis* or *M. flocculare* showed lower ADWG during the third week of the experiment and higher haptoglobin concentrations in contrast to pigs only mono-infected with *M. hyopneumoniae*. In pigs co-infected with *M. hyopneumoniae* and *M. hyorhinis*, it was interesting to observe that (i) *M. hyorhinis* was detected in bronchi of six pigs, (ii) *M. hyopneumoniae* was detected in polyserositis and (iii) there was a slight delay in the production of anti-*M. hyopneumoniae* IgG. The extent of pneumonia was not statistically different between groups. These results suggest that mycoplasmal associations appear to induce an additive effect and increase the inflammatory status in pigs, probably involving in the impairment of the immune system.

1. Introduction

The porcine respiratory disease complex (PRDC) is a multifactorial disorder, costly for swine industries worldwide, partly due to the simultaneous or sequential infection with various microorganisms. *M. hyopneumoniae* is the primary agent of PRDC; it modulates and impairs the host immune response, promoting superinfection by the PRDC-associated agents, such as viruses (e.g. the porcine reproductive and respiratory syndrome virus (PRRSV), the porcine circovirus type 2 (PCV2) or influenza viruses) or bacteria (e.g. *Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parasuis*) (Ciprian et al., 1988; Thacker et al., 1999; Opriessnig et al., 2004; Marois et al., 2009). *M. hyopneumoniae* is also the causative agent of mycoplasmal pneumonia, clinically characterized by moderate hyperthermia, coughing or anorexia, and by macroscopic catarrhal bronchopneumonia with cranio-ventral consolidation upon necropsy (Thacker, 2006). Enzootic chronic pneumonia is the progressive form of PRDC and involves other PRDC agents, leading to more severe clinical symptoms (fever, dyspnea) and extensive pulmonary lesions (Thacker and Minion, 2012). Despite progress in disease control strategies including enhanced management practices or the development of commercial vaccines, PRDC is still an economic issue, also affecting animal welfare and ultimately public health due to antimicrobial use (Thacker and Minion, 2012). Current studies tend to screen for the most threatening interactions between microorganisms to implement specific strategies to better control PRDC.

M. hyorhinis and *M. flocculare* inhabit the respiratory tract of pigs and are commonly found in lungs. A recent study showed that these

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mycoplasmal species are found in extensive gross pneumonia-like lesions in association with *M. hyopneumoniae*, but their role is not known (Fourour et al., 2018). *M. hyorhinis* is mainly described as causing serous inflammation, such as pleuritis, pericarditis and peritonitis (polyserositis) or arthritis and septicemia; it generally affects nursery pigs via direct transmission from sows (Thacker and Minion, 2012). In herds, infection with *M. hyorhinis* is associated with morbidity and weight loss or lameness (Lee et al., 2018; Thacker and Minion, 2012). The role played by *M. hyorhinis* in the development of pneumonia is controversial, although some authors maintain that this species can induce mild pneumonia in the absence of *M. hyopneumoniae* (Gois et al., 1971; Lin et al., 2006; Palzer et al., 2008; Luehrs et al., 2017). No commercial vaccine is available against *M. hyorhinis*; in general, infection is treated with antimicrobials (Martinson et al., 2018).

M. flocculare is also present in pig populations, but its prevalence is unknown. It is commonly described as a commensal agent of the respiratory tract, essentially in the nasal cavity (Thacker and Minion, 2012). *M. flocculare* and *M. hyopneumoniae* are closely related genetically, and share several virulence factors (e.g. adhesins, chaperones, hemolysin-related protein), raising the question of the potential role of *M. flocculare* as a PRDC agent (Calcutt et al., 2015; Siqueira et al., 2013; Stemke et al., 1992). However, this species is difficult to isolate, limiting knowledge on its prevalence and its pathogenicity.

The aim of this study was to determine (i) the pathogenicity of contemporary strains of *M. hyopneumoniae, M. hyorhinis* and *M. flocculare* isolated from gross pneumonia-like lesions in specific-pathogen-free (SPF) pigs, and (ii) to investigate the impact of a secondary infection by *M. hyorhinis* or *M. flocculare* in SPF pigs pre-infected with *M. hyopneumoniae*. We assessed clinical signs, macroscopic lung lesions, host immune response and identified and quantified the mycoplasmal species present.

2. Materials and methods

2.1. Mycoplasmal strains: isolation and growth conditions

Field strains of *M. hyopneumoniae* (Mhp699), *M. hyorhinis* (Mhr386) and *M. flocculare* (Mf18) were isolated from French slaughterhouses in 2016, as part of the Fourour et al. (2018) study in which lungs with severe gross pneumonia-like lesions were positive for at least two mycoplasmal species. Mhp699 and Mhr386 were isolated from the same herd (herd A), whereas Mf18 was isolated from another herd (herd B). Fifteen lungs derived from pigs in each of these herds were randomly scored for gross pneumonia-like lesions using the scoring protocol described in Madec and Kobisch (1982). In herd A, the mean pneumonia score was 10.9 ± 5.9/28 (minimum score: 3/28; maximum score: 21/28); pleuritis was observed in four lungs and no pericarditis was reported. Mhp699 and Mhr386 strains were isolated from two lungs without pleuritis, but with gross pneumonia-like lesions, with scores of 11/28 and 12/28, respectively. In herd B, the mean pneumonia score was 8.9 ± 5.3/28 (minimum score: 1/28; maximum score: 19/28) and no pleuritis was observed. *M. flocculare* was isolated from a lung with a gross pneumonia-like lesions score of 6/28.

For each strain, an initial volume of 1 ml of culture was prepared in Friis liquid medium and was incubated at 37 °C as described by Friis and Meyling (1983),until the beginning of the stationary phase, indicated by the change in the medium color from red to orange (due to the phenol red pH indicator). Then, fresh Friis liquid medium was added to the culture in a 10-fold dilution series and incubated, until a sufficient inoculum volume was obtained. Each inoculum concentration was determined by the dilution series. Therefore, the titers of mycoplasmal cultures were expressed as color-changing units per milliliter (CCU/ml) and obtained at about three weeks after the challenge. Mycoplasma identification was confirmed using species-specific multiplex qPCR (Fourour et al., 2018).

2.2. Experimental design

The experiment was performed in accordance with the animal welfare experimentation recommendations issued by the Direction Départementale de la Protection des Populations des Côtes d'Armor (ANSES registration no. C-22-754-1), and was approved by the ANSES/ENVA/UPEC ethics committee (ComEth authorization no 13/02/18-9, and authorization APAFIs #13353-2018020210309634). It was conducted in the porcine experimental unit at the ANSES Ploufragan Laboratory which produces SPF piglets in level-3 biosecurity and air-filtered facilities (Cariolet et al., 1994). These SPF pigs have good health status and are free of *M. hyopneumoniae, M. hyorhinis* and *M. flocculare* and of several respiratory pathogens, such as PRRSV, PCV-2, influenza virus, *A. pleuropneumoniae*, *P. multocida*, *Bordetella bronchiseptica* and *Haemophilus parasuis*. A total of 48 five-week-old SPF pigs were randomly assigned to six groups (Control, MHP, MHR, MF, MHPR and MHPF), and then distributed to six independent rooms containing eight pigs per room (n = 8). At six weeks of age, all pigs were intratracheally inoculated (5 ml) as previously described by Kobisch and Ross (1996), on two consecutive days, at 0 and 1 day post-inoculation (dpi). In total, the pigs received 10 ml containing 5.5×10^7 CCU of Mhp699 in rooms 2, 5 and 6 and 10 ml of Friis broth medium in rooms 1, 3 and 4. At seven weeks of age, at 7 and 8 dpi, all pigs intratracheally received 5.5×10^9 CCU of Mhr386 in rooms 3 and 5 (groups MHR and MHPR, respectively), 1×10^7 CCU of Mf18 in rooms 4 and 6 (MF and MHPF groups, respectively) and Friis broth in rooms 1 and 2 (Control and MHP groups, respectively). The experimental design is summarized in Table 1.

2.3. Clinical evaluation

Two days prior to the challenge and throughout the experiment, pigs were monitored daily for clinical signs such as lameness, dyspnea and hyperthermia (normal rectal temperature 39.5 °C in SPF pigs; hyperthermia declared at ≥40.1 °C). Episodes of coughing were counted daily during a 15 min observation period. Body weight was also recorded each week during the trial (at 0, 7, 14, 21 and 28 dpi). The average daily weight gain (ADWG) was estimated according to the

Table 1
Experimental design.

Room	Group	Number of pigs (n)	Intratracheal route	
			at 0 and 1 dpi[a]	at 7 and 8 dpi
1	Control	8	Friis medium	Friis medium
2	MHP	8	*M. hyopneumoniae strain 699*	Friis medium
3	MHR	8	Friis medium	*M. hyorhinis strain 386*
4	MF	8	Friis medium	*M. flocculare strain 18*
5	MHPR	8	*M. hyopneumoniae strain 699*	*M. hyorhinis strain 386*
6	MHPF	8	*M. hyopneumoniae strain 699*	*M. flocculare strain 18*

[a] dpi: day post-inoculation.

following formula: (1000*(body weight at week B – body weight at week A)/(days elapsed between week A and B)), week A earlier than week B.

2.4. Collection sample in live and euthanized pigs

Blood samples were collected from all pigs before inoculation, at -2 dpi in the Control, MHP, MHPR and MHPF groups and at 5 dpi in the MHR and MF groups. Post-inoculation blood samples were collected from all pigs at 2 dpi in the Control, MHP, MHPR and MHPF groups; and at 5, 7 and 9 dpi in the MHR, MF, MHP, MHPR and MHPF groups; and then at 13, 15, 19, 22 and 29 dpi in all groups. All blood samples were collected in dry tubes and EDTA tubes for serological and hematological analyses, respectively. The sera were stored at −20 °C.

Tracheal swabs were collected at 6 dpi in groups inoculated by *M. hyopneumoniae* (MHP, MHPR and MHPF). Then, tracheal (T), palatine tonsil (To) and nasal cavity (N) swabs were performed in all live pigs at 13, 15, 19, 22 and 29 dpi. “CytoBrushes” (CML, Nemours, France) were used for the nasal cavities and tonsils. Tracheas were swabbed with a sterile catheter used for tracheal intubations (Ateliers Cloup, Champigny sur Marne, France) as described in Fablet et al. (2010).

All the pigs were necropsied on 33–36 dpi. Sampling (T, To and N swabs) was performed immediately after euthanasia. Liver and spleen swabs were systematically performed for each animal before opening the rib cage. Post-mortem examinations were carried out on each animal. Lungs were extracted (i) to score pneumonia as described in Madec and Kobisch (1982) and pleuritis as described in Ostanello et al. (2007), (ii) to perform swabs, after parallel tissue incisions, in the right middle lung lobes (with pneumonia or in healthy tissue without macroscopic lesions), in the tracheobronchial lymph nodes and in the other tissues with macroscopic lesions.

2.5. Hematological and serological analyses

Red blood cells, white blood cells (leucocytes, lymphocytes) and platelets were counted from EDTA blood samples (homogenized for 1 h) using an MS9.5 hematology analyzer (Melet Schloesing Laboratoires, La Chaux de Fonds, Switzerland).

The serum concentrations of porcine acute phase inflammatory proteins—haptoglobin and pig major acute phase protein (pig MAP)—were measured with two commercial ELISA kits according to the manufacturer’s instructions (Tridelta Development Limited, Eurobio, Les Ulis, France and Kitvia, Labarthe-Inard, France, respectively). The presence of antibodies against *M. hyopneumoniae* was tested with the competitive inhibition enzyme-linked immunosorbent assay (ELISA) Oxoid™ *Mycoplasma hyopneumoniae* detection kit (Thermo Fisher Diagnostics, Dardilly, France). The inhibition percentage for each serum was calculated with the following formula: percent inhibition = 100 − [100 × (sample mean optical density (OD) / buffer control mean OD)], a sample was classified as positive if the inhibition percentage was > 50%.

2.6. Bacteriological analyses

Each swab was suspended in 2 ml of buffered peptone water broth and 500 µl were centrifuged (12,000 × *g*; 15 min; 4 °C) and resuspended in a mixture of 400 µl of lysis solution made up according to the description in Kellog and Kowk (1990), with a slight modification described in Fourour et al. (2018). All samples were directly analyzed for *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* quantification using multiplex qPCR and by extrapolating from reactions obtained with known concentrations of *M. hyopneumoniae* ATCC25095 or *M. hyorhinis* ATCC25021 or *M. flocculare* ATCC27399 DNA (three standard curves) (Fourour et al., 2018).

2.7. Statistical analysis

The data obtained for each group that followed a normal distribution with homogeneity of variance were compared simultaneously using both the ANOVA and Tukey tests. The Kruskal-Wallis and Kolmogorov-Smirnov tests were performed on variables without a normal distribution or showing heterogeneity of variance. Differences between the samples collected in live pigs (T, To, N) were assessed using a Fisher exact test ($n \leq 5$) or a chi-square test ($n > 5$) of independence in 2 by 2 tables. These tests were carried out with the Systat 9.0 program for Windows (Systat Software GmbH, Erkrath, Germany). Differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Validation of the experiment by the control group

All the pigs in the control group were negative for clinical signs, macroscopic lesions, *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* DNA upon qPCR multiplex tests and anti-*M. hyopneumoniae* IgG antibodies.

3.2. Clinical data

Quantitative data of rectal temperatures, ADWG and number of coughs were averaged over four time intervals: first (0–7 dpi), second (8–14 dpi), third (15–21 dpi) and fourth (22–30 dpi) week post-inoculation, as described in Table 2. For the first week post-inoculation, all data were split into two groups: (i) data obtained from all 24 *M. hyopneumoniae*-infected pigs (pigs in the MHP, MHPR and MHPF groups) and (ii) data of the remaining 24 pigs that received the Friis medium (pigs in the Control, MHR and MF groups). For the other time intervals, data were obtained from 8 pigs in each group.

The body temperature means in the MHP, MF, MHPR and MHPF groups were normal throughout the experiment; although, hyperthermia was observed in one pig in the MHP group at 2 dpi (40.2 °C) and in one pig in the MHPR group at 19 dpi (40.9 °C) and 20 dpi (40.6 °C), with no significant differences from the Control group. During the second week of the experiment, the mean temperature was significantly higher in the MHR group (39.5 ± 0.1 °C) than in the MHP group (39.2 ± 0.1 °C) or MHPR group (39.2 ± 0.1 °C) ($p = 0.024$ and $p = 0.031$, respectively). Four pigs effectively showed hyperthermia from 11 to 15 dpi (rectal temperatures ranged from 40.1 to 40.6 °C).

No coughing was observed in the MHR and MF groups. However, all pigs belonging to the three groups infected with *M. hyopneumoniae* (MHP, MHPR and MHPF) started coughing from 10 dpi and continued until the end of the experiment. The number of coughs counted for 15 min was higher during the third week after inoculation for the MHP and MHPF groups (i.e. 10.6 ± 2.8 and 9.8 ± 5.4, respectively), than in the MHPR group (i.e. 3.8 ± 1.8) ($p = 0.049$).

During the first week after inoculation, ADWG was significantly lower in the three groups infected with *M. hyopneumoniae* compared with the other non-infected groups ($p = 0.021$). At the second week after inoculation, ADWG was significantly lower in the group MHR (682 ± 172 g) than in the Control group (934 ± 54 g) and MF group (945 ± 43 g) ($p = 0.094$ and 0.073, respectively). At the third week after inoculation, significantly lower ADWG was observed in the MHPR and MHPF groups than in the Control and MF groups ($p < 0.05$). At the week four after inoculation, ADWG was similar in all groups.

3.3. Hematological and serological data

Hematological and serological data are presented in Figs. 1 and 2. Until 7 dpi, data were averaged by pigs infected or not by *M. hyopneumoniae* (two groups of 24 pigs). From 9 dpi, data were averaged by group ($n = 8$).

Table 2
Body temperatures, cumulative coughing and average daily weight gain (ADWG) in each group (see Table 1 for their composition) during the first (0–7 days post-inoculation (dpi)), second (7–14 dpi), third (14–21 dpi) and fourth (21–30 dpi) week after the first inoculation. Different superscript letters (A, B) indicate statistically significant differences between groups within a time interval.

	Control group	MHP	MHR group	MF group	MHPR group	MHPF group
Body temperature (°C)[a]						
0–7 dpi	39.4 ± 0.1^{A}	39.3 ± 0.2^{A}	39.4 ± 0.1^{A}	39.4 ± 0.1^{A}	39.3 ± 0.2^{A}	39.3 ± 0.2^{A}
8–14 dpi	39.3 ± 0.1^{A,B}	39.2 ± 0.1^{A}	39.5 ± 0.1^{B}	39.4 ± 0.1^{A,B}	39.2 ± 0.1^{A}	39.3 ± 0.1^{A,B}
15–21 dpi	39.2 ± 0.1^{A}	39.2 ± 0.1^{A}	39.3 ± 0.1^{A}	39.2 ± 0.1^{A}	39.3 ± 0.0^{A}	39.3 ± 0.2^{A}
22–28 dpi	39.,2 ± 0.1^{A}	39.2 ± 0.1^{A}	39.2 ± 0.1^{A}	39.1 ± 0.1^{A}	39.3 ± 0.0^{A}	39.2 ± 0.1^{A}
Coughs[b]						
0–7 dpi	NC[c]	NC	NC	NC	NC	NC
8–14 dpi	NC	2.5 ± 2.1^{A}	NC	NC	3.8 ± 2.2^{A}	2.0 ± 1.6^{A}
15–21 dpi	NC	10.6 ± 2.8^{A}	NC	NC	3.8 ± 1.8^{B}	9.8 ± 5.4^{A}
22–28 dpi	NC	4.2 ± 0.4^{A}	NC	NC	3.8 ± 1.9^{A}	5.8 ± 2.5^{A}
ADWG						
0–7 dpi	906 ± 50^{A}	755 ± 103^{B}	906 ± 50^{A}	906 ± 50^{A}	755 ± 103^{B}	755 ± 103^{B}
8–14 dpi	934 ± 54^{A}	852 ± 76^{AB}	682 ± 172^{B}	945 ± 43^{A}	723 ± 102^{AB}	859 ± 35^{AB}
15–21 dpi	984 ± 82^{A}	795 ± 64^{AB}	941 ± 101^{AB}	988 ± 42^{A}	734 ± 115^{B}	743 ± 56^{B}
22–28 dpi	965 ± 92^{A}	993 ± 66^{A}	1039 ± 92^{A}	1025 ± 27^{A}	893 ± 97^{A}	917 ± 86^{A}

[a] Average body temperature (°C).
[b] Average number of coughs per group during a 15 min observation period.
[c] NC: No coughs recorded.

White blood cell counts were lower at 9 dpi in the MHR and MHPR groups than in the Control group ($p = 0.004$), and then progressively increased in the MHR group until 15 dpi, exceeding those in the Control group ($p = 0.05$). At 9 dpi, blood lymphocyte cell counts were lower in the MHR group compared with all other groups, except the MHPR group ($p < 0.005$). Thereafter, lymphocyte counts were statistically similar to all other groups at 13 dpi. At 9 dpi, blood monocyte cell counts were lower in the MHPR group than in the MHP and MHPF groups ($p = 0.004$), but significantly higher than in the Control, MHP and/or MHPF groups at 13 and 19 dpi ($p = 0.029$ and $p = 0.022$, respectively).

Platelet cell counts were higher in pigs infected with *M. hyopneumoniae* at 5 and 7 dpi than in non-infected pigs ($p = 0.002$ for both dpi). A significantly higher platelet count was also observed in the MHP and MHPF groups at 9 dpi than in the Control group ($p = 0.008$) and at 29 dpi in the MHPR group ($p = 0.049$).

Serum pig MAP concentrations were higher in the MHR group than in all other groups at 13 dpi and in all groups except MHPR at 19 dpi ($p = 0.004$ and $p = 0.035$, respectively) (Fig. 2). Average concentrations reached 2.9 ± 1.0 mg/ml (13 dpi) and 2.0 ± 0.8 mg/ml (19

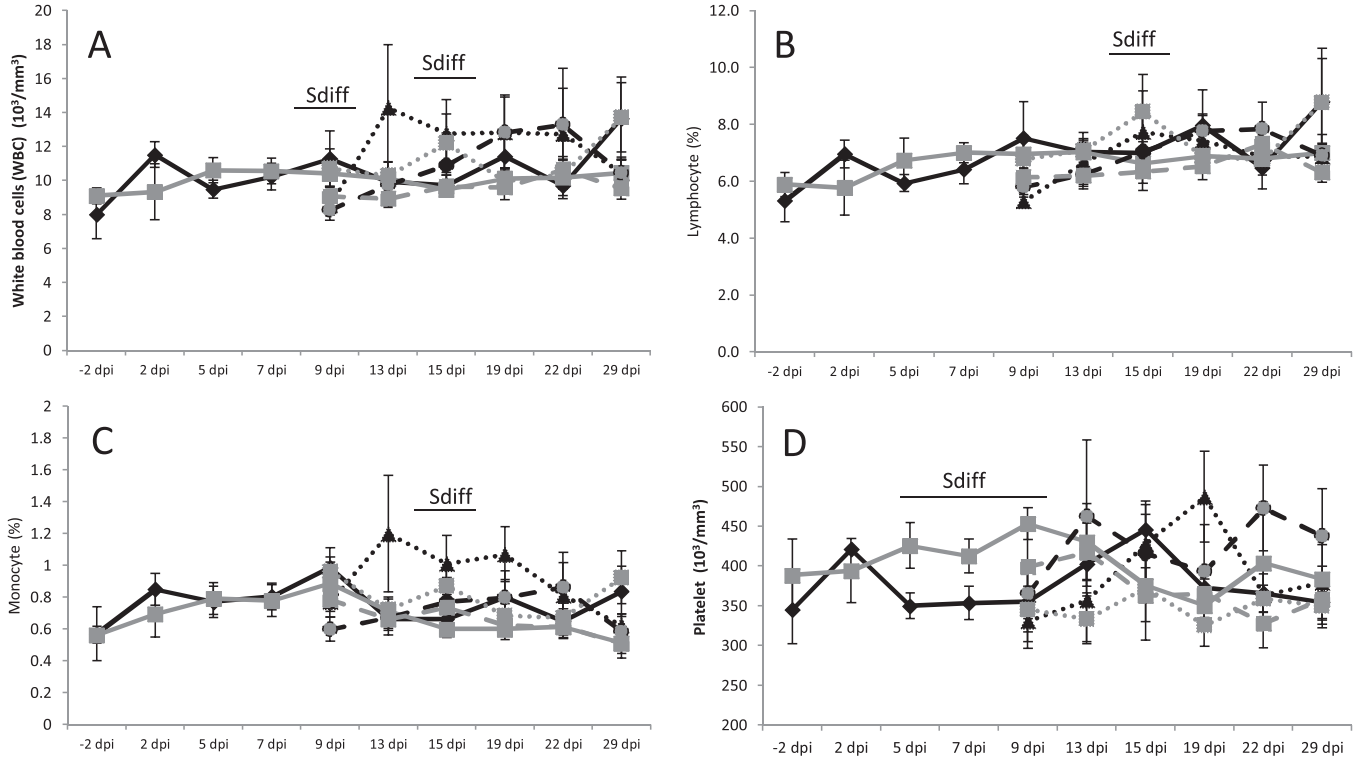

Fig. 1. Blood cell counts (A. white blood cells, B. lymphocytes, C. monocytes and D. platelets), from -2 dpi to7 days post-inoculation (dpi), data from all pigs infected with *M. hyopneumoniae* (solid gray lines and squares, MHP, MHPR and MHPF groups) and from non-infected pigs (solid black lines and diamonds, Control, MHR and MF groups). From 7 dpi, data were grouped by groups: Control (solid black lines), MHP (solid gray lines), MHR (dashed black lines and triangles), MF (dashed gray lines and squares), MHPR (dotted black lines) and MHPF (dotted gray lines). Sdiff indicates a statistically significant difference between groups on that day.

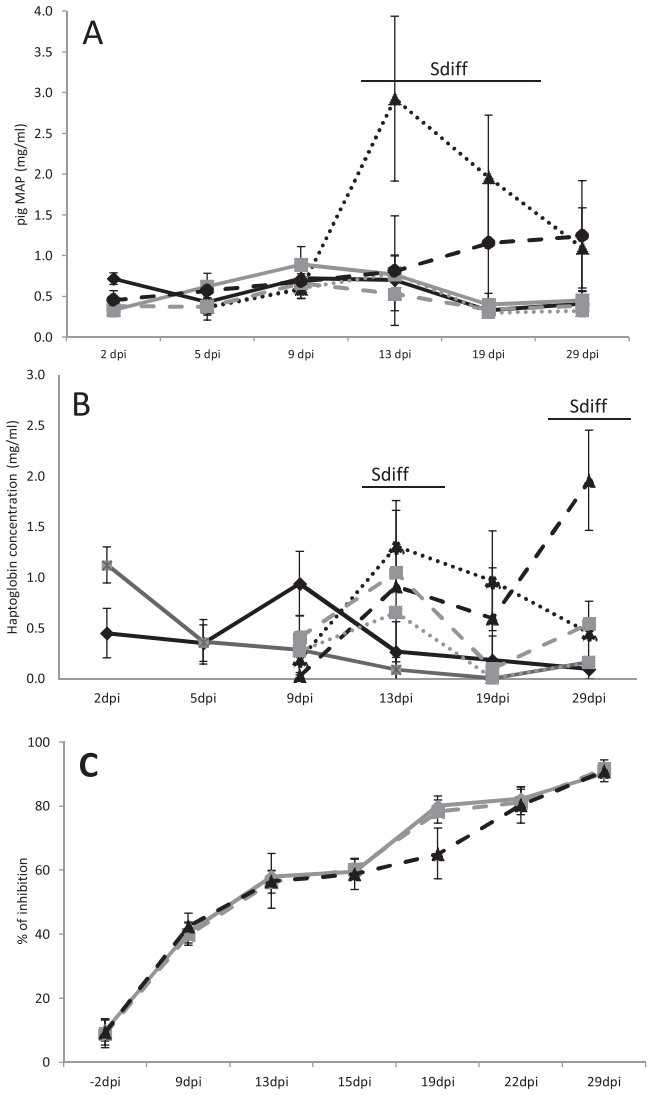

Fig. 2. Evolution of pig MAP (A) and haptoglobin (B) acute phase protein concentrations and anti-*M. hyopneumoniae* IgG production (C) for each group: Control (black lines), MHP (gray lines), MHR (dashed black lines), MF (dashed gray lines), MHPR (dotted black lines) and MHPF (dotted gray lines). Sdiff means a statistically significance between groups at corresponding day.

dpi), a four- and six-fold increase, respectively, compared with the Control group. Serum haptoglobin concentrations were higher in pigs infected with *M. hyopneumoniae* than in non-infected pigs at 2 dpi ($p = 0.001$). The average concentration reached 1.1 ± 0.2 mg/ml, meaning a roughly 2.5-fold increase compared with non-infected pigs (0.4 ± 0.2 mg/ml). At 13 dpi, concentrations in the MHR (1.3 ± 0.4 mg/ml), MHPF (1.1 ± 0.4 mg/ml) groups were higher than in the MHP group (0.1 ± 0.1 mg/ml) ($p = 0.049$), with an increase about 10- to 14-fold. At 19 dpi, serum haptoglobin concentrations were higher in the MHPR group (1.9 ± 0.5 mg/ml) than in the Control group (0.2 ± 0.1 mg/ml), with an increase about 10-fold ($p < 0.001$).

The production of anti-*M. hyopneumoniae* IgG antibodies was observed in all pigs infected with *M. hyopneumoniae*, from 13 dpi (groups: MHP = 58.0 ± 1.7% of inhibition, MHPR = 56.7 ± 8.5% of inhibition and MHPF = 56.3 ± 3.4% of inhibition) to 29 dpi (groups: MHP = 90.2 ± 3.7% of inhibition, MHPR = 90.9 ± 5.7% of inhibition and MHPF = 91.7 ± 3.9% of inhibition) without statistically significant differences between the three groups. However, a trend for delayed production was observed for the MHPR group (65.2 ± 7.8% of inhibition) compared to the MHP (80 ± 3% of inhibition) and MHPF (78.3 ± 3.6% of inhibition) groups at 19 dpi ($p = 0.071$ and 0.056, respectively). Sera collected at 29 dpi from non-infected pigs also tested negative; results showed no production of anti-*M. hyopneumoniae* IgG antibodies (Control, 17.5 ± 4.8% of inhibition; MHR, 17.0 ± 3.4% of inhibition; MF, 12.0 ± 4.2% of inhibition).

3.4. Bacteriological results of samples collected from live animals

Detection and quantification of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* using multiplex qPCR on swabs collected from all live pigs are presented in Table 3.

M. hyopneumoniae was detected in all trachea (48/48), 93% of tonsil (37/40) and in 35% of nasal (14/40) swabs in the MHP group, over all sampling times. The total number of positive trachea and tonsils in the MHPR (T: 93%, 37/48; To: 92%, 44/40) and MHPF (T: 94%, 45/48; To: 98%, 39/40) groups were not statistically different from the MHP group. However, a statistically lower number of positive nasal swabs was observed in the MHPR (8%, 3/40) and MHPF (20%, 8/40) groups than in the MHP group ($p < 0.001$). Comparing the three challenged groups, no statistically significant differences were found in the amount of *M. hyopneumoniae* at different sampling times in the trachea.

M. hyorhinis was detected in 81% (39/48) of trachea, 95% of tonsils (38/40) and 98% (39/40) nasal swabs in the MHR group, over all sampling times. The total numbers of positive tonsil (80%, 32/40) and nasal (98%, 39/40) swabs in the MHPR group were not significantly different from those of the MHR group. However, a significantly lower number of positive tracheal swabs was observed in the MHPR group than in the MHR group ($p < 0.001$): only 33% (16/48) of swabs tested positive. At different sampling times, no significant differences were found in the amount of *M. hyorhinis* in the trachea in the two challenged groups, although there was a trend for a lower amount at 19 dpi ($p = 0.071$).

M. flocculare was detected in 63% (30/48) of trachea, 68% (27/40) tonsils and 75% (30/40) nasal cavity swabs in the MF group, over all sampling times. A significantly lower number of positive trachea (0%) and tonsil (5%, 2/40) swabs was observed in the MHPF group than in the MF group ($p < 0.001$). However, the total number of positive nasal swabs (65%, 26/40) in the MHPF group was not statistically different from the MF group.

3.5. Macroscopic lesions

Macroscopic lesions observed in each group at necropsy are presented in Table 4.

Pneumonia was observed in all pigs infected by *M. hyopneumoniae* (pigs from the MHP, MHPR and MHPF groups). Pneumonia scores ranged from 7.1 ± 1.6 (MHP), 9.0 ± 2.1 (MHPR) to 9.8 ± 3.4 (MHPF), without any statistically significant differences. Considering all pigs with pneumonia, a minimum score of 2/28 was observed in the MHPR and MHPF groups, and a maximum score of 20/28 was observed in the MHPF group. Interestingly, in one pig in the MHR group that showed a pneumonia-like lesion (score 1/28), histopathological analysis of the lung lesion confirmed an interstitial pneumonia infection (data not shown, analyses performed by Labocea, Ploufragan, France).

Pleuritis was observed in pigs inoculated by *M. hyorhinis* in the MHR (4/8) and MHPR (2/8) groups, with a mean lesion score of 1.6 ± 0.9/4 and 0.8 ± 0.7/4, respectively, without any statistically significant differences. In the MHR group, among the four pigs with pleuritis, three also showed pericarditis and one of them showed both pericarditis and peritonitis. In the MHPR group, two pigs showed both pleuritis and pericarditis.

3.6. Bacteriological results of samples collected in euthanized pigs

Results of detection and quantification of *M. hyopneumoniae*, *M.*

Table 3
Detection and quantification of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* using multiplex qPCR on tracheal (T), palatine tonsils (To) and nasals (N) swabs collected in all live or necropsied pigs, and lung (L), liver, spleen and tracheobronchial lymph node (TBLN) swabs collected from necropsied pigs.

		M. hyopneumoniae qPCR						*M. hyorhinis* qPCR				*M. flocculare* qPCR			
		MHP group		MHPR group		MHPF group		MHR group		MHPR group		MF group		MHPF group	
		n[b]	Amount (g.e./ml)[c]	n	Amount (g.e./ml)	n	Amount (g.e./ml)	n	Amount (g.e./ml)	n	Amount (g.e./ml)	n	Amount (g.e/ml)	n	Amount (g.e/ml)
6 dpi[a]	T	8	6.4 ± 0.5	6	6.1 ± 1.0	8	6.3 ± 0.7	NS		NS		NS		NS	
	To	NS[e]		NS		NS		NS		NS		NS		NS	
	N	NS		NS		NS		NS		NS		NS		NS	
13 dpi	T	8	7.7 ± 1.1	8	7.5 ± 0.6	8	8.1 ± 0.2	8	5.7 ± 0.7	6	4.9 ± 1.6	1	4.1	0	
	To	7	7.1 ± 1.0	8	6.0 ± 1.5	8	6.0 ± 1.1	7	5.1 ± 0.5	7	4.8 ± 1.1	1	4.7		
	N	5	5.0 ± 0.6	0		3	3.9 ± 1.1	7	5.6 ± 1.0	8	4.6 ± 0.7	0		1	3.9
15 dpi	T	8	7.7 ± 0.8	8	7.5 ± 0.7	8	7.8 ± 0.5	8	5.9 ± 1.2	2	5.0 ± 1.2	6	4.5 ± 0.9	0	
	To	7	6.7 ± 0.9	7	5.5 ± 0.6	8	5.6 ± 1.3	8	5.6 ± 0.7	7	5.1 ± 0.7	8	3.6 ± 1.1		
	N	1	4.6	0		0		8	5.3 ± 0.3	8	5.2 ± 0.3	6	4.3 ± 0.6	3	4.5 ± 0.4
19 dpi	T	8	7.1 ± 0.6	7	7.3 ± 0.5	8	7.2 ± 0.5	7	5.8 ± 0.6	2	3.8 ± 2.0	8	5.4 ± 0.8	0	
	To	8	6.2 ± 0.8	8	5.9 ± 0.8	7	5.7 ± 0.5	8	5.6 ± 0.6	6	5.3 ± 1.2	8	3.7 ± 1.0		
	N	3	3.5 ± 0.7	2	5.6 ± 2.2	2	3.9 ± 0.5	8	5.2 ± 0.5	8	5.4 ± 0.5	8	5.2 ± 0.6	7	4.6 ± 0.7
22 dpi	T	8	7.5 ± 0.6	8	7.3 ± 0.9	8	7.4 ± 0.7	8	5.5 ± 0.5	3	5.2 ± 1.2	7	5.0 ± 0.9	0	
	To	8	6.2 ± 0.8	7	5.6 ± 1.2	8	6.0 ± 0.9	8	4.6 ± 0.9	7	5.7 ± 0.6	3	4.0 ± 0.4		
	N	2	4.8 ± 0.6	0		1	5.1	8	4.8 ± 0.4	8	5.6 ± 0.5	8	5.2 ± 0.5	8	4.6 ± 1.0
29 dpi	T	8	7.3 ± 1.0	7	7.4 ± 0.9	2	5.5 ± 1.3	8	4.8 ± 0.7	3	5.0 ± 1.1	8	5.1 ± 1.0	0	
	To	7	6.4 ± 1.4	7	5.8 ± 1	5	7.8 ± 0.3	7	4.7 ± 0.6	5	4.4 ± 1.0	7	4.3 ± 0.8	2	4.5 ± 0.4
	N	3	6.2 ± 1.6	1	7.5	4	7.1 ± 1.2	8	5.4 ± 0.5	7	4.9 ± 0.4	8	6.1 ± 0.4	7	5.4 ± 1.0
Necropsy[d]	T	8	7.5 ± 0.6	8	7.5 ± 0.6	8	7.7 ± 1.0	8	5.1 ± 1.0	0		8	5.7 ± 0.6	1	6.4
	To	8	5.9 ± 1.0	7	6.1 ± 0.9	7	6.1 ± 1.2	5	4.6 ± 0.5	5	5.0 ± 0.8	3	3.4 ± 0.9	1	5.4
	N	3	6.5 ± 0.4	2	6.7 ± 1.9	2	8.0 ± 0.2	7	5.0 ± 0.5	7	5.0 ± 0.6	8	5.8 ± 0.3	7	6.7 ± 0.5
	L	8	7.2 ± 0.6	8	7.0 ± 0.6	8	7.3 ± 0.5	1	3.4	0		7*	5.1 ± 0.6	0	
	TBLN	1	4.4	0		2	5.4 ± 1.1	1	4.5	0		0		0	
	Liver	0		0		0		1	4.3	0		0		1	5.1
	Spleen	0		0		0		1	5.4	0		0		0	

[a] dpi, day post-inoculation.
[b] Number of positive samples by qPCR multiplex (out of eight pigs).
[c] Mean amount of genome equivalents (g.e.) quantified by qPCR multiplex/ml of sample (log_{10}) ± standard deviation.
[d] Necropsy performed from 33 to 36 dpi.
[e] NS, not sampled.

Table 4
Mean, maximum and minimum pneumonia and pleuritis scores; and other macroscopic lesions observed at necropsy. Pneumonia was scored on a scale with a maximum of 28, and pleuritis on a scale with a maximum of 4. Different letters (A,B) represent statistically significant differences between groups.

Macroscopic lesions	*MHP group*	MHR group	MHPR group	MHPF group
Pneumonia (n)	8/8	1/8	8/8	8/8
Mean ± SD score (/28)	7.1 ± 1.6^{A}	0.1 ± 0.2^{B}	9.0 ± 2.1^{A}	9.8 ± 3.4^{A}
Maximum score (/28)	12	1	15	20
Minimum score (/28)	4	0	2	2
Pleuritis (n)	0/8	4/8	2/8	0/8
Mean ± SD score(/4)	–	1.6 ± 0.9 ^{A}	0.8 ± 0.7 ^{A}	–
Maximum score (/4)	–	4	3	–
Minimum score (/4)	–	0	0	–
Pericarditis (n)	–	3/8	2/8	–
Peritonitis (n)	–	1/8	–	–

hyorhinis and *M. flocculare* using multiplex qPCR on swabs collected in all euthanized pigs are presented in Table 3.

Similar to results obtained from live animals, *M. hyopneumoniae* was detected preferentially in the trachea and tonsils of necropsied animals with at least seven positive swabs out of eight animals in the MHP, MHPR and MHPF groups. However in the MHPF group, less than three nasals swabs were positive. *M. hyopneumoniae* was also detected in the lungs of all infected pigs, in the tracheobronchial lymph nodes of two pigs in the MHP and MHPF groups, and four pigs in the MHPR group. *M. hyopneumoniae* was not detected in the liver or spleen of any infected animal. In the MHPR group, *M. hyopneumoniae* was detected in pigs that showed pleuritis, pericarditis and stomach ulcers. No statistically significant differences were found among the three challenged groups in the amount of *M. hyopneumoniae* at different sampling times.

M. hyorhinis was detected in the trachea of all pigs in the MHR group, whereas no positive swabs were observed in the MHPR group. For the tonsil and nasal swabs, the same number of positive samples was found in the MHR and MHPR groups (To: 5/8 and N: 7/8). In contrast to the MHPR group with all negative samples, *M. hyorhinis* was detected in the lung, liver, spleen and tracheobronchial lymph nodes in only one pig in the MHR group (not necessarily the same pig). *M. hyorhinis* was also detected in two pigs with pleuritis in the MHR group and on pig with pericarditis in the two infected groups. No statistically significant differences were found between the two challenged groups in the amount of *M. hyorhinis* at the different sampling times.

M. flocculare was detected in the trachea and nasal cavities of all pigs in the MF group. In this group, only three tonsil swabs were positive. Seven lung sections were also positive in the MF group. In the MHPF group, *M. flocculare* was detected in the trachea and tonsils of one pig, and in the nasal cavities of seven pigs. In contrast to the MF group, all lungs were negative and one liver swab was positive. *M. flocculare* was not detected in the spleen and tracheobronchial lymph nodes of pigs in the two infected groups (MF and MHPF groups). No statistically significant differences were found between the two challenged groups in the amount of *M. flocculare* at the different sampling times.

4. Discussion

The aim of this study was to assess (i) the pathogenicity of contemporary strains of *M. hyopneumoniae, M. hyorhinis* and *M. flocculare* isolated from gross pneumonia-like lesions and (ii) to investigate the

impact of superinfection with *M. hyorhinis* and *M. flocculare* in pigs pre-infected with *M. hyopneumoniae*. Inoculation of pigs was performed by the intratracheal route, because this is the most effective route for inducing pneumonia (Garcia-Morante et al., 2016).

Pigs challenged by *M. flocculare* (MF group) did not show any clinical signs; hematological data were similar to those of the control group and no macroscopic lesions were observed upon necropsy except for a questionable point of cranio-ventral consolidation confirmed as interstitial pneumonia by histological analysis (data not shown). However, *M. flocculare* was detected in the trachea, tonsils and nasal cavities of almost all pigs about eight days after inoculation, and then in the lungs of almost all pigs. Therefore, regardless of the results, no pathogenicity was correlated with the *M. flocculare* strain used, although it had been originally isolated from gross pneumonia-like lesions.

Animals were challenged with *M. hyorhinis* at seven weeks old (MHR and MHPR groups), an age of heightened susceptibility to *M. hyorhinis*-associated polyserositis and arthritis (Martinson et al., 2017). In our study, only some pigs developed gross lesions specific to polyserositis, such as pleuritis, pericarditis and peritonitis. No pigs developed arthritis, which may be attributed to the strain's inability to induce this affliction. Given that the aim of this study was to investigate the role of *M. hyorhinis* in the initiation of pneumonia, the strain was isolated from lung bronchi showing gross pneumonia-like lesions and likely cause less polyserositis or arthritis than a strain directly isolated from animals with these afflictions. After challenge with *M. hyorhinis*, monocyte and lymphocyte counts dropped quickly. The observed difference may be due to the direct effect of *M. hyorhinis* infection, which may trigger immunosuppressive mechanisms to evade the immune system and thereby promote colonization and/or systemic spread.

M. hyorhinis was detected in the trachea, tonsils and nasal cavities of almost all pigs in the MHR group from six days after inoculation, then the average rectal temperature, monocytes cells count and pig MAP concentration were increased whereas ADWG was decreased. These results suggest the activation of the host immune system. The progressive recovery observed the following week, supports the scenario of an acute inflammatory reaction likely due to the development of polyserositis and/or septicemic spread. This hypothesis explains the detection of *M. hyorhinis* in the liver, spleen, tracheobronchial lymph nodes and pulmonary bronchi of some pigs. However, progressive recovery and the low number of pigs showing the spread of infection may suggest an effective immune response. Four out of eight challenged pigs had developed pleuritis, coupled with (i) pericarditis for three pigs, (ii) pericarditis and peritonitis for one pig, and (iii) moderate interstitial pneumonia for one pig. Regarding the mild pneumonia observed in only one pig, these results are in accordance with previous observations showing the low ability of *M. hyorhinis* to cause extensive pneumonia alone (Gois et al., 1971).

M. hyopneumoniae was detected in the trachea and tonsils of almost all challenged pigs throughout the experiment, even in the cases of co-infection with *M. hyorhinis* or *M. flocculare*. However, detection in nasal cavities was lower. Other studies have also revealed trachea or tonsils as its preferential colonization sites, whereas nasal cavities are colonized more irregularly (Marois et al., 2007; Mattsson et al., 1995; Blanchard et al., 1992; Goodwin, 1972). The appearance of a cough was preceded by an increase in platelet cell counts and was followed by the seroconversion of pigs. Platelet cells are essential for primary hemostasis, the repair of damaged endothelium and inflammation, which may explain their increase in *M. hyopneumoniae* infections resulting in tracheal injury. As reported in other studies, seroconversion occurs late and does not prevent the impairment of mucociliary clearance and the subsequent development of pneumonia (Djordjevic et al., 2004). No change in the concentration of other types of blood cells and acute phase inflammation proteins were observed in pigs challenged with *M. hyopneumoniae* only.

In co-infected pigs in the MHPF group, *M. flocculare* was primarily detected in the nasal cavities; the number of positive tracheal and tonsil swabs was statistically lower than in the MF group. As observed for *M. hyorhinis*, (ecological) niche competition likely occurred and favored *M. hyopneumoniae* in the trachea and tonsils. Although colonization of nasal cavities by *M. flocculare* was higher, the presence of *M. hyopneumoniae* allows continual transmission between pigs, causing the same symptoms in terms of coughing, platelet counts and seroconversion as in pigs infected with *M. hyopneumoniae* only. Haptoglobin was high at 13 dpi and may explain the decrease in ADWG. Likewise, Pineiro et al. (2007) suggest that acute phase proteins are elevated in situations of distress, which involve slower growth and the activation of the immune system. Haptoglobin is a useful indicator of pig health status, as already proposed (Amory et al., 2007). The highest pneumonia score observed in all *M. hyopneumoniae*-infected pigs was in the MHPF group (score of 20/28). This extent of pneumonia was also observed in lungs derived from pigs in the herd from which the *M. hyopneumoniae* strain was isolated. Further, the *M. hyopneumoniae* strain was isolated from a lung that was positive for *M. flocculare* as well.

In the MHPR group, *M. hyopneumoniae* was mainly detected in the trachea or tonsils as in the MHP group. However, a significantly lower number of MHP-positive nasal swabs was observed than in the MHP and MHPF groups. *M. hyorhinis* was essentially detected in tonsils or nasal cavities as in the MHR group, and as reported in other studies (Gomes-Neto et al., 2015). Nonetheless, only about half of the tracheal swabs were positive compared to the MHR group. Niche competition may have occurred, favoring *M. hyopneumoniae* in the trachea and *M. hyorhinis* in nasal cavities and/or *M. hyopneumoniae* impairs the multiplication of *M. hyorhinis* through secretion of toxic factors. Other studies have shown that the trachea is the preferred multiplication site of *M. hyopneumoniae* and that nasal cavities are the natural colonization site of *M. hyorhinis* (Mattsson et al., 1995; Blanchard et al., 1992). Because the transmission of *M. hyopneumoniae* may occur through nose-to-nose contact, its low ability to colonize nasal cavities, when associated with *M. hyorhinis*, may reduce the transmission between pigs and therefore reduce its binding to ciliated epithelium. This hypothesis may explain the lower number of coughing bouts and the unchanged platelet counts observed in this group at 9 dpi than in the MHP and MHPF groups. These results do not necessarily mean that an association between *M. hyopneumoniae* and *M. hyorhinis* constantly induces less coughing than infection with *M. hyopneumoniae* alone. In this study, we chose to investigate the effect of *M. hyorhinis* superinfection in pigs already infected with *M. hyopneumoniae*. However, results may have been different if *M. hyorhinis* was the first inoculated mycoplasmal species. Thus, despite the lower number of coughing cases, pigs developed macroscopic pneumonia with equivalent symptoms. Interestingly, pig MAP increased rapidly after the *M. hyorhinis* challenge in the MHR group; although there was a trend for an increase in the MHPR group, the concentration remained similar to the control group. However, haptoglobin concentration first increased at 13 dpi and more generally at 29 dpi. The production of haptoglobin is induced by pro-inflammatory cytokines, such as IL-6, which suggests an inflammatory response that potentially explains appetite loss through the weakening of the health status (Gonzalez-Ramon et al., 2000). Nevertheless, this inflammatory response is late and more extensive than in the MHR group. *M. hyorhinis* nevertheless spread throughout the body, because it was detected in the liver of one pig. In lungs, *M. hyorhinis* was detected in almost all co-infected pigs (6/8), identified through culture methods only (data not shown), in contrast to the MHR group in which this species was detected in only one lung using both multiplex qPCR and culture methods. We therefore suggest that the chronic inflammatory status of pigs co-infected with both *M. hyopneumoniae* and *M. hyorhinis* and the impairment of the immune response are due to the propagation and/or persistence of *M. hyorhinis* in pulmonary bronchi and a seeming delay in anti-*M. hyopneumoniae* antibody production. Finally, our study showed that *M. hyorhinis* does not exacerbate the pneumonia induced

by *M. hyopneumoniae*, but impairs the immune system, reflected in the increase in acute phase proteins, to limit the elimination of pathogens as recently demonstrated for the association between *M. hyopneumoniae* and the influenza virus (Deblanc et al., 2016).

5. Conclusion

Our findings indicate that superinfection of *M. hyorhinis* and *M. flocculare* in pigs previously infected with *M. hyopneumoniae* induces an inflammation response, more specifically an increase in haptoglobin concentrations, likely responsible for the decrease in ADGW. The haptoglobin concentration increased rapidly in co-infected groups, afterwards decreasing rapidly in co-infections with *M. flocculare,* but staying high until the end the experiment in co-infections with *M. hyorhinis*. Our results suggest that pigs co-infected by *M. hyopneumoniae* and *M. hyorhinis* at post-weaning may enter the fattening barn with pneumonia, polyserositis and an inflammatory status. During the fattening phase, the risk of superinfection with other PRDC agents is high, meaning that this mycoplasmal association can contribute to the overall deterioration in pig health and immunity status.

Author contributions

The experiments were designed by SF, FP, IK and CMC and performed by SF, VT, FP and CMC. The data were analyzed by SF, FP and CMC. Reagents/materials/analysis tools were contributed by SF, VT, FP, IK and CMC. SF, VT, FP, IK and CMC participated in writing the manuscript.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Conflict of interest

The authors declare no conflicts of interest.

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