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Mycoplasma hyopneumoniae vaccination at or shortly before weaning under field conditions: a randomised efficacy trial

I. Arsenakis, A. Michiels, R. Del Pozo Sacristán, F. Boyen, F. Haesebrouck, D. Maes

This study assessed the efficacy of two different *Mycoplasma hyopneumoniae* vaccination programmes in relation to the time of weaning. Eight hundred and twenty-eight piglets were randomly divided into three groups: group V1 was vaccinated three days before weaning, group V2 at weaning (21 days of age) and group NV was left non-vaccinated. Vaccinations were performed using Ingelvac MycoFLEX. After the nursery period, 306 pigs were allocated to fattening unit (F1) and 501 pigs to a second unit (F2). Efficacy was evaluated using performance parameters and pneumonia lesions at slaughter. Statistically significant differences were obtained in F2 where group V1 had a higher average daily weight gain compared to groups V2 and NV for the entire study period (17 and 18 g/day, respectively) and the fattening period (26 and 36 g/day, respectively) (P<0.05). Considering respiratory disease scores for both fattening units, group V1 was the only group where coughing severity did not increase significantly between placement and the end of the fattening period (P>0.05). Between groups, there were no statistically significant differences for the average lung lesion scores (V1=3.44; V2=4.61; NV=4.55, P>0.05) and the prevalence of pneumonia (V1=35.0 per cent; V2=38.0 per cent; NV=41.4 per cent, P>0.05). Overall, vaccination against *M hyopneumoniae* before weaning provided numerically better performance than vaccination at weaning, but did not reach statistical significance. An influenza outbreak in F1 and the presence of coexisting mixed respiratory infections in both F1 and F2 could have possibly influenced the performance of both vaccinated groups across all measured parameters.

Introduction

Mycoplasma hyopneumoniae is the primary agent of enzootic pneumonia (EP) which affects mainly grow-finishing pigs and inflicts major economic losses on the pig industry (Thacker and Minion 2012). Vaccination is a very common control measure which has been extensively proven to reduce performance losses, the severity of clinical signs and lung lesions (Maes and others 1998, 1999, Jensen and others 2002, Wilson and others 2012, Del Pozo Sacristán and others 2014).

Currently, vaccination at weaning is commonly practised as the handling of the piglets is inevitable at that time (Alarcon and others 2014, Del Pozo Sacristán and others 2014). On the other hand, weaning is one of the most stressful events in the life of a piglet (Pié and others 2004, Campbell and others 2013). It is not recommended to vaccinate animals when they are severely stressed (Chase and others 2012). For that reason, vaccinating the animals two to three days before weaning is also practised by some pig producers (Gillespie and others 2010).

Consequently, one important question that remains unaddressed is whether vaccination at the day of weaning has an influence on the efficacy of *M hyopneumoniae* vaccines. A previous study investigated the efficacy of a single *M hyopneumoniae* vaccination three days before weaning or at weaning against experimental challenge infection with a virulent *M hyopneumoniae* field strain (Arsenakis and others 2016). The results showed that the group that was vaccinated three days before weaning had the lowest macroscopic and histopathological lung lesions. A difference of three days between the two vaccination groups was chosen to allow the first and most critical steps of the immune response to develop before the stress of weaning (Kick and others 2011). However, significant differences between the vaccinated groups were only obtained for the histopathological lung lesions. Thus, it was considered that a field study would provide further insight on the same topic, since it would permit to include more animals, to test the effect under practical conditions with concurrent infections with other respiratory pathogens and to investigate performance data until slaughter age.

Veterinary Record (2017) doi: 10.1136/vr.104075

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Provenance: Not commissioned; externally peer reviewed

Accepted April 23, 2017

The objective of this study was therefore to investigate the efficacy of a one-dose *M hyopneumoniae* vaccination applied either at weaning or three days before weaning in a Belgian pig herd with mixed respiratory disease in the fattening period, where *M hyopneumoniae* has been involved as an important pathogen. Average daily weight gain (ADG) and the severity of *Mycoplasma*-like pneumonia lesions at slaughter constituted the primary efficacy parameters.

Materials and methods

Herd description

The study was conducted between September 2013 and March 2014 in a two-site Belgian pig herd, operating a four-week batch production system for the sows (Table 1). At three weeks of age, the piglets were weaned and transferred to the nursery unit located on the same site. The nursery unit consisted of six compartments with 20 pens per compartment (20 pigs per pen). Each compartment had fully slatted floors, channel ventilation and a stocking density of 0.27 m^2/pig. At 10 weeks of age, approximately 30 per cent of the pigs were sold, while the remaining ones were allocated to two different fattening units, in which they were kept until slaughter (27 weeks of age). The first fattening unit (F1) was within-site and consisted of three compartments of 26 pens each (13 pigs per pen). The second fattening unit (F2) was located 5 km from the sow herd and consisted of five compartments of six pens each (13 pigs per pen) and of two compartments of four pens each (14 pigs per pen). Both F1 and F2 had fully slatted floors, conventional mechanical ventilation systems (combining vent doors and ceiling fans) and a stocking density of 0.75 m^2/pig.

One month before the onset of the study, tracheobronchial swabs (TBS) were collected from 10-week, 16-week and 24-week-old pigs (10 for each age group) and the results showed that 7/10, 5/10 and 10/10 pigs, respectively, were positive for *M hyopneumoniae* by nested PCR (nPCR; Stärk and others 1998). Blood samples were obtained from 24-week-old pigs, and 9/10 were serologically positive for *M hyopneumoniae* (sample to positive (S/P) values higher than 0.4) using an indirect ELISA (HerdCheck *M hyo*, IDEXX). One hundred of those fattening pigs were examined at the slaughterhouse, and *Mycoplasma*-like lung lesions were present on 51 per cent of the lungs. Before the initiation of the study, the herd did not perform any vaccination of the fattening pigs, apart from the one against boar taint (Improvac, Zoetis).

TABLE 1: Herd description and health management practices

Number of sows	450
Breed of sows	Topigs 20
Breed of boars for artificial insemination	Piétrain
Vaccination of sows	
PRRS(V)	Ingelvac PRRS MLV, Boehringer
Atrophic rhinitis	Porcilis AR-T, MSD
Escherichia coli	Porcilis coli, MSD
Parvovirus+*Erysipelothrix rhusiopathiae*	Porcilis Ery-Parvo, MSD
Vaccination of gilts in quarantine unit	
PRRS(V)	Ingelvac PRRS MLV, Boehringer
Atrophic rhinitis	Porcilis AR-T, MSD
E coli	Porcilis coli, MSD
Medication in the farrowing unit	Iron (Uniferon, Pharmacosmos) at day 5 Toltrazuril orally (Baycox, Bayer) at day 5
Medication in the nursery unit (days 21–70)	In-feed zinc oxide after weaning for 14 days at 2500 ppm
Medication in the fattening unit (days 70–200)	Flubendazole at 10 weeks of age via the feed (Flubenol 5 per cent premix, Elanco Animal Health) for five days, repeated every 6 weeks Gonadotrophin-releasing factor analogue vaccine (Improvac, Zoetis) against boar taint at 10 and 18 weeks of age

PRRS(V), porcine reproductive and respiratory syndrome virus.

Study population and experimental design

In total, 828 pigs originating from one batch of sows were selected for this study. These pigs were offspring from 69 randomly selected sows. The parity distribution of the selected sows was from 1 (n=15) to 13 (n=1), with an average±sd of 2.6 ±1.8 parity number. Parity 2 sows constituted the largest group (n=34). At 14 days of age, the pigs were individually ear-tagged and randomly allocated to one of the three treatment groups. Within each litter, an equal number of pigs (n=4) was allocated to each treatment group (blocked randomisation). This experimental design was followed so that there is no difference between treatment groups in the average parity number of the sows providing the piglets. By that way, confounding factors that might influence the results could be minimised. All allocations were performed by the third author. Finally, each treatment group consisted of 276 pigs.

The three treatment groups were: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated at the day of weaning, at 21 days of age, one to three hours before the separation from the sow) and a non-vaccinated (NV) group. Pigs in groups V1 and V2 received a single-dose intramuscular injection (1 ml) of a commercial *M hyopneumoniae* bacterin vaccine (Ingelvac MycoFLEX, Boehringer Ingelheim).

In the nursery unit, piglets from maximum three different litters were regrouped according to weight and sex, while upon transfer to the fattening units, the pigs from maximum two different nursery pens were regrouped according to pen size and sex. Animals housed within the same pen in the nursery and fattening units belonged to the same treatment group. Within the nursery and fattening units, the different treatment groups were allocated alternately between the pens, until each compartment was full. In the nursery unit, only two out of the six available compartments were filled with animals included in the trial. In F1, only one compartment was used, whereas in F2, all compartments were used. During the entire trial, water and commercial feed (meal) were supplied ad libitum to the pigs. Feed (commercial meal), housing, management factors, sex distribution and prophylactic treatment were the same for the three groups. The study was approved by the ethical committee for animal experiments of the Faculty of Veterinary Medicine, Ghent University (EC2013/171).

Parameters of comparison

Performance parameters

In order to obtain the ADG (g/pig/day), all pigs were individually weighed at two weeks of age, at the end of the nursery period (10 weeks of age) and before slaughter (27 weeks of age). Weighing was performed in a blinded manner with respect to the treatment status by first and second authors and animal caretakers.

Clinical parameters

Coughing severity was measured blindly by the first author every two weeks, starting from the beginning of the fattening period. All measurements throughout the fattening period were obtained following the same pen order, each time starting at 8:00, first from F1 and then directly moving to F2. The number of pigs coughing per pen was counted for 10 minutes. Then, a respiratory disease score (RDS) was calculated by dividing the number of pigs per pen that coughed during 10 minutes by the total number of pigs in that pen, multiplied by 100 (Mateusen and others 2002, Del Pozo Sacristán and others 2012).

In case of respiratory or neurological signs, only treatment with amoxicillin by intramuscular injection or via the drinking water was allowed. Possible respiratory disease problems were monitored by paired sera from 10 coughing pigs, obtained during the outbreak and three weeks later (referred to as pre-serum and post-serum samples, respectively). The sera were analysed to detect antibodies against porcine reproductive and respiratory syndrome virus (PRRS(V)) (HerdCheck PRRS ELISA, IDEXX), porcine circovirus type 2 (PCV-2) (IgM and IgG, Ingezim PCV2

ELISA, Ingenasa), swine influenza virus (SIV) (H1N1, H1N2, H3N2, standard haemagglutination inhibition test), *M hyopneumoniae* (HerdCheck *M hyo*, IDEXX), *Haemophilus parasuis* (Hps OppA, Biocheck) and *Actinobacillus pleuropneumoniae* (serotypes 1, 9 or 11 and serotype 2; Swinecheck APP 1,9,11 and Swinecheck APP 2, Biovet). Additionally, paired nasal swabs from the same animals were collected to monitor SIV by a real-time reverse transcription PCR (rRT-PCR; VetMAX-Gold SIV Detection Kit, Life technologies) detecting type A SIV-specific RNA (Bowman and others 2016).

Postmortem examination was performed on a subset of dead pigs in order to assess the possible cause of death. In all cases, postmortem examinations were combined with bacteriological culture, antimicrobial susceptibility testing and histopathology.

Serological examination

Blood samples were collected from the same 20 randomly selected pigs per group at 2, 10, 18 and 26 weeks of age to measure antibodies against *M hyopneumoniae*, using a blocking ELISA (infectious disease enzyme immunoassay, *M hyopneumoniae* enzyme immunoassay Kit, Oxoid, UK) as previously done by Del Pozo Sacristán and others (2014). In each treatment group, equal numbers of sampled pigs were distributed across fattening units. Inhibition percentages (IP) for all sera were also calculated considering the optical density (OD) value of each serum sample as well as the negative control according to Sibila and others (2004). Classification of individual sera on the basis of IP values was as follows: IP <30 per cent, negative; IP >50 per cent, positive; IP ≥30 per cent and ≤50 per cent, equivocal.

Thirty blood samples (five for each group/fattening unit) were randomly selected from the blood samples collected at 26 weeks of age. These were additionally tested for the presence of antibodies to PRRS(V), PCV-2, SIV, *H parasuis* and *A pleuropneumoniae*. The serological tests used were the same as the ones used to investigate respiratory disease diagnostics (see Clinical parameters section).

Detection of *M hyopneumoniae* using qPCR on TBS

TBS were collected from 20 pigs per group at 10, 14 and 18 weeks of age (the same 20 pigs from which blood samples were collected). Sampled pigs were endotracheally intubated with a sterile, semirigid canine urinary catheter, as described by Vangroenweghe and others (2015). All samples were immediately cooled at 4°C and stored at −20°C until analysis. The material collected by TBS was used to quantify *M hyopneumoniae* organisms by quantitative PCR (qPCR) as previously described by Marois and others (2010) and Del Pozo Sacristán and others (2012).

Lung lesions

The prevalence of *Mycoplasma*-like pneumonia lesions, fissures and pleurisy were recorded at slaughter via a blinded manner from the second author with the assistance of the first author. The total area (percentage) of macroscopically visible *Mycoplasma*-like lung lesions (lung lesion score) was quantified according to the scoring system described by Morrison and others (1985a) and Del Pozo Sacristán and others (2012, 2014). *Mycoplasma*-like pneumonia lesions (catarrhal bronchopneumonia (CBPn)) were defined as red-purplish areas of cranioventral consolidation raised above the surface or at the surface of each lobe and with a liver-like consistency (Holyoake and Callinan 2006, Del Pozo Sacristán and others 2012, 2014). Chronic *Mycoplasma*-like pneumonia lesions (fissures) were grey to purplish cranioventral scars, shrunken below the surface of the lobes, and had a more solid texture than the unaffected neighbouring parenchyma (Sorensen and others 2006, Del Pozo Sacristán and others 2012, 2014). Pleurisy was evident as fibrous adhesions between the lung lobes and/or the lungs and thoracic wall (Del Pozo Sacristán and others 2012, 2014, Michiels and others 2015).

Statistical analysis

The number of animals in each treatment group (276) allowed to assess a difference of 19 g (sd=80) in ADG and 3.2 points (sd=13) in lung lesion score with 95 per cent certainty and 80 per cent statistical power (Win Episcope 2.0, Edinburgh, UK). The ADG and lung lesion score were the primary outcome parameters. Analysis of variance (ANOVA) was used to analyse bodyweights, ADG and RDS with treatment, compartment and sex included as fixed factor and pen as a random variable. In the combined model (including both fattening units), unit was additionally included as a random variable. Pairwise comparisons between the different treatment groups were made using Scheffe's test. Data that did not fulfil the criteria of normality and homogeneity of variances (lung lesion scores, qPCR values and serology IP) were analysed using non-parametric Kruskal-Wallis ANOVA. RDS and qPCR (time and group effect) data were analysed using repeated measures ANOVA. The average RDS summarised until 20 weeks of age and between 20 and 26 weeks of age were compared within each group using paired t-tests. Mortality rate, the percentage of pigs showing *Mycoplasma*-like lesions, fissures and pleurisy, the percentage of pigs showing *M hyopneumoniae* antibodies and the percentage positive on qPCR were analysed using logistic regression with treatment and fattening unit as predictors for the model. Statistical results were considered significant when P values were ≤0.05 (two-sided test). The statistical package SPSS V.21.0 was used to analyse the data.

Results

Performance parameters

No statistically significant differences for the average bodyweight were shown between groups at inclusion in the study (2 weeks of age) (P=0.919), end of the nursery period (10 weeks of age) (P=0.109) and before slaughter (at 27 weeks of age, taking into account both fattening units) (P=0.263) (Table 2). When taking into account each fattening unit separately (Table 3), significant differences between groups for weight gains and ADG were observed only in F2. More specifically, group V1 had significantly higher weight gains and ADG when compared with groups V2 and NV.

Clinical parameters

There was a significant difference between F1 and F2 in the overall average RDS (no group effect) between 20 and 26 weeks of age (P=0.011). Coughing severity increased towards the end of the fattening period (between 20 and 26 weeks of age) (Table 4). Taking into account the average of both fattening units, the paired t-test results revealed significant differences between the two fattening periods in groups NV and V2 (P=0.031 and P=0.007, respectively), but not in group V1 (P=0.194).

During both fattening periods from 10 to 20 and from 20 to 26 weeks of age, higher average RDS were observed in F1 compared with F2 (Table 4). The coughing severity in F1 increased at approximately 20 weeks of age among all groups (Fig 1). For that reason, all pigs in F1 were medicated for a five-day period with amoxicillin via the drinking water. Paired sera from F1 showed that 8/10 pigs had a positive post-serum titre (higher than 80) for the H3N2 subtype of SIV, with an average of 152. All pre-serum samples had a titre of 20. Moreover, all nasal swabs (10/10) collected at the same time as the pre-serum were positive for SIV type A. Serology for *M hyopneumoniae* showed an average S/P ratio of 0.81 for the pre-serum (9/10 pigs positive) and an average S/P ratio of 1.02 for the post-serum (9/10 pigs positive). Additional serology on pre-serum and post-serum samples showed that at both collection points 10/10 animals were positive for PRRS(V). Also, 10/10 pigs and 1/10 pigs were tested PCV-2 IgG and PCV-2 IgM positive, respectively.

Approximately three weeks after the occurrence of respiratory problems in F1, sporadic non-productive coughing was also

TABLE 2: Performance parameters from 2 to 27 weeks (w) of age and percentage of market pigs in the three groups with *Mycoplasma hyopneumoniae*-like lesions (*Mhyo*), fissures and pleurisy, and severity of lung lesions expressed as lung lesion score (average±sd). Average values (±sd) were calculated taking into account both fattening units (F1 and F2). Minimum and maximum (min–max) values of the lung lesion score are also presented

Performance parameters*	Age (w)	V1 n=270	V2 n=266	NV n=271	P value
Average bodyweight (kg)	2	4.28±0.78	4.32±0.77	4.31±0.76	0.919
	10	23.56±3.76	23.99±3.71	24.28±3.90	0.109
	27	106.81±12.96	105.21±13.88	104.12±15.17	0.263
Weight gain (kg)	10–27	83.26±11.44	81.46±12.41	80.10±13.42	0.094
	2–27	102.55±12.76	100.95±13.68	99.71±14.90	0.164
ADG (g/pig/day)	2–10	371±64	376±69	384±67	0.076
	10–27	718±98	701±108	688±116	0.094
	2–27	610±76	600±81	593±88	0.164
Lung lesions		n=134	n=128	n=129	
Prevalence of *Mhyo*-like lesions	Market	35.0	38.0	41.4	0.602
Prevalence of fissures	Market	37.5	38.7	39.7	0.943
Prevalence of pleurisy	Market	7.2	9.6	12.5	0.381
Lung lesion score	Market	3.44±6.77	4.61±11.11	4.55±9.99	0.617
Lung lesion score (min-max)	Market	0–32	0–64	0–66	

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Differences between groups were not statistically significant (P>0.05)
*Average daily weight gain (ADG)

TABLE 3: Performance parameters between 2 and 27 weeks (w) of age and percentage of market pigs in the three groups with *Mycoplasma hyopneumoniae*-like lesions (*Mhyo*), fissures and pleurisy, and severity of lung lesions expressed as lung lesion score (average±sd). Average values (±sd) are presented for each fattening unit (F1 and F2) separately. Minimum and maximum (min–max) values of the lung lesion score are also presented

		(F1)				(F2)			
Performance parameters*	Age (w)	V1 n=101	V2 n=102	NV n=103	P value	V1 n=169	V2 n=164	NV n=168	P value
Average bodyweight (kg)	27	98.43±11.20	100.10±14.05	98.20±13.16	0.152	111.03±11.70	108.13±13.00	108.17±15.16	0.065
Weight gain (kg)	10–27	76.12±9.30	77.15±12.48	75.25±11.64	0.124	86.80±10.74^{A}	83.96±11.69^{B}	83.25±13.60^{B}	0.011
	2–27	94.33±10.98	95.91±13.90	93.99±12.94	0.124	106.68±11.55^{A}	103.81±12.73^{B}	103.65±14.92^{B}	0.038
ADG (g/pig/day)	10–27	656±81	665±109	648±100	0.124	748±92^{A}	722±101^{B}	712±117^{B}	0.010
	2–27	562±65	570±82	559±77	0.139	634±68^{A}	617±76^{B}	616±88^{B}	0.034
Lung lesions		n=39	n=37	n=39		n=95	n=91	n=90	
Prevalence of *Mhyo*-like lesions	Market	32.4	41.0	48.7	0.357	36.1	36.9	37.6	0.980
Prevalence of fissures	Market	51.3	43.6	48.7	0.788	31.3	36.9	35.0	0.727
Prevalence of pleurisy	Market	7.5	14.6	15.0	0.529	7.0	7.6	11.2	0.579
Lung lesion score	Market	3.68±7.57	4.88±11.79	3.56±5.93	0.628	3.33±6.43	4.50±10.88	5.04±11.51	0.844
Lung lesion score (min-max)	Market	0–32	0–64	0–21		0–27	0–60	0–66	

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (nonvaccinated group). Within a row, different superscript letters indicate significant differences between values (P<0.05).
*Average daily gain (ADG).

observed in F2, mainly in group NV (Fig 1). A similar five-day treatment with amoxicillin via the drinking water was used. The serological test for *M hyopneumoniae* showed an average S/P ratio of 0.79 for the pre-serum (7/10 pigs positive) and an average S/P ratio of 1.08 for the post-serum (9/10 pigs positive). Moreover, all pre-sera and post-sera were positive for PRRS(V) and anti-PCV-2 IgG antibodies.

The numbers of dead pigs in different treatment groups are presented in Table 5. In total, 55 pigs died during the entire study period. Postmortem examination was performed on a subset of dead animals (17/55): five from the nursery and six each for F1 and F2. All pigs that underwent postmortem examination and died during the nursery period had pathological signs of fibrinopurulent pericarditis and endocarditis. *Streptococcus suis* was isolated from the heart and kidneys. The pigs that died during the fattening period showed either pathological signs of respiratory or/and wasting disease characterised by pleurisy, pericarditis and/or purulent peritonitis. Several bacteria were isolated from the lungs of those pigs: *Trueperella pyogenes* (7/12), *Pasteurella multocida* (4/12) and *S suis* (6/12). The main pathogen isolated from the pigs with pericarditis/purulent peritonitis was *S suis*.

Serological examination

The serological results for *M hyopneumoniae* at 2, 10, 18 and 26 weeks of age are presented in Table 6. Regarding the average IP values from both units, at 10, 18 and 26 weeks of age, the vaccinated groups V1 and V2 exhibited higher average values when compared with NV (P=0.004, P=0.066 and P=0.159, respectively).

The serological examination for the other pathogens revealed that in F1, at 26 weeks of age, 8/15 pigs were seropositive for the H3N2 subtype of SIV, 15/15 pigs were seropositive for PRRS(V) and that 15/15 and 6/15 pigs were tested PCV-2 IgG and PCV-2 IgM positive, respectively. In F2, 0/15 pigs were seropositive for the H3N2 subtype of SIV, 15/15 pigs were seropositive for PRRS (V), while 15/15 and 10/15 pigs were tested PCV-2 IgG and PCV-2 IgM positive, respectively.

Detection of *M hyopneumoniae* using qPCR on TBS

Group V1 had significantly lower numbers of positive animals compared with groups V2 and NV at 10 and 14 (both fattening units) weeks of age (P=0.000 and P=0.041, respectively, Table 7). The average qPCR values of *M hyopneumoniae* organisms were lower in group V1 than in groups V2 and NV, across

TABLE 4: The average (±sd) respiratory disease score (RDS) of the pigs between 10 and 20 weeks of age and between 20 and 26 weeks (w) of age. Average values (±sd) were calculated taking into account both and each fattening unit (F1 and F2) separately

Age range (w)	V1	V2	NV	P value
Average both units	n=270	n=266	n=271	
10–20	1.76±1.90^{A}	1.55±1.94^{A}	1.77±1.78^{A}	0.989
20–26	2.70±2.91^{A}	3.68±2.86^{B}	3.94±3.81^{B}	0.801
P*	0.194	0.007	0.031	
(F1)	n=101	n=102	n=103	
10–20	2.22±1.98	2.58±2.25	2.25±1.40	0.937
20–26	5.45±2.70	5.43±2.65	4.08±2.99	0.633
(F2)	n=169	n=164	n=168	
10–20	1.55±1.91	1.03±1.63	1.55±1.94	0.725
20–26	1.43±2.05	2.80±2.64	3.87±4.25	0.256

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Within a column, different superscript letters correspond to within-group significant differences between the two fattening periods (*P<0.05) (paired t-tests). The P value column indicates that there were no significant differences between groups during the two different fattening periods (P>0.05)

TABLE 5: Number of dead pigs per group between 2 and 27 weeks (w) of age. The contribution of each fattening unit (F1 and F2) on the combined (both units) mortality rates between 10 and 27 weeks of age is presented separately. All mortality rates were calculated according to the total number of animals included in each treatment group at the beginning of the study (n=276)

Age range (w)	V1	V2	NV	P value
2–10	6/276 (2.17)	10/276 (3.62)	5/276 (1.81)	0.371
10–27 (both units)	9/276 (3.26)	10/276 (3.62)	15/276 (5.43)	0.260
2–27	15/276 (5.43)	20/276 (7.24)	20/276 (7.24)	0.607
(F1)				
10–27	3/276 (1.09)	5/276 (1.81)	10/276 (3.62)	0.150
(F2)				
10–27	6/276 (2.17)	5/276 (1.81)	5/276 (1.81)	0.983

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). The P value column indicates that there were no significant differences between groups in mortality rates during the three different production periods (P>0.05)

all sampling points, apart from F1 at 18 weeks of age where group V1 had the second lowest qPCR value (2.03) when compared with groups V2 (1.78) and NV (2.28). Significant differences between groups were observed at 10, 14 and 18 (both fattening units) weeks of age (P=0.000, P=0.000 and P=0.039, respectively) (Table 7).

Lung lesions

Lung lesion evaluation at slaughter was performed on 391 pigs (n=134 V1; n=128 V2; n=129 NV). Some pigs could not be evaluated because of lost ear tags and lungs that did not reach the examination stand. Taking into account both F1 and F2 (Table 2), no statistically significant differences were found between treatment groups across all measured parameters.

Discussion

The present field study investigated the efficacy of one-dose vaccination against *M hyopneumoniae*, applied either three days before weaning (V1) or at the day of weaning (V2). The working hypothesis was that group V1 will perform better than group V2 across the different parameters investigated as the possible

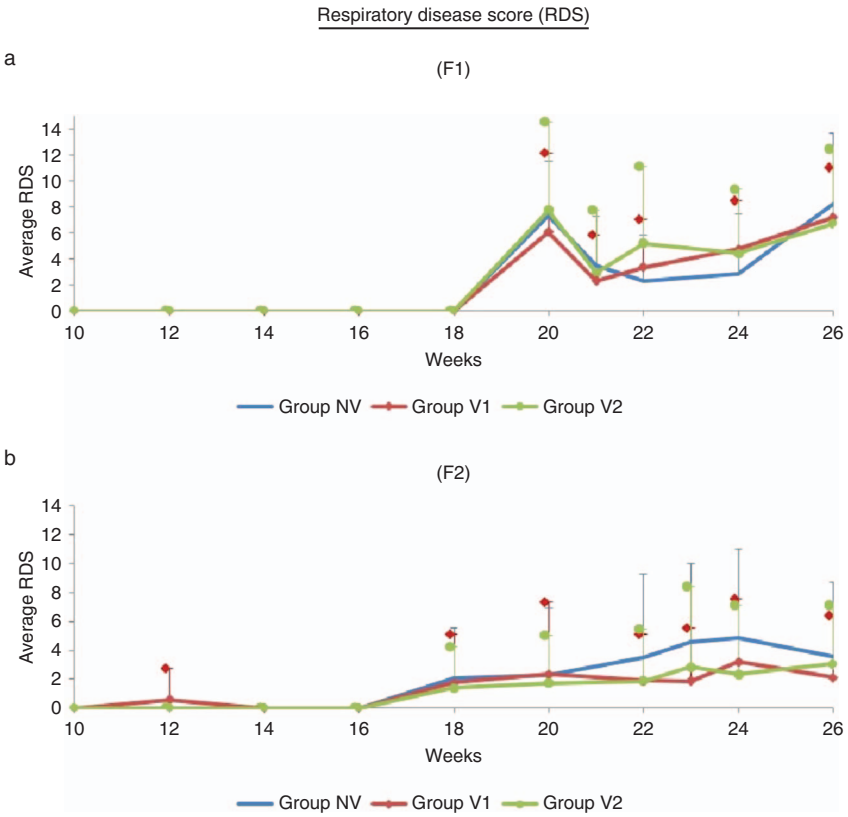

FIG. 1: Average respiratory disease score (RDS) over time in each of three groups of pigs per fattening unit (F1 and F2). Average RDS, together with the standard deviations (vertical bars), from 10 weeks of age until 26 weeks of age in group V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group).

TABLE 6: Ratios of seropositive/total number of pigs sampled per group at different time points as tested with the IDEIA, *M hyopneumoniae* EIA Kit (Oxoid) are presented together with the inhibition percentages (IP). The contribution of each fattening unit (F1 and F2) on the combined (both units) seropositivity ratios and IP at 18 and 26 weeks of age is presented separately

	Seropositive pigs for *M. hyopneumoniae*/ total number of pigs sampled				Average IP values			
Age (w)	V1	V2	NV	P value	V1	V2	NV	P value
2	7/20	10/20	9/20	0.624	44.2	42.6	45.6	0.929
10	3/20	0/20	0/20	1.000	49.9^{A}	43.4^{AB}	35.9^{B}	0.004
18 (both units)	5/20	4/20	3/20	0.735	39.0	47.4	29.3	0.066
26 (both units)	18/20	16/20	16/20	0.630	82.6	72.3	68.1	0.159
(F1)								
18	2/10	2/10	0/10	0.980	33.4^{AB}	39.1^{B}	14.3^{A}	0.011
26	9/10	9/10	7/10	0.246	84.3^{A}	77.1^{B}	52.4^{B}	0.006
(F2)								
18	3/10	2/10	3/10	0.863	44.6	55.7	44.2	0.682
26	9/10	7/10	9/10	0.421	81.0	67.6	83.8	0.358

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (nonvaccinated group). Within a row, different superscript letters correspond to significant differences in the average IP values between groups during the different sampling points (P<0.05).

TABLE 7: Ratios of qPCR positive animals for *Mycoplasma hyopneumoniae* (*Mhyo*)/total number of pigs sampled per group at different time points are presented together with the Log *Mhyo* copies/ml. The contribution of each fattening unit (F1 and F2) on the combined (both units) qPCR positivity ratios and Log Mhyo copies/ml at 14 and 18 weeks of age is presented separately

	qPCR positive pigs for *M. hyopneumoniae* / total number of pigs sampled				Log *Mhyo* copies/ml			
Age (w)	V1	V2	NV	P value	V1	V2	NV	P value
10	$0/20^{A}$	$3/20^{B}$	$17/20^{BC}$	0.000	0.58 ± 0.71^{A}	0.96 ± 0.75^{AB}	1.12 ± 0.77^{C}	0.000
14 (both units)	$2/20^{A}$	$7/20^{B}$	$10/20^{BC}$	0.041	0.85 ± 0.72^{A}	1.21 ± 1.20^{BC}	1.76 ± 1.59^{C}	0.000
18 (both units)	2/20	8/20	8/20	0.088	1.99 ± 2.45^{AB}	2.21 ± 2.44^{AC}	2.19 ± 2.35^{A}	0.039
(F1)								
14	2/10	2/10	7/10	0.073	0.91±0.25	1.05±1.21	1.62±1.38	0.257
18	1/10	6/10	4/10	0.183	2.03±2.50	1.78±2.03	2.28±2.32	0.076
(F2)								
14	0/10	5/10	3/10	0.613	0.78 ± 1.18^{A}	1.33 ± 1.17^{AB}	1.86 ± 1.69^{B}	0.001
18	1/10	2/10	4/10	0.205	1.96±2.42	2.42±2.84	2.07±2.37	0.118

Treatment groups: V1 (vaccinated before weaning at 18 days of age), V2 (vaccinated on the day of weaning at 21 days of age) and NV (non-vaccinated group). Within a row, different superscript letters indicate significant differences in the percentage of qPCR positive animals and qPCR values between groups during the different sampling points (P<0.05).

negative effect of weaning stress on vaccination would be decreased. Differences between group V1 and groups V2 and NV were mostly not statistically significant, except for the second fattening unit (F2) where group V1 had significantly higher weight gains and ADG when compared with groups V2 and NV between 10 and 27 weeks of age, as well as the whole study period. When taking into account both fattening units, group V1 had a numerically lower percentage of pigs with pneumonia, fissures and pleurisy at slaughter when compared with groups V2 and NV. The results of this study are in agreement with the previous experimental study of Arsenakis and others (2016) where group V1 performed better than V2 across all parameters concerning the assessment of lung lesions; however, differences between V1 and V2 were small and mostly statistically not significant.

In the present field study, serology and qPCR were both used to confirm the presence of pigs naturally infected with *M hyopneumoniae*. At 26 weeks of age (1 week before slaughter), the percentage of tested pigs being seropositive for *M hyopneumoniae* ranged between 70 and 90 per cent, while at 18 weeks of age, between 10 and 60 per cent of tested pigs were found to be qPCR positive. These results indicate that *M hyopneumoniae* has been involved as an important respiratory pathogen in both fattening units. Martínez and others (2009) studied the relationship between infectious factors and pneumonia at slaughter on 39 fattening herds and confirmed that vaccination of piglets against *M hyopneumoniae* did not appear to be related to the seroprevalences against the pathogen at slaughter. The qPCR results indicate that infection with *M hyopneumoniae* started already during the nursery period, which may explain the high prevalence of pulmonary fissures at slaughter. Nevertheless, the diagnostic results show that apart from *M hyopneumoniae*, there was a combination of viruses and other bacteria circulating in this herd.

In this study, it was decided to use the scoring system of Morrison and others (1985a), in order to quantify the severity of macroscopically visible *Mycoplasma*-like lung lesions. Those lesions are typically compatible with CBPn, which is the most common lung lesion associated with *M hyopneumoniae* infection (Sorensen and others 2006, Meyns and others 2011). It is characterised by well-demarcated red-purplish areas that have a poor retraction state and thus, this scoring system was considered to be able to achieve the maximum possible differentiation against other types of lung lesions that are usually induced by viral pathogens, namely PRRS(V), SIV and PCV-2. Those pathogens most often cause interstitial pneumonia (IPn; Van Alstine 2012). The main difference between CBPn and IPn is that in the latter one, the lesions are widely distributed throughout the lungs and the lung lobes maintain their rubbery consistency (Van Alstine 2012, López and Martinson 2017). It is documented that *M hyopneumoniae* predisposes to secondary pathogens such as *T pyogenes*, *P multocida* and *S suis* (Goodwin and others 1965, Morrison and others 1985b, Ciprián and others 1988, Amass and others 1994, Opriessnig and others 2011, López and Martinson 2017). By that way, the lung lesions produced by *M hyopneumoniae* are exacerbated, leading to

more severe CBPn and pleural adhesions which extend the healing period (formation of fissures). Additionally, *M hyopneumoniae* together with the aforementioned pathogens form the pathological complex of EP (Maes and others 1996, Thacker and Minion 2012). Vaccination against *M hyopneumoniae* in herds facing severe EP has been shown to reduce the extent and severity of *Mycoplasma*-like lung lesions (Maes and others 1999, Alexopoulos and others 2004, Del Pozo Sacristán and others 2014).

In the current study, the percentage of pigs being positive for *M hyopneumoniae* by qPCR increased between 14 and 18 weeks of age and the percentage of pigs seropositive for *M hyopneumoniae* increased between 18 and 26 weeks of age. Additionally, in both F1 and F2, the RDS increased between 20 and 26 weeks of age when compared with the period between 10 and 20 weeks of age. This increase in the RDS occurred almost simultaneously with the increase in the percentage of *M hyopneumoniae* seropositive animals towards the end of the fattening period. Nathues and others (2012), who examined the value of clinical examination in diagnosing EP in fattening pigs from 59 herds at 18 weeks of age, suggested that a combination of an increasing RDS towards the end of the fattening period together with a seroprevalence of more than 50 per cent against *M hyopneumoniae* is highly indicative of an EP diagnosis. In the current study, the EP diagnosis is also justified by the detection of secondary pathogens, such as *T pyogenes*, *P multocida* and *S suis*, in the pigs that underwent postmortem examination during the fattening period. The average percentage of *Mycoplasma*-like pneumonia lesions, in both F1 and F2, was considerably higher (38 per cent) than the average of 24 per cent reported by Meyns and others (2011), who collected data from 50 randomly selected batches from 60 Belgian herds at slaughter.

Of course, it is important to mention that viral pathogens, such as PRRS(V), SIV and PCV-2, were present in both fattening units (as documented by the diagnostics performed). The presence of such coinfections confirms the multifactorial nature of porcine respiratory disease complex (PRDC) in the present herd and could probably explain why in most of the measured parameters the vaccinated groups V1 and V2 did not perform significantly better than the NV group. The high percentage of *Mycoplasma*-like pneumonia lesions among all groups can likely be the result of interactions between *M hyopneumoniae* and the viral (PRRS(V), SIV and PCV-2)) pathogens observed. All tested pigs were seropositive for PRRS(V) and PCV-2 IgG at 20, 23 and 26 weeks of age, and additionally a high number of pigs were seropositive for PCV-2 IgM at 26 weeks of age. Given that all pigs participating in this trial did not receive any other vaccination apart from the one with the commercial *M hyopneumoniae* bacterin, these results prove that the herd was facing a chronic PRRS(V) infection in combination with a circulating PCV-2 infection at the nursery and fattening units.

It is generally acknowledged that most PRRS(V) and PCV-2 infections occurring at the end of the fattening period are subclinical and thus, this is the reason why in many cases no gross IPn lesions are detected at slaughter (Segalés and others 2012, Pileri and Mateu 2016). Nevertheless, the potentiating effect of the combination of viral pathogens with a *M hyopneumoniae* infection on the severity of *Mycoplasma*-like lung lesions induced has already been experimentally described by Thacker and others (1999, 2001), Opriessnig and others (2004) and Dorr and others (2007), and demonstrated under field conditions by Del Pozo Sacristán and others (2014). It is possible that in this study a better control of viral pathogens such as PRRS(V), PCV-2 and SIV through better biosecurity and vaccination at an early age would allow vaccination against *M hyopneumoniae* to demonstrate significant benefits versus the NV animals in terms of the severity of *Mycoplasma*-like lung lesions induced. Previously published studies performed in herds facing mixed infections with the above-mentioned pathogens showed that vaccination against PCV-2 (Raith and others 2015, Duivon and others 2016) and PRRS(V) (Revilla and others 2006), in combination with vaccination against *M hyopneumoniae,* further reduced the prevalence of *Mycoplasma*-like lung lesions at slaughter (Raith and others 2015, Duivon and others 2016), reduced mortality rates (Revilla and others 2006) and increased the ADG during the fattening period (Duivon and others 2016), when compared with *M hyopneumoniae* vaccination alone.

The possible effect of other vaccinations applied during the same time period on *M hyopneumoniae* vaccination or vice versa is not known. Experimental studies showed that a modified live PRRS(V) vaccine based on a US-type PRRS(V) strain (Thacker and others 2000) applied in the same time period as *M hyopneumoniae* vaccination decreased the efficacy of *M hyopneumoniae* vaccination. In contrast, this did not occur when a PRRS(V) vaccine based on an EU-type PRRS(V) strain was used (Drexler and others 2010). However, it is not known whether a possible interaction of *M hyopneumoniae* vaccination and other vaccines applied at the same time period would be different when applied at weaning or shortly before weaning.

The evolution of respiratory distress in F1 is more typical of a SIV outbreak as a clear increase in the RDS was observed at 20 weeks of age and lasted for almost a week. A similar increase in coughing severity during a SIV outbreak has already been described by Berckmans and others (2015) after using microphones for monitoring respiratory distress in a Dutch herd over a three-month period. For the remaining fattening period of up to 26 weeks of age, a significantly higher overall average RDS was observed in F1 compared with F2. Thacker and others (2001) reported that concurrent infection with *M hyopneumoniae* and SIV increased the severity and duration of EP. Van Reeth and others (1996) found that the clinical effects of PRRS(V) were exacerbated with concurrent infection with SIV. Also, it is well established that secondary infections with bacteria such as *P multocida* and *S suis* may enhance the severity of a SIV outbreak (Van Reeth and others 2012). Considering all the above and also the fact that there are many between-pathogen interactions which have not yet been fully elucidated, the higher RDS observed in F1 compared with F2 as well as the inconsistency in the results of group V1 for some of the measured parameters (such as the weight gains and ADG across the different fattening units) could possibly be attributed to the SIV outbreak. The proximity of F1 to the sow herd and the nursery unit provides a possible explanation for the origin of the SIV outbreak.

At 10 and 14 weeks of age, group V1 had significantly less animals being qPCR positive for *M hyopneumoniae* than groups V2 and NV. Nevertheless, the impact of this lower colonisation rate on the performance and clinical parameters remains unclear. A limitation of the current study is that this lower colonisation rate together with the significantly lower average qPCR values (Log *M hyopneumoniae* copies/ml) detected in group V1 compared with groups V2 and NV could neither be confirmed before slaughter, since there were no TBS obtained at 26 or 27 weeks of age. In a field study of Sibila and others (2007), which compared two different *M hyopneumoniae* vaccination programmes (two vaccine doses at 1 and 3 weeks of age versus one dose at 6 weeks of age) with a control group, vaccination was related to a reduction in the number of animals found to be nPCR positive at 25 weeks of age (37.5 per cent and 55.8 per cent of the vaccinated animals, respectively, and 70.2 per cent of the control group). However, no quantitative method such as qPCR was used. It was concluded that a qPCR would be more useful in elucidating whether vaccination reduces the bacterial load in the upper respiratory airways during the late stages of the fattening period. Possibly, the reduced bacterial load could be associated with the shedding of less organisms. This could be interesting to investigate in a future experimental trial, where vaccinated and NV fattening pigs would be purchased from a farrow-to-finish herd and then involved in a transmission experiment using direct nose-to-nose contact.

Although this study included a high number of animals, the results should be interpreted with caution, particularly when trying to draw general conclusions from a single study. It should always be taken into account that biosecurity levels, climatic

environment and management conditions as well as metaphylactic antimicrobial treatment and vaccination programmes differ between herds facing concurrent infections with *M hyopneumoniae* and other respiratory pathogens. The above-mentioned parameters influence not only mixed respiratory tract infection dynamics (Bochev 2007, 2008, Thacker and Minion 2012), but also the efficacy of the vaccination protocols applied against *M hyopneumoniae* (Maes and others 2008). Therefore, more herds with mixed respiratory disease where *M hyopneumoniae* is involved as an important pathogen need to be investigated, in order to discover the most suitable techniques for making vaccination protocols that are tailored to the individual needs of each herd.

In conclusion, the fact that in F2 group V1 had significantly higher weight gains and ADG compared with groups V2 and NV is indicative of the potential benefits of vaccinating against *M hyopneumoniae* three days before weaning. The SIV outbreak in F1 and the presence of other respiratory pathogens in both F1 and F2 likely influenced the performance of both vaccinated groups and highlight the difficulties of evaluating interventions in field settings. Thus, additional studies are necessary to further explore the possible impact of the process of weaning on the efficacy of vaccination against *M hyopneumoniae*.

Acknowledgements

The authors are grateful to Sandra Willaert, Leila Vande Velde, Ria Spiessens and Els De Fré for their assistance in numerous arrangements of the trial.

Funding The study was financed by Boehringer Ingelheim but was conducted solely by the Unit Porcine Health Management at the Faculty of Veterinary Medicine of Ghent University. The authors do not have any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Veterinary Record 2017 181: 19 originally published online June 10, 2017
doi: 10.1136/vr.104075