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Comparison of vaccination protocols against *Mycoplasma hyopneumoniae* during the gilt acclimation period

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ABSTRACT

This study evaluated different gilt vaccination protocols against *Mycoplasma (M.) hyopneumoniae* at acclimation and their effect on the genetic diversity. A total of 180 *M. hyopneumoniae* naïve gilts were selected 1 week post-entry (wpe) at the acclimation barn in a clinically affected *M. hyopneumoniae* farm. Gilts were distributed according to the *M. hyopneumoniae* antibodies levels into three different vaccination schedules: A) four doses of a *M. hyopneumoniae* commercial vaccine at 2, 4, 6 and 8 wpe; B) two vaccine doses at 2 and 6 wpe and PBS at 4 and 8 wpe; and C) four PBS doses at the same wpe. Detection of *M. hyopneumoniae* (rt-PCR) and antibodies (ELISA) were assessed in gilts at 1, 14, 27 and 34 wpe and in 6 of their piglets at weaning. Rt-PCR positive gilts were detected at 14 wpe, being the proportion significantly lower in groups A and B (3/120, 3%) than C (27/60, 45%). Seroconversion was detected at 14 wpe, showing significant differences in percentage of inhibition (PI) between groups A (median 4.9, range 3.1–19.9) and B (5.5, 3.7–13.5), and C (14.3, 3.3–53.2). Gilts remained seropositive over the study and significant differences in PI were detected between groups A and B versus C. All piglets were rt-PCR negative, but the proportion of seropositive piglets coming from vaccinated gilts was significantly higher than the non-vaccinated group. *M. hyopneumoniae* characterization showed high variability. Hence, gilt vaccination with 2 or 4 doses significantly decreased the pathogen infectious pressure, variability, and provided high antibody levels to gilts and their offspring.

1. Introduction

Mycoplasma hyopneumoniae (M. hyopneumoniae) is the primary agent of enzootic pneumonia (EP), a chronic respiratory disease that affects mainly growing and finishing pigs. This disease is an important concern to the swine industry due to economic losses generated by a reduction of performance and growth, as well as antimicrobial treatments and control/eradication costs (Thacker and Minion, 2012).

Mycoplasma hyopneumoniae is transmitted by direct contact between infected and susceptible animals (Maes et al., 1996). Since intrauterine infection has not been described, piglets are considered *M. hyopneumoniae*-free at birth. Hence, the first exposure to *M. hyopneumoniae* would occur during the lactation period when piglets are in contact with a shedding dam (Sibila et al., 2007). Taking into account that piglet colonization at weaning has been correlated with *M. hyopneumoniae* prevalence and severity of lung lesions in growing-finishing pigs (Fano et al., 2007; Sibila et al., 2007), a reduction of transmission between dams and offspring during the lactation period, together with the piglet vaccination, seem to be a key point for disease control. Additionally, bacterial shedding of gilts and young sows seems to be higher than that of older parity sows (Boonsoongnern et al., 2012). Thus, an adequate gilt acclimation focused on decreasing the bacterial shedding at first farrowing should aid in reducing piglet colonization at weaning age (Pieters and Fano, 2016).

Information on gilt acclimation practices is limited. In Europe, a previous study based on a survey identified the vaccination against *M.*

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hyopneumoniae as the acclimation strategy most used by the participants (Garza-Moreno et al., 2017). Likewise, *M. hyopneumoniae* gilt vaccination has been described as the most common practice in US (Fano and Payne, 2015). Recently, a review compared the acclimation practices carried out in Europe and America (Mexico and USA), being vaccination the main strategy used as well (Garza-Moreno et al., 2018).

Gilt vaccination schedules against *M. hyopneumoniae* can be variable among farms in terms of number of administered vaccine doses and application timings. Thus, gilt vaccination protocols based on multiple doses are becoming commonly used in some parts of the world (Alfonso et al., 2004; Yeske, 2007; Garza-Moreno et al., 2018). Nevertheless, no information regarding the efficacy of multiple gilt vaccination during the acclimation period is available in the literature. Therefore, this study aimed to compare different gilt vaccination schedules on the *M. hyopneumoniae* gilt seroconversion and shedding at different times post-vaccination, as well as piglet antibody detection and colonization at weaning. Moreover, this study also investigated the potential effect of vaccination on the genetic diversity of *M. hyopneumoniae* within the studied farm.

2. Material and methods

2.1. Farm management and housing conditions

A conventional *M. hyopneumoniae* positive, clinically affected (Garza-Moreno et al., 2018) farrow-to-finish farm introducing external negative and own positive replacement gilts was selected. The selected farm had a gilt development unit (GDU) for acclimation with a duration approximately of 10 weeks, which followed all-in/all-out management practices. After acclimation, external and own replacement gilt batches were moved to oestrus detection and synchronization unit (DSU) and were allocated sharing pens (60 gilts per pen). At the DSU, gilt oestrus was synchronized and gilts were divided in weekly batches of 90 gilts according to oestrus detection time. Gilts were artificially inseminated at the second oestrus and pregnancy diagnosis was performed 4 weeks after insemination. Once pregnancy was confirmed, pregnant gilts were moved to the gestation unit and housed in pens (60 gilts/pen). Finally, one week previous to delivery, gilts were moved to the farrowing units. In these facilites, with continuous flow and weekly batch management, a total of 50 gilts and sows of different parities were housed.

2.2. M. hyopneumoniae infectious status previous to the start of the study

To confirm *M. hyopneumoniae* gilt infection during acclimation, laryngeal swabs (LS) from a total of 20 gilts (10 gilts/batch) showing clinical signs of dry coughing from two previous batches of own replacement (FS1 and FS2) were collected at 14 weeks post-entry (wpe) in the GDU. In these samplings, 19 out of 20 (95%) gilts were *M. hyopneumoniae* positive by real-time PCR (rt-PCR), being 10 out of 10 (100%) positive gilts in FS1 and 9 out of 10 (90%) in FS2. The Ct values varied from 30.7 to 36.9 in FS1 and from 27.5 to 36.9 in FS2.

2.3. Animal selection and study design

Blood samples (BS) and LS from a total of 180 six month-old gilts coming from an external *M. hyopneumoniae* negative farm were collected at 1 wpe at the GDU (Fig. 1). Laryngeal swabs and BS were tested for detection of the pathogen and the antibodies against it, respectively. Afterwards, gilts were randomly divided into three groups (A: four vaccine doses; B: two vaccine doses and C: no vaccinated) balanced according to *M. hyopneumoniae* antibodies measured by ELISA percentage of inhibition (PI). Animals received intramuscularly 2 mL per dose of a *M. hyopneumoniae* commercial vaccine (Hyogen®, CEVA Santé Animale, Libourne, Cedex, France), indicated for pigs from 3 weeks of age or older, and/or 2 mL of phosphate-buffered saline (PBS) according to their experimental group and time points (Table 1).

Table 1
Vaccination schedules against *M. hyopneumoniae* performed during the gilt acclimation period.

Group name	No. of gilts	Vaccine doses	Vaccination schedule			
			2 wpe	4 wpe	6 wpe	8 wpe
A	60	4	Hyogen®	Hyogen®	Hyogen®	Hyogen®
B	60	2	Hyogen®	PBS	Hyogen®	PBS
C	60	0	PBS	PBS	PBS	PBS

wpe: weeks post-entry; PBS: phosphate-buffered saline.

Gilts included in this study entered at the DSU splitted into two batches (n = 90 each batch) separated by one week. Piglets were ear tagged at birth according to their maternal treatment and cross-fostering was allowed only between sows within the same treatment. One day prior to weaning, six randomly selected piglets per sow were monitored and sampled. No antimicrobials against *M. hyopneumoniae* were administrated to gilts and piglets under study.

Study procedures were approved by the Animal Experimentation Ethics Committee of the Generalitat de Catalunya (*Departament de Territori i Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural*; Reference 9336).

2.4. Sample collection and processing

Blood samples and LS were collected from gilts at 1, 14, 27 and 34 wpe, and from 6 piglets of each of them at weaning (Fig. 1). Once in the laboratory, blood was centrifuged at 1500 *g* for 10 min at 4 °C and sera was aliquoted and stored at -20 °C until used. Laryngeal swabs were re-suspended in 1 mL of PBS, vortexed, and stored at -20 °C until DNA extraction was performed.

2.5. Detection of M. hyopneumoniae-specific antibodies in serum

Sera were tested in duplicate for the presence of antibodies against *M. hyopneumoniae* by means of a commercial competitive ELISA (IDEIA™ *M. hyopneumoniae*, EIA kit, Oxoid, Thermo Fisher Scientific, UK). ELISA results were expressed as percentage of inhibition (PI). The PI was calculated considering the mean optical density (OD) of each sample and the buffer control following the formula: % PI = 100*(mean sample OD/ mean buffer control OD). Samples with PI < 50% were considered to be positive, whereas doubtful (PI from 50 to 64%) and negative samples (PI ≥ 65%) were classified as negative. PI median and ranges of tested samples were calculated.

Fig. 1. Study design, housing location and sampling points of gilts and piglets included in the study. Housing sites are gilt development unit (GDU), detection and synchronization unit (DSU), gestation unit (GU) and farrowing unit (FU). Shapes represent gilt vaccination (▲) and sampling points of gilts (●) at different weeks post-entry (wpe) into GDU indicated by numbers. Additionally, piglets (♦) were sampled at weaning (equivalent to 38 wpe of gilts).

Table 2
Proportion (%) of *M. hyopneumoniae* seropositive gilts and piglets and median (range) of percentage of inhibition (PI) at different sampling points.

Groups	Sampling points									
	1 wpe		14 wpe		27 wpe		Prefarrowing (34 wpe)		Piglets (at weaning)	
	Prop (%)	PI (Range)	Prop (%)	PI (Range)	Prop (%)	PI (Range)	Prop (%)	PI (Range)	Prop (%)	PI (Range)
A	0/60 0%[a]	67.1 (51.2-89.4)[a]	60/60 100%[a]	4.8 (3.1-19.8) [a]	52/52 100%[a]	6.8 (3.4-39.3)[a]	49/49 100%[a]	5.7 (3.6-17.8) [a]	230/252 91%[a]	15.5 (2.1-98.7)[a]
B	0/60 0%[a]	67.1 (50.6-85.5)[a]	60/60 100%[a]	5.5 (3.7-13.5)[b]	49/49 100%[a]	8.07 (2.8-22.4)[a]	47/47 100%[a]	6.2 (4.0-36.6) [a]	200/252 79%[a]	23.9 (2.0-94.3)[a]
C	0/60 0%[a]	66.9 (50.4-85.2)[a]	59/60 98%[a]	14.3 (3.3-53.2)[c]	45/51 88%[b]	31.7 (6.6-82.1)[b]	44/50 88% [b]	28.7 (5.0-61.8)[b]	85/240 35% [b]	27.3 (1.0-99.0)[b]
Total	0/180	–	179/180 (99.4)	–	146/152 (96.1)	–	140/146 (95.9)	–	515/744 (69.2)	–

wpe: week post-entry; *Prop*: Proportion; Range: minimum – maximum; Different superscripts within each column indicate significant differences among groups at different time points ($p < 0.05$).

2.6. DNA extraction and M. hyopneumoniae detection by real time PCR

DNA was extracted from 200 µL of LS suspension using MagMax™ DNA Multi-Sample Kit (Life Technologies, USA) according to the manufacturer's instructions, on the BioSprint 96 workstation (Qiagen GmbH, Germany). Two different positive extraction controls were used in each extraction: a LS spiked with *M. hyopneumoniae strain* 11 (ATCC®25095™) and a commercial internal positive control (Xeno™, included in VetMax™-Plus qPCR Master Mix kit). Negative controls (PBS) were also included to assess potential contamination during extraction.

Extracted DNA was tested by a commercial real time PCR (rt-PCR) for *M. hyopneumoniae* detection: VetMax™-Plus qPCR Master Mix (Life Technologies, USA) and VetMax™ *M. hyopneumoniae* Reagents (Life Technologies, USA), according to the manufacturer's instructions. Rt-PCR runs were carried out in ABI PRISM® 7500 machine (Applied Biosystems, Singapore). The rt-PCR threshold was set at 10% of the maximum fluorescence value of the commercial DNA positive control. Samples with cycle threshold (Ct) values equal or lower than 40 were considered positive. Ct ranges were calculated considering only rt-PCR positive samples.

2.7. M. hyopneumoniae genetic variability

Positive rt-PCR LS were genotyped by the Sanger sequencing method and the variable number of tandem repeats (VNTR) of three loci (P97, P146 and H1) were counted. Moreover, the reference strain (RF) 11 (ATCC® 25934™) was also included as technique positive control. Primers used were previously described by Vranckx et al., (2011). These three loci were individually amplified in a final volume of 50 µL. Reaction mixtures contained 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 0.4 pmol/µL of each primer, 0.03U/µL U of GoTaq® G2 Flexi DNA Polymerase (Promega, Madison, USA) and, finally, 6 µL of extracted DNA dilution (1:10). Cycling conditions were 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 53 °C and 30 s at 72 °C, then a final extension step of 7 min at 72 °C. The typing PCR products were analysed by electrophoresis on 2% agarose gel in Tris-Acetate-EDTA (TAE)-buffer and stained with ethidium bromide. These PCR products were purified by ExoSAP-IT® (Isogen Life Science, The Netherlands) according to manufacturer's instructions and sequenced using a ABI PRISM 3130xl (Applied Biosystems, Singapore) genetic analyser.

Nucleotide sequences were aligned and translated to aminoacid sequences using FingerPrinting II Informatix software (Applied Maths, Saint-Martens-Latem, Belgium). VNTR per each locus were counted and a typing variant profile (TP) was assigned according to the combination of the three loci. The TP was considered different when the combination of VNTR per each locus was unique. A minimum spanning tree (MST) was also constructed to visualize the similarity among TP.

2.8. Statistical analyses

Bivariate analysis using the Kruskal-Wallis test was applied for median comparison of PI among gilt groups (A, B, and C) at different sampling points. The homogeneity of PI values in each group through the study was evaluated by *F* values. The Chi square test was used to evaluate the proportion of positive rt-PCR samples between treatments at different sampling points. When significant results were obtained, *a posteriori* contrast analysis 2 to 2 was performed. *Post hoc* pairwise comparisons were computed using Tukey's Honestly Significant Difference. Additionally, a linear mixed model was used to assess the effect of different gilt vaccination programs on piglet colonization and humoral immunity at weaning, considering sow as a random effect. Statistical analyses were performed with SAS v9.4 (SAS Institute Inc., Cary, NC, USA). The significance level was set to $p < 0.05$.

3. Results

3.1. Detection of antibodies against M. hyopneumoniae in gilts and piglets

Studied gilts were seronegative at 1 wpe (Table 2, Fig. 2). At 14 wpe all gilts with the exception of one in group C had seroconverted (179/180, 99.4%). By 27 wpe, the number of gilts were reduced from 180 to 152 (52, 49, and 51 gilts in groups A, B and C, respectively) since 28 gilts were culled (due to lack of pregnancy and lameness, mainly). At that sampling point, all vaccinated gilts remained seropositive, but six non-vaccinated gilts were seronegative. From 146 gilts (49, 47, and 50 gilts in groups A, B and C, respectively) that reached one week prior to farrowing sampling (34 wpe), all vaccinated gilts (groups A and B) remained seropositive, whereas the percentage of non-vaccinated seropositive gilts was slightly lower (44/50, 88.0%). Statistical differences ($p < 0.05$) in terms of proportion of seropositive gilts among vaccinated (A and B) and non-vaccinated (C) groups were detected at 27 and 34 wpe.

Mean PI values (± SD) for each group at different sampling time points are detailed in Table 2. The PI values were statistically different ($p < 0.05$) between vaccinated (A and B) and non-vaccinated (C) groups at 14, 27 and 34 wpe (Table 2; Fig. 2). Statistical differences ($p < 0.05$) among all three groups (A, B and C) were detected in terms of PI at 14 wpe, showing differences between four and two vaccine doses. The *F* values showed statistical higher homogeneity of IP values (Fig. 2) through this study in vaccinated group A ($F = 1.27$) compared

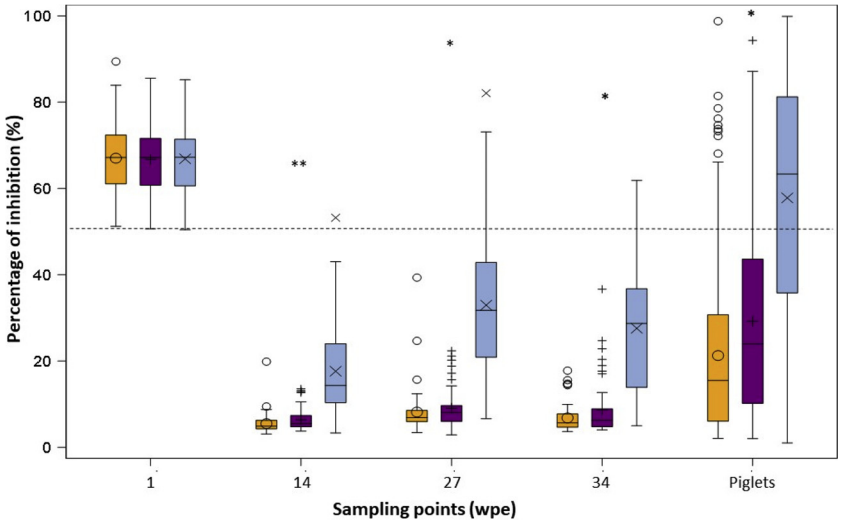

Fig. 2. Percentages of inhibition (%) of gilts from groups A (■), B (■) and C (■) at 1, 14, 27 and 34 wpe; and from their piglets at weaning. Statistically significant differences ($p < 0.05$) were observed between vaccinated (A and B) and non-vaccinated groups (C) (*) as well as among all three groups (**). The discontinuous line represents ELISA seropositivity threshold.

to group B ($F = 3.94$) and C ($F = 5.49$).

A total of 744 piglets (252 from gilts of group A [n = 42], 252 from gilts of group B [n = 42] and 240 from gilts of group C [n = 40]) born to the 124 gilts that reached farrowing were sampled one day prior to weaning. The differences in the proportion of seropositive and PI of piglets from vaccinated and non-vaccinated gilts were statistically significant. Additionally, a higher number of seropositive piglets coming from gilts from group A was detected compared to the ones from group B (Table 2). A total of 229 piglets were seronegative (30.8%), where 23 (10%) of them came from seronegative non-vaccinated sows (group C) and the remaining 206 (22 [9.6%], 52 [22.7%] and 132 [57.7%] from groups A, B and C, respectively) came from seropositive sows (Table 2).

3.2. *M. hyopneumoniae* detection in LS in gilts and piglets

M. hyopneumoniae positive shedding gilts were detected at 14 and 27 wpe (Table 3). The proportions of rt-PCR positive gilts at 14 wpe in vaccinated groups A (1/60, 1.7%) and B (2/60, 3.3%) were significantly lower ($p < 0.05$) compared to the non-vaccinated group C (27/60, 45%). However, no statistical differences were found between vaccinated groups (A and B) with different number of doses. The proportion of rt-PCR positive gilts was reduced at 27 wpe and only one positive gilt (1/51, 2.2%) was detected in the group C. Finally, none of the gilts was positive at 34 wpe for rt-PCR.

All LS samples from piglets (n = 744) were negative for *M. hyopneumoniae* by rt-PCR.

3.3. Characterization of *M. hyopneumoniae* genotypes through the study

All rt-PCR positive samples detected into previous batches (FS1 = 10 and FS2 = 19), 14 wpe (n = 1 in group A, n = 2 in group B and n = 27 in group C) and 27 wpe (n = 1 in group C) were used for *M. hyopneumoniae* genetic characterization. From these 50 samples, 43 (86.0%) were successfully sequenced by the three selected loci (Table 4). Eleven different genotypes were detected among samples. Five TP were detected in each batch FS1 (n = 20) and FS2 (n = 19), although two variants (TP4 and TP5) were identified in both batches. Three variants were detected at 14 wpe, being different from those detected in previous batches (Table 4). No TP was able to be characterized from the positive gilt from group A at 14 wpe. A MST was generated to show the genetic variation over time (Fig. 3).

Regarding VNTR per each locus, the P97 locus showed limited heterogeneity due to the fact that only two VNTR (9 and 11 repeats) were identified (Table 4). The P146 locus showed higher variability at FS1 and FS2, ranging from 38 to 47 and from 22 to 47 repeats, respectively. The VNTR of P146 at 14 wpe was homologous (20 repeats) in all identified variants. For locus H1, the VNTR varied from 4 to 7 repeats.

4. Discussion

One of the key points to control EP within farms is the *M. hyopneumoniae* transmission between the dam and her piglets. Since gilts are considered the main *M. hyopneumoniae* shedders, acclimation strategies focused on reducing the bacterial shedding at first farrowing have been

Table 3
Proportion (%) of *M. hyopneumoniae* rt-PCR positive gilts and piglets and Ct values at different sampling points of the study.

Groups	Sampling points									
	1 wpe		14 wpe		27 wpe		Prefarrowing (34 wpe)		Piglets (at weaning)	
	Prop (%)	Ct range (Max – Min)	Prop (%)	Ct range (Min– Max)	Prop (%)	Ct range (Max – Min)	Prop (%)	Ct range (Max – Min)	Prop (%)	Ct range (Max – Min)
A	0/60 0%[a]	NA	1/60 2%[a]	38.8-38.8 [a]	0/52 0%[a]	NA	0/49 0%[a]	NA	0/252 0%[a]	NA
B	0/60 0%[a]	NA	2/60 3%[a]	32.2-37.8[a]	0/49 0%[a]	NA	0/47 0%[a]	NA	0/252 0%[a]	NA
C	0/60 0% [a]	NA	27/60 45%[b]	29-1-38.7 [a]	1/51 2%[a]	37.5-37.5	0/50 0%[a]	NA	0/240 0%[a]	NA
Total	0/180	–	30/180 (16.7)	–	1/152 (0.6)	–	0/146	–	0/744	–

wpe: week post-entry; *Prop*: Proportion; *Na:* Non – applicable; Different superscript within each column indicate significant differences among groups ($p < 0.05$). Ct range has been calculated considering only rt-PCR positive animals.

Table 4
Variable number of tandem repeat (VNTR) profiles and typing profile (TP) in *M. hyopneumoniae* positive gilts using three loci.

Sampling point	Number of gilts carrying the variant (%)	VNTR			TP	Group	
		P97	P146	H1		Treatment	n
FS1	1 (2.3)	11	38	6	1	NA	10
	1 (2.3)	11	42	6	2		
	2 (4.5)	11	45	6	3		
	5 (11.3)	11	46	6	4		
	1 (2.3)	11	47	6	5		
FS2	1 (2.3)	11	22	4	6	NA	9
	1 (2.3)	11	43	6	7		
	5 (11.3)	11	46	6	4		
	1 (2.3)	11	47	6	5		
	1 (2.3)	9	22	6	8		
14 wpe	1 (2.3)	9	20	4	9	C	1
	19 (43.2)	9	20	6	10	B	2
						C	17
	4 (9.0)	9	20	7	11	C	4
RF	1 (2.3)	14	21	10	RF	NA	1
Total	44 (100.0)	NA	NA	NA	12	NA	44

TP: Typing profile assigned; *wpe*: week post-entry; RF: Reference strain; *NA:* Non-applicable.

proposed (Pieters and Fano, 2016). Vaccination has been determined as the most used strategy for acclimation as well as elimination, although different number of doses and application timings are being used (Garza-Moreno et al., 2018). However, its effect on gilt shedding, humoral immune response, as well as the maternal derived immunity transfer to piglets was not assessed in any of those studies. The number of vaccine doses and the application timing that has been proposed in the past has been 2 doses at 1 and 3 wpe to GDU (Yeske, 2007), or 3 doses at 55 and 220 days of age of gilts, and another last dose 2 weeks prior to farrowing (Alfonso et al., 2004). On the other hand, the effect of sow vaccination against *M. hyopneumoniae* infection at 8 and 4 (Grosse-Beilage and Schereiber, 2005), or 5 and 3 weeks prior to farrowing (Ruiz et al., 2003; Sibila et al., 2008) has been evaluated. All the three studies agreed that sow vaccination against *M. hyopneumoniae* enhanced levels of antibodies in vaccinated sows as well as in their piglets. In addition, Ruiz et al. (2003) and Sibila et al. (2008) concluded that sow vaccination could reduce, numerically, the prevalence of piglet colonization, whereas Grosse-Beilage and Schereiber (2005) did not evaluate this parameter. Therefore, the objective of the present study was to evaluate the effect of gilt vaccination against *M. hyopneumoniae* using different vaccination programs during the acclimation period on gilt shedding, and consequently, on piglet's colonization.

The current study used vaccination protocols based on multiple doses (four and two) since a number of field studies have hypothesized that a high number of vaccine doses could induce a strong immune response, reduce colonization, and better control the disease (Alfonso et al., 2004; Yeske, 2007). The interval between the first and booster vaccination in the present study was 2 weeks to complete the vaccination protocol within a rather usual timing of acclimation at GDU (Garza-Moreno et al., 2017). In order to minimize the effect of the previous humoral immune status, gilts were distributed into three groups according to ELISA PI values at entry. At 14 wpe, all vaccinated gilts had seroconverted, as expected with commercial vaccines. From the non-vaccinated group, all gilts but one also seroconverted, indicating that *M. hyopneumoniae* natural exposure occurred soon after entry. Significant differences of humoral responses in terms of PI were detected between vaccinated and non-vaccinated groups at all sampling points, as previously described by Kristensen et al. (2004) in sows. Interestingly, all vaccinated gilts remained seropositive during all study duration. On the contrary, the proportion of seropositive gilts in the non-vaccinated group decreased slightly over time (from 98% at 1 wpe to 88% in both 27 and 34 wpe). These findings suggest that vaccination may provide a longer duration of humoral immunity compared to that of natural infection. Furthermore, the statistically significant differences found between animals vaccinated with four or two vaccine doses at 14 wpe suggests that repeated vaccination elicited stronger immune response (lower PI). Indeed, PI values from vaccinated gilts with four doses remained statistically more homogeneous (lower *F* values) than those vaccinated with two doses followed by the non-vaccinated group, suggesting that vaccination helped homogenizing the immune status of the studied population.

A significantly higher percentage of seropositive piglets at weaning from gilts vaccinated four or two times was detected compared to those from non-vaccinated gilts. This finding is in agreement with other studies in sows (Kristensen et al., 1981; Grosse-Beilage and Schereiber, 2005), fitting with the hypothesis that vaccinated gilts show higher antibody levels against *M. hyopneumoniae* (lower PI) than non-vaccinated gilts in colostrum. Nevertheless, no statistical differences were identified between piglets coming from gilts from groups A and B, suggesting the passive humoral transfer might be fairly independent of 2 or 4 doses applied. Additionally, 206 seronegative piglets were from seropositive sows at 34 wpe. This fact could be explained by a poor

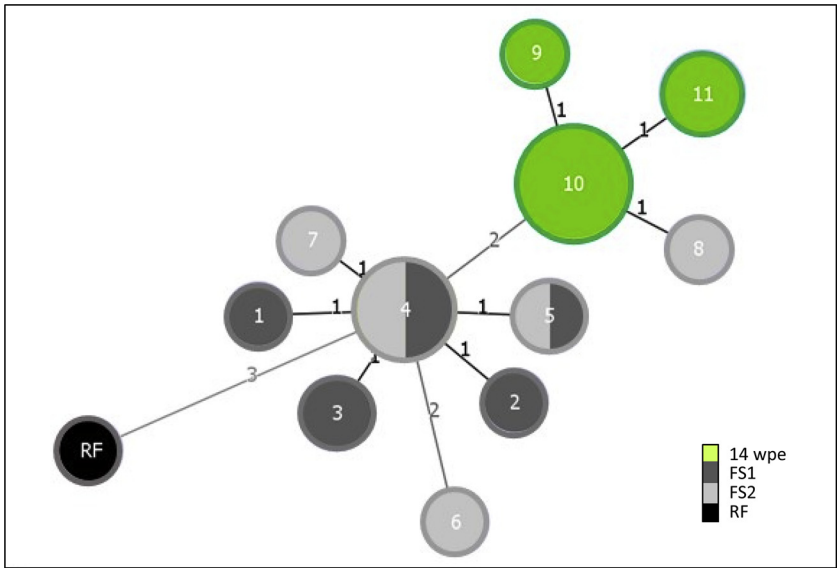

Fig. 3. Minimum spanning tree showing different *M. hyopneumoniae* variant profiles detected. RF: Reference strain 11 used as technique control. Each circle represents a variant profile. The size of the circle is proportional with the number of samples belonging to each variant profile. Absolute distances among variant profiles are represented by link label.

colostrum intake (Quesnel, 2011) and/or the decay of *M. hyopneumoniae* maternal antibodies since previous studies reported a median half-live of maternally derived antibodies of 15 days and, therefore, the amount of transferred antibodies was dependent on the dam's serological status (Morris et al., 1995).

M. hyopneumoniae shedding was first detected at 14 wpe, with the proportion of shedding gilts significantly lower in the vaccinated than in non-vaccinated groups. This finding could be associated with the reduction of *M. hyopneumoniae* bacterial load by vaccination, as previously reported by Woolley et al. (2014). Moreover, an absolute reduction of shedding gilts was detected at 27 wpe in vaccinated groups, and only one non-vaccinated gilt was a shedder. *M. hyopneumoniae* was not detected in any studied group at 34 wpe. The fact that all gilts from the non-vaccinated group seroconverted indicates that *M. hyopneumoniae* infection occurred, and this probably took place in a relatively short period of time, since by 27 wpe only one gilt was detected as PCR positive. Taking this into account, gilts from this trial could have been naturally exposed to *M. hyopneumoniae* around 9–12 wpe, shortly after mixing them with the positive own replacement. The peak of shedding might have been reached at 14 wpe (5 weeks after this exposure) and the complete cease of shedding would have occurred between 27 and 34 wpe (18–25 weeks after exposure). The duration of gilt shedding in this study seemed to be shorter compared to a previous study that detected shedders up to 200 days post-infection under experimental conditions (Pieters et al., 2009). The difference in the duration of shedding may be explained by the lower *M. hyopneumoniae* load in naturally infected gilts under field conditions compared to experimental conditions, where the bacterial load administered endo-tracheally might be much higher. Moreover, given the limited number of samplings over time of this study, the proposed timings about *M. hyopneumoniae* infection dynamics within this breeding herd should be considered as an estimation. Further research is needed to gain insight into infection dynamics of gilts under field conditions.

Previous studies have suggested that *M. hyopneumoniae* piglet colonization at weaning is correlated with respiratory disease and lung lesions at fattening stages (Fano et al., 2007; Sibila et al., 2007). However, in the present field study, *M. hyopneumoniae* gilt shedding one week prior to farrowing (34 wpe) was not detected by rt-PCR and all piglets from vaccinated and non-vaccinated gilts were rt-PCR negative. Thus, association between gilt shedding at farrowing and piglet colonization at weaning could not be assessed. Obtained results are in agreement with previous studies with similar *M. hyopneumoniae* infection dynamics in which piglets were also negative at weaning (Takeuti et al., 2017a, 2017b).

Taking all these results together, the usage of two or four vaccination dose protocols in gilts seemed to be effective strategies for decreasing *M. hyopneumoniae* shedding and infectious pressure within a farm. Furthermore, results using four doses were slightly better (from a numeric point of view) than using two doses regarding ELISA results. Notwithstanding, these differences were only statistically significant in terms of PI at 14 wpe, suggesting that vaccination with these two extra doses is not apparently justified from an infection and seroconversion points of view. Moreover, further studies would be needed to further compare these two doses with a single dose.

The intra-farm genetic diversity of *M. hyopneumoniae* has been previously described using different methods and bacterium loci and thus, results are diverse. Several studies described a high intra-farm genetic diversity (Stakenborg et al., 2006; Nathues et al., 2011; dos Santos et al., 2015; Tamiozzo et al., 2015; Michiels et al., 2017; Takeuti et al., 2017a) whereas others concluded that the variability was limited within the same farm (Stakenborg et al., 2005; Mayor et al., 2007, 2008; Vranckx et al., 2012; Charlebois et al., 2014; Galina-Pantoja et al., 2016). Results from the current study showed high *M. hyopneumoniae* variability within the farm. In the previous gilts batches (FS1 and FS2), higher variability was detected compared to monitored gilts at 14 wpe. Despite variants being different, similarity between TP 8 from FS2 and the more prevalent variant at 14 wpe (TP 10) might indicate that TP 10 was the result of mutations for each loci along this study. These findings are in agreement with previous reports that concluded that Mollicutes can exhibit high mutation and recombination rates by modification of environmental conditions (Razin et al., 1998). Furthermore, this similarity also suggests that the source of infection of gilts included in the study at entry was the own replacement (infected already based on data from FS1 and FS2) located in the same GDU. Unfortunately, information about *M. hyopneumoniae* variants harboured by own replacement over time was not assessed in the present study. Finally, analysing the VNTR obtained per locus, results of P97 and P146 loci are in accordance with previously published reports in which Spanish *M. hyopneumoniae* strains showed approximately 20 repeats in P97 and more than 30 repeats in P146 (dos Santos et al., 2015). No previous data regarding VNTR for H1 are available.

In conclusion, the present study showed that *M. hyopneumoniae* gilt vaccination at acclimation period significantly reduced the *M. hyopneumoniae* shedding of gilts at 14 wpe and increased antibody levels (low PI) of dams and their piglets. Since *M. hyopneumoniae* shedding in gilts was not detected at 34 wpe, the lack of bacterial piglet colonization was expectable. Despite the fact that *M. hyopneumoniae* vaccination does not provide full protection, the infectious pressure within the gilt population of the studied herd was significantly reduced. Gilt vaccination protocol with four doses showed slightly better numerical results than the protocol with two doses during all study. Therefore, these results suggested that the vaccination with two doses seems to be sufficient to reduce the infectious pressure and to induce strong and humoral immune response in gilts. Based on obtained results, a higher number of vaccine doses does not seem to be justified. Finally, the characterization of *M. hyopneumoniae* strains confirmed high genetic variability of this bacterium within the studied farm.

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Conflicts of interest

Laura Garza-Moreno, Roman Krejci, and Marta Carmona are employees of Ceva Santé Animale.

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