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Research article

Sow vaccination modulates the colonization of piglets by *Haemophilus parasuis*

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ABSTRACT

Haemophilus parasuis is the etiologic agent of Glässer's disease in pigs and a colonizer of the upper respiratory tract of healthy pigs. A good balance between colonization and immunity is important to avoid a disease outbreak. This work studied the colonization of *H. parasuis* in healthy piglets coming from vaccinated and non-vaccinated sows. Piglets from vaccinated sows had higher IgG levels at early time points and subsequently were colonized later and to a lower degree than piglets from non-vaccinated ones. The variability of *H. parasuis* isolates was investigated by 2 genotyping methods: enterobacterial repetitive intergenic consensus (ERIC)-PCR and multilocus sequence typing (MLST). A high turnover of strains was found in both groups of piglets, with few strains found on more than one sampling occasion. We found a higher number of *H. parasuis* strains (16 strains) within a given farm than previously thought. Overall, more *H. parasuis* diversity was found in piglets from non-vaccinated sows than in those from vaccinated sows. These results indicate that vaccination of sows in a farm delays the colonization of piglets and reduces the carriage and heterogeneity of *H. parasuis* strains.

1. Introduction

Some microorganisms colonize the upper respiratory tract of piglets early after birth. Some of these "early colonizers" are also pathogens under particular conditions. *Haemophilus parasuis* is one of these colonizers and the etiological agent of the systemic disease, Glässer's disease. Thus, *H. parasuis* can be found in the nose of healthy pigs, but those strains are often non-virulent or virulent but controlled by the immune system (Harris et al., 1969; Møller and Kilian, 1990; Oliveira and Pijoan, 2004).

Classically, *H. parasuis* has been classified by serotyping; however a significant percentage of isolates are non-typeable by this method (Oliveira and Pijoan, 2004). Therefore, genotyping methods have been developed (Olvera et al., 2007b). Enterobacterial repetitive intergenic consensus (ERIC)-PCR, a fingerprinting method, is especially useful to assess the *H. parasuis* strain diversity within a farm (Oliveira et al., 2003; Ruiz et al., 2001), but also sequencing methods have been developed to perform global epidemiological studies (Olvera et al., 2006a,b).

To prevent disease, commercial bacterins can be used directly in the affected piglets or, alternatively, in the sows. Vaccination of sows is less laborious and intends to increase the level of antibodies in the colostrum to be taken by the piglets. However, it is not known how this vaccination affects the colonization of piglets by *H. parasuis*.

Previous studies have shown that several strains can be isolated from conventional farms (Oliveira et al., 2003; Olvera

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et al., 2006a,b; Ruiz et al., 2001; Smart et al., 1993, 1988). However, to our knowledge, there are no reports on the dynamics of colonization of *H. parasuis* over time. Thus, the present work aimed to elucidate the long term colonization process in healthy piglets and the effects of sow vaccination on the dynamics of this colonization by *H. parasuis*.

2. Materials and methods

2.1. Study design

All procedures involving animals followed EU normative (Council Directive 86/609/EEC) and were performed with institutional authorization. The study was performed in a conventional 250-sow farm with a continuous flow production system. The farm was performing the vaccination of sows with a commercial *H. parasuis* bacterin (Hiprasuis®-Glässer, Laboratorios Hipra S.A., Spain), which includes serovars 1 and 6, 20 days before delivery. The farm did not have problems of Glässer's disease during the study. When the study started, 12 sows with different parity numbers (ranging from 3 to 9) were selected: six sows were re-vaccinated and the other six were not vaccinated at that gestation period. For clarity, throughout the text, this will be indicated as vaccinated and non-vaccinated sows. At birth, 4 piglets per sow were randomly selected and labeled. Therefore, 24 piglets per group were included, for a total of 48 piglets in the study. To avoid possible deviations in the study, handling conditions, facilities, environment and feeding conditions were the same for all the animals. Piglets were weaned between 15 and 21 days of age and moved to nursery units where they were mixed with other piglets. Later, at about 10 weeks of age, pigs were transferred to the growing-finishing units.

Sows were sampled once, on the delivery day, including nasal, vaginal and blood samples. Nasal swabs and blood samples from piglets from vaccinated sows were taken on days 0, 3, 7, 14, 24, 60, 90 and 170 after birth. Piglets from non-vaccinated sows were sampled on days 0, 2, 7, 12, 24, 60, 90 and 156 after birth. Sera were obtained from the blood and stored at −80 °C until use.

2.2. H. parasuis detection

Swabs (Deltalab, Barcelona, Spain) from all sows and piglets were obtained in duplicate; one swab was used for bacterial isolation and the other for PCR. Swabs were transported immediately under refrigeration to the laboratory. For bacterial isolation, swabs were plated on chocolate agar (bioMérieux, Madrid, Spain) to isolate colonies. After 2–3 days at 37 °C with 5% CO_2, up to 5 *H. parasuis*-like colonies per agar plate were selected for further identification (Møller and Kilian, 1990). The second set of swabs was processed for DNA extraction with the Nucleospin® blood kit (Macherey-Nagel) following manufacturer instructions. The extracted DNA was used in a species-specific PCR to identify *H. parasuis* (Oliveira et al., 2001).

2.3. Genotyping of H. parasuis isolates

In order to discriminate different strains, all *H. parasuis* isolates were analyzed by enterobacterial repetitive intergenic consensus (ERIC)-PCR following a previously described protocol (Rafiee et al., 2000). ERIC-PCR band patterns were normalized, and Pearson correlation similarity matrices were calculated using Fingerprinting II v3.0 software (Bio-Rad). Cluster analysis of ERIC-PCR fingerprints was performed by the unweighted-pair group method using average linkages (UPGMA) as previously recommended (Ooyen, 2001). The threshold for 2 isolates to be considered the same or different strains was set at 90%.

Representative isolates from the different ERIC-PCR patterns (which included all the different genotypes identified) were analyzed by multilocus sequence typing (MLST) as previously described (Olvera et al., 2006b). Fingerprinting II v.3.0 software (Bio-Rad) was used to edit, assemble and align the sequences and to carry out allele assignment. Cluster analysis was performed by unweighted-pair group method with arithmetic mean (UPGMA) using the matrix of pairwise differences with the START program (Jolley et al., 2001). In addition, a maximum parsimony network among isolates was estimated by the statistical parsimony method of Templeton et al. (1992) using the TCS computer program (Clement et al., 2000) with a presence–absence matrix of the different alleles (columns) in every isolate (row).

Diversity of *H. parasuis* strains at each time point was estimated using the Simpson's Index of Diversity $= 1 - \sum (n/N)^2$, where n is the number of isolates of a particular genotype and N the total number of isolates.

2.4. Detection of antibodies against H. parasuis

Antibodies against *H. parasuis* were evaluated by an in-house capture ELISA. Briefly, 96-well plates were coated with a rabbit hyperimmune serum against the *H. parasuis* strain Nagasaki (1:10,000 in 0.05 M carbonate buffer [pH 9.6]). After overnight incubation at 4 °C, the plates were washed 3 times with 0.05% Tween 20 in PBS (PBS-Tween) to eliminate unbound antibody. Then, a soluble extract of *H. parasuis* Nagasaki was added to the wells (1.5 μg per well in 2% skim milk PBS-Tween) and incubated for 1 h at 37 °C. After 3 washes with PBS-Tween, dilutions of the test sera (1:50 in PBS-Tween and 2% milk) were added and incubated for 1 h at 37 °C. After 3 washes with PBS-Tween, the antibodies were detected with a goat anti-pig-peroxidase conjugate (1/20,000 dilution, 1 h at 37 °C), followed by 50 μl TMB (Sigma–Aldrich, Spain) as substrate. After stopping the reaction with 0.5 M sulfuric acid, plates were read at 450 nm.

2.5. H. parasuis serotyping

All the different strains isolated in the farm (i.e., one isolate of each genotype) were serotyped. Serotype determination was performed by indirect haemagglutination at the Department of Animal Health of the Veterinary School at the University of León (Spain) following a previously published protocol (Del Rio et al., 2003).

3. Results and discussion

At the delivery day, vaccinated sows showed slightly higher level of antibodies in ELISA than non-vaccinated

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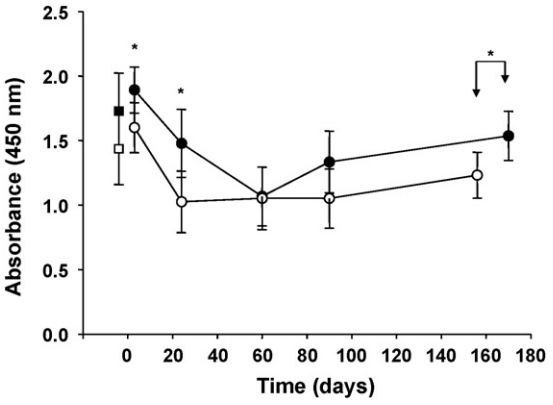

Fig. 1. *H. parasuis* antibodies, IgG, in the sera of piglets from vaccinated (●) or non-vaccinated (○) sows during 6 months of life as measured by ELISA. The level of IgGs in the sows at the time of delivery is also included (■, vaccinated sows; □, non-vaccinated sows). Results are expressed as mean ± standard deviation. *Statistical differences at the indicated time point (Student's *T*-test, $P \leq 0.01$).

ones, although this difference was not statistically significant (Fig. 1; Student's *T*-test, $P = 0.398$). However, piglets from vaccinated sows had significant higher levels of antibodies at 3 and 24 days after delivery (Fig. 1). These results indicate that the level of antibodies in colostrum (indirectly measured by the level of antibodies in piglets at 3 days of age) reflects better the effect of sow vaccination than serum antibodies.

Vaccination of the sows had an effect on the subsequent colonization of the piglets. Piglets from vaccinated sows were colonized later and to a lower degree than piglets from non-vaccinated ones (Fig. 2A and B). Bacterial isolation from nasal swabs showed that 50% of piglets from non-vaccinated sows carried *H. parasuis* at 7 days of age in sufficient quantity to yield a positive culture, while just 25.8% of piglets from vaccinated sows were culture-positive at a later time, 24 days of age. The highest level of nasal colonization by *H. parasuis* in both groups of piglets was found at 60 days of age. At that time, *H. parasuis* was isolated from 100% of the piglets from non-vaccinated sows and from 85.7% of the piglets from vaccinated ones (Fig. 2A). Concurrently with an increase in colonization, a decrease in *H. parasuis* antibodies in the piglets was detected. The level of antibodies in piglets from non-vaccinated sows dropped earlier and was at its minimum level at 24 days of age, while the antibodies in the piglets from vaccinated sows reached its lowest level later, at 60 days (Fig. 1).

By direct PCR of nasal swabs, *H. parasuis* DNA was detected at day 7 in piglets from vaccinated sows and at day 2 in piglets from non-vaccinated ones (Fig. 2B). Sixty days after birth, all nasal swabs from both groups of piglets were positive in the PCR test; such high detection was maintained until the end of the study. These results indicate that all the piglets are eventually colonized by *H. parasuis* (as detected by PCR), but the quantity of bacteria in piglets from vaccinated sows is lower and cannot be detected by bacterial isolation in all of them. As age increased, the level of colonization showed a reduction and at the end of the experiment only 21% and 45% of the piglets from vaccinated and non-vaccinated sows, respectively, were positive by bacterial culture (Fig. 2A). This difference in bacterial isolation went along with a difference in the level of IgGs in both groups of piglets (Fig. 1). The higher level of IgGs in piglets from non-vaccinated sows at this time point does not have a clear explanation, but it might be the effect of immunological priming by other transferred maternal immune components. The reduced bacterial isolation in older animals was in agreement with the lack of isolation of *H. parasuis* from nasal swabs from both groups of sows (vaccinated or non-vaccinated), which was only detected by PCR in 4 out of 6 sows (66.6%), in both groups. In addition, *H. parasuis* was not isolated or detected by PCR from vaginal swabs from either group of sows, indicating that the colonization of the piglets does not occur during delivery, but rather after contact with the sow and receiving the bacteria from the upper respiratory tract.

Taking together, these data indicate that the level of antibodies in a pig modulates the timing and level of colonization by *H. parasuis*. Moreover, vaccination of the sows induced a higher level of antibodies in these animals, which were then transferred to their litters. The presence of higher level of antibodies in the piglets reduced their

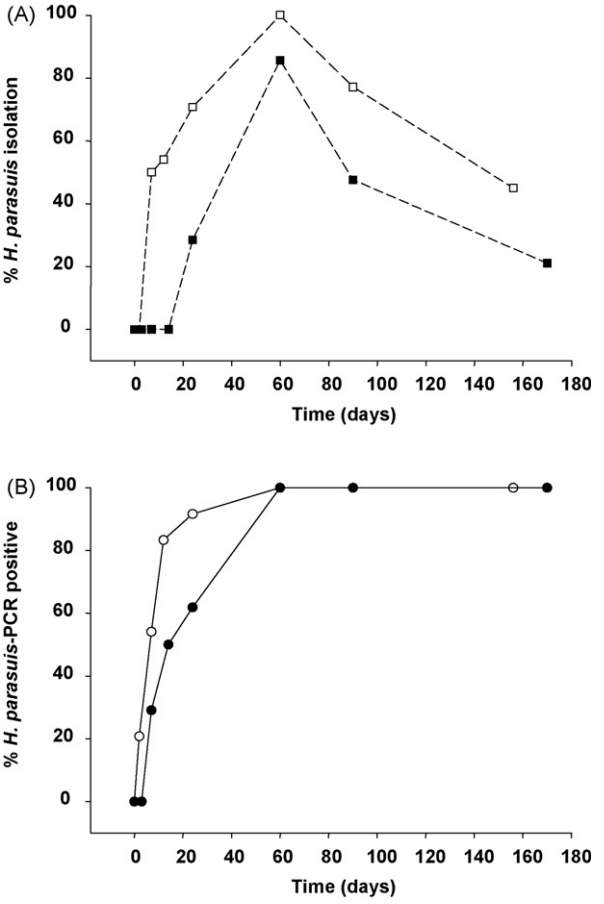

Fig. 2. Detection of *H. parasuis* in nasal swabs from piglets from vaccinated (black symbols) and non-vaccinated (white symbols) sows by bacterial culture isolation (A) and PCR detection (B).

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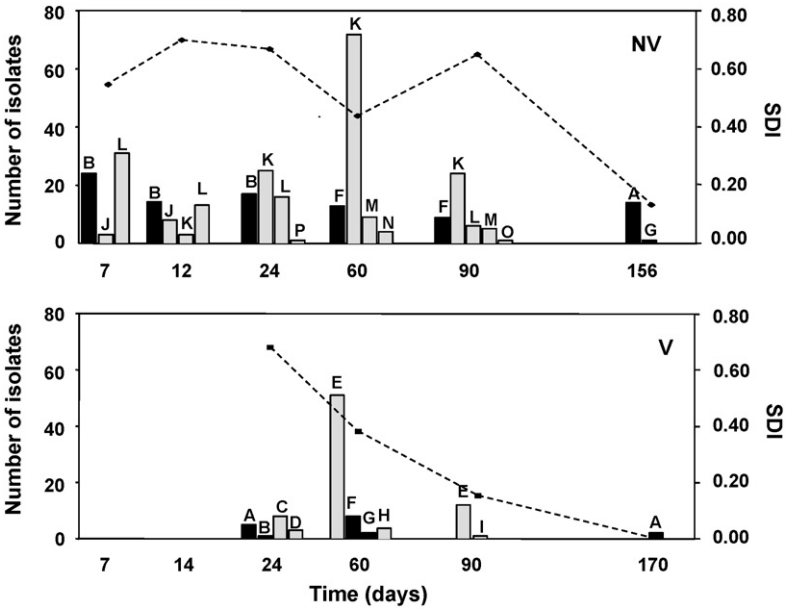

Fig. 3. Number of *H. parasuis* isolates from the different genotypes (each genotype is indicated by a letter from A to P) determined by enterobacterial repetitive intergenic consensus (ERIC)-PCR and multilocus sequence typing (MLST) from piglets from non-vaccinated (NV, top panel) or vaccinated (V, bottom panel) sows. Genotypes isolated from both groups of animals are indicated in black bars. Simpson's diversity index (SDI) is also shown in both panels in dashed lines.

nasal colonization. This reduction in the amount of *H. parasuis* might represent a lower risk for future development of disease by this bacterium. This phenomenon has been already described for other respiratory pathogens, such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* in humans (Faden et al., 1997, 1991; Harabuchi et al., 1994) or *Mycoplasma hyopneumoniae* in pigs (Fano et al., 2007; Sibila et al., 2007), where the colonization of the upper respiratory tract by these pathogens correlates with the probability of disease development.

In a previous report, Kirkwood et al. (2001) found that vaccination of the sows with an autogenous *H. parasuis* bacterin increased the level of specific antibodies in the piglets. However, they did not detect an effect of the vaccination on the timing or proportion of animals colonized by *H. parasuis*. This difference could be due to the different conditions of the study: use of an autogenous bacterin, shorter study time frame (14–28 days of age), and the way the results were analyzed, since they considered positive colonization when 50% of the litter was positive.

It is already known that different strains can be found in a single farm (Oliveira et al., 2003; Olvera et al., 2006b, 2007a; Smart et al., 1993), but the evolution of the strains over time had not been studied yet. Therefore, all the *H. parasuis* isolates from this study (436 isolates) were analyzed by ERIC-PCR to determine the different strains in the animals. Sixteen different strains were detected and each of them was then genotyped by MLST and assigned a code (letters A–P). The distribution of the strains was examined in piglets from both vaccinated and non-vaccinated sows (Fig. 3). During the length of the experiment, 16 different strains were detected on the farm, but the number of different strains was lower (between 2 and 7) at a given time point. A slightly higher number of strains were found in piglets from non-vaccinated sows (11 different genotypes) than in those from vaccinated sows (9 different genotypes). Four strains, labeled A, B, F and G, were found in both groups of piglets, but many strains were detected only in one of the groups (Fig. 3). Interestingly, the highest Simpson's diversity index was found at 24 days while the number of isolates increased to a maximum at 60 days (Fig. 3). These results correlated with the predominance of genotype K (72/98 isolates) in non-vaccinated animals and genotype E (51/65 isolates) in vaccinated animals and it could be indicative of the higher fitness of these genotypes.

Our results indicate that there is more variability of *H. parasuis* strains within a given farm than previously thought. This high strain diversity suggests a rapid strain turnover, which indicates that *H. parasuis* colonization is an active, dynamic process. A rapid turnover and high diversity of strains colonizing the upper respiratory tract of children has also been observed in *H. influenzae* (Dhooge et al., 2000; Lacross et al., 2008; Samuelson et al., 1995), however, no association was found between carriage of multiple *H. influenzae* strains and history of frequent ear infections (Samuelson et al., 1995; St Sauver et al., 2000).

We did not detect a clear relationship between the different strains or any association to their origin (from vaccinated or not vaccinated sows) or isolation time. Fig. 4 shows the statistical parsimony network among isolates. A dispersal distribution of strains was reported, indicating the lack of relationship between the different strains, their origin and the isolation time. Also, a remarkable point was the appearance and disappearance in different points of the network of particular alleles from several of the genes used in the analysis (i.e. *atpD6*, *rpoB27*, *6pgd20 frdB11*,

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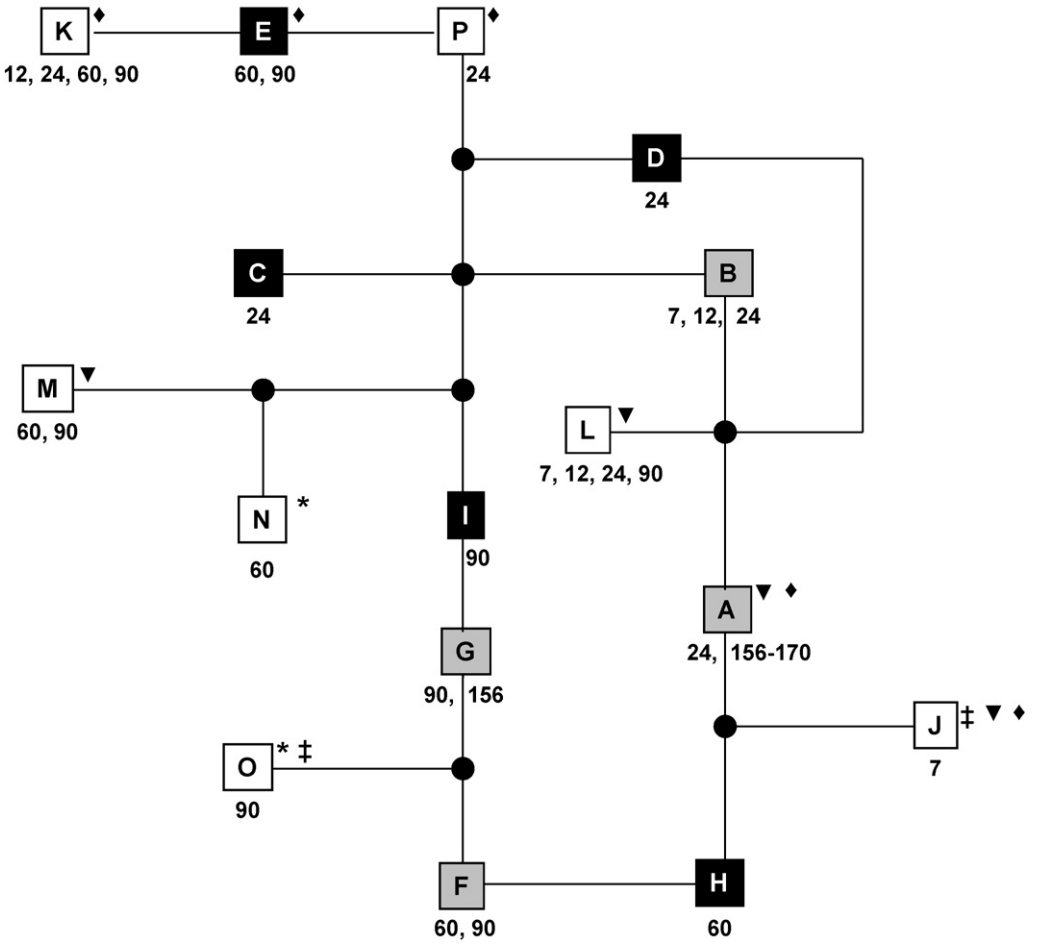

Fig. 4. Statistical parsimony network among isolates from piglets from vaccinated and non-vaccinated sows. Each genotype is indicated by a letter from A to P. Genotypes isolated only from piglets from vaccinated sows are indicated in black squares; genotypes isolated only from piglets from non vaccinated sows are shown in white squares; genotypes isolated from both groups, are shown in grey. Alleles that suggest lateral transfer of genetic material are indicated by symbols beside some of the genotypes. *, allele *rpoB27*; ‡, *6pgd20*; ▼, *atpD6* and ◆, *frdB11*. Numbers under each genotype indicate isolation time in days.

Fig. 4). Although it is possible that marginal genotypes were not isolated and the network is incomplete, our observation is indicative of the existence of lateral gene transfer among the strains and agrees with previous observations supporting this phenomenon in *H. parasuis* (Kehrenberg et al., 2005; Olvera et al., 2006a; Pina et al., 2009).

In addition, the strains were compared with other *H. parasuis* strains by MLST and cluster analysis by unweighted-pair group method with arithmetic mean (UPGMA). While a single strain (genotype H, isolated from piglets from vaccinated sows) grouped with strains of systemic origin, nine strains grouped with those of nasal origin and six with a cluster with no clinical association (data not shown). The most abundant genotypes, B, L, K and E, clustered with other nasal isolates.

Limited information was obtained from the serotyping data since a high percentage (65%) of the strains were non-typeable (NT) (Table 1). Although it is common to find a significant percentage of NT *H. parasuis* strains in prevalence studies (Angen et al., 2004; Cai et al., 2005; Oliveira et al., 2003; Rubies et al., 1999), the percentage

Table 1
Correspondence between genotypes and serovars in the different strains isolated in the farm.

Genotype	Serovar
$A^{nv,v}$	NT[a]
$B^{nv,v}$	15
C^{v}	NT
D^{v}	15
E^{v}	NT
$F^{nv,v}$	2
$G^{nv,v}$	15
H^{v}	NT
I^{v}	NT
J^{nv}	NT
K^{nv}	NT
L^{nv}	NT
M^{nv}	15
N^{nv}	NT
O^{nv}	15
P^{nv}	NT

nv: isolated from piglets from non-vaccinated sows. v: isolated from piglets from vaccinated sows.
[a] Non-typeable.

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found in this study is unusually high. This result may be explained by the "uncommon" origin of the strains, nasal cavity, which is not included in prevalence studies where only clinical isolates are studied. The rest of strains were assigned to serovars 15 (5 strains) and 2 (1 strain). Strains from serovars 15 and 2 were found in both groups of piglets. Thus, we could not detect any association between the application of the vaccine and the elimination of specific serotypes.

4. Conclusion

The diversity of *H. parasuis* within a farm is higher than previously thought. Vaccination of sows reduced not only the colonization of the upper respiratory tract of piglets, but also the variability of strains of *H. parasuis* colonizing the piglets. This reduction of *H. parasuis* carriage in the piglets could be the underlying mechanism to control disease in the piglets by vaccinating the sows.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with people or organisations that could inappropriately influence or bias the content of this study.

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