Colonization of Suckling Pigs by *Streptococcus suis* with Particular Reference to Pathogenic Serotype 2 Strains

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ABSTRACT

Three swine commercial farms with high mortality rates in nursery pigs due to *Streptococcus suis* serotype 2 were studied. Brain samples from diseased animals were collected for a period of 6 to 10 mo and used to isolate the strain that was responsible for the mortality (virulent strain) in each farm. Tonsil swabs from piglets at 5, 10 and 15 d were taken to assess both total colonization and colonization by the virulent strain. The effect of sow vaccination against *S. suis* on colonization was evaluated in 1 of the farms. All suspect tonsil isolates were identified biochemically and then tested against serotype 2. The genomic patterns of serotype 2 isolates were compared to that of the virulent strain using Rep-PCR. Results showed that total colonization by *S. suis* occurred very early in the pigs' life, with most animals being colonized by weaning age. Prevalence of colonization by serotype 2 strains was much lower than total colonization. After comparing serotype 2 isolates with the virulent strains, only 1 tonsillar isolate had the same genomic pattern as the virulent strain and it belonged to a 4-week-old weaned pig. The genomic pattern of the virulent strain was not found in any tonsillar isolate from 15-day-old or younger pigs. Although limited by sample size, sow vaccination against *S. suis* increased total colonization at the same time significantly decreasing colonization by serotype 2 strains.

Even though most pigs are colonized early in age by *S. suis*, colonization by the virulent strain is of low prevalence and delayed in time. This could constitute a risk factor for developing the disease later in time, because animals would be colonized when maternal immunity is no longer present, allowing the organism to become systemic.

RÉSUMÉ

Des échantillons de cerveau furent prélevés pendant une période de 6 à 10 mois chez des porcelets malades en pouponnière en provenance de trois élevages commerciaux où des taux de mortalité élevés dus à *Streptococcus suis* sérotype 2 étaient observés. Un écouvillonnage des amygdales de porcelets aux âges de 5, 10 et 15 jours fut effectué afin d'évaluer la colonisation totale ainsi que la colonisation par la souche virulente. L'influence de la vaccination des truies contre *S. suis* sur la colonisation fut évaluée dans une des fermes. Tous les isolats suspects provenant des amygdales étaient identifiés biochimiquement de même qu'à l'aide d'un antisérum dirigé contre le sérotype 2. Les patrons génomiques des isolats de sérotype 2 furent comparés à celui de la souche virulente. Les résultats démontrent que la colonisation totale par *S. suis* survient très tôt dans la vie du porcelet et que la majorité des animaux étaient colonisés à l'âge du sevrage. La prévalence de la colonisation par le sérotype 2 était beaucoup plus faible que la colonisation totale. Après comparaison des isolats de sérotype 2 avec les souches virulentes, seulement un isolat des amygdales avait le même patron que la souche virulente et il provenait d'un porcelet sevré âgé de quatre semaines. Le patron génomique de la souche virulente ne fut pas retrouvé parmi aucun des isolats provenant des amygdales des animaux âgés de 15 jours ou moins. Bien que la taille du groupe soit restreinte, la vaccination des truies envers *S. suis* semble augmenter la colonisation totale mais en même temps semble réduire de façon significative la colonisation par les souches su sérotype 2. Même si la plupart des porcs sont colonisés en bas âge par *S. suis*, la colonisation par la souche virulente est de prévalence faible et retardée dans le temps. Ceci pourrait constituer un facteur de risque au développement de la maladie à un âge plus avancé, les animaux étant colonisés alors que l'immunité maternelle n'est plus présente, permettrait ainsi au micro-organisme de devenir systémique.

(Traduit par docteur Serge Messier)

INTRODUCTION

Streptococcus suis serotype 2 is one of the most important etiological agents of meningitis in swine. Its distribution has been reported worldwide and its incidence has increased since pigs are raised in intensive systems (1). Outbreaks of *S. suis* meningitis can be seen in pigs of all ages, but nursery pigs are more commonly affected. Nursery mortalities normally range between 4 and 6% but values up to 14% have also been reported. Meningitis is the predominant sign in affected animals, with arthritis, endocarditis and pneumonia also occasionally seen (2). There are at least 35 serotypes of *S. suis* (3–6). Previous reports have shown a variable prevalence of these different serotypes (7–9). Serotypes 2, 3, 4, 5 and 7 are

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the most prevalent when only serotypes isolated from clinical cases are considered (7).

Modern trends in swine production are towards raising pigs under high health conditions. To accomplish this, pigs are weaned early and separated from the sows in off-site nurseries. Efforts to eliminate *S. suis* using segregated early weaning (SEW) techniques have failed, since pigs are already colonized by 5 d of age (10,11). *S. suis* is considered a part of the normal pig's upper respiratory flora (12), with animals harboring more than 1 serotype in their tonsils (13–15). The pathogenesis of *S. suis* is not fully understood. Differences in virulence have been observed among serotypes and within strains of the same serotype (16–19). Recently, an increase in incidence has been observed in high health farms that wean between 14 and 18 d. In an attempt to explain this increase in mortality, it has been proposed that the percentage of colonized animals at weaning is a critical factor for the pigs to develop the disease (20). According to this theory, only a few animals are colonized at weaning, with the majority of pigs being colonized later in time when maternal antibodies are no longer present and the animals have not developed their own immunity. However, colonization by *S. suis* occurs very early in the pigs' life. By the time these animals are weaned, most of them yield *S. suis* from their tonsils. Previous studies on *S. suis* colonization have not addressed specific colonization by the problem strain that causes mortality in a particular farm. Mogollon et al (13) demonstrated that in outbreaks of meningitis, there was a predominant strain responsible for most of this mortality. This strain, called the "virulent strain," is apparently established in each farm and can be isolated from the brain of most necropsied animals. It is possible therefore, that colonization by the virulent strain proceeds more slowly than that seen with all *S. suis* strains, with resulting low prevalence of colonized pigs being weaned into separated facilities.

The objective of this paper was to study both total colonization and colonization by the virulent strain in pigs before weaning. The studies were performed in swine commercial farms with a high percentage of mortality due to *S. suis* in nursery pigs.

MATERIALS AND METHODS

SWINE FARMS AND VIRULENT STRAINS

Three swine farms with nursery mortalities ranging from 4 to 12% were selected. Diseased pigs had clinical signs of meningitis showing recumbency, paddling and incoordination. Arthritis was also present in some of the pigs. Bacteriological culture of brain samples from the affected animals yielded *S. suis* serotype 2 at necropsy.

The farms A, B, and C had weaning ages between 14 and 20 d. Farm A was a 1000-sow, 2-site unit with farrowing and nursery rooms in 1 site and finishing in another site, 24 km away. Farms B and C had 700 and 400 sows respectively, with 3 off-site production units separated from each other. Weekly groups of pigs were raised under strict all-in/all-out measures, which minimized microbial transmission between groups. The farms were independent from each other, but farms A and B had purchased boars from the same genetic company at about the same time.

Brain samples from dead animals were taken over time. Seven *S. suis* serotype 2 isolates were recovered from farm A through a period of 8 mo. Eight isolates were recovered from farm B through a period of 4 mo but only 1 isolate was available for farm C. The *S. suis* strains isolated from brain samples are referred as "virulent strain" for each farm. The genetic patterns of these virulent strains were compared by a Rep-PCR technique in order to establish whether the virulent strain in each farm remained the same or had changed overtime. These virulent strains were further characterized and studied for the presence of phenotypic virulence markers. The strains were tested for the presence of MRP (muraminidase release protein) and EF (extracelullar factor) proteins and production of hemolysin as described elsewhere (21,22).

SAMPLING CONDITIONS AND SOW VACCINATION

Tonsillar swab samples were taken from piglets at 5, 10 and 15 d (± 1 d) of age. Between 10 and 30 pigs were sampled in each age group at each farm. Three different samplings were performed in farm A at different times and under different disease and management conditions. In the first sampling (A1), health conditions were stable, but the farm had about 5% postweaning mortality attributed to *S. suis*. The sow herd had experienced a reproductive outbreak of PRRS virus with stillborns, mummifications and premature farrowings 5 mo before the beginning of the trial. By the time the trial took place, the PRRS virus outbreak was under control in the sow herd, but virus was still circulating in the nursery pigs. In the 2nd sampling (A2), the sampled piglets came from sows vaccinated against *S. suis*. The vaccine used was a commercial product prepared with a serotype 2 isolate. It had an oil-in-water adjuvant and was administered intramuscularly at 77 and 98 d of gestation. The 3rd sampling (A3) corresponded to samples from 11 pigs from nonvaccinated sows, 1 wk after weaning (about 25 d of age) that were kept in isolation for other purposes. Only 1 sampling was done in farms B and C. Farm B was very similar to farm A, with a similar history of PRRS virus infection in sows and high nursery mortality attributed to *S. suis*. Farm C was different, in that sows were seronegative to PRRS virus and *S. suis*-associated mortality was seen in the grow-finisher at 12–14 wk of age. Neither farms B or C used sow vaccination against *S. suis* at the time of the study.

S. suis IDENTIFICATION

Tonsillar swabs were cultured onto CNA (Colistin-Nalidixic) blood agar plates. Usually 3 alpha-hemolytic colonies were chosen for further identification. Gram-staining, catalase, amylase, growth in 6.5% of NaCl and Voges-Proskawer (VP) were used as identification tests (23,24). Isolates that were cocci, gram-positive, catalase-negative, amylase-positive, VP-negative and did not grow in 6.5% of NaCl were considered to be *S. suis*. Isolates were serotyped, but only against serotype 2. This was due to the fact that the virulent strain isolated from these farms was always serotype 2. The genomic patterns of the tonsillar isolates that were confirmed as *S. suis* serotype 2 were

compared to the virulent strain for each farm, using Rep-PCR.

REP-PCR

The primers used were ERIC 1R and ERIC 2 (Ransom Hill Bioscience, Ramona, California, USA). PCR amplifications were carried out in a thermocycler (Perkin-Elmer, GeneAmp PCR system 2400) with a final reaction volume of 25 μL, containing 1 μM of each primer, 100 ng of template DNA, 0.3 mM of each 4 dNTPs (Boehringer Gmb, Mannheim, Germany), 3 mM $MgCl_2$ and 1U of Taq polymerase (Boehringer Gmb, Mannheim, Germany) in a 1 × PCR buffer. The PCR amplification conditions were as follows: initial denaturation step, 94°C for 5 min; 30 cycles of 90°C for 30 s; 42°C for 2 min; 72°C for 2 min; and a final extension step of 72°C for 7 min. The PCR products were run on a 2% agarose gel containing 0.5 μL/mL of ethidium bromide, at 73 V for 75 min (25,26). The gel was visualized and photographed using the Eagle eye system (Stratagene, La Joya, California, USA), images were processed using Adobe Photoshop 3.0.

All the *S. suis* isolated from brain samples of diseased animals were analyzed using this technique. Comparison of the genetic patterns of these brain isolates allowed the determination of the genetic pattern of the virulent strain for each farm. Serotype 2 tonsillar isolates were also analyzed by this procedure and their genetic pattern was compared to the virulent strain for each farm.

COLONIZATION PERCENTAGES

The percentage of total colonization was calculated as the number of pigs from which *S. suis* was isolated divided by the total number of sampled pigs in each group. Colonization by *S. suis* serotype 2 strains was calculated as the number of pigs with *S. suis* serotype 2 strains divided by the total number of pigs that were sampled in each group.

STATISTICS

Differences in serotype 2 colonization percentages between piglets coming from vaccinated or nonvaccinated sows were analyzed using logistic regression analysis. Significant differences were considered under $P < 0.05$.

Table I. Results of total colonization by *Streptococcus suis*

	Farm A				
	A1	A2	A3	Farm B	Farm C
0–5 d	19/24[a] (79%)[b]	23/24 (96%)	nt[c]	2/15 (13%)	12/20 (60%)
6–10 d	14/27 (52%)	15/20 (75%)	nt	3/11 (27%)	11/20 (55%)
11–15 d	3/10 (30%)	16/20 (80%)	nt	7/20 (35%)	6/20 (30%)
25 d	nt	nt	9/11 (81%)	nt	nt

[a] Number of animals from which *S. suis* was isolated/total number of animals sampled
[b] Percentage of positive animals (or total colonization percentage)
[c] nt: Not tested
A1: Before *S. suis* sow vaccination; A2: After *S. suis* sow vaccination; A3: Weaned pigs from unvaccinated sows. Farms B and C did not vaccinate sows against *S. suis* at the moment of the samplings.

Table II. Results of colonization by serotype 2 strains

	Farm A				
	A1[1]	A2[2]	A3	Farm B	Farm C
0–5 d	13/24[a] (54%)[b]	5/24 (21%)	nt	0/15 (0%)	7/20 (35%)
6–10 d	4/27 (15%)	0/20 (0%)	nt	1/11 (9%)	0/20 (30%)
11–15 d	0/10 (0%)	1/20 (5%)	nt	2/20 (10%)	3/20 (15%)
25 d	nt	nt	9/11 (81%)	nt	nt

[a] Number of animals from which *S. suis* serotype 2 was isolated/total number of animals sampled
[b] Percentage of positive animals (or percentage of colonization by serotype 2 strains)
[c] nt: Not tested
[1,2] Differences in superscript numbers are statistically significant at $P < 0.05$.
A1: Before *S. suis* sow vaccination; A2: After *S. suis* sow vaccination; A3: Weaned pigs from unvaccinated sows. Farms B and C did not vaccinate sows against *S. suis* at the moment of the samplings.

RESULTS

Total colonization percentages are shown in Table I. Samplings A1, A2 and C had similar results, with higher colonization percentages than those found in sampling B. Colonization percentages increased with age in farm B, but percentages in samplings A1 and C decreased during the first 15 d of life. Percentages in sampling A2 remained about the same over time. The percentage of colonization by serotype 2 strains was always lower than total colonization (Table II). The relationship between total colonization and colonization by serotype 2 strains appeared to be proportional, since higher serotype 2 colonization values were found when total colonization values were also higher. Average values for serotype 2 colonization ranged between 0 and 20%. Two of the samplings had unexpected high values of 54 and 35% at 5 d of age respectively, but in these 2 groups, colonization at 15 d dropped to 0 and 15% respectively. Differences in these values could be attributed to differences in sampling size between ages within each group or to differences between weekly groups of pigs.

Only 1 limited sampling was taken from weaned pigs. These animals were 4 wk old at the time of sampling. *S. suis* was isolated from 9 out of 11 pigs. Serotyping of these isolates showed that all were serotype 2.

Sow vaccination had a small effect of increasing on total *S. suis* colonization. However, when colonization by serotype 2 strains was analyzed, sow vaccination had a significant ($P = 0.005$) effect of reducing colonization by these strains.

Phenotypic studies for the presence of virulence markers revealed that virulent strains from farms A and B were MRP− EF− and hemolysin negative. However, the virulent strain from farm C was MRP+ EF+ and hemolysin positive. The genomic patterns of these virulent strains can be seen in Figure 1. All but one of the isolates from diseased animals in farm A had the same genetic pattern. The genetic patterns of the virulent strains from farms A and B were very similar. Only 1 isolate was available from farm C, with a genetic pattern that was different from those found in farms A and B.

Among brain isolates for each farm, the virulent strain was defined as the strain with a genomic pattern repeated

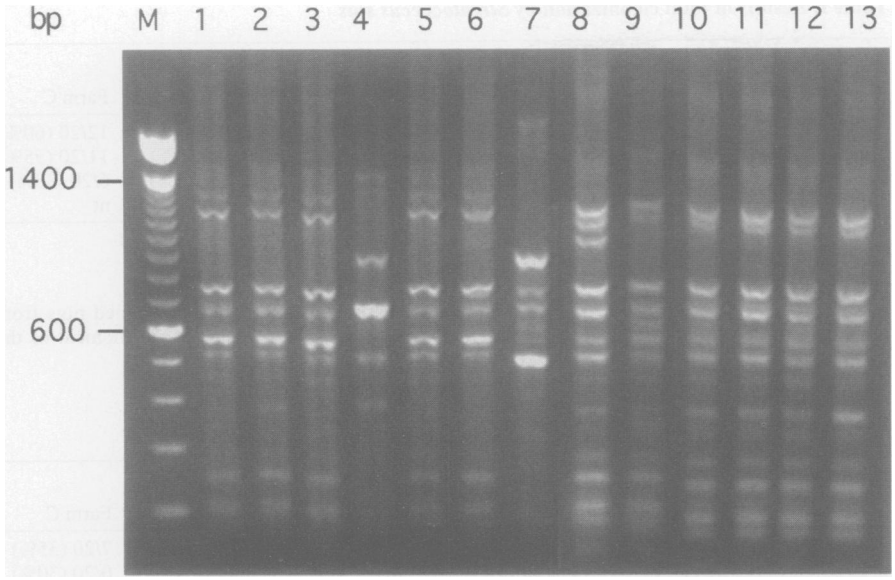

Figure 1. Rep PCR, using ERIC 1R and ERIC 2 primers of virulent strains recovered from farms A, B and C. bp: base pairs. Lane M:100 bp marker. Lanes 1–6: virulent strains from farm A. Lane 7: virulent strains from farm C. Lanes 8–13: virulent strains from farm B.

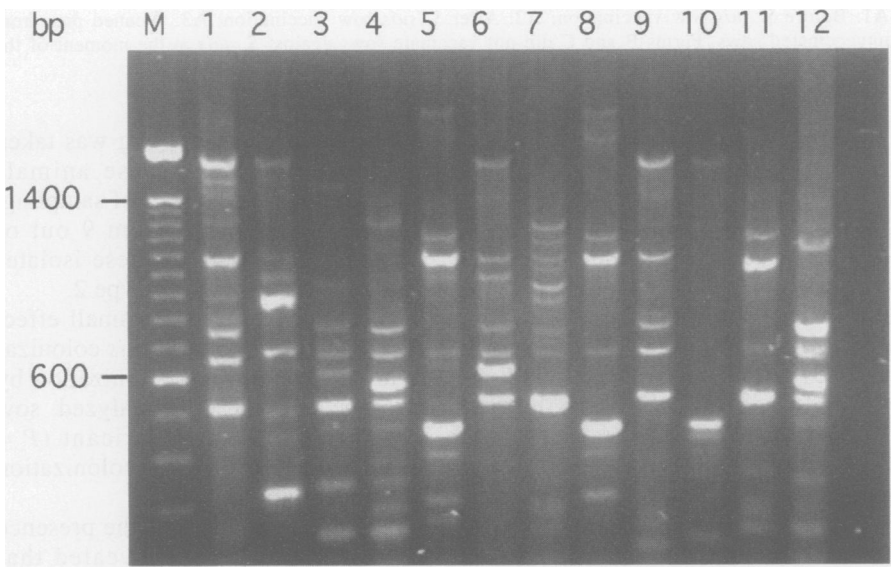

Figure 2. Rep-PCR, using ERIC 1R and ERIC 2 primers, comparing the virulent strain of farm A with the serotype 2 tonsillar isolates from the same farm. bp: base pairs. Lane M: 100 bp marker. Lanes 1–3, 5–12: serotype 2 tonsillar isolates from farm A. Lane 4: virulent strain from farm A.

over time. Results comparing the genomic patterns of the serotype 2 tonsil isolates with the virulent strain from each farm, showed only 1 tonsillar isolate with a very similar genomic pattern as the virulent strain (Figure 2, lane 12). This isolate was recovered from a weaned pig. The genetic pattern of the virulent strain was not found in any of the tonsillar isolates from piglets sampled before 15 days of age.

DISCUSSION

Differences in colonization percentages between samplings from the same farm or between different farms can be attributed to differences in the prevalence of *S. suis*, the amount of bacteria present in each pig, the immune and infectious status of the sows, the effect of management or disease practices and the bacteriological procedures used to isolate *S. suis*.

Previous studies have shown that prevalence of *S. suis* is high in swine commercial farms (13) and that pigs are colonized very early in their lives. Some authors have shown that *S. suis* can be found in tonsils of 1-day-old pigs and that by the time they are weaned most of the animals are colonized. Attempts to eliminate *S. suis* from swine commercial farms using medicated early weaning have failed, again suggesting that *S. suis* colonization takes place very early in life (10,11). *S. suis* is often isolated from tonsils of healthy animals which is why it is considered a normal inhabitant of the pig's upper respiratory tract (12).

In order to take a more specific approach to the study of colonization by potentially pathogenic strains, previous studies have tried to evaluate the prevalence of serotype 2 strains. In some cases, the studies population was much older than the population at risk. Seventy-six percent of pigs were found to be carriers of *S. suis* serotype 2 at slaughter (27). Also, the prevalence of serotype 2 strains in weaned pigs was higher in farms with *S. suis* serotype 2 problems when compared to farms were *S. suis* serotype 2 had not been isolated in the previous years (27,28). Prevalence of *S. suis* serotype 2 in weaned pigs of the affected farms ranged between 0% and 50%. In non-affected farms, only 3 out of 23 farms had animals with serotype 2 strains and prevalence in those farms ranged between 2% and 19% (28). However, since this study was performed in different farms, it is difficult to conclude if the higher colonization prevalence was indeed related to the increase in clinical cases.

In an effort to further study colonization by *S. suis*, we focused on determining colonization by both the *S. suis* serotype 2 strains as well as the virulent strain for each farm. These virulent strains, in the farms studied, were all serotype 2. The percentages of colonization by serotype 2 strains were always lower than those for total colonization. However, colonization by the virulent strain, as determined by the genomic patterns of serotype 2 tonsillar isolates, could not be shown in pigs 15 d old or younger. This could be a reflection of the isolation technique used, the sampling size or it might truly suggest that the

prevalence of the virulent strain before weaning was very low and that most of the colonization of the pigs by the virulent strain occurred after weaning. It is interesting to note that one serotype 2 strain isolate had a different genomic pattern to the virulent. This emphasizes that care should be taken under analyzing data were serotyping is the only factor considered.

Previous reports have shown the usefulness of molecular techniques to study the epidemiology of *S. suis* infections in a farm (29). In this study we chose to use the Rep-PCR technique, which successfully differentiated between serotype 2 strains. We believe that the use of this technique in the study of *S. suis* epidemiology has not previously reported.

The virulent strains were also analyzed phenotypically for the presence of the virulence markers, MRP and EF. The virulent strain for farm C had these markers, but none of the virulent isolates from farms A and B had the markers, even though they were repeatedly isolated from the meninges of acutely ill pigs. Some recent reports on virulence factors have indicated that the MRP and EF proteins may not be the sole virulence markers. A *S. suis* serotype 2 mutant deleted for MRP and EF proteins showed no differences in virulence with the wild type strain after infection of susceptible pigs (30). The present study supports the notion that virulence in *S. suis* may not only depend on the presence of virulence factors but also of the ability to colonize late in time, when maternal antibodies are no longer present. It must also be noted, however, that farms A and B, although geographically distant and not related, purchased boars from the same genetic company. Their virulent strain had the same genomic and phenotypic patterns, suggesting that it might be a strain from a common origin.

Conclusions on the effect of sow vaccination are limited by the fact that only one sampling was performed. However, vaccination appeared to reduce colonization by serotype 2 strains. In the case of *P. haemolytica* (31), it has been shown that cow vaccination reduces colonization. In the present study, colonization by serotype 2 strains was reduced, but total colonization was not affected or slightly increased. The vaccine used in this study was a commercial vaccine containing only a serotype 2 isolate. Since cross-protection among *S. suis* serotypes has not been shown in previous vaccination studies (32), this could explain why total colonization was not affected.

In conclusion, total colonization by *S. suis* followed a similar pattern in all the farms studied. Pigs were colonized early in their lives and by the time they were weaned most of them carried *S. suis* in their tonsils. Colonization by serotype 2 strains appeared to be lower than total colonization. Colonization by the virulent strain was delayed since it could not be detected in pigs 15 day-old or younger, and it was only detected in 1 weaned pig.

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