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Genetic and virulence-phenotype characterization of serotypes 2 and 9 of *Streptococcus suis* swine isolates

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Summary. The aim of this study was to analyze the genetic characteristics and virulence phenotypes of *Streptococcus suis*, specifically, in clinical isolates of serotypes 2 and 9 (n = 195), obtained from diverse geographical areas across Spain. Pulsed-field gel electrophoresis (PFGE) typing identified 97 genetic profiles, 68% of which were represented by single isolates, indicative of a substantial genetic diversity among the *S. suis* isolates analyzed. Five PFGE profiles accounted for 33.3% of the isolates and were isolated from 38% of the herds in nine different provinces, indicative of the bacterium's widespread distribution in the Spanish swine population. Representative isolates of the most prevalent PFGE profiles of both serotypes were subjected to multilocus sequence typing (MLST) analysis. The results indicated that serotypes 2 and 9 have distinct genetic backgrounds. Serotype 2 isolates belong to the ST1 complex, a highly successful clone that has spread over most European countries. In accordance with isolates of this complex, most serotype 2 isolates also expressed the phenotype $MRP^+EF^+SLY^+$. Serotype 9 isolates belong to the ST61 complex, which is distantly related to the widespread European ST87 clone. Also, in contrast to most isolates of the European ST87 clone, which express the large variant MRP*, the majority of serotype 9 isolates (97.9%) did not express the protein. [**Int Microbiol** 2009; 12(3):161-166]

Keywords: *Streptoccoccus suis* · swine · genetic typing · PFGE · MLST · virulence-related factors

Introduction

Streptococcus suis is an important swine pathogen that is widespread among the swine population worldwide and endemic in most pig-rearing countries [4,11,21]. The pathogen is responsible for severe clinical disease in pigs, including meningitis, septicemia, arthritis, bronchopneumonia, endocarditis, abortions, and abscesses [1,9]. The organism can be carried in the tonsils and nasal cavities of apparently healthy animals, which plays an essential role in *S. suis* transmission [9]. *S. suis* is also a zoonotic agent that can cause meningitis, septicemia, arthritis, endocarditis, and a toxic-shock-like syndrome in people in close contact with infected pigs or pork-derived products [12].

Different capsular serotypes can be distinguished in *S. suis*, designated as types 1–31, 33 and 1/2 [9]. Serotype 2 is the most prevalent type associated with swine disease worldwide, although other serotypes also cause disease in pigs [1,8]. The distribution of the different serotypes changes

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depending on the geographical origin of the isolates. In Europe. However, *S. suis* serotype 2 is the most prevalent type isolated from diseased pigs in many countries. Serotype 9 is the most prevalent type in Germany, Belgium, and the Netherlands [1,24] while serotypes 1 and 14 are the most prevalent in the UK [8]. In Spain, serotype 2 has long been the dominant serotype isolated from diseased pigs [13,21], but serotype 9 has emerged as responsible for pig infections [22].

Serotyping, however, is not sufficiently discriminative to distinguish among *S. suis* isolates, and several molecular typing techniques are frequently used, sometimes in combination with virulence genotyping, to understand the population structure and epidemiology of this bacterium [11,14,15, 18,23]. Research on the virulence-associated factors of this pathogen usually focus on the muraminidase-released protein (MRP), extracellular factor (EF), and suilysin (SLY) [4,18,19,24]. These studies have confirmed the existence of widespread clones of *S. suis*. Thus, serotype 2 strains of the ST1 complex that are *sly*+ *mrp*+ *ef*+ are widespread across Europe [20,24], whereas the majority of serotype 9 isolates from Central Europe belong to the ST87 complex and express a larger variant of MRP (MRP*) [19,24].

In the present study, the two most prevalent serotypes of *S. suis* circulating in the Spanish swine population, clinical isolates of *S. suis* of serotypes 2 and 9, obtained from diverse geographical areas across Spain, were characterized with respect to their virulence-associated phenotypes, pulsed-field gel electrophoresis (PFGE) and multilocus sequence (MLST) typing. The results further our understanding of the changes in the epidemiology of this pathogen in Spain and facilitate investigations of the relationship between *S. suis* Spanish isolates and the prevalent European clones.

Materials and methods

***Streptococcus suis* isolates.** One hundred and ninety five *S. suis* clinical isolates of serotype 2 (n = 101) and serotype 9 (n = 94) recovered from diseased pigs during a 5-year period were used in this study. Isolates were obtained from organs of diseased pigs with pneumonia (n = 48) and systemic infections (meningitis, arthritis, or septicemia; n = 74, 15, and 58, respectively) from 83 different herds located in 20 provinces of Spain.

Isolation, identification, and serotyping. *S. suis* was isolated on Columbia agar containing 5% defibrinated sheep blood (bioMérieux, Spain) and supplemented with colistin sulfate (10 μg/ml) and nalidixic acid (15 μg/ml) (Oxoid, Basingstoke, Hampshire, UK). All agar plates were incubated under oxic conditions at 37°C for 24 h. The resulting α-hemolytic, gram-positive and catalase-negative cocci were biochemically identified by conventional tests [13] and the Rapid ID32 Strept (bioMérieux) system according to the manufacturer's instructions. Biochemical identification was further confirmed by PCR amplification of a 688-bp glutamate dehydrogenase gene fragment [16]. Capsular typing was performed by slide agglutination [5] using specific rabbit antisera against the reference strains of serotypes 2 and 9 (kindly supplied by Dr. M. Gottschalk, Groupe de Recherche sur les Maladies Infectieuses du Porc, GREMIP, Montréal, Canada).

Production of MRP, EF, and SLY proteins. The production of MRP and EF proteins was determined by Western blotting, as previously described [13], using monoclonal antibodies kindly supplied by Dr. H.J. Wisselink (DLO-Institute for Animal Science and Health, Lelystad, the Netherlands). The hemolytic activity of SLY was determined by a microtitration assay, as described previously [21].

PFGE typing. Genetic typing of *S. suis* isolates was done by PFGE after genomic DNA digestion with *Bsp*120I, as described previously [23]. The PFGE patterns were examined visually and similarities between restriction endonuclease digestion profiles were expressed using the Jaccard similarity index, with the numerical taxonomy program BioNumerics (Applied Maths BVBA, Belgium). A similarity matrix was computed and transformed into an agglomerative cluster using the unweighted pair group method with arithmetic averages (UPGMA). Genetic diversity (GD) was calculated as the ratio between the total number of PFGE patterns and the total number of isolates [15].

Multilocus sequence typing (MLST). MLST was carried out as described by King et al. [11]. MLST alleles and the resulting sequence types (STs) were assigned through submission of the respective data to the *S. suis* MLST database [http://ssuis.mlst.net]. The MLST complexes were analyzed with eBURST [http://www.mlst.net] [6].

Statistical analysis. The chi-square test was used to analyze the relationship between *S. suis* PFGE profiles and capsular types, and the Fisher test to determine the relationship between *S. suis* and virulence phenotypes. Both analyses were carried out using the SPSS 12.0 software for Windows. Differences were considered significant at $P < 0.05$.

Results and Discussion

The isolates examined in the present study were properly identified by conventional tests and the Rapid ID 32 Strep strips, and their biochemical identification further confirmed by PCR. All of the isolates gave the expected amplification product of 688 bp, specific for *S. suis* [16]. After PFGE typing, 97 profiles were identified (GD 0.50), most of them (68%) represented by single isolates (Fig. 1) and thus indicative of a substantial genetic diversity among the *S. suis* isolates analyzed. Both serotypes exhibited similar GD values. Isolates of serotypes 2 and 9 yielded 60 and 41 PFGE profiles, corresponding to a genetic diversity of 0.60 and 0.47,

Fig 1. Dendogram showing the genetic relationship, based on UPGMA cluster analysis, and the distribution of virulence factors of the *Streptococcus suis* serotypes 2 and 9 isolates examined in this study. At a 65% similarity level, two main clusters (**A** and **B**) were observed, which correlated ($P < 0.05$) with isolates of capsular types 9 and 2, respectively.

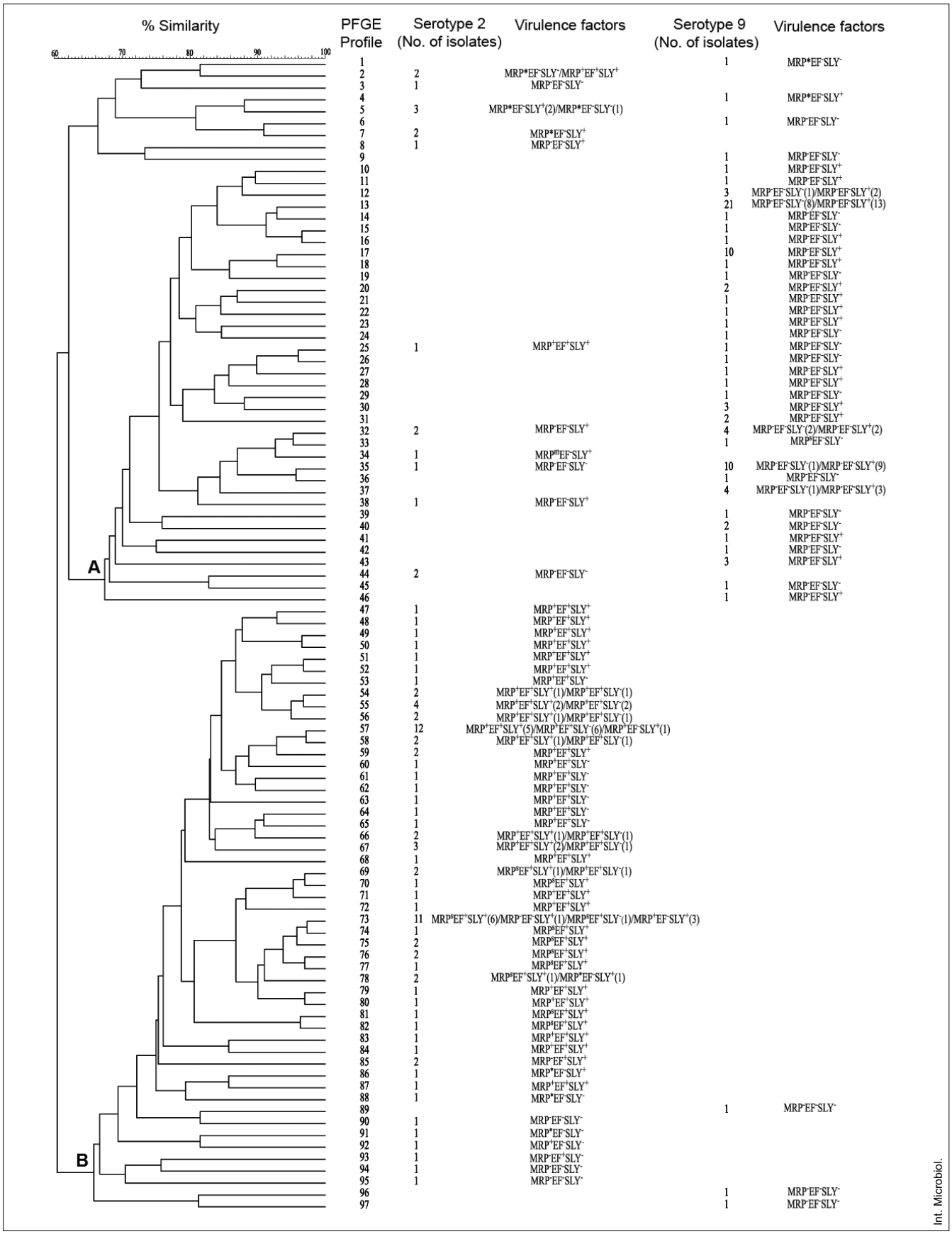
% Similarity
60
70
80
90
100
A
B
PFGE Profile
Serotype 2 (No. of isolates)
Virulence factors
Serotype 9 (No. of isolates)
Virulence factors
1 1 MRP*EF-SLY-
2 2 MRP*EF-SLY-/MRP+EF+SLY+
3 1 MRP+EF-SLY-
4 1 MRP*EF-SLY+
5 3 MRP*EF-SLY+(2)/MRP*EF-SLY-(1)
6 1 MRP-EF-SLY-
7 2 MRP*EF-SLY+
8 1 MRP-EF-SLY+
9 1 MRP-EF-SLY-
10 1 MRP-EF-SLY+
11 1 MRP-EF-SLY+
12 3 MRP-EF-SLY-(1)/MRP-EF-SLY+(2)
13 21 MRP-EF-SLY-(8)/MRP-EF-SLY+(13)
14 1 MRP-EF-SLY-
15 1 MRP-EF-SLY-
16 1 MRP-EF-SLY+
17 10 MRP-EF-SLY+
18 1 MRP-EF-SLY+
19 1 MRP-EF-SLY-
20 2 MRP-EF-SLY+
21 1 MRP-EF-SLY+
22 1 MRP-EF-SLY+
23 1 MRP-EF-SLY+
24 1 MRP-EF-SLY-
25 1 MRP+EF+SLY+ 1 MRP-EF-SLY-
26 1 MRP-EF-SLY-
27 1 MRP-EF-SLY+
28 1 MRP-EF-SLY+
29 1 MRP-EF-SLY-
30 3 MRP-EF-SLY+
31 2 MRP-EF-SLY+
32 2 MRP-EF-SLY+ 4 MRP-EF-SLY-(2)/MRP-EF-SLY+(2)
33 1 MRPS EF-SLY-
34 1 MRPm EF-SLY+
35 1 MRP-EF-SLY- 10 MRP-EF-SLY-(1)/MRP-EF-SLY+(9)
36 1 MRP-EF-SLY-
37 4 MRP-EF-SLY-(1)/MRP-EF-SLY+(3)
38 1 MRP-EF-SLY+
39 1 MRP-EF-SLY-
40 2 MRP-EF-SLY-
41 1 MRP-EF-SLY+
42 1 MRP-EF-SLY-
43 3 MRP-EF-SLY+
44 2 MRP-EF-SLY-
45 1 MRP-EF-SLY-
46 1 MRP-EF-SLY+
47 1 MRP+EF+SLY+
48 1 MRP+EF+SLY+
49 1 MRP+EF+SLY+
50 1 MRP+EF+SLY+
51 1 MRP+EF+SLY+
52 1 MRP+EF+SLY+
53 1 MRP+EF+SLY-
54 2 MRP+EF-SLY+(1)/MRP+EF+SLY-(1)
55 4 MRP+EF+SLY+(2)/MRP+EF+SLY-(2)
56 2 MRP+EF+SLY+(1)/MRP+EF+SLY-(1)
57 12 MRP+EF+SLY+(5)/MRP+EF+SLY-(6)/MRP+EF-SLY+(1)
58 2 MRP+EF+SLY+(1)/MRP+EF+SLY-(1)
59 2 MRP+EF+SLY+
60 1 MRP+EF+SLY-
61 1 MRP+EF+SLY-
62 1 MRP+EF+SLY-
63 1 MRP+EF+SLY-
64 1 MRP+EF+SLY-
65 1 MRP+EF+SLY-
66 2 MRP+EF-SLY+(1)/MRP+EF+SLY-(1)
67 3 MRP+EF+SLY+(2)/MRP+EF+SLY-(1)
68 1 MRP+EF+SLY+
69 2 MRPS EF+SLY+(1)/MRP+EF+SLY-(1)
70 1 MRPS EF+SLY+
71 1 MRP+EF+SLY+
72 1 MRP+EF+SLY+
73 11 MRPS EF+SLY+(6)/MRP-EF-SLY+(1)/MRPS EF+SLY-(1)/MRP+EF-SLY+(3)
74 1 MRPS EF+SLY+
75 2 MRPS EF+SLY+
76 2 MRPS EF+SLY+
77 1 MRPS EF+SLY+
78 2 MRPS EF+SLY+(1)/MRP*EF-SLY+(1)
79 1 MRP-EF-SLY+
80 1 MRP+EF+SLY+
81 1 MRPS EF+SLY+
82 1 MRPS EF+SLY+
83 1 MRP+EF+SLY+
84 1 MRP+EF+SLY+
85 2 MRP+EF+SLY+
86 1 MRP*EF-SLY+
87 1 MRP+EF+SLY+
88 1 MRP*EF-SLY-
89 1 MRP-EF-SLY-
90 1 MRP-EF-SLY-
91 1 MRP*EF-SLY-
92 1 MRP+EF-SLY-
93 1 MRP-EF+SLY-
94 1 MRP-EF-SLY-
95 1 MRP-EF-SLY-
96 1 MRP-EF-SLY-
97 1 MRP-EF-SLY-
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Table 1. Multilocus sequence types (STs) for selected isolates of the most frequently isolated PFGE profiles of *Streptococcus suis* serotype 2 and 9

		Locus							
Serotype	ST (No. of isolates)	*aroA*	*cpn60*	*dpr*	*gki*	*mutS*	*recA*	*thrA*	ST complex
2	1 (12)	1	1	1	1	1	1	1	1
	124[a] (1)	1	1	1	12	1	1	1	1
9	123[a] (8)	17	21	5	45	44	22	4	61
	125[a] (9)	17	21	5	62[b]	44	22	4	61

[a]Novel STs identified in this study.
[b]Novel allele identified in this study. GenBank accession number FM878659.

respectively ($P > 0.05$). This relatively large variety of genotypes was not unexpected considering the large number of herds from which the isolates were obtained and the diverse geographical areas across Spain where the herds were located. In addition, it is in agreement with the genomic variability reported for this pathogen [23]. PFGE results suggested distinct genetic backgrounds for both serotypes. At the 65% similarity level, most of the isolates of serotypes 2 and 9 (93.4%) could be grouped within two main PFGE clusters (Fig. 1A,B). Clusters A and B comprised 87 (92.6%) and 84 (83.2%) of serotype 9 and 2 isolates, respectively ($P < 0.05$). In addition to differences in their genetic backgrounds, the isolates of serotypes 2 and 9 differed in the expression of their virulence-associated factors (Fig. 1). Phenotypes $MRP^+EF^+SLY^+$ and $MRP^+EF^+SLY^-$ were the most frequently detected (33.7 and 20.8%, respectively) among the serotype 2 isolates, while $MRP^-EF^-SLY^+$ and $MRP^-EF^-SLY^-$ were the predominant phenotypes (64.9 and 31.9%, respectively) among isolates of serotype 9. These differences between isolates of the two serotypes with respect to the expression frequencies of the most prevalent virulence phenotypes were statistically significant ($P < 0.05$).

Despite the genetic diversity observed, some of the PFGE profiles of the two serotypes were more frequently isolated than others. Thus, three PFGE profiles (13, 17, and 35; Fig. 1) of serotype 9 and two serotype 2 (57 and 73; Fig. 1) accounted for 33.3% of the isolates examined. These five prevalent PFGE profiles were isolated from 38.6% of the herds in nine different provinces (Coruña, Segovia, Toledo, Sevilla, Granada, Málaga, Jaén, Huesca, and Lleida), which is indicative of their widespread distribution in the Spanish swine population. Isolates of these prevalent PFGE profiles, selected to represent isolates from different animals, diverse clinical backgrounds, geographical areas, and herds, were subjected to MLST analysis (Table 1). Nearly all serotype 2 isolates were assigned to the allelic profile ST1. Only one isolate carried a different allele for the gen *gki* (encoding glucose kinase) and was thus assigned to a new allelic profile (ST124) representing a single-locus variant of ST1. Serotype 9 isolates were assigned to two new allelic profiles (ST123 and S125). The sequence of the novel allele and the allelic profiles of novel STs identified in this study are available [http://ssuis.mlst.net]. Based on the eBURST results with the most stringent group definition [6], serotypes 2 and 9 isolates were assigned to the ST1 and ST61 complexes, respectively [11]. These MLST results lend further support to the distinct genetic backgrounds of serotypes 2 and 9.

The ST1 complex represents a highly successful clone that has spread throughout most European countries [11,18]. Isolates of the ST1 complex typically express the phenotype $MRP^+EF^+SLY^+$ [11,24], which was also the most frequent phenotype expressed by serotype 2 isolates in the present study. Considering that Spain imports pigs from countries of Central Europe, it was expected that isolates of serotype 9 would belong to the prevalent ST87 clone circulating in Europe [11,18]. However, analysis of the allelic profiles of the ST123 and ST125 genotypes indicated that they represent a single-locus or double-loci variant/s of two Spanish serotype 9 isolates of the ST61 complex detected in Spain before 2002 [11] and share only three alleles with genotypes comprising the ST87 complex [11,18]. Therefore, genotypes ST123 and ST125 are distinct from the widespread European ST87 clone. These genotypes differ also from isolates of the ST87 clone regarding the expression of virulence factors. Most serotype 9 isolates examined in the present study expressed SLY (66%) and none expressed MRP and EF, in accordance with previous data [1,3,18,24]. However, only 2.1% expressed the large variant MRP*, which is very low

compared to serotype 9 isolates of other European countries, the majority of which express the protein [18,19,24].

The comprehensive characterization of *S. suis* isolated from diseased pigs in this study showed that the serotype 2 strains, which, according to the PFGE results, have a greater distribution in the Spanish pig population, belong to the widespread European ST1 clone, while the serotype 9 isolates were derived from a clone (ST61 complex) previously detected in Spain before 2002. This result suggests that the increase in the number of cases of infections in pigs caused by serotype 9 isolates in Spain [22] is related to the emergence and dissemination of the ST61 clone among the pig population. The absence of heterologous protection [10] and the selective pressure imposed by increasing immunity to the prevalent serotype 2 may have contributed to the spread of the ST161 clone. These results could help to delineate effective measures for the prevention and control of *S. suis* infections affecting the Spanish swine industry.

Spain is one of several European countries that have dedicated intense efforts to research in veterinary sciences [2,7,17]. It is also one of the largest pig-producing countries in the world. Therefore, the results of the present study not only contribute to understanding the recent changes in the epidemiology of *S. suis* in Spain, but also to clarifying differences between *S. suis* isolates circulating elsewhere in Europe.

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