New Sequence Types and Antimicrobial Drug–Resistant Strains of *Streptococcus suis* in Diseased Pigs, Italy, 2017–2019

Lucilla Cucco, Marta Paniccià, Francesca Romana Massacci, Alessandra Morelli, Massimo Ancora, Iolanda Mangone, Adriano Di Pasquale, Andrea Luppi, Denis Vio, Cesare Cammà, Chiara Francesca Magistrali

Streptococcus suis is a pathogen associated with severe diseases in pigs and humans. Human infections have a zoonotic origin in pigs. To assess circulating strains, we characterized the serotypes, sequence types, and antimicrobial susceptibility of 78 *S. suis* isolates from diseased farmed pigs in Italy during 2017–2019. Almost 60% of infections were caused by serotypes 1/2 and 9. All but 1 of the serotype 2 and 1/2 isolates were confined to a single cluster, and serotype 9 isolates were distributed along the phylogenetic tree. Besides sequence type (ST) 1, the serotype 2 cluster included ST7, which caused severe human infections in China in 1998 and 2005. A large proportion of serotype 9 isolates, assigned to ST123, were resistant to penicillin. The emergence of this clone threatens the successful treatment of *S. suis* infection. Characterizing *S. suis* isolates from pigs will promote earlier detection of emerging clones.

Streptococcus suis is a major pathogen in pigs and an emerging zoonotic agent (*1*–*4*). This bacterium is a natural inhabitant of the upper respiratory tract of pigs and is endemic to all pig-production countries. In pigs, *S. suis* causes meningitis, septicemia, polyserositis, arthritis, and endocarditis, mainly during the postweaning period; it is a source of concern for farmers because of potential economic losses and its effects on the welfare of infected pigs (*2*). Human infection is acquired through occupational contact or ingestion of undercooked pork-derived products and is associated with meningitis, endocarditis, septicemia, deafness, and death (*5*).

S. suis is a heterogeneous species. Until 2005, *S. suis* was divided into 35 serotypes (1–34 and 1/2), based on capsular polysaccharides, but 6 serotypes were recently reclassified as belonging to other *Streptococcus* species, leaving 29 currently recognized *S. suis* serotypes (*6*,*7*). Most *S. suis* infections in humans and pigs are caused by serotype 2, but the predominant serotypes causing invasive disease in pigs vary according to time and region (*8*). In some countries in Europe, serotype 9 has emerged as the leading cause of invasive diseases in pigs (*2*,*8*–*10*); prevalence of this serotype has also recently increased in China (*5*).

Since 2002, the introduction of a standard multilocus sequence typing (MLST) scheme has improved the description of the epidemiology of *S. suis* infection (*8*). Sequence types (STs), determined by MLST, are also better predictors of the pathogenicity of a particular isolate than are serotypes (*11*). Among serotype 2 isolates from pigs, ST1, a highly successful clone associated with most human infections globally, is prevalent in Europe (*8*). Another sequence type of serotype 2, ST7, was responsible for major *S. suis* epidemics among humans in 1998 and 2005 in China (*12*). Serotypes other than 2 are less frequently responsible for human infections (*8*). Of note, despite the increased frequency of pig infections caused by serotype 9, the first human case of serotype 9 infection was documented in Thailand in 2015 (*13*). That strain was assigned to ST16, an emerging sequence type known for its increased virulence potential and predominance in invasive *S. suis* infections in pigs in the Netherlands (*14*).

Author affiliations: Istituto Zooprofilattico Sperimentale dell' Umbria e delle Marche 'Togo Rosati,' Perugia, Italy (L. Cucco, M. Paniccià, F.R. Massacci, A. Morelli, C.F. Magistrali); Istituto Zooprofilattico Sperimentale di Abruzzo e Molise 'Giuseppe Caporale,' Teramo, Italy (M. Ancora, I. Mangone, A. Di Pasquale, C. Cammà); Istituto Zooprofilattico Sperimentale della Lombardia e della Emilia-Romagna 'Bruno Ubertini,' Brescia, Italy (A. Luppi); Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy (D. Vio)

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On-farm management options for controlling *S. suis* infections include improving environmental conditions (e.g., providing correct temperature, providing correct air humidity, and reducing overcrowding and pig mixing) (*15*). The control of viral infections, particularly porcine reproductive and respiratory virus, is also essential because they are well-known predisposing factors for the disease (*16*). Another tool for protecting against infection is vaccination, but available vaccines are based on bacterins and provide only nonheterologous protection (*17*). Thus, in many countries in Europe, including Italy, control of *S. suis* infections in pigs is based mainly on antimicrobial treatment (*18*). *S. suis* is generally susceptible to β-lactams, the main class of antimicrobials administered to control the infection on pig farms. Conversely, *S. suis* is almost always resistant to tetracycline; macrolide-lincosamide-streptogramin B; and, less frequently, aminoglycosides, chloramphenicol, vancomycin, and linezolid (*15*). In *S. suis*, genes encoding antimicrobial resistance (AMR) are often carried on mobile genetic elements that can be transferred to other members of the genus, including human pathogens (*4*,*19*). Thus, *S. suis* can be considered a public health concern because of its zoonotic potential (a leading cause of antimicrobial drug use in pig farming) and a reservoir of AMR genes (*4*,*19*).

Information about circulating strains is lacking in many countries, including Italy, which is one of the most prominent pig-production countries in Europe (*8*). We characterized the serotypes, sequence types, and antimicrobial susceptibility of 78 *S. suis* isolates from infected pigs in Italy. By providing updated epidemiologic information about *S. suis* infection, we aim to drive the use of autogenous vaccines, reduce antibiotic consumption, and protect animal health. We also assessed presence of *S. suis* clones with zoonotic potential.

Materials and Methods

Bacterial Isolates

We investigated isolates collected from pigs with clinical *S. suis* infection on pig farms in northern/central Italy during 2017–2019. To avoid redundancy, we included only 1 isolate per year and farm. A total of 78 *S. suis* isolates were collected from piglets with meningitis (49), pericarditis (1), arthritis (3), septicemia (17), and pneumonia (8) (Appendix 1, https://wwwnc.cdc.gov/EID/article/28/1/21-0816-App1.xlsx).

The samples were cultured on 5% sheep blood agar (Biolife Italiana Srl, http://www.biolifeit.com) at 5% CO_2, 37°C, for 24–48 h. We used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics GmbH, https://www.bruker.com) and PCR to confirmed selected suspected α-hemolytic colonies as belonging to the *S. suis* species (*20*).

Serotyping and Virulence Genotyping

We identified serotype and virulence-associated genes by using PCR (Appendix 2 Table 1, https://wwwnc.cdc.gov/EID/article/28/1/21-0816-App2.pdf). To discriminate between different variants of *mrp*, we used whole-genome sequencing (*21*–*23*).

Antimicrobial Susceptibility Testing

We assessed MICs by using a commercially prepared microtiter MIC panel (BOP06F, Sensititre; Trek Diagnostic Systems Inc., https://www.thermofisher.com) according to the manufacturer's instructions and by using *Streptococcus pneumoniae* ATCC 49619 as a quality control strain. We interpreted MIC results by using the breakpoints recommended by the Clinical Laboratory Standards Institute (*24*) for swine respiratory *S. suis*. The interpretative criteria for trimethoprim/sulfamethoxazole and clindamycin were those recommended for human *S. pneumoniae* (*25*).

Whole-Genome Sequencing

We prepared genomic DNA from all 78 *S. suis* isolates. We extracted pure cultures from 1 mL of logarithmic-phase broth cultures by using QIAamp DNA Mini Kit (QIAGEN, https://www.qiagen.com) according to the manufacturer's instructions and then quantified the DNA by using the Qubit fluorometer (Thermo Fisher Scientific, https://www.thermofisher.com). We prepared the libraries by using the Nextera XT Library Prep kit (Illumina Inc., https://www.illumina.com) and then loaded them onto an Illumina NextSeq 500/550 Mid Output Reagent Cartridge version 2 kit (300 cycles) and sequenced them on an Illumina NextSeq 500 platform to generate 150-bp paired-end reads.

Sequence Analyses

Raw data were checked for quality, trimmed by using Trimmomatic version 0.36 (*26*), and assembled by using SPAdes genome assembler version 3.11.1 (*27*). To determine distinct sequence types, we performed MLST. The allele sequences and profiles were obtained from the *S. suis* MLST database (https://pubmlst.org/ssuis). We uploaded sequences for new MLST allele variations to the same database for assignment of allele identification and then uploaded final allele combinations for assignment of new

MLSTs. We submitted the raw sequencing data to the National Center for Biotechnology Information Sequence Read Archive repository (BioProject PRJNA717238, Biosample SUB9357225; accession nos. SAMN18490763–SAMN18490790).

To identify potential clonal complexes and founders, we performed global optimal eBURST (http://www.phyloviz.net/goeburst analysis). The entire *S. suis* MLST database was displayed as a single goeBURST diagram by setting the double-locus variants level and the group definition to 0 of 7 shared alleles. We conducted minimum core-genome sequence typing in silico (*28*).

We annotated genomes by using Prokka (https://github.com) and constructed a maximum-likelihood phylogenetic tree, based on the final alignment of core genome from Roary analysis, by using FastTree 2.1.11 (*29*). Manual annotation of the tree was performed in iTOL (v.5.7) (*30*). We identified AMR genes by using ABRicate (https://github.com) against the following databases: AMRFinderPlus, CARD, RESfinder, ARG-ANNOT (*31*–*34*).

To research putative virulence genes, we created a database containing 91 previously described genes (*2*,*3*) (Appendix 2 Table 1) and searched by using BLASTN version 2.5.0+ (*35*). According to O'Dea et al., (*17*), only genes with ≥95% coverage and ≥99% identity were considered present. We investigated the null hypothesis of a random distribution of the number of virulence factors among the different sequence types and excluded sequence types represented by a small set of isolates (≤3), resulting in 8 sequence types and 65 isolates. To show the distribution of the putative virulence genes across the sequence types, we selected the putative virulence genes that were present in <90% or in >10% of isolates. After checking the normality of the data by using the Shapiro-Wilk normality test, we performed Kruskal-Wallis rank-sum testing, followed by pairwise comparisons using the Dunn test for multiple comparisons of independent samples. To investigate the distribution of genes encoding putative virulence factors, we constructed a heat map based on the distance metric "euclidean" and complete linkage method. We performed all analyses in R (*36*).

Results

Molecular Serotyping, Virulence Genotyping, and MLST

We identified 13 serotypes: 1, 2, 1/2, 3, 4, 5, 7, 8, 9, 10, 15, 19, and 23. The most prevalent were serotypes 9, accounting for 34.6% (n = 27) of isolates, and 1/2, accounting for 25.6% (n = 20) of isolates. These serotypes were followed by 10 (n = 7, 9.0%), 2 (n = 7, 9.0%), and 7 (n = 6, 7.7%) (Table 1).

MLST analysis revealed that 59 (75.6%) isolates belonged to 9 sequence types (ST1, ST7, ST11, ST16, ST28, ST29, ST94, ST108, and ST123) in the *S. suis*

Table 1. Combination of putative virulence genes among sequence types and minimum core genome groups of *Streptococcus suis* from diseased pigs, Italy, 2017–2019*

Sequence type	MCG group	Serotype	Virulence profile	No. isolates/total no. isolates for each sequence type (%)
ST1	1	2	*mrp*^{EU}*/sly/epf*	3/17 (17.6)
ST1	1	1/2	*mrp*^{EU}*/sly/epf*	14/17 (82.4)
ST7	1	1/2	*mrp*^{EU} */sly/epf*	6/9 (66.6)
ST7	1	2	*mrp*^{EU} */sly/epf*	3/9 (33.3)
ST11	N	1	*mrp/sly/epf*	2/2 (100)
ST16	1	9	*mrp*/sly*	3/3 (100)
ST28	4	2	*mrp*^{NA1}*/sly*	1/1 (100)
ST29	4	7	*mrp**	3/6 (50)
			mrp^{NA1}	3/6 (50)
ST94	3	4	*mrp*^{NA1}*/sly*	2/3 (66.7)
		9	*mrp*^{NA1}*/sly*	1/3 (33.3)
ST108	3	23	*mrp*^{NA1}*/sly*	1/1 (100)
ST123	3	9	*mrp*^{NA1}*/sly*	17/17 (100)
ST1540	N	9	-	3/3 (100)
ST1541	1	9	-	1/1 (100)
ST1542	N	3	-	1/1 (100)
ST1543	3	4	*mrp*^{NA1}*/sly*	1/1 (100)
ST1544	3	4	*mrp*^{NA1}*/sly*	1/2 (50)
		5	*mrp*^{NA1}*/sly*	1/2 (50)
ST1545	1	8	*mrp**	1/1 (100)
ST1546	1	8	*mrp/sly*	1/1 (100)
ST1547	1	10	-	7/7 (100)
ST1548	N	15	*sly*	1/1 (100)
ST1549	N	19	-	1/1 (100)

*Dashes indicate absence of putative virulence genes according to PCR. *mrp** is the *mrp* variant (*22*). MCG, minimum core genome; N, not groupable; ST, sequence type.

MLST database, and 10 new sequence types were identified as ST1540–1549 (ID2702-ID2711; https://pubmlst.org/organisms/streptococcus-suis). Most were singletons, except for ST1547 (n = 7, all serotype 10), ST1540 (n = 3, serotype 9), and ST1544 (n = 2, serotypes 4 and 5). With 17 isolates each, the predominant STs were ST1 and ST123, which accounted for 43% of all isolates. All isolates belonging to ST29 were serotype 7, and all isolates belonging to ST11 were serotype 1.

AMR Phenotypes and Genotypes

A total of 7 (9.0%) *S. suis* isolates were resistant to antimicrobial drugs, usually tetracycline (6/7) (Table 2). Most (48/78, 61.5%) isolates were resistant to 2 antimicrobials, generally (45/48) clindamycin and tetracycline. Of the 78 isolates, 23 (29.5%) were resistant to ≥3 antimicrobials and were classified as multiresistant. Multiresistance was detected in 4/17 (23%) ST1, 1/9 (11%) ST7, and 12/17 (71%) ST123 isolates. Of 17 ST123 isolates, 14 (82%) were resistant to penicillin (Tables 1, 3; Figure 1).

Phylogenetic Analyses

A total of 1,156 genes, corresponding to 19.88% of the pangenome (Appendix 2), comprised the core genome. Phylogenetic analysis of the collected isolates showed 4 major clusters and 2 singletons. The first cluster was composed of serotypes 1, 1/2, and 2 isolates and was characterized by low heterogeneity, even though these isolates originated from different regions and body sites. We found no relationship among the 4 isolates of the second cluster, belonging to serotypes 8 and 9. Cluster 3 recognized 2 sister groups: the first group comprised serotypes 9, 15, and 19; the second group included serotype 10 isolates. The serotype SS10 isolates were derived from 7 outbreaks of meningitis, 6 of which occurred in the Piedmont region of northern Italy in 2018. The fourth cluster included the highest number of isolates from our collection, belonging to 6 serotypes. We found no correlations with geographic location, year, or site of origin for members of this cluster. All penicillin-resistant SS9 isolates were grouped within this cluster (Figure 1). goeBurst analysis (http://www.phyloviz.net/goeburst) showed 5 major clusters. ST1543 and ST1544 were related to the ST94 subgroup, ST1546 to the ST1521 subgroup, and ST1545 to clonal complex (CC) 1; the other sequence types occurred as singletons (Appendix 2 Figure 1).

Putative Virulence Genes

When we investigated the distribution of putative virulence genes in a subset of 65 isolates belonging to 8 sequence types (ST1, ST7, ST16, ST29, ST94, ST123, ST1540, and ST1547), we found 61 putative virulence genes in >10% and <90% of the isolates and included them in the heat map. Putative virulence genes were not randomly distributed across the 8 sequence types ($p<0.001$ by Kruskal-Wallis test; Figure 2). The number of putative virulence genes in ST1 and ST7 isolates did not differ. The number of putative virulence genes in ST1 and ST7 isolates differed from the number in ST123, ST29, ST1540, and ST1547 ($p<0.05$ by Dunn test). A block of 38 putative virulence genes was characteristic of ST1 and ST7 isolates. This block included genes encoding components of the cell wall, proteases, and molecules related to adhesion (*cps2E*, *cps2F*, *cps2C*, *neuB*, *fbps*, *sbp2*, *pgdA*, *dppIV*, *igaP*, *ssnA*, *srtF*, and *gnd*) and putative virulence genes involved in the regulation of metabolic pathways (*ccpA*, *lspA*, *ssu1889*, *revS*, *virA*, *guaB*, *sodA*, *adcR*, *purA*, *nadR*, *stp*, *stk*, *vapE*, *lysS*, *Ssads*, *fhs*, *apuA*, *aroK*, *flps*, *proA*, *scrB*, *ofs*, *prtP*, *gtfA*, *perR*, and *fur*). Virulence genes harbored by the 89-kb pathogenicity island, including *SalK/SalR* and *tetM*, were not found in ST7 isolates. ST123 was

Table 2. Distribution of MICs among 78 *Streptococcus suis* isolates from diseased pigs, Italy, 2017–2019*

Antibiotic molecule	No. (%) isolates by MIC, μg/mL												
	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Penicillin G	49 (63)	7 (9)	5 (6)	14 (18)	3 (4)								
Ampicillin		73 (94)	2 (3)	3 (4)									
Enrofloxacin		1 (1)	17 (22)	57 (73)	2 (3)	1 (1)							
Tetracycline				1 (1)	3 (4)	1 (1)		73 (94)					
Florfenicol		1 (1)	3 (4)	40 (51)	32 (41)		2 (3)						
TMP/SXT					67 (86)	11 (14)							
Clindamycin†		8 (10)				4 (5)	4 (5)	10 (13)	50 (64)				
Tylosin†			15 (19)							61 (78)			
Neomicin†						22 (28)	27 (35)	15 (19)	7 (9)	5 (6)			
Gentamicin†				12 (15)	39 (50)	17 (22)	6 (8)		2 (3)				
Sulfadimethoxine†											19 (24)		57 (73)

*Gray shading indicates range of values actually tested for each antibiotic. Black vertical bars indicate threshold values for clinical resistance, according to the Clinical and Laboratory Standards Institute (https://clsi.org). TMP/SXT, trimethoprim/sulfamethoxazole.
†Two isolates were not identified.

Table 3. Antimicrobial resistance genes identified in the 78 *Streptococcus suis* isolates from diseased pigs, Italy, 2017–2019, by sequence type

Antimicrobial resistance genes	Sequence type									Total
	ST1	ST123	ST1547	ST29	ST7	ST16	ST94	ST1540	Other*	
ermb, *tet(O)*	11	15	3	3	8	3	1		2	46
tet(O)	1		4		1					6
None				1			1		3	5
Cv ermb, *tet(O)*, *dfr(F)*	5									5
aac6-aph2, *ant6-ia*, *aph3-iiia*, *spw*, *ermb*, *tet(40)*, *tet(W)*, *tet(O)*, *tet(O/W/32/O)*, *tet (W/N/N)*								3	1	4
tet(M)				1						1
ermb, *tet(M)*				1						1
ermb, *tet(W)*, *tet (O/W/32/O)*, *tet(W/N/N)*									1	1
ant6ia,*aadE*, *ermb*, *tet(O)*									1	1
aac6-aph2		1								1
ant6ia, *aph3-iiia*, *spw*, *cat*							1			1
ant6ia, *aph3-iiia*, *apmA*, *ermb. optrA*, *tet(40)*, *spw*									1	1
aac6-aph2, *ermb*, *tet(O)*									1	1
ant6-ia, *aadE*, *ermb*, *tet(W)*, *tet(O)*, *tet(O/W/32/O)*, *tet(W/N/N)*									1	1
ant6-ia,*aadE*, *ermb*, *tet(40)*, *tet(O)*, *tet(O/32/O)*									1	1
ant6-ia, *spw*, *lnuB*, *lsaE*, *tet(O)*									1	1
aac-aph2, *aad(6)*, *spw*, *ermb*, *erm(47)*, *lnuB*, *lsaE*, *tet(40)*, *tet(T)*		1								1
Total	17	17	7	6	9	3	3	3	13	78

*Sequence types (STs) represented by <3 isolates.

characterized by the presence of another group of 12 putative virulence genes related to adhesion (*murM*) or involved in metabolic pathways (*htpsC*, *ppc*, *troA*, *pyrF*, *nox*, *purD*, *msmK*, *gloA*, *rgg*, and *yhbU_2*, *lysM*) (Appendix 2 Figure 3).

Discussion

Among 78 *S. suis* isolates from diseased pigs in Italy, we identified the major serotypes associated with *S. suis* infections as serotypes 9 and SS1/2, responsible for almost 60% of cases. Previous studies in Italy have shown a predominance of serotype 2 infections, which were a minority in our study (*1*,*37*). Until 2020, PCR testing for serotyping did not differentiate serotype 2 from serotype 1/2, and many studies reported these 2 serotypes as belonging to the same category. However, such distinction is relevant because serotype 1/2 is associated with pig infections; however, different from serotype 2, its role as a zoonotic agent is still uncertain (*38*). With this study, we confirmed increased serotype 9 infections at pig farms, which has already been described for other countries in Europe (*10*). The proportion of isolates belonging to serotype 7, the third most common serotype, remained more or less stable compared with data from 2000, thus confirming the trend observed in Germany (*7*,*37*). We also detected serotypes 10 and 15 in our collection. These serotypes have not previously been detected in Italy but have been identified in Spain and the United Kingdom (*37*). Given the variability of serotypes and the low prevalence of serotype 2 observed in our study, complete characterization of isolates is essential for the successful implementation of autogenous vaccines. Indeed, autogenous vaccines are well-established tools for preventing serotype 2 infections, but data about their efficacy for other serotypes, including 1/2, are still lacking (*16*).

All serotype 2 and 1/2 isolates, except 1, were confined to a single cluster of the phylogenetic tree. This cluster was composed of ST1 and, unexpectedly, ST7, which is a subgroup founder related to CC1. The number of putative virulence factors was higher in ST7 and ST1 isolates than in other STs in our collection, which was expected, because ST1 is the predominant sequence type associated with invasive infections in pigs in Europe. Cases of *S. suis* infection in humans in Italy are sporadic and are caused by serotype 2, ST1 (*1*,*39*). ST7, which differs from ST1 at a single locus, has not been detected in pigs in Europe (*8*). However, ST7 isolates are prevalent among diseased pigs in China (*40*–*42*). The epidemic strain ST7, which is characterized by the presence of an 89-kb pathogenicity island, the insertion of a 128-kb ICE (integrative and conjugative element)–phage tandem mobile genetic element, is responsible for the 2 largest outbreaks of human *S. suis* infection, which occurred in 1998 and 2005 (*42*). The ST7 isolates from our study lacked the virulence genes harbored by the 89-kb pathogenicity island; thus, their zoonotic potential may be lower than that of the ST7 epidemic strain in China. Moreover, they did not cluster with the newly described lineage III of ST7 (Appendix 2 Tables 2–4, Figure 2) (*43*). Further analysis is necessary to explain the presence of ST7 in Italy. New *S. suis* strains may

be imported by living animals or traveling humans, or they may have been derived from an early mixing of pig breeds, as previously hypothesized (*3*,*40*).

All serotype 7 isolates belonged to ST29, grouped in cluster 4, and had 2 *mrp* gene variants. The same characteristics were described for serotype 7 ST29 isolates from recent severe outbreaks among piglets in Germany and Austria (*18*). Thus, ST29 has been suggested as an emerging virulent sequence type in Europe (*18*).

In contrast to the serotype 2 and 1/2 isolates, serotype 9 isolates were distributed among different clusters in the phylogenetic tree, grouping with isolates belonging to other serotypes. High heterogeneity has been reported for serotype 9 (*11*,*44*). Three isolates from our collection were typed as serotype 9 ST16, a dominant clone in diseased pigs from the Netherlands (*3*). Although most cases in humans have been attributed to ST1 isolates, ST16 has recently been associated with cases of *S. suis* infection in humans in Thailand (*13*). It has been suggested that the zoonotic and virulence potential may be higher for ST16 than for other strains. In our study, the ST16 subgroup was related to CC1, harbored *mrp* and *sly* genes, and was close to ST1 and ST7 in the phylogenetic tree, in accordance with the results reported by Zheng et al. (*10*). The presence of ST16 in Italy suggests the need for monitoring and typing *S. suis* from diseased pigs and infected humans in a One Health scenario.

A large proportion of serotype 9 isolates were assigned to ST123 and grouped into cluster 4 in the phylogenetic tree. This sequence type was prevalent in our collection and was found in 5 regions of Italy and

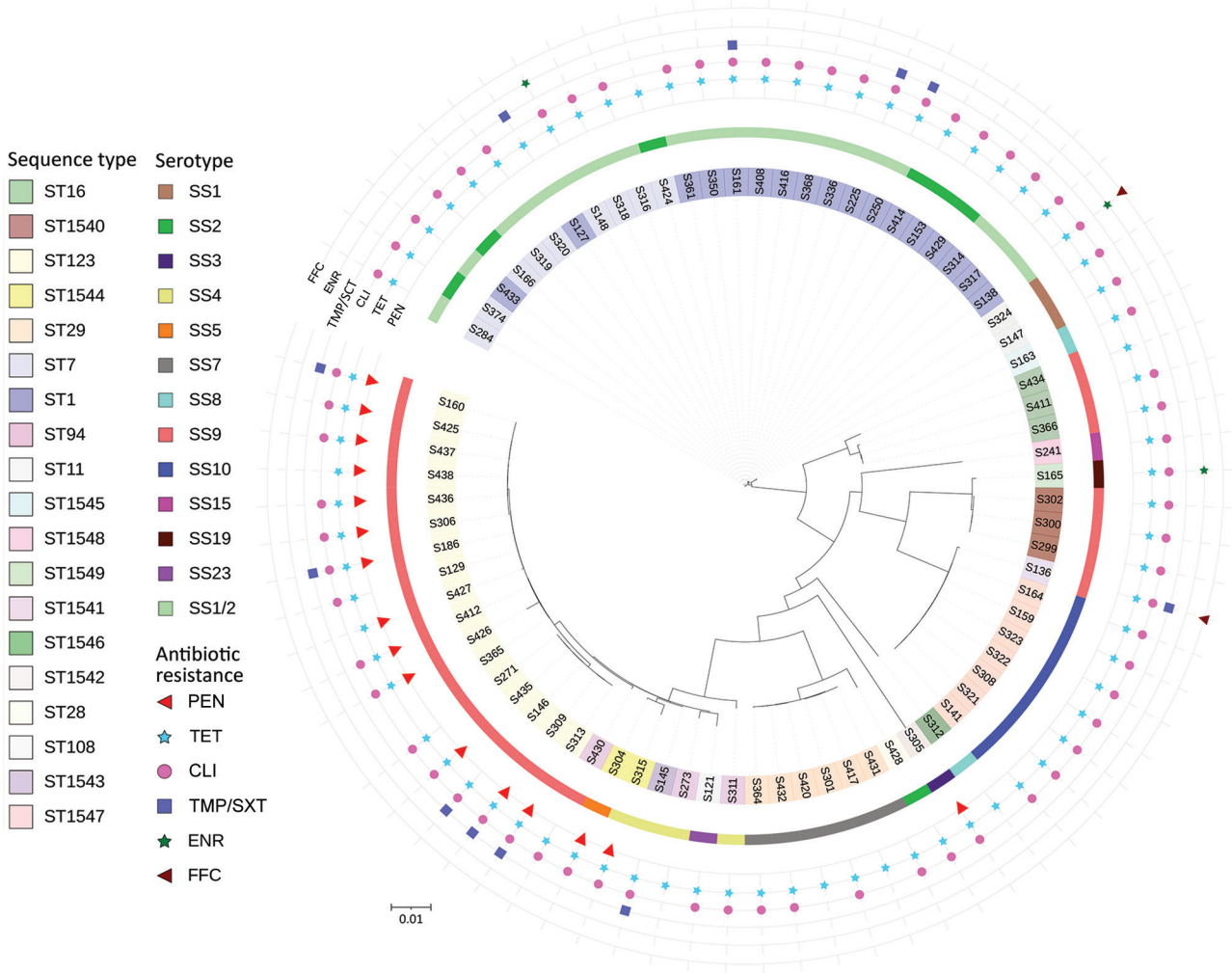

Figure 1. Circular phylogenetic tree containing 78 *Streptococcus suis* isolates from diseased pigs, Italy, 2017–2019. The tree was inferred by using the iTOL interactive user interface (https://itol.embl.de). Shading over tip labels indicates sequence types. The serotypes of each isolate are also shown. The antimicrobial-resistant molecules are annotated by colors and shapes. Scale bar indicates substitutions per site. CLI, clindamycin; ENR, enrofloxacin; FFC, florfenicol; PEN, penicillin; ST, sequence type; TET, tetracycline; TMP/SXT, trimethoprim/sulfamethoxazole.

in pigs from different production companies. Most ST123 isolates were resistant to penicillin. ST123 was reported in Spain in 2009 (*9*). As already observed in Spain, the ST123 isolates from our study were related to the ST94 subgroup and were characterized by the presence of *sly* and *mrp*^{NA1} genes (*10*).

The number of live pigs imported into Italy has increased over the past 10 years, almost doubling from 2013 to 2018 (http://www.anas.it). Pigs are imported from other countries in Europe, predominantly the Netherlands, Denmark, and Germany, and, to a lesser extent, from Spain and countries in eastern Europe. Imported live pigs can be carriers of new *S. suis* clones, which may then be transferred to other animals at the receiving farm (*11*). The differences in the *S. suis* population in our study compared with those in previous studies may result from this intensive exchange of live pigs between Italy and other countries in Europe.

We confirmed widespread resistance to tetracycline and clindamycin, as previously reported for *S. suis* isolates globally. Resistance to tetracycline was mainly associated with the presence of *tetO* and, to a lesser extent, other *tet* genes, including *tetM* and the mosaic gene *tet (O/W/32/O)*, which was first described in *S. suis* isolates in Italy (*1*). Resistance to clindamycin was coupled with high MICs for tylosin and the diffuse presence of *ermB*, suggesting a macrolide/lincosamide/streptogramin B profile. Resistance to florfenicol was detected in 2 multiresistant isolates. This type of resistance is emerging in *S. suis* species (*45*). One of the florfenicol-resistant isolates was positive for *optrA*, an oxazolidinone/phenicol resistance determinant carried by mobile genetic elements. *optrA* in *S. suis* isolates from China has been previously described and is frequently detected in *Enterococcus* isolates from pig farms in Italy (*46*,*47*). The high levels of AMR and the detection of emerging drug-resistance determinants are a consequence of selective pressure caused by antibiotic overuse. Despite the declining trend in antibiotic consumption, the use of antibiotics in veterinary medicine is still more frequent in Italy than in other countries in Europe (*48*).

We observed a high level of resistance to penicillin; ≈1 in 5 isolates showed reduced susceptibility to this antimicrobial. This finding contrasts with previous observations from other countries in Europe (*15*,*49*). Resistance to ampicillin was not observed, thus confirming the hypothesis of incomplete cross-resistance between these 2 antimicrobials (*49*). Resistance to penicillin was mostly detected in serotype 9 isolates and was particularly frequent in ST123 isolates. Blume et al. (*9*) suggested that the spread of *S. suis* serotype 9 is favored by the selective advantage conferred by the absence of heterologous immunity induced by the dominant serotype 2 clone (*9*). Our data suggest that penicillin resistance may also be a driver of the expansion of *S. suis* serotype 9.

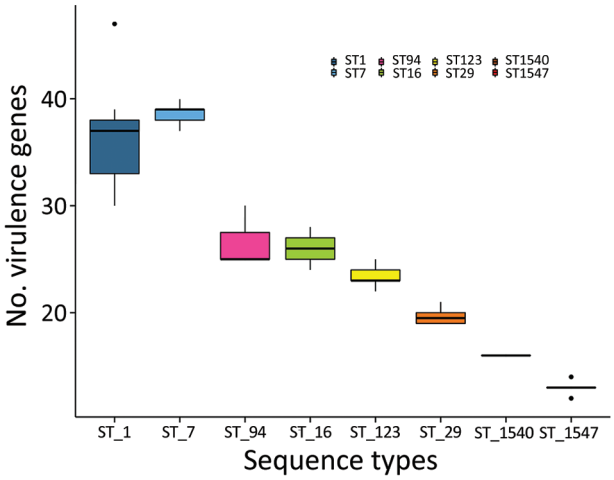

Figure 2. Distribution of the putative virulence genes detected among different sequence types of *Streptococcus suis* isolates from diseased pigs, Italy, 2017–2019. Box tops and bottoms indicate interquartile ranges, horizontal lines within boxes indicate means, whiskers indicate 95% CIs, and dots indicate outliers. ST, sequence type.

The emergence of a penicillin-resistant clone among the *S. suis* population threatens the successful treatment of *S. suis* infections in pigs. Penicillin resistance in *S. suis* may favor the prescription of critical classes of antimicrobial drugs, which should be limited in veterinary medicine. Penicillin resistance in a zoonotic agent raises concerns about hampering the treatment of infections.

In conclusion, our study highlights the value of characterizing *S. suis* isolates from pigs for monitoring trends in AMR and enabling early detection of emerging clones. In addition, our data strongly suggest the need for preventive strategies to limit the spread of penicillin-resistant *S. suis* among pig populations in Italy.

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About the Author

Dr. Cucco is a microbiologist at the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, in Perugia, Italy. Her primary research interests include epidemiology, AMR, and genomics of bacterial pathogens in veterinary medicine.

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Address for correspondence: Chiara Francesca Magistrali, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche 'Togo Rosati,' via Salvemini, 1, 06126 Perugia, Italy; email: c.magistrali@izsum.it

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New Sequence Types and Antimicrobial Drug–Resistant Strains of *Streptococcus suis* in Diseased Pigs, Italy, 2017–2019

Appendix 2

Read quality, number of contigs and coverage of Whole-Genome Sequencing data

The final assembly of the 78 sequences resulted in an average read quality after trimming of 34.60% (min 34.04, max 34.89) and 3,165,852 read pairs (min 1,043,985; max 5,077,416). The average number of contigs was 68 (min 25, max 177), with a mean length of 2,114,359 nucleotides (min 2,026,262; max 2,264,709). The average vertical coverage was 211 (min, 69; max, 339). The mean N50 and L50 values were 149,502 nucleotides (min 371, 66; max 293,044) and 6.95 (min 3, max 21), respectively.

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Appendix 2 Table 1. Primers used to identify the *S. suis* serotypes and virulence factors by PCR*

Target	Primer sequences (5′ to 3′) Forward	Reverse	Reference
For grouping PCR			
I	TGGTTCAAATATCAATGCTC	ATTGGTTGTGAGTGCATTG	(*1*)
II	TCAAAATACGCACCTAAGGC	CACTCACCTGCCCCAAGAC	
III	TGATTTGGGTGAGACCATG	CTCATGCTGGATAACACGT	
IV	ACAGTCGGTCAAGATAATCG	TCAGCTTGGGTAATATCTGG	
V	GGAAAGATGGAGGACCAGC	CCAACCAGACTCATATCCCC	
III and VI	GATGCCCCAAGCGATATGCC (F1)	GGACCAACAATGGCCATCTC (R1)	
	GACGCACCAAGTGATATGCC (F2)	GGTCCGACAATAGCCATTTC (R2)	
For typing PCR			
SS group I			(*1*)
3	GGTTTTGATTGGTCTAGTTG	CTCTAAAGCTCGATATCTAC	
13	TATGGTTAAAGGTGGAACTG	CCTTGTATATATTCCCTCCA	
18	TAATGGGATAGTTGCGTTAC	ATACATAAAGTTGTCCTGCG	
SS group II			(*1*)
2	TTAGCAACGTTGCCAATAAG	AATCCTCCATTAAAACCCTG	
6	GCTCACTATTTTTACATTACAC	TATTACTCCGCCAAATACAG	
1 and 14	TTAGACAGACACCTTATAGG	CTAGCTTCGTTACTTGATTC	
16	AAGGTTATCCACGAAAGATG	TCCGGCAATATTCTTTCAAG	
27	AGACACTGCTTGCATTATTG	TCAGAATTACTTCCTGTTGC	
1 and 1/2	ATCGCTTTGTGGTGGCCTTT	AGAAGCTTCTTTTGCTGTTTGC	(*2*)
1 and 14	ATCGCTTTGTGGTGGCCTTG		
SS group III			(*1*)
21	TATCATATTGAGAATCTTCCC	TTGCGTAGCATACAAAGTTC	
28	ATTATGTTGGTTGCAGAAGG	CGACTCAATTGTTGTAGTAG	
29	TTCTGGGATTTTAGGAATGC	CATGAAATACGCACTTGTAC	
30	TATTGCACTAGCTTCAGAAC	TGCATCCATAGTTGTATTCG	
SS group IV			(*1*)
4	GACTATCTGTATACCCAAAC	TCCTTCCAAGTATTCTCTAG	
5	ATCTTAGGAATGATTCGGAC	ACCAGATATCTGAGCAAATG	
7	AACTACCTACCTGAACTTTG	AGTCTAAAAGTGATCGAGTC	
17	TAGCATCAGTTTATACGAGG	TAGTTTATCTGTGACACACC	
19	GTGTCGCAAATCAAGTATTG	AAGCTAGTACAACAAGCATG	
23	TAATGTATGCTCTGTCACTG	AACGAAACGGAATAGTTTGC	
SS group V			(*1*)
8	AAATAAGGTAGGAGCTACTC	ATCCAACCTTAGCTTTCTGT	
15	ATCGTTTTGAGATTGAGTGG	TAAACGGATTCGGTTACTCA	
20	TGTGGATTTCTGGGATAATC	TGTGGACGAATTACTACTTG	
22	GCATTATCAGGATTCTTTCC	CCAATTGGGTGTTCAAAAAG	
25	GTTTGCTCCGATCATAATAG	CCAGTAAAAGGACTCAATAC	
SS group VI			(*1*)
9	GAAAGTAGGTATATCTCAGC	GGGCTATTAAAACTCCTATC	
10	TTTCCCATTTGCTTATGGAC	GGAATAAAAACGATTGGGAG	
11	ATGCGATTGCAACAATTGAC	AGGCATGAGTAATACATAGG	
12	AACAGGTATTTCAGGATTGC	CTCGGATAAAGATAATCAGC	
24	TACTGAGATTTATTGGGACG	AAGCGATTGGATTACATTGC	
26	TTATACCGAAATTTTGTTGCC	CGTCAATCATATAAAGTGGG	
33	GATGTTTTCAACAGGTGTAC	CAAAGTACCTATTTTCAGCG	
SS group VII			(*1*)
31	ACAATCGTTTCTGCAATACG	GATGAAAACATCGTTGGTAG	
	ATCAGTAGTGGGAATAGTTG	TTTACTGTTTTTCGACCGTG	
32	AACCGCTGTTGAATTAAGAG	TTCGTTAGTTGAACTGTTCC	
	TAGGACTATGGTTCCTAATG	TATTCTAGTTCAAGTCGCTC	
34	AAGTTTCATTCGAGGACTTC	GTATATAACACCGCAAGAAG	
	ATACAGTGATGTCTTGCAAC	ATTGCTTTTTGACAATCGGC	
For virulence factors			
mrp	GACAGATGGTGAGGAAAATGG	TGAGCTTTACCTGAAGCGGT	(*3*)
epf	GCTACGACGGCCTCAGAAATC	TGGATCAACCACTGGTGTTAC	(*4*)
Sly	CAGCTCGTTGCCTTGTACTA	ACTCTATCACCTCATCCGC	(*5*)

*The DNA for SS3 and SS14 were kindly provided by Marcelo Gottschalk (University of Montreal, Canada) and used as positive control. *S. suis* strain V20 from a previous study conducted in Italy (*6*) was used as control.

Appendix 2 Table 2. Genes lacking in Lineage III and Lineage I ST7 reference strains, but present in the ST7 isolates of this study

Gene	Annotation
group_16	putative autolysin SsaALP
group_2145	IS630 family transposase ISSsu3
elaA	Protein ElaA
group_324	hypothetical protein
group_325	hypothetical protein
group_326	hypothetical protein
group_327	hypothetical protein
pdxK	Pyridoxine kinase
hmpT	Thiamine precursor transporter HmpT
group_330	hypothetical protein
group_331	hypothetical protein
baeS	Signal transduction histidine-protein kinase BaeS
walR_2	Transcriptional regulatory protein WalR
group_334	hypothetical protein
mco	Multicopper oxidase mco
group_336	IS3 family transposase ISStin7
group_337	hypothetical protein
group_338	hypothetical protein
group_339	hypothetical protein
group_340	hypothetical protein
immR	HTH-type transcriptional regulator ImmR
mhqD	Putative hydrolase MhqD
mhqA	Putative ring-cleaving dioxygenase MhqA
pgl_1	6-phosphogluconolactonase
group_347	hypothetical protein
lgt_1	Phosphatidylglycerol--prolipoprotein diacylglyceryl transferase
group_349	hypothetical protein
group_350	hypothetical protein
copB	Copper-exporting P-type ATPase B
group_352	hypothetical protein
actP	Copper-transporting P-type ATPase
copY_2	Transcriptional repressor CopY
cadC_1	Cadmium resistance transcriptional regulatory protein CadC
group_356	hypothetical protein
group_357	hypothetical protein
group_358	hypothetical protein
group_359	hypothetical protein
group_360	hypothetical protein
cadC_2	Cadmium resistance transcriptional regulatory protein CadC
cadA	putative cadmium-transporting ATPase
yadH	Inner membrane transport permease YadH
yadG	putative ABC transporter ATP-binding protein YadG
cdr_1	Coenzyme A disulfide reductase
acr3	Arsenical-resistance protein Acr3
arsA	Arsenical pump-driving ATPase
group_368	hypothetical protein
arsD	Arsenical resistance operon trans-acting repressor ArsD
group_37	Deoxyguanosinetriphosphate triphosphohydrolase-like protein
group_370	IS6 family transposase IS1216V
group_371	hypothetical protein
group_372	hypothetical protein
dnaG_1	DNA primase
group_374	hypothetical protein
group_375	hypothetical protein
group_376	hypothetical protein
group_377	hypothetical protein
ltrA	Group II intron-encoded protein LtrA
group_379	hypothetical protein
group_380	hypothetical protein
group_381	hypothetical protein
group_382	hypothetical protein
group_383	hypothetical protein
group_45	hypothetical protein
yeeO_3	putative FMN/FAD exporter YeeO
group_82	hypothetical protein
sdpR_1	Transcriptional repressor SdpR

Appendix 2 Table 3. Genes lacking in in the ST7 isolates of this study, but present in Lineage I ST7 reference strains

Gene	Annotation
group_310	Tyrosine recombinase XerC
group_311	hypothetical protein
group_312	hypothetical protein
group_313	hypothetical protein
group_314	hypothetical protein
group_315	hypothetical protein
group_316	hypothetical protein
group_317	hypothetical protein
group_318	hypothetical protein
group_319	hypothetical protein
group_320	hypothetical protein
group_321	hypothetical protein
group_22	hypothetical protein
group_395	hypothetical protein
group_396	Adaptive-response sensory-kinase SasA
group_397	Response regulator ArlR
group_398	hypothetical protein
group_399	hypothetical protein
yxlF_2	putative ABC transporter ATP-binding protein YxlF
lmrA	Multidrug resistance ABC transporter ATP-binding and permease protein
group_402	hypothetical protein
group_403	hypothetical protein
group_404	hypothetical protein
group_405	hypothetical protein
group_406	hypothetical protein
group_407	hypothetical protein
group_408	hypothetical protein
group_409	hypothetical protein
Int-Tn	Transposase from transposon Tn916
group_411	hypothetical protein
group_412	hypothetical protein
group_413	hypothetical protein
group_414	hypothetical protein
tet(M)	tetracycline resistance ribosomal protection protein Tet(M)
group_416	hypothetical protein
group_417	hypothetical protein
group_418	hypothetical protein
group_419	hypothetical protein
group_420	hypothetical protein
group_421	hypothetical protein
group_422	hypothetical protein
group_423	hypothetical protein
group_424	hypothetical protein
group_425	hypothetical protein
group_426	hypothetical protein
pcrA_2	ATP-dependent DNA helicase PcrA
group_428	hypothetical protein
pezT	Toxin PezT
group_430	hypothetical protein
group_431	DNA primase
degU	Transcriptional regulatory protein DegU
group_433	hypothetical protein
group_434	hypothetical protein
group_435	hypothetical protein
bcrA_2	Bacitracin transport ATP-binding protein BcrA
lagD	Lactococcin-G-processing and transport ATP-binding protein LagD
group_438	hypothetical protein
group_439	hypothetical protein
group_440	hypothetical protein
group_441	hypothetical protein
group_442	hypothetical protein
group_443	hypothetical protein
group_444	2-methoxy-6-polyprenyl-1,4-benzoquinol methylase, mitochondrial
aadK	Aminoglycoside 6-adenylyltransferase
apt_1	Adenine phosphoribosyltransferase
group_447	IS1380 family transposase ISSsu5

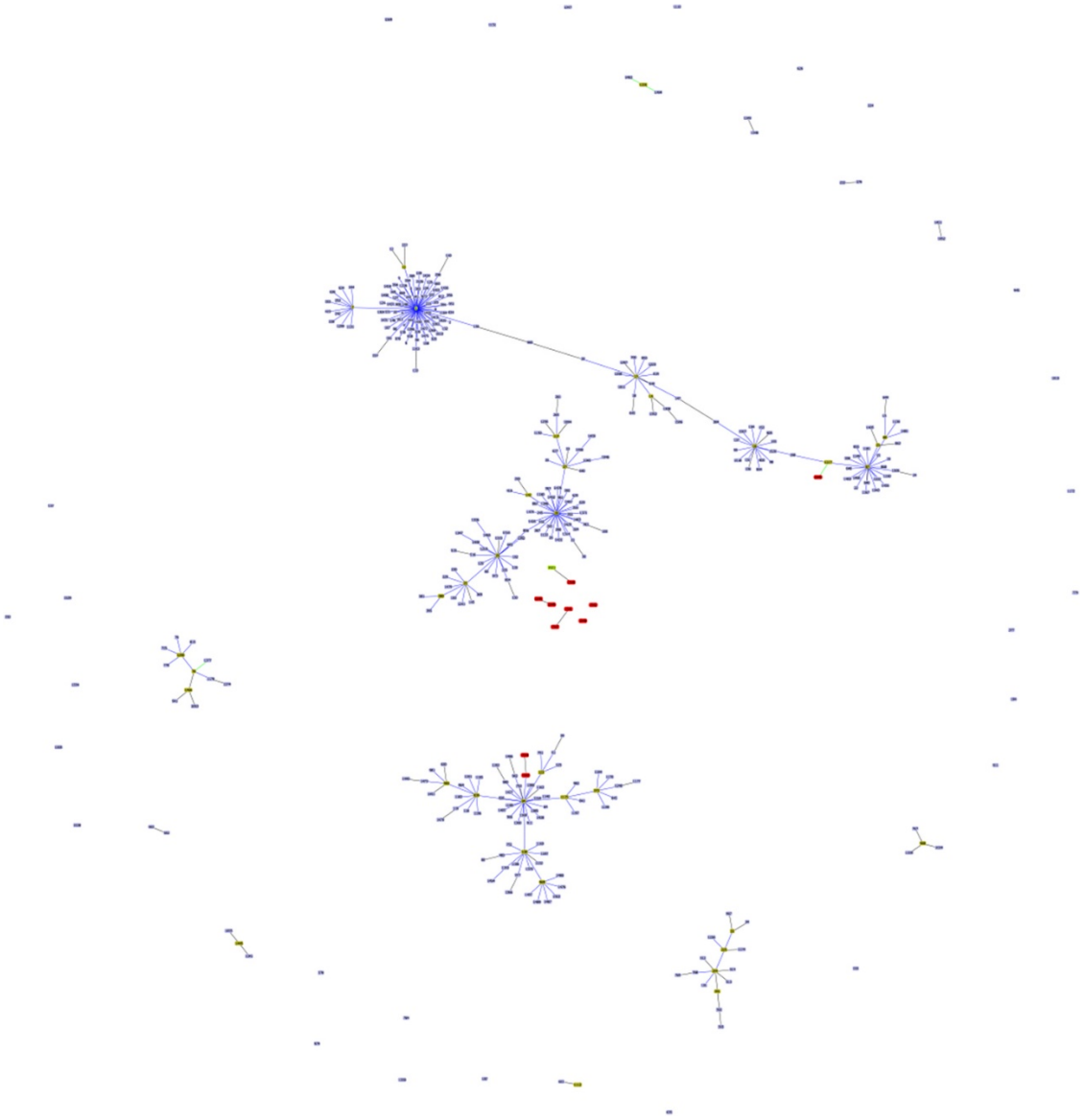
Gene	Annotation
group_448	hypothetical protein
group_449	Lantibiotic macedovicin
group_450	hypothetical protein
group_451	hypothetical protein
group_452	hypothetical protein
group_453	hypothetical protein
glgP	Glycogen phosphorylase

Appendix 2 Table 4. Genes lacking in in the ST7 isolates of this study, but present in Lineage III ST7 reference strains

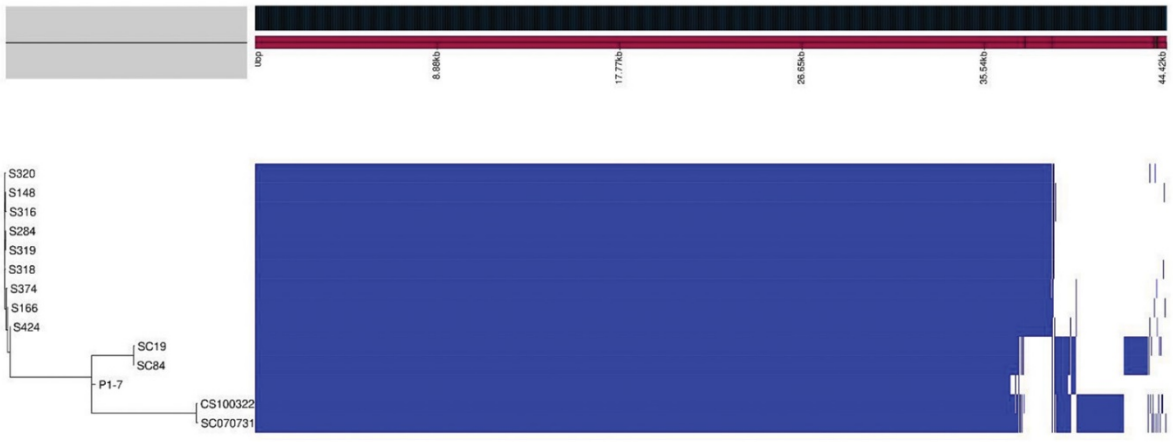
Gene	Annotation
group_173	hypothetical protein
group_127	hypothetical protein
group_179	hypothetical protein
group_18	hypothetical protein
entS	Enterobactin exporter EntS
msr(D)	ABC-F type ribosomal protection protein Msr(D)
group_182	hypothetical protein
group_183	hypothetical protein
dinB_2	DNA polymerase IV
group_185	hypothetical protein
group_186	hypothetical protein
group_187	hypothetical protein
group_188	hypothetical protein
group_189	hypothetical protein
group_190	hypothetical protein
group_191	hypothetical protein
group_192	hypothetical protein
group_193	hypothetical protein
group_194	hypothetical protein
group_195	hypothetical protein
group_196	hypothetical protein
group_197	hypothetical protein
group_198	hypothetical protein
group_199	hypothetical protein
group_200	hypothetical protein
metK_1	S-adenosylmethionine synthase
group_202	hypothetical protein
group_203	hypothetical protein
group_204	hypothetical protein
group_205	hypothetical protein
group_206	hypothetical protein
group_207	hypothetical protein
group_208	hypothetical protein
group_209	hypothetical protein
group_210	hypothetical protein
group_211	hypothetical protein
group_212	hypothetical protein
group_213	hypothetical protein
group_214	hypothetical protein
group_215	hypothetical protein
clpP_1	ATP-dependent Clp protease proteolytic subunit
group_217	hypothetical protein
group_218	hypothetical protein
group_219	hypothetical protein
group_220	hypothetical protein
group_221	hypothetical protein
group_222	hypothetical protein
group_223	hypothetical protein
smc_1	Chromosome partition protein Smc
group_225	hypothetical protein
group_226	hypothetical protein
group_227	hypothetical protein
group_228	hypothetical protein
group_230	hypothetical protein
group_231	hypothetical protein
group_232	hypothetical protein
group_233	hypothetical protein
group_234	hypothetical protein

Gene	Annotation
group_235	hypothetical protein
aphA	Aminoglycoside 3'-phosphotransferase
satA	Streptothricin acetyltransferase A
group_238	Aminoglycoside 6-adenylyltransferase
group_239	hypothetical protein
group_241	hypothetical protein
group_242	hypothetical protein
group_243	hypothetical protein
group_244	hypothetical protein
group_245	hypothetical protein
group_250	hypothetical protein
group_251	hypothetical protein
group_253	hypothetical protein
group_254	hypothetical protein
group_255	hypothetical protein
group_256	hypothetical protein
group_257	hypothetical protein
group_258	hypothetical protein
noc	Nucleoid occlusion protein
group_260	hypothetical protein
group_261	hypothetical protein
group_262	hypothetical protein
group_263	hypothetical protein
group_264	hypothetical protein
group_265	DNA primase
group_267	Toxin PezT
group_268	hypothetical protein
group_269	hypothetical protein
group_270	hypothetical protein
group_271	hypothetical protein
group_272	hypothetical protein
nisP	Nisin leader peptide-processing serine protease NisP
regX3	Sensory transduction protein regX3
creC	Sensor protein CreC
bcrA_1	Bacitracin transport ATP-binding protein BcrA
group_277	hypothetical protein
group_278	hypothetical protein
nsuA	Lantibiotic nisin-U
nisB	Nisin biosynthesis protein NisB
group_281	putative ABC transporter ATP-binding protein
nisC	Nisin biosynthesis protein NisC
nisI_1	Nisin immunity protein
group_284	hypothetical protein
group_289	hypothetical protein
group_292	hypothetical protein
group_293	hypothetical protein
group_294	hypothetical protein
group_295	PTS system mannose-specific EIID component
sorC_2	PTS system sorbose-specific EIIC component
sorB_2	PTS system sorbose-specific EIIB component
group_298	hypothetical protein
group_299	hypothetical protein
xylB	Xylulose kinase
xylA	Xylose isomerase
nagC	N-acetylglucosamine repressor
group_303	ISL3 family transposase ISSsu13
group_304	hypothetical protein
group_32	IS110 family transposase ISCARN28
group_71	hypothetical protein
group_78	hypothetical protein
gmuD_3	6-phospho-beta-glucosidase GmuD
group_15	hypothetical protein
purD_2	Phosphoribosylamine--glycine ligase
group_290	hypothetical protein
group_3	IS110 family transposase ISSsu7
group_31	IS110 family transposase ISCARN28
group_34	IS110 family transposase ISCARN28
group_35	IS110 family transposase ISCARN28
group_39	hypothetical protein

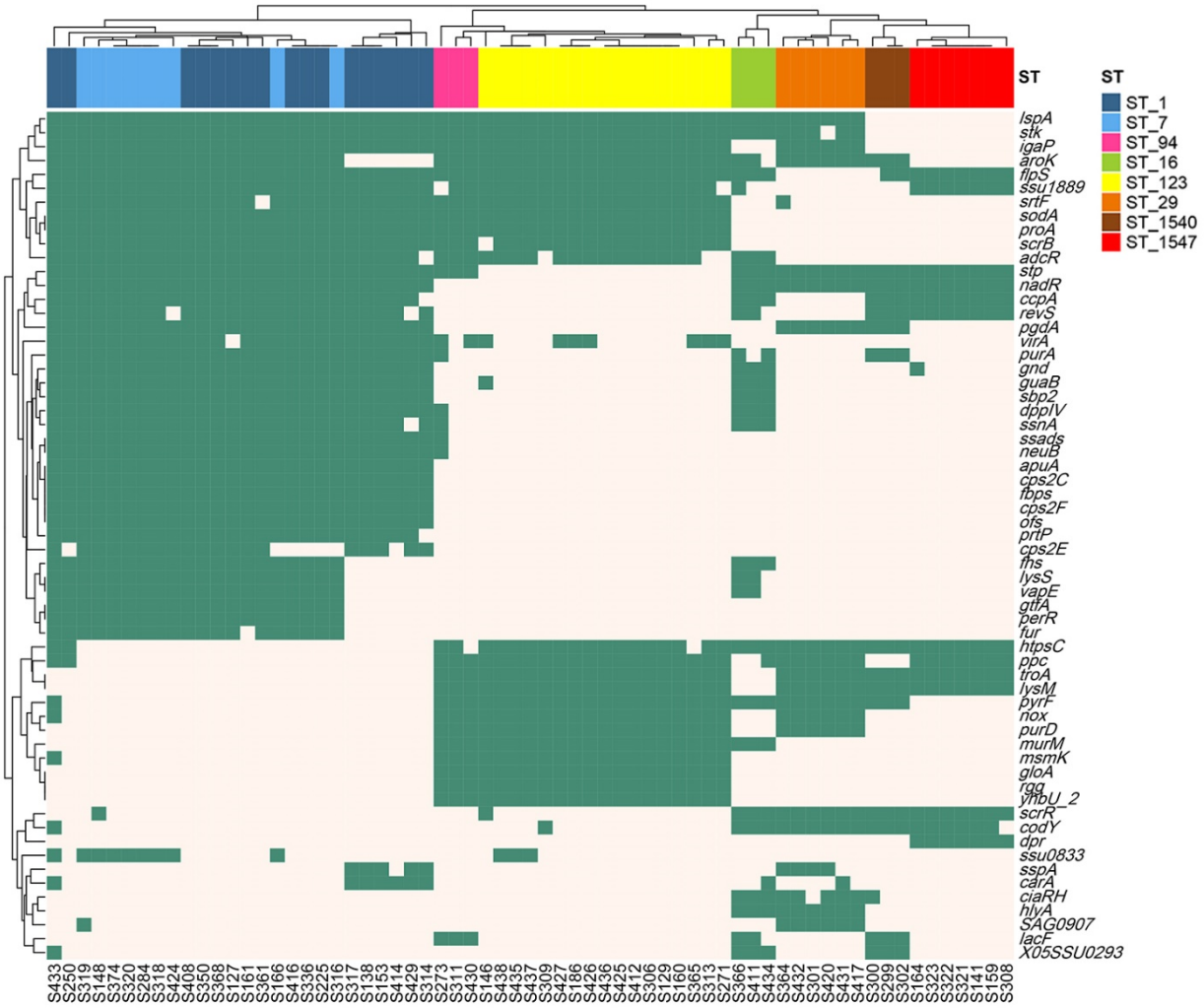
Gene	Annotation
mraY_1	Phospho-N-acetylmuramoyl-pentapeptide-transferase
group_4	IS110 family transposase ISSsu7
group_43	hypothetical protein
glf_1	UDP-galactopyranose mutase
group_61	hypothetical protein
group_65	hypothetical protein
group_67	hypothetical protein
group_7	IS110 family transposase ISSsu7
group_96	IS4 family transposase ISSsu2



Appendix 2 Figure 1. e-BURST illustration of the Streptococcus suis population was used together with the complete MLST database to cluster the sequence types (STs) into major clonal complexes (CCs). The representation of groups was performed with the double-locus variants (DLVs) parameters. Primary founders (green) are positioned at the center of the cluster and subgroup founders are showed in yellow. The new STs described in our study are highlighted in red.



Appendix 2 Figure 2. Results of comparison between the genomes of our ST7 with the genomes of CS100322 and SC070731, two novel ST7 types described by Dong et al. (*7*) as belonging to lineage III, with SC84 and SC19, two ST7 lineage I strains, and with P1-7 as reference strain. The figure was generated using Phandango, an interactive viewer for bacterial population genomics (https://jameshadfield.github.io/phandango/#/) using as input the file generated by Roary (gene_precence_absence.csv and accessory_binary_genes.fa.newick). Genes are shown as light blue bricks along the top and are sorted left to right by the proportion of isolates they are observed in. Presence (blue) and absence (white) of genes are plotted considering the phylogenetic placement of each isolates.


ST
ST
ST_1
ST_7
ST_94
ST_16
ST_123
ST_29
ST_1540
ST_1547
lspA
stk
igaP
aroK
flpS
ssu1889
srtF
sodA
proA
scrB
adcR
stp
nadR
ccpA
revS
pgdA
virA
purA
gnd
guaB
sbp2
dppIV
ssnA
ssads
neuB
apuA
cps2C
fbps
cps2F
ofs
prtP
cps2E
fhs
lysS
vapE
gtfA
perR
fur
htpsC
ppc
troA
lysM
pyrF
nox
purD
murM
msmK
gloA
rgg
yhbU_2
scrR
codY
dpr
ssu0833
sspA
carA
ciaRH
hlyA
SAG0907
lacF
X05SSU0293
S433
S250
S319
S148
S374
S320
S284
S318
S424
S408
S350
S368
S127
S161
S361
S166
S336
S225
S316
S317
S138
S153
S414
S429
S314
S273
S311
S430
S146
S438
S435
S437
S309
S427
S186
S426
S436
S425
S412
S306
S129
S160
S365
S313
S271
S366
S411
S434
S364
S432
S301
S420
S431
S417
S300
S299
S302
S164
S323
S322
S321
S141
S159
S308