RESEARCH REPORT
SWINE HEALTH

Title: Comparing the whole genome sequences of historical and recent *Streptococcus suis* strains to identify virulence factors, genes or markers responsible for pathogenesis**, NPB#17-129**

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Industry Summary:

Over the past few years, *Streptococcus suis* has become a reemerging bacteria causing significant economic losses for the swine industry. However, the reason for the reemergence is unclear. As both commensal and pathogenic strains of *S. suis* exist, numerous serotypes and multilocus sequence types (ST) have been identified and are used to differentiate *S. suis* strains. The goal of our study was to characterize historical strains of *S. suis* to identify any shift in the historical isolates that may possibly explain its reemergence.

A total of 114 historical isolates were selected for characterization from clinical diagnostic cases between 1999 and 2013 and partitioned into time frames consisting of 5 years to investigate trends. The serotype was identified for 93% of the isolates, with 14 serotypes identified, while 7% of the isolates were nontypable (NT). The predominant serotypes were 1/2 , 2, and 7. Within the predominant serotypes, serotype 2 slightly decreased while serotype 7 remained relatively constant over the 3 time periods. Serotype 1/2 nearly doubled between the first and last time period. The ST was identified in 68% of the isolates with 14 STs identified. ST28 was the predominant sequence type (24%) while 25% of the isolates had novel ST profiles. ST28 remained relatively constant over time. Interestingly, only a single ST1 was identified within the first 10 years while five isolates were identified within the last five years of the study. The number of novel ST and ST87 isolates decreased during the third time period.

In Europe, MRP, EF, and SLY have been associated with some pathogenic strains of *S. suis.* Only five and six isolates contained the MRP and EF gene, respectively, and most of the isolates were from 2009-2013. The gene SLY was identified in 61% of the isolates. Three genes were recently identified as indicators of pathogenicity in a contemporary set of *S. suis* strains. In this historical set, 95-100% of the isolates contained these three genes, indicating the new virulence genes have not changed overtime. AMR genes were investigated in the historical *S. suis* isolates, and 93% of the *S. suis* isolates contain an AMR profile of ErmB, tet32, and tetO or tet32 and tetO. The reduction of the ErmB gene occurred over time, which contributed to an increase of the tet32 and tetO profile over time. ErmB confers resistance to macrolides, lincosamides, & streptogramin while the tet32 and tetO genes confer resistance to tetracycline. Only two isolates had AMR genes to lincosamide and nucleoside antibiotics while a single isolate had AMR gene to lincosamides & pleuromutilins. Four isolates had AMR genes to aminoglycosides. AMR genes to beta-lactams were not identified in the isolates.

Recently, our research group published results on a contemporary set of pathogenic *S. suis* isolates (1). From the previous study, the predominant serotype was serotype 1/2, which is consistent with the increase of serotype 1/2 in this study. This historical study suggests an increase in serotype 1 and decrease in serotype 7, indicating a shift in *S. suis* isolates. In the contemporary set of *S. suis* isolates, ST28 was the dominant ST followed by ST1, ST94, and ST108. Therefore, ST28 was predominant in both studies. The increase of ST1 in the historical study may align with ST1 being one of the dominant STs in the contemporary set of *S. suis* isolates. In both the historical and contemporary *S. suis* isolate sets, MRP, EF, and SLY genes are not indicators of virulence while the new genes predicting pathogenesis were present in both sets of *S. suis* isolates. Interestingly, there was a reduction of

the AMR gene ErmR in the historical set, indicating an increase of multi-resistant *S. suis* isolates was not occurring over time. The comparison between historical and contemporary *S. suis* isolates provides reference and highlights that other factors may be contributing to the reemergence of *S. suis* in the United States.

1. Estrada AA, Gottschalk M, Rossow S, Rendahl A, Gebhart C, Marthaler DG. Serotype and Genotype (Multilocus Sequence Type) of Streptococcus suis Isolates from the United States Serve as Predictors of Pathotype. J Clin Microbiol. 2019 Jun 26.

Keywords: *Streptococcus suis, serotype, multilocus sequence type, antimicrobial resistance genes*

Scientific Abstract:

Streptococcus suis has become a reemerging bacterium causing significant economic losses for the swine industry. As both commensal and pathogenic strains of *S. suis* exist, numerous serotypes (n=35) and multilocus sequence types (ST, n=1161) have been identified and are used to differentiate *S. suis* strains. The goal of our study was to characterize 114 historical isolates of *S. suis* from clinical diagnostic cases between 1999 and 2013 to identify any shift in the historical isolates of *S. suis.* The predominant serotypes were 1/2 (n=21), 2 (n=20), and 7 (n=20). Serotype 2 slightly decreased while serotype 7 remained relatively constant within the 3 time periods. Serotype 1/2 nearly doubled between the first and last time period. The predominant sequence type was ST28 (24%) while 25% of the isolates had novel ST profiles. Interestingly, only a single ST1 isolate was identified within the first 10 years while five isolates were identified within the last five years of the study.

The virulence genes MRP, EF, and SLY have been associated with some pathogenic strains of *S. suis,* and only five and six isolates contained the MRP and EF gene, respectively. The gene SLY was identified in 61% of the isolates. Three genes were recently identified as indicators of pathogenicity in a contemporary set of *S. suis* strains from the United States, and 95-100% of the isolates contained these three genes, indicating the new virulence genes have not changed over time. AMR genes were investigated in the historical *S. suis* isolates. A majority (93%) of the *S. suis* isolates contain an AMR profile of ErmB, tet32, and tetO (69.3%) or tet32 and tetO (23.7%). The reduction of the ErmB gene occurred, which contributed to an increase of the tet32 and tetO profile over time. AMR genes to beta-lactams were not identified in the isolates. This historical study suggests an increase in serotype 1 and decrease in serotype 7, indicating a shift in *S. suis* isolates. While this finding may be an artifact of the study, the comparison between historical and contemporary *S. suis* isolates provides reference and highlights other factors may be contributing to the reemergence of *S. suis* in the United States.

Introduction:

Streptococcus suis is a significant pathogen of swine and emerging zoonotic disease in southeast and east Asia. Composed of both commensal and pathogenic strains, pathogenic strains of *S. suis* causes severe economic losses for the swine industry by producing a variety of diseases including, arthritis, endocarditis, polyserositis, meningitis, and septicemia in piglets and growing pigs (1,2). Pigs commonly carry commensal strains in the upper respiratory tract and are colonized during the early stages of life (1,3,4). *S. suis* strains can be opportunistic bacteria, enhancing clinical disease when co-infected with other pathogens (2,5).

S. suis is an encapsulated, gram-positive bacterium, and 35 serotypes have been determined globally based on capsular polysaccharides (CPS), though some of the serotypes represent different *Streptococcus* species (6–8). Historical studies date back to the 1980's and provide the foundation for investigating the prevalence of serotypes and multilocus sequence types (MLST) and the emergence of virulence factors. Between January 2003 and December 2005, 100 *S. suis* isolates from the United States were serotyped, revealing 15 different serotypes while three isolates were untypable (9). Untypeable *S. suis* strains occur due to loss of their CPS, and the loss of the CPS increases their affinity to mammalian cells, and biofilms can form in a variety of tissues including brain, joints, heart, and lungs, contributing to severe clinical disease (10).

Globally, multiple efforts in clinical microbiology have been proposed to identify and distinguish different clinical *S. suis* strains to understand their epidemiological implications. Multilocus sequencing typing (MLST) is a typing method based on seven housekeeping genes, and 1161 sequence type (ST) profiles have been recognized as of February 28th, 2019 (11). While the predominant swine STs are ST1, ST25, and ST28, only ST25 and ST28 have been reported as the pathogenic strains in North America while ST1 is more prevalent in Europe and Asia (12–16). In Europe, the *S. suis* virulence-associated genes (VAGs) muramidase-released protein (MRP), extracellular protein factor (EF), and thiol-activated hemolysin (SLY) have been associated with pathogenic strains (12,17). However, their association with pathogenesis is unclear as their presence or absence varied among virulent strains of various serotypes. In addition, the prevalence of VAGs may be dependent on geographical location, similar to serotype. Antimicrobial resistance (AMR) has also been recorded and compared among serotypes, but a correlation between them is lacking (2). While previous reports indicated a majority of strains were penicillin resistant, later studies

noted resistance to one or more antibiotics such as tetracycline and macrolides.

Most recently, whole-genome sequencing of bacteria using next generation sequencing (NGS) has been deployed to identify the serotype, ST, VAGs, and AMR genes, which is extremely cost-effective as information on four areas of interest can be investigated at the same time. In this study, we used NGS to determine the serotype, ST, VAGs, and AMR genes in a historical set of *S. suis* isolates to determine if a profile shift occurred over time.

Objectives:

Characterization and comparison of historical to contemporary *S. suis* isolates to identify serotype, ST, VAGs, and AMR profile changes associated with pathogenesis.

Materials & Methods:

A total of 114 historical *S. suis* isolates were retrieved from Zoetis since the storage of bacterial isolates at the veterinary diagnostic laboratories is limited. The historical isolates came from clinical cases submitted between 1999 and 2013, with an average of 8 isolates per year. Upon receipt of the *S. suis* isolates from Zoetis, the isolates were re-cultured on blood agar plates (Thermo Fisher Scientific, Waltham, MA, USA) and verified as *S. suis* using the Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Microflex device, Bruker Daltonics GmbH, Germany). While in the logarithmic growth (24-48 hours), the DNA from the isolates was extracted using the QIAamp DNA kit with the protocol for cultured cells (Qiagen Inc., Germantown, MD, USA). The DNA underwent library preparation using Nexture TX (Illumina, San Diego, CA) and next generation sequencing on an Illumina HiSeq 2500 (Illumina) with 250 bp paired-end reads at the University of Minnesota Genomic Center (UMGC, St. Paul, MN, USA).

The low quality reads and adaptors were removed using Trimmomatic. *In-silico* serotype and MLST for isolates were obtained targeting the *cpsK* gene (*Streptococcus suis* NSUI002, Accession CP011419), using the *S. suis* serotyping pipeline (https://github.com/streplab/SsuisSerotyping_pipeline) and the Short Read Sequence Typing for Bacterial Pathogens (SRST2) program (http://katholt.github.io/srst2), respectively. Isolates without a serotype or ST allele were reported as NT (nontypeable) and NF (not found), respectively. New ST profiles were reported as Novel. Previous research in Europe identified mrp, ef, and sly as virulence genes and our research group identified 3 genes (srftD, manN, and ssnA) as predictors of pathogenesis. The reads were mapped to the six genes using Bowtie to determine the absence or presence of the genes. To investigate antimicrobial resistance (AMR) genes, Spades (18) de novo assembled contigs were blasted against the reference nucleotide sequences in Nucleotide_fasta_protein_homolog_model from CARD Database version 3.0.2 (19). Presences of AMR genes were determined by an identity cutoff of >=90% and E-value<$1x10^{-50}$. Subsequent, frequentist analysis was done using R software (20).

Results:

Serotyping and MLST distribution of the historical S. suis isolates

A total of 114 historical isolates were selected for characterization from clinical diagnostic cases between 1999 and 2013, and study period was partitioned into time frames consisting of 5 years to investigate trends. *In-silico* serotyping analysis identified 93% of the isolates (n=106) within 14 serotypes while 7% of the isolates (n=8) were nontypable (NT) by WGS (Table 1). The predominant serotypes were 1/2 (n=21), 2 (n=20), and 7 (n=20). Within the predominant serotypes, serotype 2 slightly decreased while serotype 7 remained relatively constant within the 3 time periods. Interestingly, serotype 1/2 nearly doubled between the first and last time period.

Table 1. Serotypes identified in the historical *S. suis* isolates.

Serotype	1999-2003	2004-2008	2009-2013	Total	Percentage
1	0	0	1	1	1%
2	8	7	5	20	18%
3	2	5	2	9	8%
4	2	0	3	5	4%
5	1	3	1	5	4%
7	7	7	6	20	18%
8	6	3	3	12	11%
9	0	1	1	2	2%
10	2	1	1	4	4%
14	0	0	1	1	1%
19	1	0	1	2	2%
23	1	1	0	2	2%
31	1	0	1	2	2%
1/2	6	4	11	21	18%
NT	1	5	2	8	7%
Total	38	37	39	114	

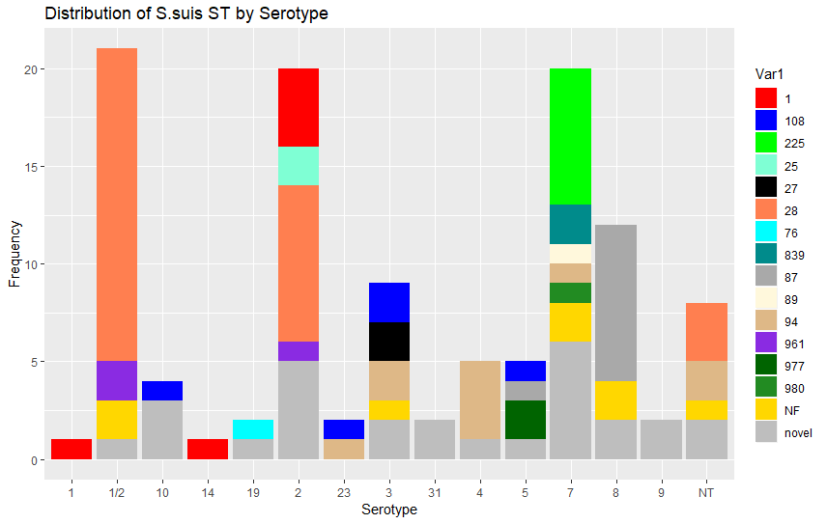
In-silico MLST analysis identified 68% of the isolates (n=78) within 14 STs (Table 2). The ST was not found (NF) in 7% of the isolates (n=8). Interestingly, 25% of the isolates (n=28) had novel ST profiles. ST28 was the predominant sequence type (n=27, 24%) and remained relatively constant over time. Only a single ST1 was identified within the first 10 years of the study while 5 isolates were identified within the last 5 years of the study. Interestingly, the number of novel ST and ST87 isolates decreased during the third time period.

Table 2. STs identified in the historical *S. suis* isolates.

ST	1999-2003	2004-2008	2009-2013	Total	Percentage
ST1	1	0	5	6	5%
ST25	0	2	0	2	2%
ST27	0	2	0	2	2%
ST28	10	7	10	27	24%
ST76	0	0	1	1	1%
ST87	5	3	1	9	8%
ST89	0	1	0	1	1%
ST94	4	3	3	10	9%
ST108	2	3	0	5	4%
ST225	4	1	2	7	6%
ST839	1	0	1	2	2%
ST961	0	1	2	3	3%
ST977	1	0	1	2	2%
ST980	0	0	1	1	1%
NF	0	3	5	8	7%
Novel	10	11	7	28	25%
Total	38	37	39	114	

Relationship of Serotyping and ST

The relationships between serotype and ST were investigated. The predominant serotype 1/2 was primarily composed of ST28 (n=16/21). Of the 20 isolates identified as serotype 2, a large proportion were ST28 (n=8) followed by novel STs (n=5) and ST1 (n=4). Also composed of 20 isolates, serotype 7 contained all the ST225 isolates from the study (n=7) followed by novel STs (n=6). Serotype 7 contained the most STs (n=6), and ST225 isolates (n=7), ST839 (n=2), and ST980 (n=1) were only identified as serotype 7. Also, the ST87 isolates (n=9) only belonged to serotype 8.



Virulence gene/predictors of pathogenicity

In Europe, MRP, EF, and SLY have been associated with some pathogenic strains of *S. suis*, and the presence of these genes was investigated in the historical data set. Only five and six isolates contained the MRP and EF gene, respectively (Table 3). Interestingly, almost of the isolates were from 2009-2013. The gene SLY was identified in 61% of the isolates, and the frequency of the gene remained constant over time. Three genes (A, B, and C) were recently identified as indicators of pathogenicity in a contemporary set of *S. suis* strains. In the historical set, 95-100% of the isolates contained these three genes.

Table 3. Virulence genes in the historical *S. suis* isolates.

Gene	1999-2003	2004-2008	2009-2013	Total	Percentage
mrp	1	0	4	5	4.4%
ef	1	0	5	6	5.3%
sly	23	24	23	70	61.4%
A	35	37	37	109	95.6%
B	37	37	38	112	98.2%
C	38	37	39	114	100.0%

AMR Profiles

The AMR genes were investigated in the historical *S. suis* isolates using *in silico* analysis. Of the 114, a majority (93%) of the *S. suis* isolates contain either an AMR profile of ErmB, tet32, and tetO (69.3%) or tet32 and tetO (23.7%). A reduction of the ErmB gene occurred, which contributed to an increase of the tet32, tetO profile over time. Only a single strain lacked any AMR genes and only seven isolates contained a unique profile. Only four isolates contained AMR genes to aminoglycoside, two isolates contain AMR genes to lincosamide or nucleoside antibiotics, and a single isolate contained AMR genes to lincosamides and pleuromutilin antibiotics. The presence of lincosamide, pleuromutilin, and nucleoside antibiotics appeared in the latter half of the sampling period (after 2007). AMR genes to beta-lactams were not identified in the isolates.

Table 4. AMR profile in the historical *S. suis* isolates.

AMR Profile	1999-2003	2004-2008	2009-2013	Total	Percentage
ErmB, tet32, and tetO	29	30	20	79	69.3%
tet32 and tetO	6	6	15	27	23.7%
Unique	3	1	3	7	6.1%

Lacks	0	0	1	1	0.9%
Total	38	37	39	114	100%

Table 5. Unique AMR profiles in the historical *S. suis* isolates.

	aminoglycoside			tetracycline						lincosamide		lincosamide & pleuromutilin	nucleoside	macrolide, lincosamide, & streptogramin
Year	aad(6)	ANT(6)-Ia	APH(3')-IIIa	tet (W/N/W)	tet32	tet44	tetM	tetO	tetW	lnuB	lnuC	lsaE	SAT-4	ErmB
1999		X			X			X						X
2003				X					X					X
2003				X	X			X	X					X
2007	X		X		X	X		X			X		X	X
2009	X	X	X		X			X					X	X
2011							X							
2013			X		X			X		X		X		

Discussion:

The reason for the recent reemergence of *S. suis* in the U.S. swine industry remains unknown, but the characterization of historical strains allows for the investigation and comparisons to contemporary pathogenic strains, especially as little information is available for known historical pathogenic strains of *S. suis*. In this study, we used NGS to determine the serotype, ST, VAGs, and AMR genes in 114 historical *S. suis* isolates from the United States. Our study identified the predominant serotypes of 1/2 (n=21), 2 (n=20), and 7 (n=20), which have been reported as predominant serotypes in North America (9,21–23). This historical study suggests an increase in serotype 1 and decrease in serotype 7, indicating a shift in *S. suis* isolates. Recently, our research group published results on a contemporary set of pathogenic *S. suis* isolates and the predominant serotype was 1/2 (24), which is consistent with the increase of serotype 1/2 in this study. In the contemporary set of *S. suis* isolates, ST28 was the dominant ST followed by ST1, ST94, and ST108. Therefore, ST28 was the dominant in both studies. The increase of ST1 in the historical study may align with ST1 being a dominant type in the contemporary *S. suis* isolates.

In both the historical and contemporary *S. suis* isolate sets, MRP, EF, and SLY genes were not indicators of virulence while the new genes predicting pathogenesis were present in both sets of *S. suis* isolates. Interestingly, there was a reduction of the AMR gene, ErmR, in the historical set. Only four *S. suis* isolates contained AMR genes for multiple antibiotics, indicating multi-resistant *S. suis* isolates was not increasing over time. Moreover, AMR genes to beta-lactams were not identified in the isolates. While this finding may be an artifact of the study, the comparison between historical and contemporary *S. suis* isolates provides reference and highlights other factors may be contributing to the reemergence of *S. suis* in the United States.

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