RESEARCHREPORT

SWINE HEALTH

pork checkoff

Title: Prospective investigation of Streptococcus suis isolates from diseased pigs and healthy pen-matched controls in outbreaks of Streptococcus-associated neurologic disease using metagenomics, serotyping and sequencing. ***#17-117***

Investigator: Bailey Arruda

Institution: Iowa State University

Date Submitted: 12/21/2020

Industry Summary:
The impact of *Streptococcus suis*-associated neurologic disease has substantially increased since first described more than half a century ago. It has emerged as a common, serious clinical challenge and is a major reason for antibiotic therapy in post-weaning pigs. It is estimated that this pathogen costs the US swine industry more than $300 million annually.

There are many (over 25) different serotypes of *S. suis* that can be isolated from diseased pigs, yet the majority of published research focuses on serotype 2 which fundamentally limits extrapolation between serotypes. The virulence factors and the pathogenesis of *S. suis* are complex and poorly understood. Moreover, the lack of uniformity between the discrimination parameters of virulent and avirulent strains, as well as virulence discrepancies within isolates of the same strain, impairs a consensus regarding important virulence markers. Limited information is available concerning the immunopathogenesis and virulence of serotypes. Discrepancies in characterization from field cases, including genetic sequence analysis of isolates from affected pigs and healthy cohorts, make it difficult to understand the ecology, pathogenesis, distribution and serotype prevalence among clinical and healthy pigs. Further complicating matters is the lack of confirmatory testing of many of the clinical cases by histopathology, which compromises accuracy of assessing causation. There is also a lack of precise and targeted collection of isolates. In total, generalizations made about *S. suis*-associated disease in the United States swine herds without evaluation or analysis of a representative sample of well-characterized isolates/strains are unlikely to be valid. Therefore, the goals of this project are to identify the presence of virulence factors, genes or markers in isolates derived from diseased versus healthy pigs; determine if these 'markers' can be used to predict virulence; and to investigate the immunopathogenesis of *S. suis*-associated neurologic disease using a well-defined, aseptically collected set of samples.

Over 60 crossbred, commercially raised pigs from multiple herds with a history of *S. suis*-associated neurologic disease were necropsied. Four to seven live pigs (affected and pen- or litter-matched controls) from each outbreak were necropsied with two to six pigs meeting the case definition. Aseptically collected samples submitted for bacterial culture included a meningeal swab, bronchial lavage, tonsil scrape, nasal swab, small intestine, large intestine, cerebrospinal fluid as

These research results were submitted in fulfillment of checkoff-funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer-reviewed.

For more information contact:
National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

well as swabs of any grossly visible serofibrinous exudates in joints or on serosal surfaces. Serotyping was performed per the Iowa State University Veterinary Diagnostic Laboratory protocol. The total number of *S. suis* isolates collected was 324. These isolates were serotyped using a PCR-based method and traditional co-agglutination. Later, 125 isolates were screened for 3 virulence-associated genes (mrp, epf and sly). The most common serotypes identified in descending order were 10, 21, 7, 1 and 4. The serotype that was identified as causing meningitis in a majority of the cases was serotype 1, followed by, in descending order, serotypes 2 or 1/2, 10, 4, 5, 11 and 14. Over 20% of the pigs had *S. suis* isolated from the alimentary tract and the enteric system of three animals was colonized by same strain causing disease in the herd and/or this individual. Fifty-seven *S. suis* isolates (both from diseased and non-diseased pigs) obtained during this study as well as 13 *S. suis* isolates from pigs with histologic evidence of meningitis submitted to the ISU VDL were sequenced and compared to identify virulence factors, markers, and genes.

Key findings of this study are directly applicable to herd surveillance and monitoring, herds can be screened by nasal and/or tonsil swab to identify carriage once a virulent strain has been identified from aseptically collected samples from either the joint or brain. Litter-matched healthy piglets were commonly colonized by the same *S. suis* strain causing disease further supporting the sow as an early source of *S. suis* colonization. The use of genotyping tools in addition to serotyping could aid the control of *S. suis* disease in swine herds and expand the knowledge of critical factors involved in the epidemiology and pathogenesis of infections by *S. suis*. *S. suis* isolates of the same serotype originating from systemic sites with bacterial lesions and the nasal/tonsil of diseased pigs and healthy carriers in a single diagnostic case submission are consistently the same strain when characterized by whole genome sequence. The site of isolation can be a confounding factor when cataloguing isolates as commensal or pathogenic and trying to discern virulence factors as virulent strains can be found in the tonsil and nasal cavity of healthy carriers. Cerebrospinal fluid offers a relatively easy and aseptic sample to collect for *S. suis* isolation. Given the large genome size and extensive genome variability between *S. suis* strains as well as the lack of circularized *S. suis* genomes, identification of virulence 'markers' using genetic information of *S. suis*, if at all possible, is going to necessitate innovative approaches that may entail machine learning in combination with a very well-characterized set of isolates.

Although not initially a focus of this project but as a direct result of it, a cerebrospinal fluid collection method and a proposed isolate characterization score and sample collection scheme for autogenous production were developed and shared with diagnosticians and swine veterinarians. Additional *S. suis* research is being conducted to characterize nearly 1000 *S. suis* isolates from case submissions with *S. suis* meningitis in order to gain additional insight into the serotype and genetic variability of disease causing *S. suis* in US swine herds to facilitate vaccine development.

Bailey Arruda, DMV PhD
Iowa State University
1850 Christensen Dr.
Ames, IA 50014
Phone: 515-294-5750
Email: wilberts@iastate.edu

Key Findings:

- *S. suis* isolates of the same serotype originating from systemic sites with bacterial lesions and the nasal/tonsil of diseased pigs and healthy carriers in a single diagnostic case submission are consistently the same strain when characterized by whole genome sequence.
- Once a virulent strain has been identified from aseptically collected samples from either the joint or brain, the herd population could be screened by nasal and/or tonsil swab to identify carriage.
- The use of genotyping tools in addition to serotyping could aid the control of *S. suis* disease

in swine herds and expand the knowledge of critical factors involved in the epidemiology and pathogenesis of infections by *S. suis*.

- Site of isolation can be a confounding factor when cataloguing isolates as commensal or pathogenic as virulent strains can be found in the tonsil and nasal cavity of healthy carriers.
- Litter-matched healthy piglets were commonly colonized by the same *S. suis* strain causing disease further supporting the sow as an early source of *S. suis* colonization.
- Cerebrospinal fluid offers a relatively easy and aseptic sample to collect for *S. suis* isolation.
- Given the large genome size and extensive genome variability between *S. suis* strains as well as the lack of circularized *S. suis* genomes, identification of virulence 'markers' using genetic information of *S. suis*, if at all possible, is going to necessitate innovative approaches that may entail machine learning in combination with a very well-characterized set of isolates.

Keywords: *Streptococcus suis,* serotyping, virulence markers, whole-genome sequencing, genotyping

Scientific Abstract:
The impact of *Streptococcus suis*-associated neurologic disease has substantially increased since first described and has emerged as a common and serious clinical challenge. The goals of this project were to identify the presence of virulence factors, genes or markers in isolates derived from diseased versus healthy pigs; determine if these 'markers' can be used to predict virulence; and to investigate the immunopathogenesis of *S. suis*-associated neurologic disease using a well-defined, aseptically collected set of samples. Over 60 crossbred, commercially raised pigs from multiple herds with a history of *S. suis*-associated neurologic disease were necropsied. Four to seven live pigs (affected and pen- or litter-matched controls) from each outbreak were necropsied with two to six pigs meeting the case definition. Aseptically collected samples submitted for bacterial culture included a meningeal swab, bronchial lavage, tonsil scrape, nasal swab, small intestine, large intestine, cerebrospinal fluid as well as swabs of any grossly visible serofibrinous exudates in joints or on serosal surfaces. Serotyping was performed per the Iowa State University Veterinary Diagnostic Laboratory protocol. The total number of *S. suis* isolates obtained and saved was 324 of which155 isolates originated from pen-matched controls and 169 isolates were recovered from pigs with bacterial meningitis. These isolates were serotyped by PCR-based serotyping and traditional co-agglutination tests. The most common serotypes identified in descending order were 10, 21, 7, 1 and 4. The serotype that was identified as causing meningitis in a majority of the cases was serotype 1, followed by, in descending order, serotypes 2 or 1/2, 10, 4, 5, 11 and 14. Over 20% of the pigs had *S. suis* isolated from the alimentary tract and the enteric system of three animals was colonized by same strain causing disease in the herd and/or the individual. The majority of *S. suis* isolates originated from the tonsil and nasal cavity, making up 39% and 37% of the isolates, respectively. One hundred and twenty-five isolates were screened for 3 virulence-associated genes (mrp, epf and sly) and only serotypes 1 and 14 were positive for all three genes. Fifty-seven *S. suis* isolates (both from diseased and non-diseased pigs) obtained during this study as well as 13 *S. suis* isolates from pigs with histologic evidence of meningitis submitted to the ISU VDL were sequenced and compared to identify virulence factors, markers, and genes. Based on the results of this study, *S. suis* isolates of the same serotype originating from systemic sites with bacterial lesions and the nasal/tonsil of diseased pigs and healthy carriers in a single diagnostic case submission are consistently the same strain when characterized by whole genome sequence. Accordingly, herds can be screened by nasal and/or tonsil swab to identify carriage once a virulent strain has been identified from aseptically collected samples from either the joint or brain. Litter-matched healthy piglets were commonly colonized by the same *S. suis* strain causing disease further supporting the sow as an early source of *S. suis* colonization. These findings also further confirm that the site of isolation can be a confounding factor when cataloguing isolates and trying to discern virulence factors as commensal or pathogenic as virulent strains can be found in the tonsil and nasal cavity of healthy carriers. In the future, the use of genotyping tools in addition to serotyping could aid the control of *S. suis* disease in swine herds and expand the knowledge of critical factors involved in the epidemiology and pathogenesis of infections by *S. suis*. Given the large genome size and extensive genome variability between *S. suis* strains as

well as the lack of circularized *S. suis* genomes and lack of critical clinicopathologic information of *S. suis* strains, identification of virulence 'markers' using genetic information of *S. suis*, if at all possible, is going to necessitate innovative approaches that may entail machine learning in combination with a large very well-characterized set of isolates.

Introduction:

Streptococcus suis is a common porcine bacterial pathogen causing septicemia and deaths and is a major justification for aggressive use of antimicrobials in growing swine. Streptococcosis is one of the primary causes of death in postweaned pigs from 5 to 10 weeks of age and is common across production types even in the absence of concurrent disease risk factors including PRRSV and IAV. Death losses, production losses and interventions cost the United States swine industry more than $300 million annually.

At least 35 serotypes of *Streptococcus suis* have been reported based on capsular polysaccharide(CPS) antigens; however, some of the newer classification techniques suggest a portion may be misclassified or inaccurately identified. Nontypable strains are also isolated from pigs and not all strains are thought to be pathogenic. Part of the confusion arises because *S. suis* is diverse, very common flora and can be easily isolated from most pigs but not all reports have clear and accurate diagnostic criteria for disease status. Consequently, the reports of strain variation, serotype diversity, or distribution of serotypes from surveys are suspect because organisms tested were not confirmed as originating from diseased tissue with clear criteria or confidence. The scientific literature suggests that serotype 2 is the most virulent and the most commonly isolated serotype from diseased pigs, it is unclear where the diversity, origin and case definition associated with this dogma was generated.

Streptococcus suis strains have also been evaluated and classified into sequence types (ST) by multilocus sequence typing and, more recently, by whole genome sequencing. Yet, a majority of these isolates are decades old and are not well-characterized pathologically which impacts the validity of conclusions. Again, there is little to no information concerning the presence of consistent histologic lesions, concurrent disease status, location from which the isolate was recovered, or collection technique (*S. suis* is a ubiquitous bacteria and contamination is a significant problem during sample collection).

The natural ecologic niche of *S. suis* is the upper respiratory tract (tonsil and nasal cavity), as well as the genital and intestinal tracts of pigs. In general, the main route of infection is oro-nasal[1]; however, the intestinal tract cannot be excluded as a portal of entry. Numerous bacterial components have been implicated in *S. suis* virulence, yet the precise role of each component remains unclear. So far, some of the virulence-associated factors identified include components of the CPS, extracellular factor, muraminidase-released protein, suilysin, adhesions, 38 kDa protein, glutamate dehydrogenase (GDH), fibronectin-binding protein (FBP), and arginine deiminase.

Limited studies have been performed with North American strains hence knowledge of the ecology, animal carriage, pathogenesis, virulence markers, immunopathogenesis and disease expression of *S. suis* is profoundly deficient. As a result, the primary intervention for disease is widespread administration of antibiotics. An improved understanding of *S. suis*-associated disease is required to improve disease preventative efforts which include development of an efficacious vaccine to mitigate antibiotic usage.

The impact of Streptococcus suis-associated neurologic disease has substantially increased since first described multiple decades ago. It has emerged as a common and serious clinical challenge over the last few years in US swine herds and is a major reason for antibiotic therapy in post-weaning pigs. Historically, sporadic serious outbreaks of streptococcal septicemia and meningitis occurred in confined rearing situations, but in most outbreaks, mortality usually did not exceed 0.3%. More recently, it is not uncommon for *S. suis* to result in a spiking mortality of 3% virtually overnight, with additional losses incurred after initiation of treatment. Losses due to *S. suis* can reach 4 to 5%, even in herds that are porcine reproductive and respiratory syndrome virus and

Influenza A virus negative; mortality is further increased in herds with these co-infections. It is estimated that this pathogen costs the US swine industry more than $300 million annually.

There are many (over 25) different serotypes of *S. suis* that can be isolated from diseased pigs, yet the majority of published research focuses on serotype 2 which fundamentally limits extrapolation between serotypes. The virulence factors and the pathogenesis of *S. suis* are complex and poorly understood. Moreover, the lack of uniformity between the discrimination parameters of virulent and avirulent strains, as well as virulence discrepancies within isolates of the same strain, impairs a consensus regarding important virulence markers. Limited information is available concerning the immunopathogenesis and virulence of serotypes. Discrepancies in characterization from field cases, including genetic sequence analysis of isolates from affected pigs and healthy cohorts, make it difficult to understand the ecology, pathogenesis, distribution and serotype prevalence amongclinical and healthy pigs. Further complicating matters is the lack of confirmatory testing of many of the clinical cases by histopathology, which compromises accuracy of assessing causation. There is also a lack of precise and targeted collection of isolates. In total, generalizations made about *S. suis*-associated disease in the United States swine herds without evaluation or analysis of a representative sample of well-characterized isolates/strains are unlikely to be valid.

Due to the endemic nature of this bacteria and nearly ubiquitous colonization of mucosal surfaces of most pigs, the diagnostic process must include a concise case definition of specified and compatible clinical signs, compatible gross and histologic lesions, and confirmation of the organism within the affected tissue by bacterial isolation or other validated detection methods. Failure to accurately diagnose the disease state of infection can lead to erroneous generalizations or conclusions, and may not accurately estimate the impact or importance of certain strains/isolates of *S. suis*-associated or disease in US swine herds. Accordingly, the goals of this project are to identify the presence of virulence factors, genes or markers in isolates derived from diseased versus healthy pigs; determine if these 'markers' can be used to predict virulence; and to investigate the immunopathogenesis of *S. suis*-associated neurologic disease using a well-defined, aseptically collected set of samples.

Objectives:

Objective 1: Generation of a well-defined, precisely acquired set of *S. suis* isolates and panel of samples from diseased and healthy pigs.

Aim 1. Obtain a set of *S. suis* isolates from diseased pigs and from healthy pen-matched cohorts. Samples from pigs which have met qualifying criteria will be aseptically collected from multiple body locations, minimally to include brain, tonsil, lung, nasal cavity, large intestine, and small intestine, by swine diagnostic pathologists. These samples will be invaluable for this and other research projects focusing on *S. suis*. Such samples are currently unavailable.

Aim 2. Perform 16S metagenomic analysis from the lung, tonsil and intestinal luminal content samples to assess the potential impact of other bacterial etiologies on the ecopathogenesis of *S. suis*.

Aim 3. Acquisition and processing of samples for potential shotgun sequencing (detect viruses) and transcriptomics (detect gene expression if sequencing reveals limited information) pending initial results.

Aim 4. Characterize gross and histologic lesions present in diseased and healthy pigs in context of specific attributes of detected *S. suis*.

Aim 5. Collect acute and convalescent serum pigs which meet clinical criteria, receive appropriate intervention and survive.

Meeting these aims will provide the samples necessary to identify: the presence of virulence factors, genes or markers; determine if these 'markers' can be used to predict virulence; and assess if the

potential of cross-protection between serotypes.

Objective 2: Identify the presence of virulence factors, genes or markers in isolates from diseased versus healthy pigs and determine if these 'markers' can be used to predict virulence.

Aim 1. Sequence 48 isolates selected from the set established in Aim 1 from diseased and healthy pen-matched controls.

Aim 2. Correlate sequencing results with gross and histologic evaluation performed in Aim 4.

Meeting these aims will provide pertinent information to practitioners concerning the identification of virulent *S. suis* resulting in improved management strategies for swine production systems that will enhance production efficiency, animal well-being, and improve animal health.

Objective 3: Investigate the immunopathogenesis of *S. suis*-associated disease.

Aim 1. Assess if the potential of cross-protection between serotypes exists using convalescent serum.

Aim 2. Develop an opsonophagocytic assay for *S. suis* similar to those developed for *S. pneumoniae.* Multiple studies have documented the importance of antibody and complement mediated opsonophagocytosis in the protection against invasive pneumococcal disease in humans which led to the development of well controlled OPAs. There is currently no well controlled OPA for *S. suis*. Such as assay would provide an *in vitro* technique to assess protection following vaccination/exposure. An improved understanding of *S. suis*-associated disease is required to undertake disease preventative efforts.

Materials & Methods:
A total of 65 crossbred, commercially raised pigs from herds with a history of *S. suis*-associated neurologic disease were necropsied. A total of 35 crossbred, commercially raised pigs were used for collection of isolates as not all necropsied pigs met the case definition. Four to seven live pigs (affected and pen- or litter-matched controls) from each outbreak were necropsied with two to six pigs meeting the case definition. Lung and lymph node from the affected and healthy pigs were pooled by group and tested by PCR for PRRSV, IAV, and PCV2. Aseptically collected samples submitted for bacterial culture included a meningeal swab, bronchial lavage, tonsil scrape, nasal swab, small intestine, large intestine, cerebrospinal fluid as well as swabs of any grossly visible serofibrinous exudates in joints or on serosal surfaces. Serotyping was performed per the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) protocol on the predominate *S. suis* detected from privileged locations and on four *S. suis* isolates of varying morphology, if possible, from the tonsil and nasal swab from across blood agar plates. All isolates were serotyped by co-agglutination and PCR-based serotyping (mPCR), and subsequently 125 isolates were screened for 3 virulence-associated genes (mrp, epf and sly). Thirty-five *S. suis* reference strains were used as positive controls. All isolates were saved at -80°C for further analysis.

Fifty-seven *S. suis* isolates (both from diseased and non-diseased pigs) obtained during this study as well as 13 *S. suis* isolates from pigs with histologic evidence of meningitis submitted to the ISU VDL were sequenced to identify virulence factors, markers, and genes. Full genome sequencing of these isolates was performed on an Illumina Miseq. *De novo* assembly of samples was performed using SPADES. Alignment of *S.suis* contigs was done to reference with RaGOO. Annotation of genes was conducted using PROKKA. The Maximum Likelihood tree was generated using FASTTREE. Tree visualization was done in R with GGTree. For a subset of analyses, only 46 isolates were compared as based on whole genome sequencing, the same strain was identified in multiple anatomic sites and pigs within a single case submission.

Histopathologic evaluation include cerebrum, cerebellum, brainstem, lung, tonsil, turbinate, spleen,

liver, synovium and intestine. Samples from the lung, tonsil, and luminal content from the ileum and apex of the spiral colon was taken for 16S metagenomics analysis and maintained at -80°C until sample processing. Briefly, DNA extraction will be done utilizing the commercially available ZymoBIOMICS DNA Microprep Kit, DNA concentration will be measured by Qubit dsDNA BR Assay kit and total DNA will be amplified using primers specific to the V2 and V4 regions of bacterial 16S genes. Amplicons will be submitted to Argonne National Laboratory and run for 150 cycles on an Illumina MiSeq instrument. To date, whole DNA was extract from twenty-five samples using ZymoBIOMICS DNA Microprep Kit. These samples included colon content, ileum content, BAL and tonsil from five different animals to test the DNA extraction protocol. Tonsil samples were divided and extracted using different protocols to determine which protocol provides the best protocol by which to assess the microbiome. First protocol was directed to scrape half of the surface of the tonsil, and proceed with the standard bead beating protocol. The second protocol was done with the other half of the tonsil, which was incubated with tissue buffer and proteinase K for an hour, later followed the standard bead beating protocol. Samples were collected and saved for potential analysis using shotgun sequencing (detect viruses) and transcriptomics (detect gene expression if sequencing reveals limited information). Unfortunately, participating farms have not had an interest in pursuing acute and convalescent serum.

Results:
A total of 65 pigs were necropsied. Pigs that met the study criteria originated from 8 different farms totaling 35 pigs from which *S. suis* was isolated and samples were collected. *S. suis* isolates varied from 21 to 65 per case. The total number of *S. suis* isolates obtained and saved was 324 of which 155 isolates originated from pen-matched controls and 169 isolates were recovered from pigs with bacterial meningitis. The number of isolates obtained varied by site and included the tonsil (39.2%), nasal cavity (37%), bronchoalveolar fluid (6.4%), brain (5.5%), joint (3.7%), ileum (3%), colon (1.8%), cerebrospinal fluid (1.8%), pericardial fluid (0.6%), abscess (0.3%) and lung (0.3%; Figure 1).

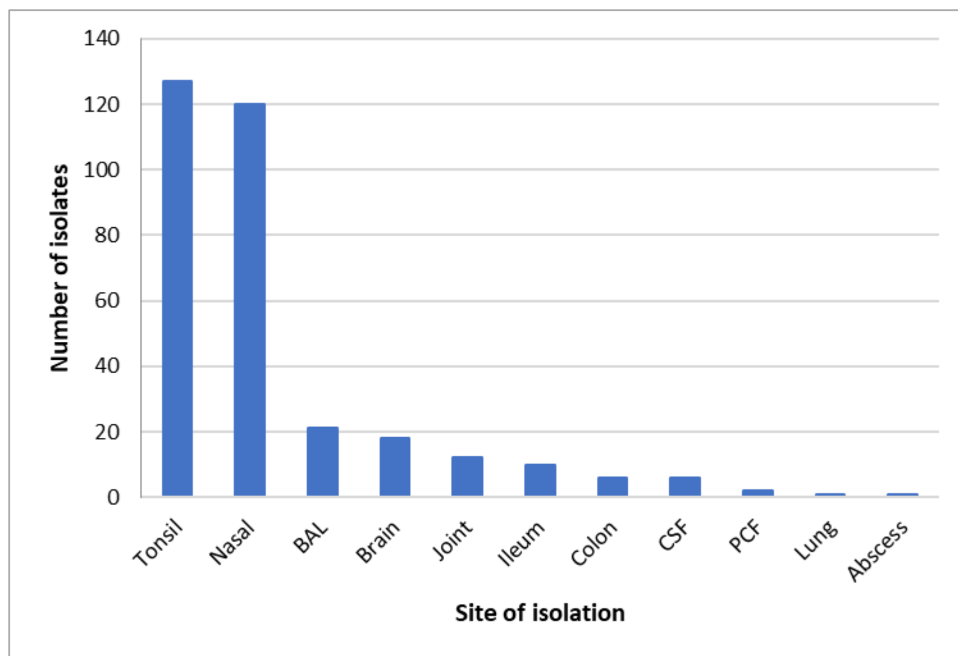

Figure 1. Number of *S. suis* isolates by site of isolation. Cerebrospinal fluid (CSF), Pericardial fluid (PCF).

Untypable *S. suis* isolates were the most common in both the nasal cavity and tonsil of diseased and healthy pigs. Serotype 10 (25/324, 7.7%) was the most frequently identifiable serotype by mPCR and co-agglutination combined, especially from the nasal cavity (24%) of healthy pigs, tonsil (24%) of both groups and bronchoalveolar fluid (16%) of diseased pigs, followed by serotypes 21 (24/324, 7.4%), 7 (22/324, 6.8%), 1 (21/324, 6.4%) and 4 (17/324, 5.2%; Figure 2 and 3). Serotypes identified less

frequently (<5%) included 11, 16, 8, 15, 28, 5, 2 or1/2, 29, 30, 22, 25, 3, 12, 13, 14, 18, 27, 31, 9, 1or14, 1/2 and 2. The diversity of identifiable serotypes colonizing an individual pig varied in the upper respiratory system. Nine percent of pigs had no identifiable serotype in their nasal cavity with 46.8% being colonized by a single serotype and 43.7% colonized by 2 or more different serotypes. One pig had no *S. suis* isolates recovered from the tonsil while 54.5% of pigs were colonized by 2 different serotypes and 24.2% were colonized by 3 or more different serotypes. When nasal and tonsil samples were combined by pig, one pig was found to be colonized by only one identifiable *S. suis* serotype while another was colonized by up to 6 different serotypes in the upper respiratory tract.

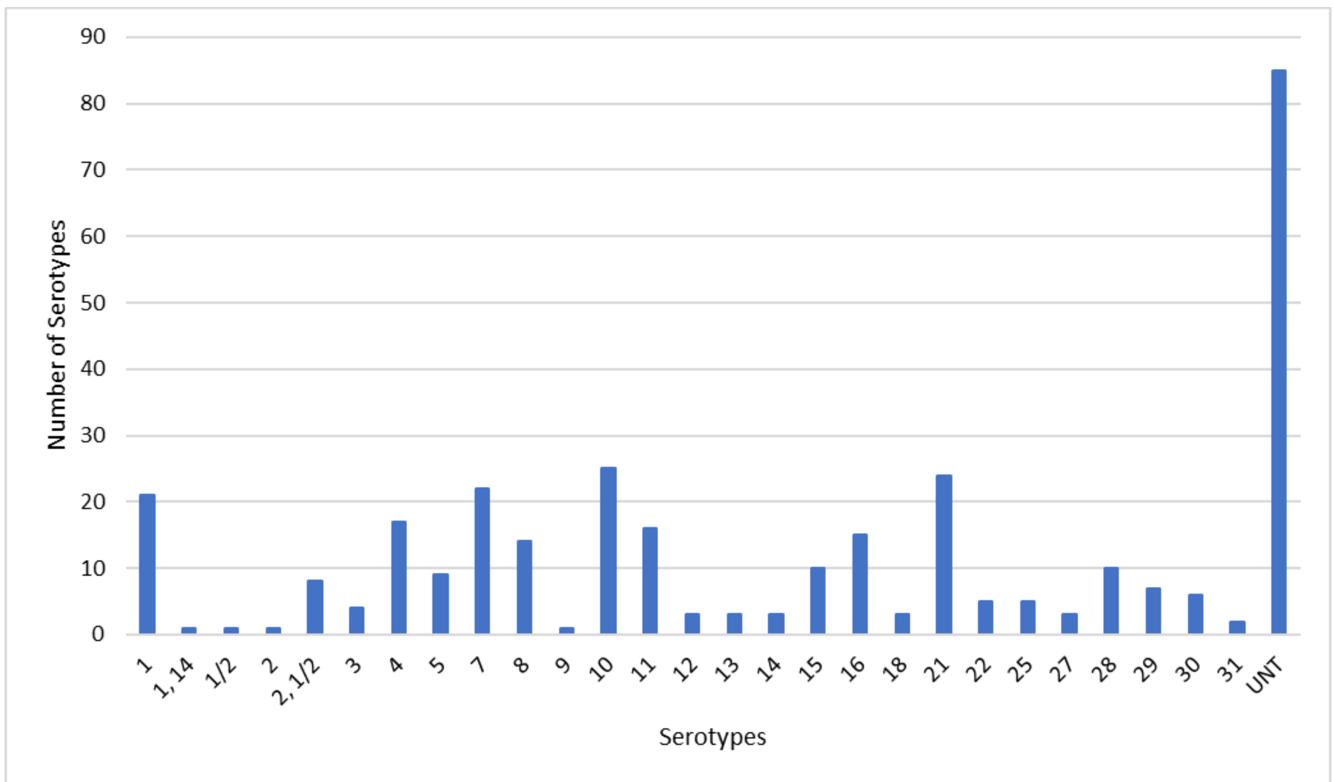

Figure 2. Number of *S. suis* serotypes that were typed by mPCR and co-agglutination.

Figure 3. The most common *S. suis* serotypes by group and site of isolation. Cerebrospinal fluid (CSF), Pericardial fluid (PCF).

S. suis serotype 1 was the most common serotype causing streptococcal meningitis. This serotype/strain was also isolated from the tonsil of diseased and litter-matched controls. Streptococcal meningitis was also caused, in descending order, by serotypes 2 or 1/2, 10, 4, 5, 11 and 14. In the case in which serotype 5 was the cause of meningitis, serotype 5 isolates were also recovered from almost all cultured samples, except tonsil and BAL. Over 20% of the pigs had *S. suis* isolated from the alimentary tract and the enteric system of three animals was colonized by same strain causing disease in the herd and/or this individual. Of the 125 isolates screened for 3 virulence-associated genes (mrp, epf and sly), Serotypes 1 and 14 were the only serotypes positive for all three virulence genes tested, most of them from diseased pigs (10/35, 28%; Figure 4).

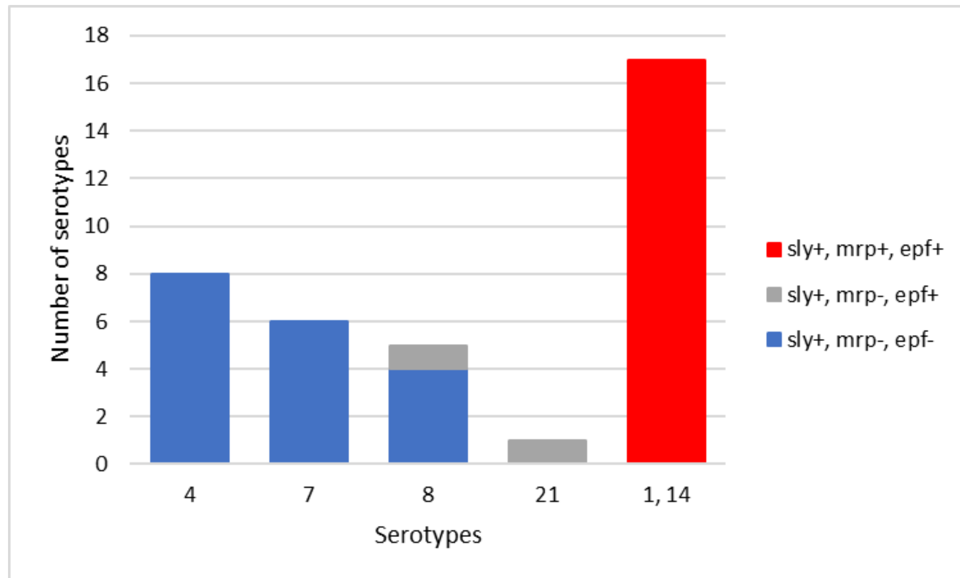

Figure 3. Number of *S. suis* isolates that were screened for three virulence genes (sly, mrp and epf). Isolates that were negative for all three genes were not included.

To date, whole DNA has been extract from twenty-five samples using ZymoBIOMICS DNA Microprep Kit. Colon content ranged between 63.9 µg/µl to 160 µg/µl. Ileal content ranged between 0.51 µg/µl to 78.7 µg/µl. BAL ranged between 14.2 µg/µl to 84.3 µg/µl. The first tonsil protocol recovered less DNA (62.7 µg/µl to 87.6 µg/µl) than the second tonsil protocol (87.7 µg/µl to 149 µg/µl). The total DNA extract from the positive control was 44.3 µg/µl.

Whole genome sequencing identified a total of 6546 genes with only 725 core genes, approximately 11% of the total genes identified. A summary of the number of genes by gene type in presented in Table 1. A tree based on the core genome of *S. suis* isolates revealed no obvious clustering of *S. suis* isolates categorized as pathogenic or commensal (Figure 5). A similarity matrix of core SNPs is presented in Figure 6; the color in the heat map represents SNP rate as shown in the legend at the top left. The presence or absence and percent identity of putative virulence markers of isolates are presented in Table 2 and 3. The presence or absence of putative virulence markers and resistance genes of isolates are presented in Figure 7.

Table 1. Number of genes by gene type following whole-genome sequencing of *S. suis* isolates.

Gene Category	**Definition Used**	**Number of Genes**
Core	99% ≤ strains ≤ 100%	725
Soft core	95% ≤ strains < 99%	624
Shell	15% ≤ strains < 95%	961
Cloud	0% ≤ strains < 15%	4236
Total	0% ≤ strains ≤ 100%	6546

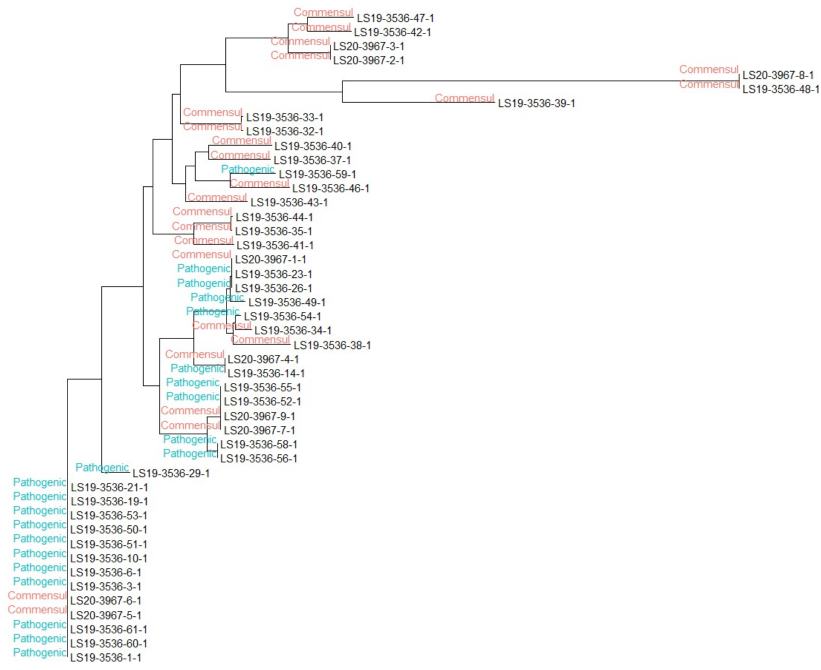

Figure 5. Core genome tree of *S. suis* isolates with no clear grouping of isolates by pathotype.

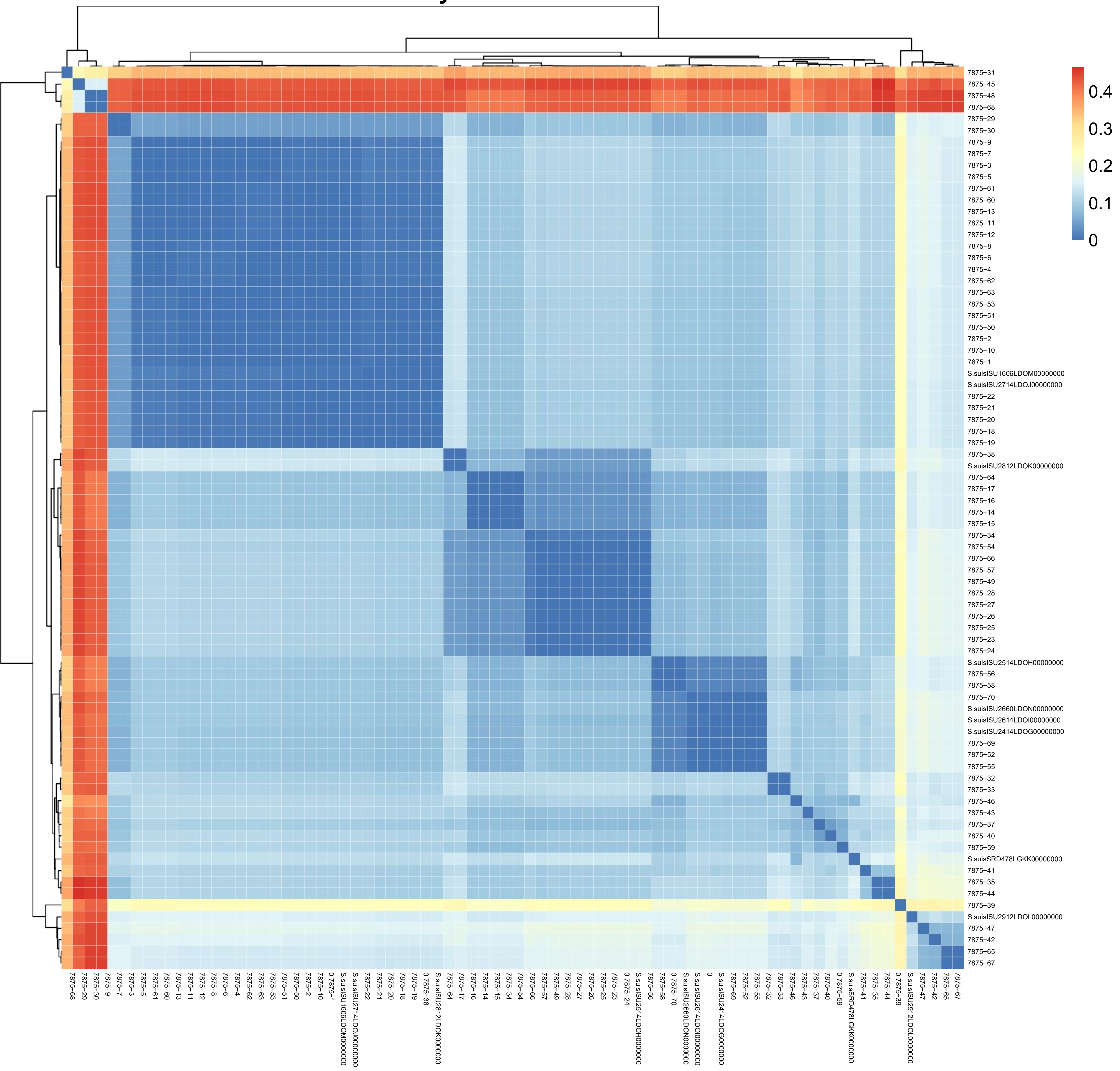
Similarity matrix of core SNPs
0.4
0.3
0.2
0.1
0
7875−31
7875−45
7875−48
7875−68
7875−29
7875−30
7875−9
7875−7
7875−3
7875−5
7875−61
7875−60
7875−13
7875−11
7875−12
7875−8
7875−6
7875−4
7875−62
7875−63
7875−53
7875−51
7875−50
7875−2
7875−10
7875−1
S.suisISU1606LDOM00000000
S.suisISU2714LDOJ00000000
7875−22
7875−21
7875−20
7875−18
7875−19
7875−38
S.suisISU2812LDOK00000000
7875−64
7875−17
7875−16
7875−14
7875−15
7875−34
7875−54
7875−66
7875−57
7875−49
7875−28
7875−27
7875−26
7875−25
7875−23
7875−24
S.suisISU2514LDOH00000000
7875−56
7875−58
7875−70
S.suisISU2660LDON00000000
S.suisISU2614LDOI00000000
S.suisISU2414LDOG00000000
7875−69
7875−52
7875−55
7875−32
7875−33
7875−46
7875−43
7875−37
7875−40
7875−59
S.suisSRD478LGKK00000000
7875−41
7875−35
7875−44
7875−39
S.suisISU2912LDOL00000000
7875−47
7875−42
7875−65
7875−67

Table 2. Presence or absence and percent identity of putative virulence factor by isolate.

Silva Virulence									
Isolate ID	**cps2J**	**cps1J**	**cps9**	**cps7**	**gdh**	**arcA**	**mrp**	**epf**	**sly**
LS19-3536-10-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-1-1_ssuis		99.9			100	99.92	99.97	99.92	100
LS19-3536-11-1_ssuis		99.79			100	99.92	100	99.92	100
LS19-3536-12-1_ssuis		99.69			100	99.92	100	99.92	100
LS19-3536-13-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-14-1_ssuis					97.33	98.21			99.6
LS19-3536-15-1_ssuis					97.33	98.21			99.6
LS19-3536-16-1_ssuis					97.33	98.21			99.6
LS19-3536-17-1_ssuis					97.33	98.21			99.6
LS19-3536-18-1_ssuis	99				100	99.92	99.97	99.92	100
LS19-3536-19-1_ssuis	99.1				100	99.92	99.97	99.92	100
LS19-3536-20-1_ssuis	99				100	99.92	99.97	99.92	100
LS19-3536-2-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-21-1_ssuis	99.1				100	99.92	100	99.92	100
LS19-3536-22-1_ssuis	99.1				100	99.92	100	99.92	100
LS19-3536-23-1_ssuis					97.33	98.21			98.99
LS19-3536-24-1_ssuis					97.33	98.21			99.6
LS19-3536-25-1_ssuis					97.33	98.21			
LS19-3536-26-1_ssuis					97.33	98.21	87.63		99.6
LS19-3536-27-1_ssuis					97.33	98.21	87.66		99.6
LS19-3536-28-1_ssuis					97.33	98.21	87.63		99.6
LS19-3536-29-1_ssuis					95.32	97.72		91.16	99.93
LS19-3536-30-1_ssuis					95.32	97.72		91.16	99.93
LS19-3536-3-1_ssuis		99.9			100	99.92	99.97	99.92	100
LS19-3536-32-1_ssuis				99.84	97.4	98.21			
LS19-3536-33-1_ssuis					97.4	98.21			
LS19-3536-34-1_ssuis					97.33				
LS19-3536-35-1_ssuis					95.7	98.21		88.13	99.67
LS19-3536-37-1_ssuis					97.62	97.72			
LS19-3536-38-1_ssuis					97.33	97.89	86.97		99.67
LS19-3536-39-1_ssuis					94.59	87.57			
LS19-3536-40-1_ssuis					97.25	97.89			
LS19-3536-4-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-41-1_ssuis					95.62	98.29	86.23		99.67
LS19-3536-42-1_ssuis					96.44	97.89			
LS19-3536-43-1_ssuis					97.18	97.64			
LS19-3536-44-1_ssuis					95.7	98.21		89.23	99.67
LS19-3536-46-1_ssuis			99.54		96.96	98.37			
LS19-3536-47-1_ssuis					96.36	97.89			
LS19-3536-48-1_ssuis					88.67				
LS19-3536-49-1_ssuis				100	97.33	98.21	87.81		99.67
LS19-3536-50-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-5-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-51-1_ssuis		99.79			100	99.92	99.97	99.92	100
LS19-3536-52-1_ssuis	98.9				96.81	97.97	88.24		
LS19-3536-53-1_ssuis		99.79			100	99.92	99.97	99.92	100
LS19-3536-54-1_ssuis				100	97.25	98.21	87.63		99.67
LS19-3536-55-1_ssuis	98.8				96.88	97.97			
LS19-3536-56-1_ssuis	99.1				97.62	97.81			
LS19-3536-58-1_ssuis	99.1				97.62	97.81			
LS19-3536-59-1_ssuis					97.18	97.81			
LS19-3536-60-1_ssuis		99.79			100	99.92	100	99.92	100
LS19-3536-6-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-61-1_ssuis		99.9			100	99.92	99.97	99.92	100
LS19-3536-7-1_ssuis		99.9			100	99.92	99.97	99.92	100
LS19-3536-8-1_ssuis		99.9			100	99.92	100	99.92	100
LS19-3536-9-1_ssuis		99.9			100	99.92	100	99.92	100
LS20-3967-1-1_ssuis					97.33	98.21			99.6
LS20-3967-2-1_ssuis					96.44	97.89			
LS20-3967-3-1_ssuis					96.44	97.89			
LS20-3967-4-1_ssuis					97.33	98.21			99.6
LS20-3967-5-1_ssuis					100	99.92	100	99.92	100
LS20-3967-6-1_ssuis		99.9			100	99.92	100	99.92	100
LS20-3967-7-1_ssuis	98.9				96.88	97.97	88.24		
LS20-3967-8-1_ssuis					88.67				
LS20-3967-9-1_ssuis	98.9				96.88	97.97	88.24		

Table 3. Presence or absence and percent identity of putative virulence factor by isolate.

Pathotyping							
Isolate ID	**ABC_Trans**	**ATPase_1**	**Sporulation**	**Type-1-Restrict**	**mrp**	**epf**	**sly**
LS19-3536-10-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-1-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-11-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-12-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-13-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-14-1_ssuis		90.93	99.13				99.6
LS19-3536-15-1_ssuis		90.93	99.13				99.6
LS19-3536-16-1_ssuis		90.93	99.13				99.6
LS19-3536-17-1_ssuis		90.93	99.13				99.6
LS19-3536-18-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-19-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-20-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-2-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-21-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-22-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-23-1_ssuis		89.23	99.13				98.99
LS19-3536-24-1_ssuis		89.23	99.13				99.6
LS19-3536-25-1_ssuis		89.23	99.13				
LS19-3536-26-1_ssuis		89.23	99.13		87.63		99.6
LS19-3536-27-1_ssuis		89.23	99.13		87.66		99.6
LS19-3536-28-1_ssuis		89.23	99.13		87.63		99.6
LS19-3536-29-1_ssuis		95.715	99.24			91.16	99.93
LS19-3536-30-1_ssuis		95.715	99.24			91.16	99.93
LS19-3536-3-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-32-1_ssuis		91.95	99.13				
LS19-3536-33-1_ssuis		91.95	99.13				
LS19-3536-34-1_ssuis		89.23	99.13				
LS19-3536-35-1_ssuis	99.38	91.33	99.35			88.13	99.67
LS19-3536-37-1_ssuis	99.59		99.56				
LS19-3536-38-1_ssuis		88.51	99.13		86.97		99.67
LS19-3536-39-1_ssuis			80.4				
LS19-3536-40-1_ssuis		92.49	98.26				
LS19-3536-4-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-41-1_ssuis	99.45		99.35		86.23		99.67
LS19-3536-42-1_ssuis			98.8				
LS19-3536-43-1_ssuis			99.02				
LS19-3536-44-1_ssuis	99.45	91.33	99.35			89.23	99.67
LS19-3536-46-1_ssuis			98.15				
LS19-3536-47-1_ssuis			98.69				
LS19-3536-48-1_ssuis		90.75					
LS19-3536-49-1_ssuis		89.23	99.13		87.81		99.67
LS19-3536-50-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-5-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-51-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-52-1_ssuis		90.11	99.13		88.24		
LS19-3536-53-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-54-1_ssuis		89.23	99.13		87.63		99.67
LS19-3536-55-1_ssuis		90.11	99.13				
LS19-3536-56-1_ssuis		90.35	99.13				
LS19-3536-58-1_ssuis		90.35	99.13				
LS19-3536-59-1_ssuis	99.59		99.35				
LS19-3536-60-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-6-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-61-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-7-1_ssuis		95.955	100	100	99.97	99.92	100
LS19-3536-8-1_ssuis		95.955	100	100	100	99.92	100
LS19-3536-9-1_ssuis		95.955	100	100	100	99.92	100
LS20-3967-1-1_ssuis		89.23	99.13				99.6
LS20-3967-2-1_ssuis		87.28	99.35				
LS20-3967-3-1_ssuis		87.28	99.35				
LS20-3967-4-1_ssuis		90.93	99.13				99.6
LS20-3967-5-1_ssuis		95.955	100	100	100	99.92	100
LS20-3967-6-1_ssuis		95.955	100	100	100	99.92	100
LS20-3967-7-1_ssuis		90.11	99.13		88.24		
LS20-3967-8-1_ssuis		90.75					
LS20-3967-9-1_ssuis		90.11	99.13		88.24		

Tree scale: 0.1

Gene Typing

- Resistance Gene
- Virulence Factor

Aac6-Aph2_AGly, Ant6-Ia_AGly, Aph2"Ic_AGly, Aph3-III_AGly, ErmB_MLS, LinB_MLS, LnuC_MLS, MsrD_MLS, Sat4A_AGly, TetL_Tet, TetM_Tet, TetO_Tet

Agglutinin_receptor, Capsule_related, Copper_exporting_ATPase1, EF, EndAsuis, IdeSsuis, Muramidase-released_protein, Type_I_restriction-modification_sysrem_S_protein, cbp40, cbpD, cpsB, cpsC, cpsE, cpsF, cpsG, cpsJ, cpsL, dppIV, eno, epf, fbp54, fbps, fhb, fhbp, hhly3, hylA, iga, ltaA, murM, murN, neuB, neuC, oatA, ofs, pdgA, plr/gapA, sbp2, sipF, sly, spf1, spf2, srtA, srtF, ssa, ssnA, sspA, tig/ropA

	MLST	Isolate_ID	Location	Age	Ass.Lesions	Serotype Agg/PCR	State
7875-11	1	Joint C	Joint	3 weeks	Arthritis	1	IA
7875-10	1	Brain C	Brain	3 weeks	Meningitis	1	IA
7875-63	1	Tonsil B1	Tonsil	3 weeks	no lesions	1	IA
7875-12	1	Brain D	Brain	3 weeks	Meningitis	1	IA
7875-13	1	Joint D	Joint	3 weeks	Arthritis	1	IA
7875-60	1	Brain	Brain	21 Days	Meningoencephalitis, suppurative	1	IN
7875-61	1	Joint	Joint	21 Days	Synovitis, fibrinosuppurative	1	IN
7875-53	1	Brain C	Brain	13 Days	fibrinosuppurative meningitis	14	IA
7875-2	1	Joint C	Joint	3 weeks	Arthritis	14	MN
7875-1	1	Brain C	Brain	3 weeks	Meningitis	1	MN
7875-50	1	Brain A	Brain	13 Days	Meningitis, purulent	1	IA
7875-51	1	Brain B	Brain	25 Days	Pyogranulomatous meningoencephalitis	14	IL
7875-62	1	Brain A1	Brain	3 weeks	no lesions	1	MN
7875-5	1	Brain D	Brain	3 weeks	Meningitis	1	MN
7875-3	1	Brain C	Brain	3 weeks	Meningitis	1	MN
7875-4	1	Tonsil C4	Tonsil	3 weeks	No lesions	1	MN
7875-7	1	Tonsil B3	Tonsil	3 weeks	No lesions	1	MN
7875-9	1	Joint D	Joint	3 weeks	Arthritis	1	MN
7875-6	1	Brain B	Brain	3 weeks	Meningitis	1	MN
7875-8	1	Brain D	Brain	3 weeks	Meningitis	1	MN
S.suisISU1606LDOM00000000	1					2	
S.suisISU2714LDOJ00000000	1					2	
7875-21	1	Brain	Brain	10 weeks	Purulent Meningitis	1	IA
7875-22	1	Joint	Joint	10 weeks	Fibrinopurulent Arthritis	1	IA
7875-19	1	Joint F	Joint	3 - 6 weeks	Fibrinopurulent arthritis	1	IA
7875-20	1	Tonsil F1	Tonsil	3 - 6 weeks	No lesions	1	IA
7875-18	1	Brain F	Brain	3 - 6 weeks	No lesions	1	IA
7875-29	17	Brain C	Brain	5 weeks	Purulent Meningoencephalitis	4	IA
7875-30	17	Nasal C1	Nasal	5 weeks	No lesions	4	IA
7875-41	NF	Tonsil A2	Tonsil	5 weeks	no lesions	30	IA
7875-44	NF	Tonsil B2	Tonsil	3 weeks	no lesions	UNT/28	MN
7875-35	NF	Tonsil B4	Tonsil	3 weeks	no lesions	21	IA
S.suisSRD478LGKK00000000	NF					unknown	
7875-32	NF	Nasal C1	Nasal	3 weeks	no lesions	7	MN
7875-33	NF	Tonsil A3	Tonsil	3 weeks	no lesions	8	MN
7875-43	NF	Ileum C1	Ileum	5 weeks	no lesions	UNT	IA
7875-40	NF	Colon C	Colon	4 weeks	no lesions	UNT	IA
7875-37	NF	Tonsil C1	Tonsil	3 - 6 weeks	no lesions	15	IA
7875-59	NF	Brain 1-2	Brain	3 Weeks	Meningitis, purulent	9	MO
7875-46	NF	Ileum D1	Ileum	3 weeks	no lesions	33/9	MN
S.suisISU2912LDOL00000000	NF					unknown	
7875-42	NF	Nasal B	Nasal	5 weeks	no lesions	28	IA
7875-47	NF	Nasal C1	Nasal	3 weeks	no lesions	UNT/21	IA
7875-65	NF	Nasal A1	Nasal	4 weeks	no lesions	11	IA
7875-67	NF	Nasal B4	Nasal	4 weeks	no lesions	11	IA
7875-39	NF	BAL B1	BAL	3 - 6 weeks	no lesions	UNT	IA
7875-31	NF	Tonsil A1	Tonsil	3 weeks	no lesions	16	MN
7875-45	NF	Nasal D3	Nasal	3 weeks	no lesions	UNT/UNT	MN
7875-68	NF	Colon B	Colon	4 weeks	no lesions	11	IA
7875-48	NF	Colon B	Colon	4 weeks	no lesions	11/UNT	IA
S.suisISU2514LDOH00000000	25					2	
7875-58	25	Brain A	Brain	7 Weeks	Suppurative meningitis	2	IL
7875-56	25	Brain A	Brain	59 Days	Purulent meningitis	1-2	IA
S.suisISU2660LDON00000000	787					2	
S.suisISU2614LDOI00000000	28					unknown	
7875-52	28	Brain B	Brain	3 Weeks	Suppurative encephalitis	1/2	PA
7875-69	28	Tonsil D2	Tonsil	5 weeks	no lesions	1/2	IA
S.suisISU2414LDOG00000000	NF					2	
7875-55	28	Brain A	Brain	42 Days	Meningitis, suppurative	2	IA
7875-70	28	Tonsil D1	Tonsil	3 - 6 weeks	no lesions	2	IA
S.suisISU2812LDOK00000000	76					unknown	
7875-38	76	Nasal B2	Nasal	3 - 6 weeks	no lesions	18	IA
7875-17	1170	Brain F	Brain	3 - 6 weeks	very mild meningitis	10	IA
7875-64	1170	Nasal B3	Nasal	3 - 6 weeks	no lesions	10	IA
7875-15	1170	Joint E	Joint	3 - 6 weeks	pronounced arthritis	10	IA
7875-16	1170	Tonsil E3	Tonsil	3 - 6 weeks	No lesions	10	IA
7875-14	1170	Brain E	Brain	3 - 6 weeks	mild meningitis	10	IA
7875-49	839	Brain	Brain	5 Weeks	Meningitis, purulent	7	IA
7875-57	839	Brain A	Brain	50 Days	Streptococcal meningitis	7	IA
7875-34	962	Tonsil C2	Tonsil	3 weeks	no lesions	4	MN
7875-54	94	Brain A	Brain	17 Days	Streptococcal meningitis	7	SD
7875-27	119	Joint D	Joint	4 weeks	Purulent Arthritis	5	IA
7875-28	119	Colon D2	Colon	4 weeks	No lesions	5	IA
7875-26	119	Brain D	Brain	4 weeks	Purulent Meningitis	5	IA
7875-25	119	Tonsil C1	Tonsil	4 weeks	No lesions	11	IA
7875-23	119	Brain C	Brain	4 weeks	Purulent Meningitis	11	IA
7875-24	119	Joint C	Joint	4 weeks	Purulent Arthritis	11	IA
7875-66	119	Brain A	Brain	4 weeks	no lesions	11	IA

Discussion:
S. suis is and will continue to be an important pathogen in growing swine resulting in significant economic losses and antibiotic usage in herds even in the absence of PRRSV. To date, there is limited data available concerning the serotypes and strains associated with disease in US swine herds. This significantly hampers disease prevention and mitigation efforts including cross-serotype/strain vaccine development and efficacy evaluation. This study demonstrated that there is a greater variation in serotypes/strains causing bacterial meningitis in US swine herds than previously documented. Accordingly, additional research on the serotype/strain diversity in the US needs to be conducted and reported to better inform vaccine development/evaluation.

Key findings of this study are directly applicable to herd surveillance and monitoring, *S. suis* isolates of the same serotype originating from systemic sites with bacterial lesions and the nasal/tonsil of diseased pigs and healthy carriers in a single diagnostic case submission are consistently the same strain when characterized by whole genome sequence. Herds can be screened by nasal and/or tonsil swab to identify carriage once a virulent strain has been identified from aseptically collected samples from either the joint or brain. Litter-matched healthy piglets were commonly colonized by the same *S. suis* strain causing disease further supporting the sow as an early source of *S. suis* colonization. The use of genotyping tools in addition to serotyping could aid the control of *S. suis* disease in swine herds and expand the knowledge of critical factors involved in the epidemiology and pathogenesis of infections by *S. suis*. Cerebrospinal fluid offers a relatively easy and aseptic sample to collect for *S. suis* isolation.

S. suis strains are highly genetically diverse with nearly a 1000 published genomes of various serotypes and sequence types; however, a vast majority of these are incomplete annotations with very few being circularized closed genomes and incomplete information concerning the clinicopathologic validity of the isolate; both of which limit analysis and knowledge generation. The site of isolation can be a confounding factor when cataloguing isolates and trying to discern virulence factors as commensal or pathogenic as virulent strains can be found in the tonsil and nasal cavity of healthy carriers. Given the large genome size and extensive genome variability between *S. suis* strains as well as the lack of circularized *S. suis* genomes, identification of virulence 'markers' using genetic information of *S. suis*, if at all possible, is going to necessitate innovative approaches that may entail machine learning in combination with a large very well-characterized set of isolates.