RESEARCH REPORT
SWINE HEALTH

Title: **Comparative genomic and virulence analysis of *Streptococcus suis* isolates - #17-084 – IPPA**

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Industry Summary: *Streptococcus suis* is a key swine pathogen responsible for significant economic losses to the swine industry worldwide. *S. suis* is capable of causing a wide variety of clinical diseases in pigs including pneumonia, meningitis, septicemia, and endocarditis. Additionally, *S. suis* is a zoonotic pathogen causing severe infections in people due to penetrating injuries associated with occupational exposure or consumption of raw or undercooked pork products. Despite the significant impact on swine health and public health implications, the strategies and mechanisms used by *S. suis* to colonize and cause disease remain unknown. More importantly, vaccines and/or intervention strategies that do not rely on broad spectrum antibiotics currently do not exist to mitigate *S. suis* disease burden. The overall goal of this project was to use an unbiased and comprehensive approach to identify genomic and/ or transcriptional differences responsible for the spectrum of virulent capacities that occur among *S. suis* strains. Whole genome sequencing followed by comparative genomic analyses revealed several notable regions of difference, including regions encoding secreted and membrane-associated factors, which likely contributed to the spectrum of clinical disease observed. Transcriptome sequencing was performed on virulent and nonvirulent isolates following incubation in whole pig blood. Numerous laboratory assays were performed to test the capacity of these strains to adhere, survive, and/or persist within conditions that mimic various host microenvironments. Collectively, these results provide a foundation for understanding the genomic attributes responsible for the spectrum of virulent phenotypes that exist among *S. suis* isolates. This information is paramount to designing effective vaccines needed by the swine industry to mitigate *S. suis* disease and decrease public health concerns.

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Keywords: *Streptococcus suis*, closed whole-genome sequence, comparative genomic analyses, RNA sequencing, transcriptional response

Scientific Abstract: *Streptococcus suis* is a major swine pathogen responsible for significant economic losses to the swine industry worldwide. *S. suis* is capable of causing a wide variety of clinical diseases in pigs including pneumonia, meningitis, septicemia, and endocarditis. Additionally, *S. suis* is a zoonotic agent causing severe infections to people in close contact with infected pigs or pork-derived products. Despite the significant impact on swine health and public health implications, the strategies and mechanisms used by *S. suis* to colonize and cause disease remain unknown. More importantly, vaccines and/or intervention strategies that do not rely on broad spectrum antibiotics currently do not exist to mitigate *S. suis* disease burden. The overall goal of this project was to use an unbiased and comprehensive approach to identify genomic and/or transcriptional differences responsible for the spectrum of virulent capacities that occur among *S. suis* strains. The specific objectives of this study were to 1) Obtain closed whole-genome sequences of *S. suis* isolates that are known to exhibit different pathogenic capacities to perform comparative genomic analyses. 2) Perform RNA sequencing of *S. suis* isolates to generate a more comprehensive assessment of the adaptive transcriptional response of these strains. 3) Use *in vitro* adherence, invasion, and biofilm assays to test the capacity of these strains to adhere, survive and/or persist within conditions that mimic various host microenvironments. Nine genetically diverse strains recently isolated within the U.S. were chosen and compared to a well-characterized highly virulent reference *S. suis* strain by whole genome sequence analysis and by virulence assessment following intranasal challenge. A spectrum of virulence phenotypes were observed among *S. suis* isolates following intranasal challenge of pigs. Whole genome sequencing followed by comparative genomic analyses revealed several notable regions of difference, including regions encoding secreted and membrane-associated factors, which likely contributed to the spectrum of clinical disease observed. These analyses revealed nucleotide diversity of genes encoding proposed virulence factors such as *codY*, *neuB*, and SSU0854 Hemolysin. Chromosomal gene content among all strains was analyzed and allowed for the determination of core genes (present in all strains), accessory genes (present in 2-9), and unique genes (present in 1). Transcriptome sequencing was performed on virulent and nonvirulent isolates following incubation in whole pig blood. Numerous laboratory assays were performed to test the capacity of these strains to adhere, survive, and/or persist within conditions that mimic various host microenvironments. However, no *in vitro* assay tested correlated with *in vivo* virulence phenotype observed following intranasal challenge of pigs. Collectively, these results provide a foundation for understanding the genomic attributes responsible for the spectrum of virulent phenotypes that exist among *S. suis* isolates. These results obtained should aid in the development of effective vaccines needed by the swine industry to mitigate *S. suis* disease and decrease public health concerns.

Introduction: *S. suis* is a Gram positive bacterium commonly carried in the tonsil and nasal cavity of swine that causes systemic and respiratory disease. *S. suis* is also a zoonotic agent capable of causing meningitis in humans, and although historically sporadic in nature, there have been recent outbreaks in China and Vietnam with high levels of mortality (1-3). *S. suis* strains are typed based on a serological reaction against the capsular polysaccharide, encoded by genes located in the capsule locus (4, 5). To date, 35 capsular serotypes of *S. suis* have been described (6, 7). The most frequently isolated serotype from both diseased swine and humans is serotype 2, and therefore, is considered to be the most virulent (7). However, direct experimental evidence to support this assumption is lacking and other serotypes are routinely isolated from pigs exhibiting

disease (7, 8). More importantly, it is know that *S. suis* isolates can exhibit different virulence capabilities ranging from highly virulent to completely non-virulent (7, 8). In pigs, the earliest sign of disease is usually fever followed by bacteremia, which can result in meningitis with central nervous system signs, arthritis with lameness, polyserositis, endocarditis, and pneumonia. Virulence factors are not well characterized (9). Several potential virulence factors of *S. suis* have been identified such as the capsular polysaccharide, muramidase-released protein (*mrp*), extracellular protein factor (*ef*), and suilysin (*sly*) (9). However, none of these factors appears to completely correlate with the ability to cause systemic disease and therefore virulence is thought to be multifactorial (9-11). There are no effective approaches to eradicate *S. suis* from pig herds and controlling outbreaks has proven difficult. Although vaccines have been developed, most show poor efficacy and/or safety profiles so consequently no broadly protective vaccines or intervention strategies exist. Current treatment for *S. suis* is broad spectrum antibiotics, which are expensive and are believed to increase the risk of resistant strain development. Additionally, with increased pressure to limit antibiotic use in agriculture, alternative approaches and biotherapeutics are desperately needed to reduce disease burden and economic losses caused by *S. suis* (12, 13). The overall goal of this project was to use an unbiased and comprehensive approach to identify genomic and/ or transcriptional differences responsible for the spectrum of virulent capacities that occur among *S. suis* strains.

Objectives:
Objective 1: Obtain closed whole-genome sequences of *S. suis* isolates that are known to exhibit different pathogenic capacities to perform comparative genomic analyses.
Objective 2: Perform RNA sequencing of *S. suis* isolates to generate a more comprehensive assessment of the adaptive transcriptional response of these strains.
Objective 3: Use *in vitro* adherence, invasion, and biofilm assays to test the capacity of these strains to adhere, survive and/or persist within conditions that mimic various host microenvironments.

Materials & Methods:
Genome sequencing and annotation. Strains were cultured in THY medium (Todd-Hewitt broth supplemented with 0.2% yeast extract) supplemented with 5% filtered heat-inactivated horse serum at 37°C. Total genomic DNA was extracted using the MasterPure Gram-positive purification kit (Lucigen Co., WI). Whole genome sequencing was performed using both the Pacific Biosciences (PacBio) and Illumina MiSeq platforms. Library preparation for PacBio sequencing was performed following the PacBio 10-kb insert library preparation protocol available online at (http://www.pacb.com/wp-content/uploads/2015/09/Procedure-Checklist-10-kb-Template-Preparation-and-Sequencing.pdf). The 10 kb library for each strain was sequenced using the PacBio RSII platform with two SMRT® cells for each isolate. Indexed libraries for the MiSeq protocol were generated with the Nextera XT DNA sample preparation and index kits (Illumina, San Diego, CA), pooled, and sequenced using MiSeq v2 500-Cycle reagent kit yielding 2 x 250-bp paired-end reads (Illumina, San Diego, CA). Whole genome assemblies were generated using the PacBio smrtanalysis v. 2.3.0 (https://www.pacb.com/products-and-services/analytical-software/smrt-analysis/) and CANU v. 1.3 (14) software for ISU2912. Whole genome assemblies for all other isolates were generated using the bacterial genome assembly software Unicycler (15). Assembling the PacBio data for each strain resulted in a fully sequenced closed circular chromosome, which was subsequently oriented to start at the *dnaA* gene and trimmed by removing any overlapping sequence. Genomes were then polished and error corrected using the Broad Institute's Pilon v 1.18 (16) and Illumina data. The closed

genome for each strain was then annotated using NCBI's Prokaryotic Genome Annotation Pipeline (PGAP) and additional curation was performed using the Prokka annotation software (version 1.12) (17) along with a *Streptococcus suis*-specific custom database. Roary (18) was used for clustering and BLAST to identify conserved genes and assigns the genes from each strain to homologous groups. Nucleotide sequences of selected genes encoding previously proposed virulence factors (9) from all *S. suis* isolates were compared to genes from P 1/7 or from P5/11/88 for *hylA* using BLASTN.

Virulence study. Morbidity and mortality were evaluated following intranasal challenge of Caesarean-derived, colostrum-deprived (CDCD) pigs, a reference model free from many confounding factors, such as coinfection with other pathogens and existing immunity, inherent in alternative model systems. Groups of 4-5, 8-week-old CDCD pigs were intranasally inoculated with 2 ml (1 ml per nostril) of approximately 1 x 10^9 CFU/ml of each strain.

Whole-blood incubation and RNA Sequencing. Overnight cultures from single colonies were diluted to an OD600 of ~ 0.05 in THY broth supplemented with 5% horse serum and cultivated at 37°C with shaking until exponential phase (OD600 ~ 0.5). Equal volumes of fresh blood was collected in heparinized tubes from 3 different healthy pigs on-site and then pooled. Bacteria were collected by centrifugation and resuspended in original volume of PBS. For whole-blood treatment, 1 mL bacterial culture was added to a flask containing 9 mL heparinized whole-blood. For THY medium treatment, 1 mL bacterial culture was added to a flask containing 9 mL THY medium. Both flasks were then incubated for 1 hour at 37°C with gentle agitation (125 rpm) and 5% CO2. Two volumes of RNA protect was added to each flask and incubated at room temperature for 30 min. Samples were then centrifuged for 5 min at 500 xg to pellet somatic cells. Supernatants were transferred to a new tube and then centrifuged for 10 min at 4,000 xg to pellet bacterial cells. Cell pellets were stored at −80°C. RiboPure Bacteria RNA purification kit (ThermoFisher Scientific, Waltham, MA) was used to extract RNA from the bacterial cells as per the manufacturer's directions. RNA samples were further processed using the MICROBEnrich™ Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufactures recommendation to remove any eukaryotic RNA. Genomic DNA was subsequently removed using Turbo DNA-free DNase (ThermoFisher Scientific, Waltham, MA, USA) as per the manufacturer's directions. Quantitation of total RNA was performed using a NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). RNA quality was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). rRNA was depleted using a Ribo-Zero rRNA Removal kit (Bacteria) according to the manufacturer's instructions (Illumina, Inc., San Diego, CA), and rRNA removal was verified on the 2100 Bioanalyzer. The cDNA libraries were constructed using TruSeq Stranded Total RNA Library Prep kits and sequenced on a HiSeq 3000 using a 150-cycle single-end run (Illumina, Inc., San Diego, CA) at the Iowa State University DNA core facility.

Growth curve for *S. suis* isolates. A single colony was inoculated in THY broth supplemented with 5% serum at 37ºC with shaking. To ensure similar inocula, bacteria were then subcultured at a starting OD600 of 0.02 into a 300 µL fresh broth. Plates were then incubated at 37°C for 24 hours and the OD600 of each well recorded every 15 minutes after 5 seconds of shaking using a Bioscreen C Microbiological Growth Analyzer (ThermoLabsystems, Basingstoke, UK). Data points represent averages obtained from three independent experiments.

Adherence of *S. suis* isolates to BEAS2B cells. Adherence *in vitro* to BEAS2B cells (human lung/bronchus epithelial cell line) is expressed as the proportion of bacteria in the original inoculum found to be adherent after centrifugation and a 2 hour incubation period, using an MOI of 100. The data are the means $\pm$ standard errors of three independent experiments.

Adherence of *S. suis* isolates to macrophages. Adherence *in vitro* to J774.16 cells (murine macrophage-like cell line cell line) is expressed as the proportion of bacteria in the original inoculum found to be adherent after centrifugation and a 2 hour incubation period, using an MOI of 100. The data are the means ± standard errors of three independent experiments.

Biofilm formation by *S. suis* isolates. *S. suis* isolates were cultivated for 24 hours in 96-well plates. After incubation, the OD_{600} of each well was measured and the biofilm biomass was quantified using a standard crystal violet assay and visualized by OD_{538}. All OD_{538} values were normalized by OD_{600} values. The data are the means ± standard errors of three independent experiments.

Oxidative stress survival by *S. suis* isolates. Overnight cultures from single colonies were diluted to a starting OD_{600} of 0.02 into 5 mL fresh THY broth supplemented with 5% serum and cultivated at 37ºC with shaking until exponential phase (approximate OD_{600} of 0.5). Cultures were then divided and hydrogen peroxide (10 mM final concentration) was added to the treated cultures, while an equal volume of water was added to the untreated cultures. Cultures were then incubated at 37°C for 15 minutes followed by catalase addition (10 µg/ml final concentration) and plated to determine CFU counts. Percent survival was calculated as the proportion of treated samples to untreated samples, expressed as a percentage. Data are the means ± standard errors of at least three independent experiments.

Secreted Nuclease Activity. *S. suis* isolates were cultivated overnight at 37ºC. Strains were normalized to ensure an equivalent OD_{600}. Cultures were normalized to ensure an equivalent OD_{600} and pelleted to collect the supernatant. An equivalent volume of bacterial supernatant was incubated with 2 µM FRET substrate for 10 minutes. Nuclease activity was the quantified by measuring the change in fluorescence (ex. 552 nm/ em. 580 nm). The data are the means ± standard errors of three independent experiments.

Cell-Associated Nuclease Activity. *S. suis* isolates were cultivated overnight at 37ºC. Strains were normalized to ensure an equivalent OD_{600}. Cultures were normalized to ensure an equivalent OD_{600} and pelleted to remove the supernatant. Bacterial pellets were resuspended in fresh media and an equivalent volume of bacterial sample was incubated with 2 µM FRET substrate for 10 minutes. Nuclease activity was the quantified by measuring the change in fluorescence (ex. 552 nm/ em. 580 nm). The data are the means ± standard errors of three independent experiments.

Hemolysis assay. *S. suis* isolates were cultivated overnight at 37ºC in complex media containing pullulan (CM-P). Bacteria were pelleted by centrifugation, washed with PBS, resuspended in 5 ml PBS, and pelleted again to collect the supernatant. DTT (final concentration of 5 mM) was added to supernatants and two-fold dilutions were prepared in CM-P + 5 mM DTT. 100 µl of the supernatant dilution was added to 100 µl 2% RBCs and incubated for 2 hours at 37°C. Unlysed RBCs were pelleted by centrifugation (1500 x g, 10 minutes) and 100 µl of the supernatant transferred to a fresh 96-well plate. Absorbance was read at 540 nm. Lysis by 1% Triton X-100 was used as a reference for 100% lysis. Hemolytic activity was calculated as the highest dilution that induced at least 50% hemolysis. One Hemolytic units was defined as the reciprocal of the highest dilution that induced at least 50% lysis of erythrocytes.

Oxidative stress. Overnight cultures from single colonies were diluted to a starting OD_{600} of 0.02 into 5 mL fresh THY broth supplemented with 5% serum and cultivated at 37ºC with shaking until exponential phase (approximate OD_{600} of 0.5). Cultures were then divided into two 2.5 ml cultures and hydrogen peroxide (10 mM final concentration) was added to the treated cultures, while an equal volume of water was added to the untreated cultures. Cultures were then incubated at 37°C for 15 minutes followed by catalase addition (10 µg/ml final concentration)

and serial dilutions were subsequently plated to determine CFU counts. Percent survival was calculated as the proportion of treated samples to untreated samples, expressed as a percentage.
Survival in Whole-blood. Overnight cultures from single colonies were diluted to an OD600 of ~ 0.05 in THY broth supplemented with 5% horse serum and cultivated at 37°C with shaking until exponential phase (OD600 ~ 0.5). In a 96 well plate, 10 µl bacterial culture was added to 90 µl heparinized whole blood collected from a healthy pig and incubated for 1 hour at 37°C. Serial dilutions were prepared in PBS and plated for CFU counts. Percent survival is expressed as a percentage of the original inoculum. The data are the means ± standard errors of at least three independent experiments.
Serum sensitivity. Overnight cultures from single colonies were diluted to a starting OD_{600} of 0.02 into 5 mL fresh THY broth supplemented with 5% serum and cultivated at 37ºC with shaking until exponential phase (approximate OD_{600} of 0.5). Bacteria were pelleted by centrifugation, washed with PBS, and resuspended in 5 ml PBS. 10 µl bacterial cells were then added to one of the following: 90 µl guinea pig serum (Quidel Corp., San Diego, CA) for treated samples, 90 µl heat-inactivated guinea pig serum for treated samples or 90 µl PBS for untreated samples and then incubated for 1 hour at 37°C and serial dilutions were subsequently plated to determine CFU counts. Serum-sensitive *Haemophilus parasuis* H465 was used as a control. Percent survival was calculated as the proportion of treated samples to untreated samples, expressed as a percentage.

Results:
Objective 1: Obtain closed whole-genome sequences of *S. suis* isolates that are known to exhibit different pathogenic capacities to perform comparative genomic analyses.

Nine genetically diverse strains recently isolated within the U.S. were chosen for whole genome sequence analysis and virulence assessment following intranasal challenge and compared to a well-characterized and highly virulent reference *S. suis* strain. A spectrum of virulence phenotypes were observed among *S. suis* isolates following intranasal challenge of pigs. Specifically, *S. suis* strains ISU2614 and ISU1606 exhibited a high level of virulence with all pigs (5 out of 5) in each of these groups developing systemic clinical disease within 8 days post-challenge (Figure 1). *S. suis* strains ISU2714, ISU2660, and ISU2514 were moderately virulent with 3 out of 5 pigs challenged with ISU2714 developing neurologic signs and/or lameness, while only 2 out of 5 pigs challenged with ISU2660 developed lameness (Figure 1). 1 out of 5 pigs challenged with ISU2514 developed neurologic signs and 2 out of 5 developed lameness (Figure 1). *S. suis* strains ISU2414, ISU2812, ISU2912, and SRD478 were completely avirulent and all pigs in these groups remained healthy and exhibited no signs of clinical disease (Figure 1). The complete genome assembly and annotation of all nine *S. suis* isolates used in this study was completed (Table 1). *S. suis* strains ISU2614, ISU2514, and ISU2812 all harbored a single plasmid, while SRD478 harbored 4 plasmids (Table 1).

Gene content among all strains was analyzed using Roary (18), which uses clustering and BLAST to identify conserved genes and assigns the genes from each strain to homologous groups. A gene was designated as a Core gene if it is present in all 10 strains with a protein sequence identity greater than or equal to 90%. Accessory genes are present in 2 – 9 of the strains (≥ 90% protein sequence identity). Unique genes are those that cannot be assigned to a homologous group and are either unique to a single strain or their sequences are less than 90% identical to genes from the other strains (Figure 2). Nucleotide diversity of genes encoding proposed virulence factors from the previous comprehensive review by Fittipaldi et al. (9) were compared to genes from reference *S. suis* strain P 1/7 or from *S. suis* strain P5/11/88 for *hylA*.

This analysis revealed variable nucleotide diversity of genes encoding proposed virulence factors such as *codY*, *neuB*, and SSU0854 Hemolysin (Figure 3).

Objective 2: Perform RNA sequencing of *S. suis* isolates to generate a more comprehensive assessment of the adaptive transcriptional response of these strains.

Fresh whole-blood collected from three healthy pigs housed on-site was pooled to diminish any host variability effects and all nine *S. suis* isolates, along with reference stain P 1/7 were incubated in either pooled blood for one hour at 37ºC or in THY medium. Bacteria were subsequently collected and RNA was isolated and then sequenced. Overall, excellent coverage of the sequencing reads from all strains and under both conditions was observed. Gene set enrichment analysis for KEGG classes differently expressed between whole-blood and medium conditions for each strain is still ongoing. However, early analyses suggest that genes encoding products involved in the arginine deiminase pathway comprise a gene set outside of capsule loci genes that are largely effected.

Objective 3: Use *in vitro* binding assays to test the capacity of the ST5 strains, which genome sequence information will be obtained, to adhere to human and porcine keratinocytes.

Numerous laboratory assays were performed to test the capacity of these strains to adhere, survive, and/or persist within conditions that mimic various host microenvironments with the goal of identifying an *in vitro* condition in which the results or phenotype correlated with *in vivo* virulence phenotype observed following intranasal challenge of pigs. First, *in vitro* growth kinetics for all isolates was measured over the course of 24 hours (Figure 4). No growth rate observed correlated with either a virulent or non-virulent *in vivo* phenotype (Figure 4). Next, adherence to both BEAS2B cells (Figure 5) and macrophages (Figure 6) was measured. No adherence capacity observed by any of the isolates correlated with either a virulent or non-virulent *in vivo* phenotype (Figure 5 and 6). The ability *S. suis* isolates to form or live in a biofilm can increase survival and persistence within the respiratory tract of swine. Therefore, the capacity to form biofilms by all the *S. suis* isolates was measured (Figure 7). Variations in the ability to form biofilms was observed among all the isolates tested, however, no correlation with either a virulent or non-virulent *in vivo* phenotype was observed (Figure 7). The ability to survive oxidative stress was measured for the *S. suis* isolates and no correlation with either a virulent or non-virulent *in vivo* phenotype was observed (Figure 8). Both secreted (Figure 9) and cell-associated nuclease activity (figure 10) by *S. suis* isolates was measured and no correlation with either a virulent or non-virulent *in vivo* phenotype was observed (Figure 9 and 10). Hemolytic activity (Figure 11) exhibited by S. suis isolates was measured and both P1/7 and SRD478 exhibited the highest hemolytic activity (Figure 11). Survival in whole-blood by *S. suis* isolates. (Figure 12) and no correlation with either a virulent or non-virulent *in vivo* phenotype was observed for the isolates tested. Sensitivity to serum was tested for virulent P 1/7 and non-virulent SRD478 and no statistical difference was observed (Figure 13). In summary, no *in vitro* assay tested correlated with *in vivo* virulence phenotype observed following intranasal challenge of pigs.

Discussion: Despite the significant impact on the swine industry, mechanisms used by *S. suis* to colonize and cause disease remain poorly understood and no broadly protective vaccine or intervention strategy exist. In addition to decreasing the disease burden and economic loss caused by *S. suis*, another top priority for swine producers is decreasing the prevalence of zoonotic agents from the swine production environment and fully elucidating any potential public health risks associated with human occupational exposure. The majority of studies, if not

all, investigating the mechanisms used by *S. suis* to colonize and cause disease focus on characterizing a deletion mutant relative to its wild-type parental strain for the purpose of evaluating the contribution of the inactivated factor to a specific disease process. While these studies are invaluable for understanding the disease process caused by most pathogens, the usefulness is diminished for *S. suis* and similar pathogens where different isolates are known to cause a spectrum of disease outcomes ranging from highly virulent to completely non-virulent.

This study began with the establishment of virulence of nine *S. suis* strains recently isolated within the U.S. compared to a virulent reference *S. suis* isolate following intranasal challenge of CDCD pigs, a reference model free from many confounding factors, such as coinfection with other pathogens and existing immunity, inherent in alternative model systems. *S. suis* strains ISU2614 and ISU1606 were observed to be virulent, *S. suis* strains ISU2714, ISU2660, and ISU2514 were moderately virulent, and ISU2414, ISU2812, ISU2912, and SRD478 were completely avirulent. The complete genome assembly and annotation of all nine *S. suis* isolates used in this study was completed. *S. suis* strains ISU2614, ISU2514, and ISU2812 all harbored a single plasmid, while SRD478 harbored 4 plasmids. Nucleotide diversity of genes encoding previously proposed virulence factors were compared and revealed variable diversity in genes *codY*, *neuB*, and SSU0854 Hemolysin. Genome reduction through the loss of genes, potentially interfering with host infection, has also been proposed in *S. suis* (19) based on the observation that invasive *S. suis* isolates harbored smaller genomes compared to non-virulent isolates. However, this trend was not observed in this study. In fact the non-virulent SRD478 isolate harbors a genome that is very similar to the genome size of virulent strains P1/7 and ISU1606. RNA sequencing was performed on all nine *S. suis* isolates, along with reference stain P 1/7, following incubation in either fresh whole-blood or in THY medium. Analyses are still ongoing, but preliminary analyses suggest that genes encoding products involved in the arginine deiminase pathway comprise a gene set outside of capsule loci genes that are largely effected. Numerous laboratory assays were performed to test the capacity of these strains to adhere, survive, and/or persist within conditions that mimic various host microenvironments. However, no *in vitro* assay tested correlated with *in vivo* virulence phenotype observed following intranasal challenge of pigs. Collectively, these results provide a foundation for understanding the genomic attributes responsible for the spectrum of virulent phenotypes that exist among *S. suis* isolates. These results obtained should aid in the development of effective vaccines needed by the swine industry to mitigate *S. suis* disease and decrease public health concerns.

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Table 1. Sequencing status for genomes of *S. suis* strains used in study.

Strain	Virulence phenoype	Status	Chromosome Contigs	Size (bp)	Plasmid Size
P 1/7	Virulent	Published reference strain (2)	1	2,007,491	None
ISU2614	Virulent	Assembled with Unicycler	1	2,163,384	4,984
ISU1606	Virulent	Assembled with Unicycler	1	2,073,988	None
ISU2714	Moderatley virulent	Assembled with Unicycler	1	2,063,877	None
ISU2660	Moderatley virulent	Assembled with Unicycler	1	2,182,487	None
ISU2514	Moderatley virulent	Assembled with Unicycler	1	2,248,415	5,581
ISU2414	Nonvirulent	Assembled with Unicycler	1	2,222,543	None
ISU2812	Nonvirulent	Assembled with Unicycler	1	2,563,853	5,586
ISU2912	Nonvirulent	Assembled with Smrtanalysis + canu	1	2,720,381	None
SRD478	Nonvirulent	Assembled with Unicycler	1	2,063,454	15,154 4,723 3,972 2,945

Figure 1. Survival rates of pigs intranasally challenged with 10 different strains of *S. suis*. Morbidity and mortality were evaluated following intranasal challenge of Caesarean-derived, colostrum-deprived (CDCD) pigs, a reference model free from many confounding factors, such as coinfection with other pathogens and existing immunity, inherent in alternative model systems. Groups of 4-5, 8-week-old CDCD pigs were intranasally inoculated with 2 ml (1 ml per nostril) of approximately 1 x 10^9 CFU/ml of each strain.

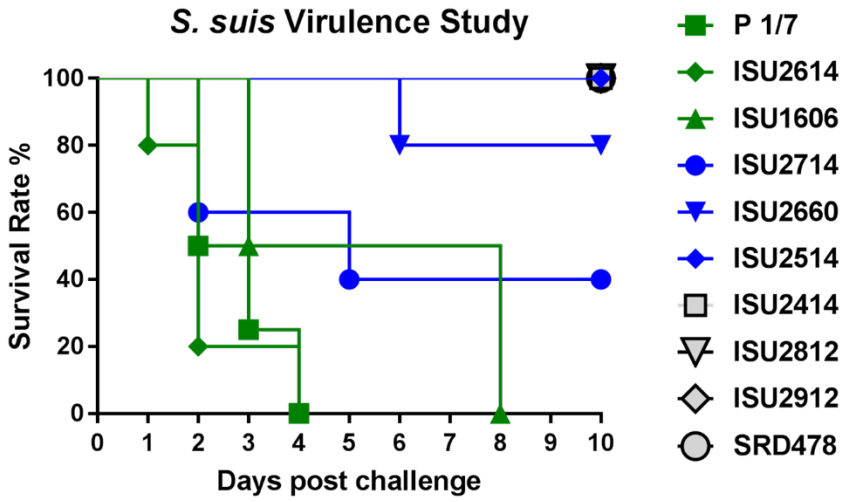

Figure 2. Gene content among *S. suis* isolates. Gene content was evaluated using Roary (18) and designated as core genes, accessory genes, or unique genes.

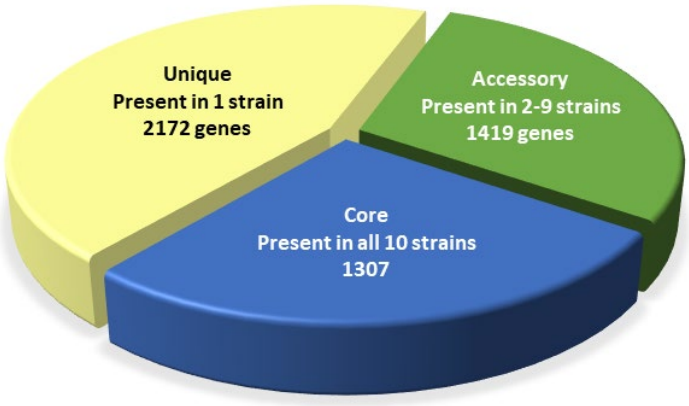

Figure 3. Nucleotide diversity of genes encoding proposed virulence factors. Nucleotide sequences of selected genes from *S. suis* isolates (names listed at top) were compared to genes from P 1/7 or from P5/11/88 for *hylA* using BLASTN. Heatmap generated based on percent nucleotide identity of genes (rows) from each strain (columns, name of strain listed at top) using the color scale at top; white= genes not present within a strain; #, &, or * = pseudogene.

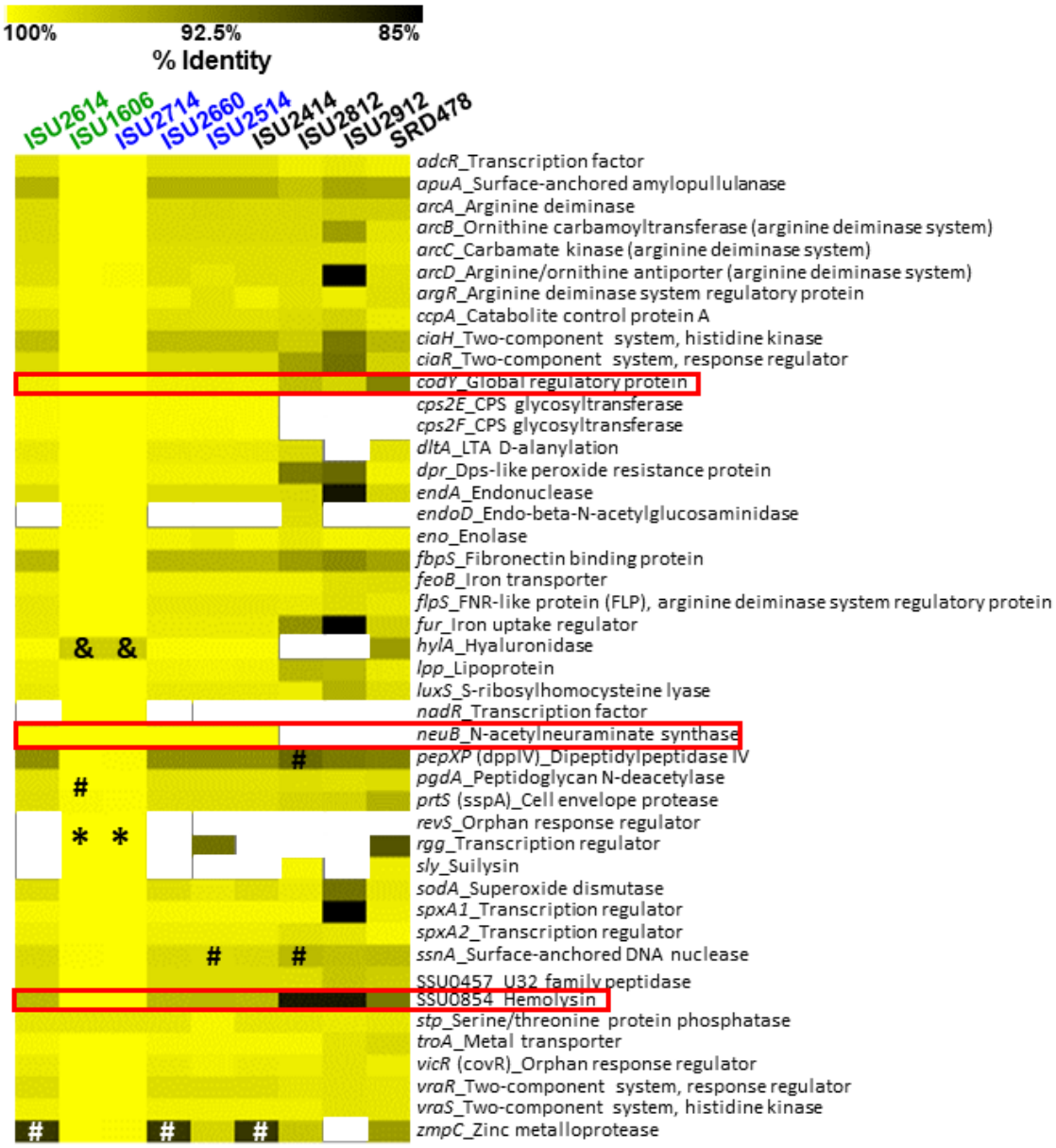

Figure 4. Growth curve for *S. suis* isolates. Bacteria were subcultured at a starting OD600 of 0.02 into a 300 µL THY medium and incubated at 37°C for 24 hours. OD600 was recorded every 15 minutes. Data points represent averages obtained from three independent experiments.

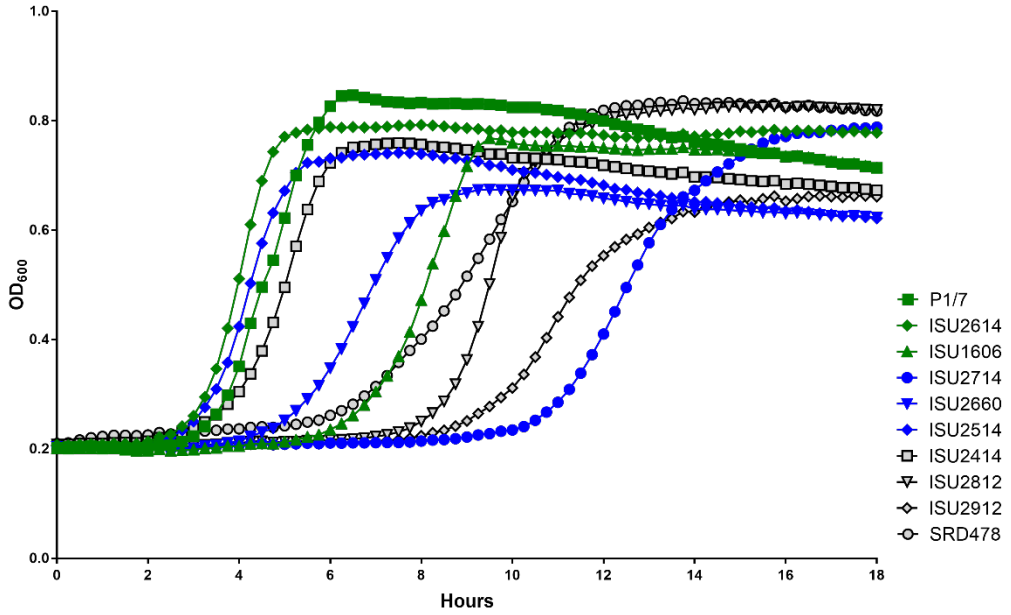

Figure 5. Adherence of *S. suis* isolates to BEAS2B cells. Adherence *in vitro* to BEAS2B cells (human lung/bronchus epithelial cell line) is expressed as the proportion of bacteria in the original inoculum found to be adherent after centrifugation and a 2 hour incubation period, using an MOI of 100. Data are the means ± standard errors of three independent experiments.

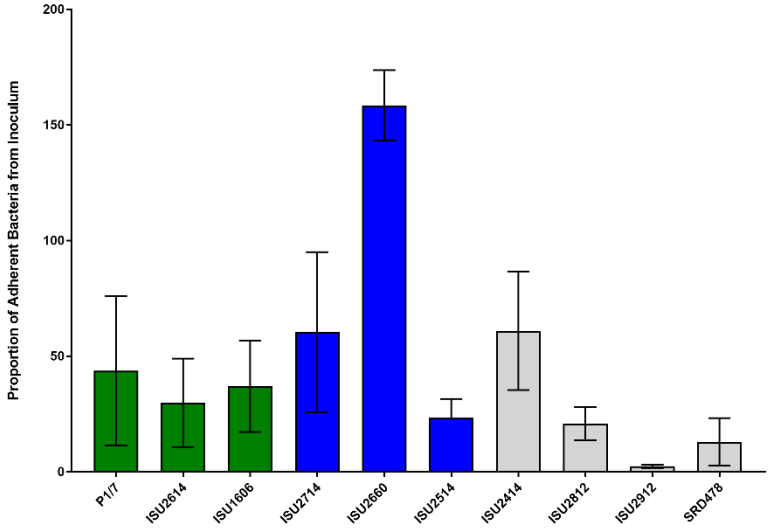

Figure 6. Adherence of *S. suis* isolates to macrophages. Adherence *in vitro* to J774.16 cells (murine macrophage-like cell line cell line) is expressed as the proportion of bacteria in the original inoculum found to be adherent after centrifugation and a 2 hour incubation period, using an MOI of 100. Data are the means + standard errors of three independent experiments.

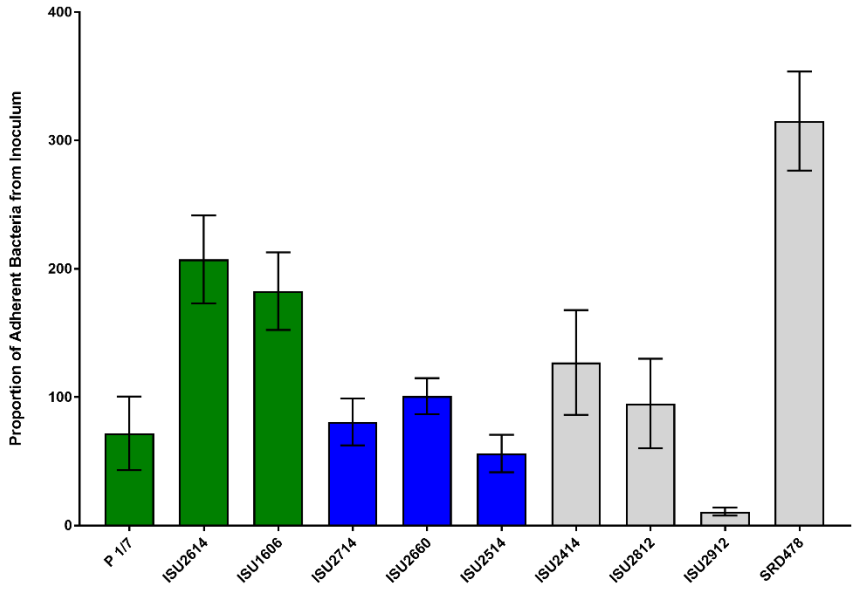

Figure 7. Biofilm formation by *S. suis* isolates. *S. suis* isolates were cultivated for 24 hours in 96-well plates. After incubation, the OD_{600} of each well was measured and the biofilm biomass was quantified using a standard crystal violet assay and visualized by OD_{538}. All OD_{538} values were normalized by OD_{600} values. Data are the means $\pm$ standard errors of three independent experiments.

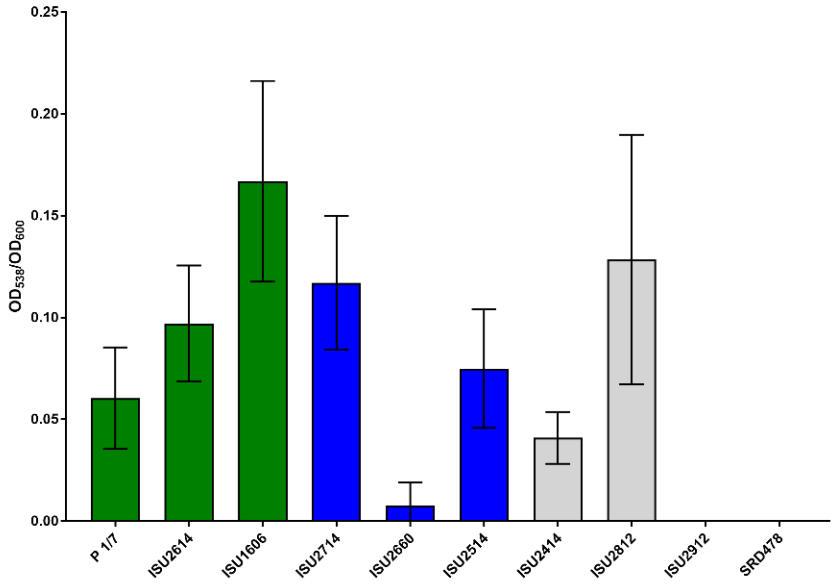

Figure 8. Oxidative stress survival by *S. suis* isolates. Exponential phase cultures were divided and hydrogen peroxide (10 mM final concentration) was added to the treated cultures, while an equal volume of water was added to the untreated cultures. Cultures were then incubated at 37°C for 15 minutes followed by catalase addition (10 µg/ml final concentration) and plated to determine CFU counts. Percent survival was calculated as the proportion of treated samples to untreated samples, expressed as a percentage. Data are the means ± standard errors of at least three independent experiments.

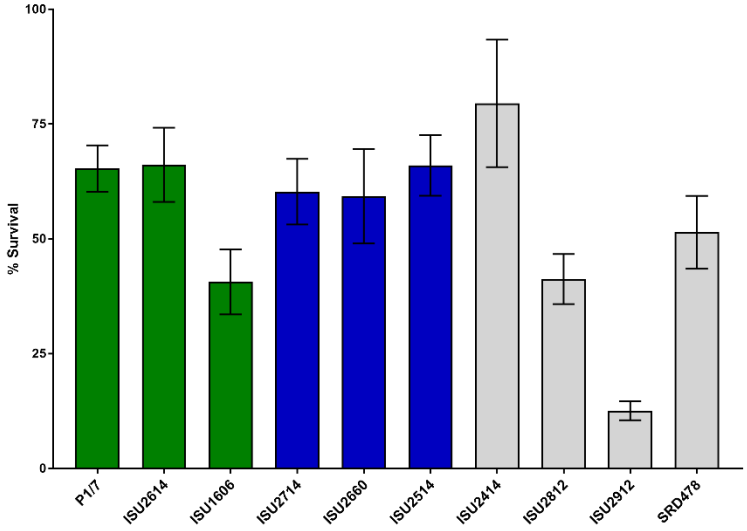

Figure 9. Secreted Nuclease Activity by *S. suis* isolates. *S. suis* isolates were cultivated overnight at 37°C, normalized to an equivalent OD_{600} and pelleted to collect the supernatant. An equivalent volume of bacterial supernatant was incubated with 2 µM FRET substrate for 10 minutes. Nuclease activity was the quantified by measuring the change in fluorescence (ex. 552 nm/ em. 580 nm). Data are the means ± standard errors of three independent experiments.

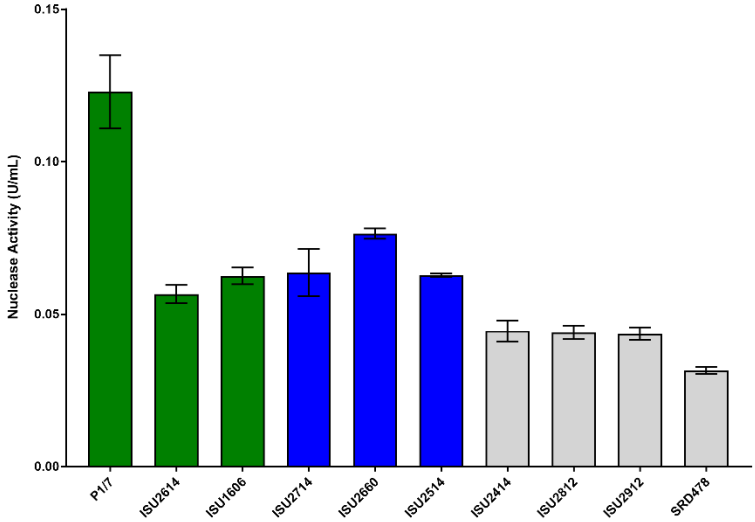

Figure 10. Cell-Associated Nuclease Activity by *S. suis* isolates. *S. suis* isolates were cultivated overnight at 37ºC, normalized to ensure an equivalent OD_{600} and pelleted to remove the supernatant. Bacterial pellets were resuspended in fresh media and an equivalent volume of bacterial sample was incubated with 2 µM FRET substrate for 10 minutes. Nuclease activity was the quantified by measuring the change in fluorescence (ex. 552 nm/ em. 580 nm). The data are the means ± standard errors of three independent experiments.

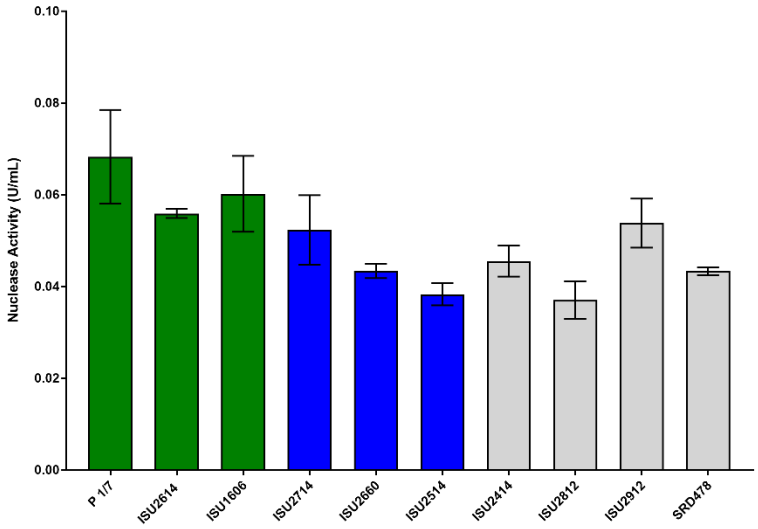

Figure 11. Hemolytic activity by *S. suis* isolates. *S. suis* supernatants and two-fold dilutions were prepared and added to RBCs and incubated for 2 hours at 37°C. Unlysed RBCs were pelleted by centrifugation, supernatants transferred to a new plate, and absorbance was read at 540 nm. Hemolytic activity was calculated as the highest dilution that induced at least 50% hemolysis. Lysis by 1% Triton X-100 was used as a reference for 100% lysis. One Hemolytic units was defined as the reciprocal of the highest dilution that induced at least 50% lysis of erythrocytes.

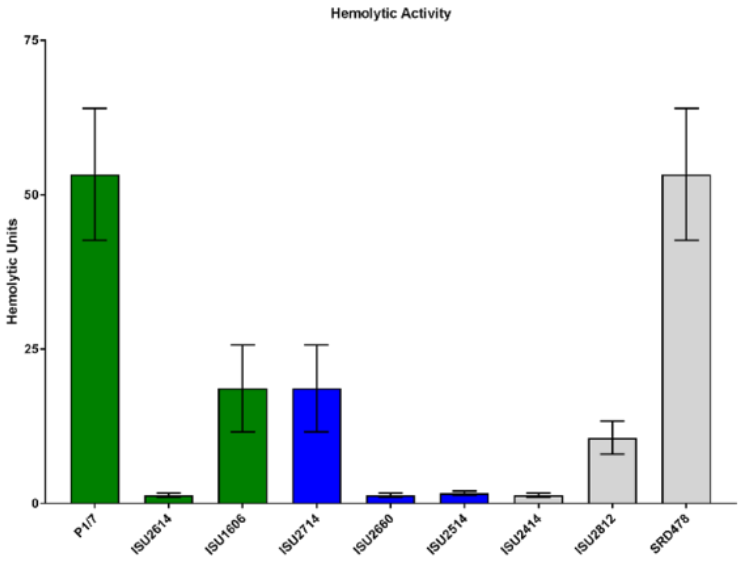

Figure 12. Survival in whole-blood by *S. suis* isolates. In a 96 well plate, 10 µl *S. suis* culture was added to 90 µl heparinized whole blood collected from a healthy pig and incubated for 1 hour at 37°C. Serial dilutions were prepared in PBS and plated for CFU counts. Percent survival is expressed as a percentage of the original inoculum. Data are the means ± standard errors of at least three independent experiments.

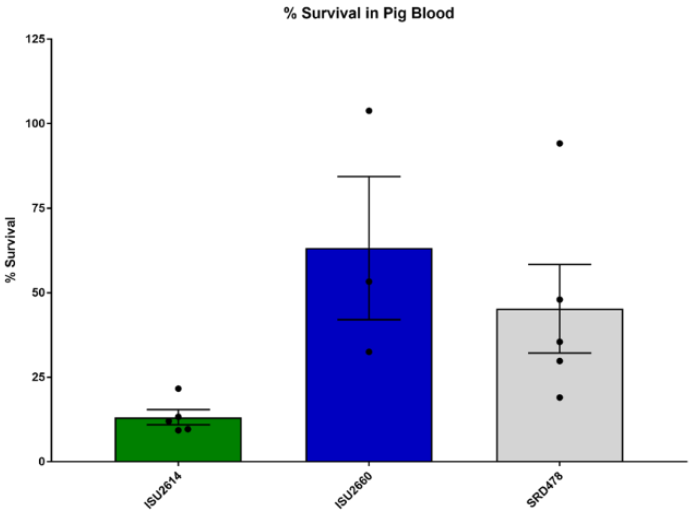

Figure 13. Serum sensitivity by *S. suis* isolates. Percent survival was calculated as the proportion of treated samples to untreated samples, expressed as a percentage. Serum-sensitive *Haemophilus parasuis* H465 was used as a control. Data are the means ± standard errors of at least three independent experiments.