RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Origin, evolution, and zoonotic potential of livestock-associated methicillin-resistant *Staphylococcus aureus* found in US swine farms - **NPB #14-124**

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Industry Summary: Reports claiming that livestock, particularly pigs, could serve as a reservoir for the 'superbug' methicillin resistant *Staphylococcus aureus* (MRSA) shocked the medical and veterinary worlds when first discovered in Europe around 2004. Early studies identified a novel type of MRSA (ST398) in pigs in Europe, however the situation has become a bit more complicated since other varieties (e.g., ST9 in Asia and ST5 in North America, among others), have also been found in pigs. Unlike ST398 and ST9, which do not appear to contribute a significant role on human health in the USA, ST5 MRSA are among the most common type causing human clinical infections. Several studies have demonstrated a genetic basis for why ST9398 strains have a reduced ability to cause disease in humans. Until recently, similar studies had not been done on MRSA ST5 strains and so it remained unclear whether or not ST5 strains have the capacity to colonize and cause disease in humans. To address this question, this project obtained whole-genome sequences for 156 ST5 strains encompassing both MSSA and MRSA strains from both human and swine related sources. Additionally, this project tested the ability of these ST5 strains to adhere to skin cells obtained from humans. While working to finish the genome assemblies, we undertook a separate genetic comparison of our ST5 strains and found that the swine associated ST5 isolates uniformly lacked a set of genes known as the immune-evasion cluster (IEC) genes. These genes are central to the ability of *S. aureus* to cause serious infections in people, and are transmitted by a specific bacteriophage (virus infecting bacteria). This phage was also uniformly absent from the swine related isolates but present, along with IEC genes, in over 90% of the human ST5 isolates. The lack of IEC genes has been documented in ST398 MRSA and is thought to be related to the adaptation of the bacteria to animal hosts. Our conclusion from this study is that ST5 from swine related sources have a diminished capability to cause human disease compared with types circulating in the human population. Phylogenetic or family trees have been constructed using the core genome sequences from these ST5 strains. Additionally, *in vitro* binding assays were used to test the capacity of the ST5 strains to adhere to human keratinocytes. Combined the data from this study provide a comprehensive understanding of the origin, evolution, and zoonotic potential of LA-MRSA ST5 strains associated with swine to both the public health and veterinary communities.

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Scientific Abstract: Since its first detection associated with swine industry, livestock associated methicillin-resistant *S. aureus* (LA-MRSA) has drawn concern from the public health community for two main reasons. The first reason is that these isolates are the single largest reservoir of MRSA outside of hospitals settings and secondly, similar to all MRSA strains, LA-MRSA strains tend to contain a high number of antimicrobial resistance genes. The adaption and evolution of *S. aureus* is largely due to the acquisition of large segments of DNA referred to as mobile genetic elements (MGEs) that carry genes encoding factors enabling *S. aureus* to cause disease and antimicrobial resistance (AMR). The first sequence type (ST) of LA-MRSA associated with swine is ST398. Studies have demonstrated a genetic basis for reduced human infection with ST398. In the US, MRSA ST5 is more commonly being found in swine facilities. Genetic studies similar to the ones performed on ST398 strains have not been conducted on these MRSA ST5 strains. The objectives of this study were to 1) obtain draft whole-genome sequences of 110 ST5 strains isolated from human, animal, and environmental samples collected from swine farms in the U.S. 2) compare the draft genome sequences obtained for these strains to each other and to other MSSA and MRSA publicly available genome sequences to generate a more comprehensive assessment of the origin, evolution, and zoonotic potential of these strains. 3) use *in vitro* binding assays to test the capacity of these strains, which draft genome sequence information will be obtained, to adhere to human and porcine keratinocytes. To date we have obtained whole-genome sequence for 155 ST5 strains encompassing both MSSA and MRSA strains from both human and swine related sources. Additionally, we have chosen a subset of these strains that encompasses the diversity based on spa type, host, and origin or location of isolation and used these strains in *in vitro* binding assays. The genome assemblies for the ST5 strains is now complete along with the phylogenetic and other comparative genomic analyses. One of the comparative genomic analyses that is now completed is the screening of these ST5 isolates for the prevalence of the IEC genes carried by β-hemolysin converting bacteriophages, whose absence in LA-MRSA ST398 is thought to contribute to reduced rates of human infection and transmission associated with this lineage. We found IEC genes absent from any of the ST5 strains from agricultural sources and the β-hemolysin gene was intact in these strains, indicating the bacteriophage's absence. In contrast, the prevalence of the β-hemolysin converting bacteriophage in ST5 strains from humans with no exposure to swine was 90.4%. The absence of β-hemolysin converting bacteriophage in LA-MRSA ST5 isolates is consistent with previous reports evaluating ST398 strains and provides genetic evidence indicating LA-MRSA ST5 isolates may harbor a reduced capacity to cause severe disease in immunocompetent humans.

Introduction: *Staphylococcus aureus (S. aureus)* is a pathogen of both humans and animals that is most commonly associated with skin and soft tissue infections. *S. aureus* harboring the *mecA* gene are resistant to methicillin and other β-lactam antimicrobials and are referred to as methicillin-resistant *S. aureus* (MRSA) (13). *S. aureus* resistance to methicillin was first described in 1961 (2), and since then MRSA has spread worldwide, with dramatic increases in prevalence occurring in the last two decades (11, 13, 22). MRSA isolates from human disease cases can be grouped into either hospital acquired (HA) or community acquired (CA) (13, 32). HA-MRSA strains are generally less virulent than CA-MRSA strains and infections are often associated with a predisposing risk factor or illness such as hospitalization, surgery, dialysis, long-term care, and indwelling

devices (11, 13, 32). In contrast, CA-MRSA infections occur in otherwise healthy individuals with no predisposing risk factors. CA-MRSA strains are genetically distinct from HA-MRSA strains and are typically more virulent, owing to the presence of a variety of toxins, such as Panton-Valentine leukocidin (PVL) (9, 11, 13, 32).

Recently a new lineage has been found in associated with livestock and livestock workers and classified as livestock associated (LA-MRSA). A particular sequence type (ST) of LA-MRSA, ST398, associated with swine was originally reported in a study of swine farmers in France (1). Since then a number of studies in Europe (3, 10, 12, 16, 20, 23, 33, 40, 41), Canada (21) and the United States (36) have reported findings of ST398 among swine, swine production facilities, and swine workers. The main reason why LA-MRSA strains are drawing concern from the public health community is that these isolates are the single largest reservoir of MRSA outside of a hospital setting (35). However, several studies have shown LA-MRSA ST398 strains to be less pathogenic towards humans than HA-MRSA and CA-MRSA isolates (4, 8, 38). Genome level differences between human MRSA and LA-MRSA isolates include MGEs, such as the IEC genes carried on a specific family of bacteriophage, which are found in nearly all human isolates, but rarely found in LA-MRSA isolates (27, 34, 38). The absent of these bacteriophage among LA-MRSA strains likely accounts for the decreased transmission and pathogenesis of these strains in human hosts (34).

Another reason why LA-MRSA strains are drawing attention and concern from the public health community is the high number of AMR genes these strains, like all MRSA strains, tend to contain. A common finding associated with swine ST398 is increase resistance to tetracycline and zinc (5, 34). Since these antimicrobials are used in swine production, it has been speculated that antimicrobial use in swine production has promoted the development and establishment of ST398 in swine populations (34). Evidence cited to support this hypothesis is lacking. To date, one study has applied comparative genome sequence analysis in an attempt to characterize a collection of ST398 strains (34). The authors conclude that LA-MRSA ST398 originated as MSSA in humans and then transferred to livestock and this transition was subsequently accompanied by the acquisition of tetracycline and methicillin resistance (34). It is important to note that although the authors used comparative genome sequence analysis on a collection of LA-MRSA strains, this study only used ST398 strains and did not include LA-MRSA strains from different STs.

A recent study investigating the prevalence of LA-MRSA strains associated with swine in the US have shown that the most common ST was ST5, not ST398 (14). This is a potential cause of concern because ST5 is the most successful human-associated lineages and one of the most globally disseminated lineages. This success is thought to be due to ST5 strains being more genetically predisposed to the acquisition of AMR and virulence or host-adapted genes by bacteriophage (24). A study investigating the evolutionary history of the MRSA strains in poultry industry found the majority of the strains isolated from poultry belonged to the ST5 lineage and resulted from a human-to-poultry jump (18). After the host jump these strains underwent genetic diversification resulting in the acquisition of avian-specific genes and inactivation of human-specific virulence genes (18). A similar adaption process may be occurring with ST5 strains in swine.

The goal of this study was to perform whole-genome sequence comparative analysis on 110 ST5 strains encompassing both MSSA and MRSA strains from both human and swine populations. These ST5 isolates were obtained from swine, swine farms and production facilities, individuals who transiently visited swine farms (veterinary students), and from individuals with long-term exposure to diverse swine farms (veterinarians). In addition to comparative sequence analysis, we also planned to test the capacity of these ST5 strains to adhere to human and porcine keratinocytes using *in vitro* binding assays. Our hypothesis was that ST5 strains contain bacteriophage that are unique to LA-MRSA strains and do not carry MGEs encoding the ability to cause disease in humans. Additionally, we hypothesized that AMR genes are maintained separately among LA-MRSA strains and are not transferred to human MSSA or MRSA strains. Our expectation was that the combination of comparative sequence analysis along with phenotypic attachment assays would provide a comprehensive assessment of the origin, evolution, and zoonotic potential of LA-MRSA ST5 strains associated with swine.

Objectives:
Objective 1: Obtain whole-genome sequences of 110 ST5 strains isolated from human, animal, and environmental samples collected from diverse swine farms in the U. S.
Objective 2: Compare the genome sequences obtained for the 110 ST5 strains to each other and to other MSSA and MRSA publicly available genome sequences to generate a more comprehensive assessment of the origin, evolution, and zoonotic potential of these strains.
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.

Materials & Methods:

Strain acquisition. Swine associated isolates were obtained from Iowa State University (14) and the University of Minnesota. Sources for these isolates were swine (38 isolates), the environment within swine facilities (26 isolates), humans with short-term contact with swine (9 isolates), and swine veterinarians representing humans with long-term contact with swine (9 isolates). Clinical isolates from humans with no swine contact, representing both HA- and CA-MRSA, were obtained from the University of California Irvine (64 isolates) (19) and the University of California San Francisco (9 isolates). All isolates were MLST and Staphylococcal protein A (spa) typed prior to acquisition. ATCC strains Mu3 (ATCC #700698), Mu50 (ATCC #700699), Newman (ATCC #25904) were obtained for use as controls for the IEC genes, and a ST398 isolate from Iowa State University was used as a control for a strain encoding an intact β-hemolysin gene.
Genomic DNA Isolation and Sequencing. Genomic DNA was isolated from overnight cultures using High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's directions and quantified with PicoGreen (Molecular Probes, Eugene, OR). Nextera XT DNA sample preparation and index kits (Illumina, San Diego, CA) were used to convert genomic DNA into indexed libraries suitable for sequencing. Indexed libraries were pooled and sequenced on the Illumina MiSeq platform (Illumina, Inc. 5200 Illumina Way, San Diego, CA, USA), using the MiSeq v2 500 Cycle reagent kit yielding 2 × 250-bp paired-end reads, giving an estimated average coverage of 90X.
Genome Sequence Assembly, Annotation and Analysis. Sequence quality filtering, format conversion and assembly was performed using NextGENe® software v2.00 (Softgenetics, State College, PA). Draft *de novo* genome assemblies were generated using MIRA v. 4.0.2 (6) and submitted to the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) for annotation.
Cell culture and binding assays. Human keratinocytes were purchased from ATCC and maintained keratinocyte basal medium with appropriate supplements. Primary porcine keratinocytes were obtained by trypsinizing the top layer of skin collected from a CDCD pig maintained on site at the NADC and maintained in serum-supplemented medium. ST5 strains, along with USA300 (TCH1516) and USA100 (MRSA252) strains as a positive controls for adherence to human keratinocytes, were grown overnight in tryptic soy broth (TSB). Cultures were diluted, adjusted to same optical density, and added to human keratinocytes in triplicate. Samples were then incubated for 1 hour at 37^{o}C, washed, and collected using a sterile cell scraper. Serial dilutions were then be plated on agar plates for enumeration.
Antimicrobial Susceptibility Testing. Antimicrobial susceptibility performed as previously described (14).
PCR reactions. Primers were designed based on a multiple sequence alignment of Mu3, Mu50, and Newman. PCR screening was conducted in 50 uL reaction volume using either AmpliTaq (Applied Biosystems, Carlsbad, CA) or AccuPrime Taq (Invitrogen, Carlsbad, CA) depending on the primer set. An MJ Research PCT-200 DNA Engine thermocycler (GMI, Ramsey, MN) was used for amplification using the following settings: 30 cycles of 30 second denaturation at 94º C, 30 seconds annealing at the temperature listed in Table from Hau et al. (17), 1 minute extension at 72ºC (AmpliTaq) or 68ºC (AccuPrime). PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light. Nucleotide sequence determination of PCR products was completed by Sanger sequencing methods.
Southern blotting. Genomic DNA (500ng) was digested overnight with BamH1 in a 25 uL reaction volume and run on a 1% agarose gel. Each gel was depurinated in 0.2M HCl for 10 minutes and rinsed with distilled

water. It was then placed in denaturing solution for 1 hour followed by neutralizing solution for 1 hour. Each gel was set up for transfer via capillary action to a nylon membrane overnight. The DNA was crosslinked in UV light to the membrane. Prehybridization was done at 42ºC for 2 hours and hybridized at 42ºC overnight using a DIG labeled probe (Roche, Mannheim, Germany). The membrane was washed with 2X and 0.5X wash solution and blocked for 1 hour with maelic acid solution containing 5% powdered milk. It was then probed with anti-DIG antibody (Roche, Mannheim, Germany) at 1:10,000 in 5% powdered milk in maelic acid solution. The membrane was washed in washing buffer to remove excess antibody and CSPD (Roche, Mannheim, Germany) was added for visualization. Imaging was done using a myECLImager (Life Technologies, Grand Island, NY) or x-ray film development.

Results:

Objective 1: Obtain whole-genome sequences of 110 ST5 strains isolated from human, animal, and environmental samples collected from diverse swine farms in the U. S.

We expanded our collection of ST5 strains to 155 isolates. Swine associated isolates were obtained from Iowa State University (14) and the University of Minnesota. Sources for these isolates were swine (38 isolates), the environment within swine facilities (26 isolates), humans with short-term contact with swine (9 isolates), and swine veterinarians representing humans with long-term contact with swine (9 isolates). Clinical isolates from humans with no swine contact, representing both HA- and CA-MRSA, were obtained from the University of California Irvine (64 isolates) (19) and the University of California San Francisco (9 isolates). Draft *de novo* genome assemblies and annotations have been completed for all of these isolates.

Objective 2: Compare the genome sequences obtained for the 110 ST5 strains to each other and to other MSSA and MRSA publicly available genome sequences to generate a more comprehensive assessment of the origin, evolution, and zoonotic potential of these strains.

One of the comparative genomic analyses that is now completed and published (17) is the screening of our ST5 strains for the prevalence of the IEC genes carried by β-hemolysin converting bacteriophages, whose absence in LA-MRSA ST398 is thought to contribute to reduced rates of human infection and transmission associated with this lineage. We found IEC genes absent from any of the ST5 strains from agricultural sources and Southern blotting demonstrated that the β-hemolysin gene was intact in these strains, indicating the bacteriophage's absence (17). In contrast, the prevalence of the β-hemolysin converting bacteriophage in ST5 strains from humans with no exposure to swine was 90.4% (17). The absence of β-hemolysin converting bacteriophage in LA-MRSA ST5 isolates is consistent with previous reports evaluating ST398 strains and provides genetic evidence indicating LA-MRSA ST5 isolates may harbor a reduced capacity to cause severe disease in immunocompetent humans. Comparison of 155 genomes yielded a total of 761 SNPs and phylogenetic or family trees have been constructed using the core genome sequences from these ST5 strains (Figure 1). Additionally, identification of mobile genetic elements, including phages, has been completed along with the identification of all AMR genes carried by these strains.

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.

We encountered numerous problems isolating and propagating porcine epidermal keratinocytes. We have recently contacted Nancy A. Monteiro-Riviere from Kansas State University who has graciously provided a protocol developed in her lab for culturing and propagating epidermal keratinocytes isolated from pigs. After numerous attempts with using this protocol we were unsuccessful to isolate and propagate epidermal keratinocytes isolated from pigs. We have had a greater success in propagating and using human keratinocytes and using these cells in *in vitro* attachment assays with our ST5 strains. Replicates of these assays are completed and shown in Figure 2 and 3.

Discussion: It has long been known that *S. aureus* commonly colonizes many mammalian and avian species and particular lineages are more adapted to different host species (31). The recent recognition that livestock may represent a substantial reservoir of MRSA and people having regular contact with animals were commonly colonized with LA-MRSA isolates represented a shift in MRSA epidemiology and raised urgent questions about the public health significance of these organisms. Fundamental questions remain about the ability of *S. aureus* lineages adapted to animals to both colonize and cause disease in humans. The capacity of ST398 LA-MRSA to cause clinical disease in humans is established, but reports of severe infections in people with occupational exposure to livestock remain uncommon, despite continued exposure to these organisms. There is increasing evidence that ST398 MRSA isolates of animal origin are less likely to be transmitted between people and are less likely to be associated with severe infections than are human adapted variants. Genomic studies have indicated that distinct livestock and human variants are identifiable even within a given sequence type and spa type, such as ST398/t571 and ST1/t127 (15, 38). More specifically, the absence of MGE associated virulence factors, including IEC genes has been linked to host adaptation and loss of virulence in ST398 LA-MRSA (7, 27, 34).

The MRSA lineages ST398 and ST9, which predominate in swine populations in Europe and Asia respectively, do not appear to have a significant impact on human health in the US. Unlike in Europe and Asia where ST5 MRSA have only rarely been reported, several studies indicated that ST5 S. aureus (both MRSA and MSSA) are relatively common in the North American swine industry (14, 21, 25, 29, 42). Because the ST5 lineage is a major contributor to both hospital and community associated MRSA and MSSA infections in this country and worldwide (28, 30), it is important to address the question about the potential contribution, if any, of the swine reservoir to the burden of clinical disease associated with ST5 *S. aureus*.

At this time, no human disease due to swine-associated LA-MRSA ST5 isolates has been reported. This may be due to differences in the composition of the accessory genome seen in LA-MRSA versus HA- and CA-MRSA isolates, similar to that previously noted in ST398 isolates (7, 34). HA- and CA-MRSA ST5 isolates are known to carry several MGE that enhance their virulence and antibiotic resistance, which have contributed to their dissemination and pathogenicity (30).

This proposal is the first large scale study investigating the evolution and zoonotic potential of LA-MRSA ST5 strains associated with swine. Thanks to the funding received, we now have whole-genome draft sequences for 155 ST5 isolates from both human and swine related sources. We have completed the construction of phylogenetic trees based on single-nucleotide polymorphisms (SNPs) within the core genome of these strains and have identified the AMR genes harbored by these strains. During the assembly and annotation process, we completed and published a report examining the prevalence of the IEC genes carried by β-hemolysin converting bacteriophages among our ST5 isolates from both human and swine related sources (17). This was the first report examining β-hemolysin converting bacteriophage prevalence within human clinical MRSA ST5 isolates specifically. We found the incidence of prophage integration in ST5 isolates obtained from humans with no swine contact was consistent with that found in previous reports of human clinical *S. aureus* isolates, both MRSA and methicillin-susceptible (7, 34, 39). Additionally, this was the first study investigating the prevalence of the β-hemolysin converting bacteriophage in LA-MRSA ST5 isolates. Our results were consistent with previous publications, and comparative statistical analysis showed no significant difference ($p = 0.3987$) in the prevalence of β-hemolysin converting bacteriophages between LA-MRSA ST5 isolates evaluated in this study (0/82) and LA-MRSA ST398 isolates evaluated previously (1/63) by Price and colleagues (34).

Due to the restricted host specificity of the most prevalent genes (*sak*, *scn*, *chp*), it has been suggested that this prophage may be absent in MRSA isolates after adapting to a livestock niche (34). These genes would not confer an advantage during colonization or disease development in livestock species and are therefore unnecessary to retain within the genome of LA-MRSA isolates. The loss of these important virulence factors is likely one of the reasons LA-MRSA isolates are rarely known to cause invasive disease in immunocompetent humans. The absence of IEC genes carried by β-hemolysin converting bacteriophages in LA-MRSA ST398 and ST5 strains parallels the findings for poultry adapted ST5 strains in that the human-specific IEC genes were lost and subsequently replaced by genes encoding avian-specific factors after the human-to-poultry transition (26). However, unlike poultry adapted ST5 strains, swine-associated LA-MRSA ST398 and ST5 strains harbor an

intact β-hemolysin gene, indicating that the bacteriophage is absent from these strains rather than being replaced by genes encoding swine-specific factors.

ST5 *S. aureus* appear to be widespread in the North American swine population and have likely been endemic in this reservoir for some time, yet livestock contact has not been identified as a risk factor for clinical staphylococcal disease (37). Our study reported clear genomic differences between ST5 MRSA isolates linked to swine and isolates from human clinical infections (39). These differences parallel previous observations with ST398 isolates. We hypothesize that the genetic changes observed may reflect general processes related to host adaptation of *S. aureus* to pigs. In summary, we are incredibly grateful to have received funding from the National Pork Board to initiate and complete this work. The data arising from this project is extremely interesting and is paving the way for many future projects.

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Figure 1. Maximum parsimony tree of 155 ST5 strains encompassing both MSSA and MRSA strains from both human and swine related sources. Comparison of 155 genomes yielded a total of 761 SNPs. Only nodes with bootstrap values below 100 are listed.

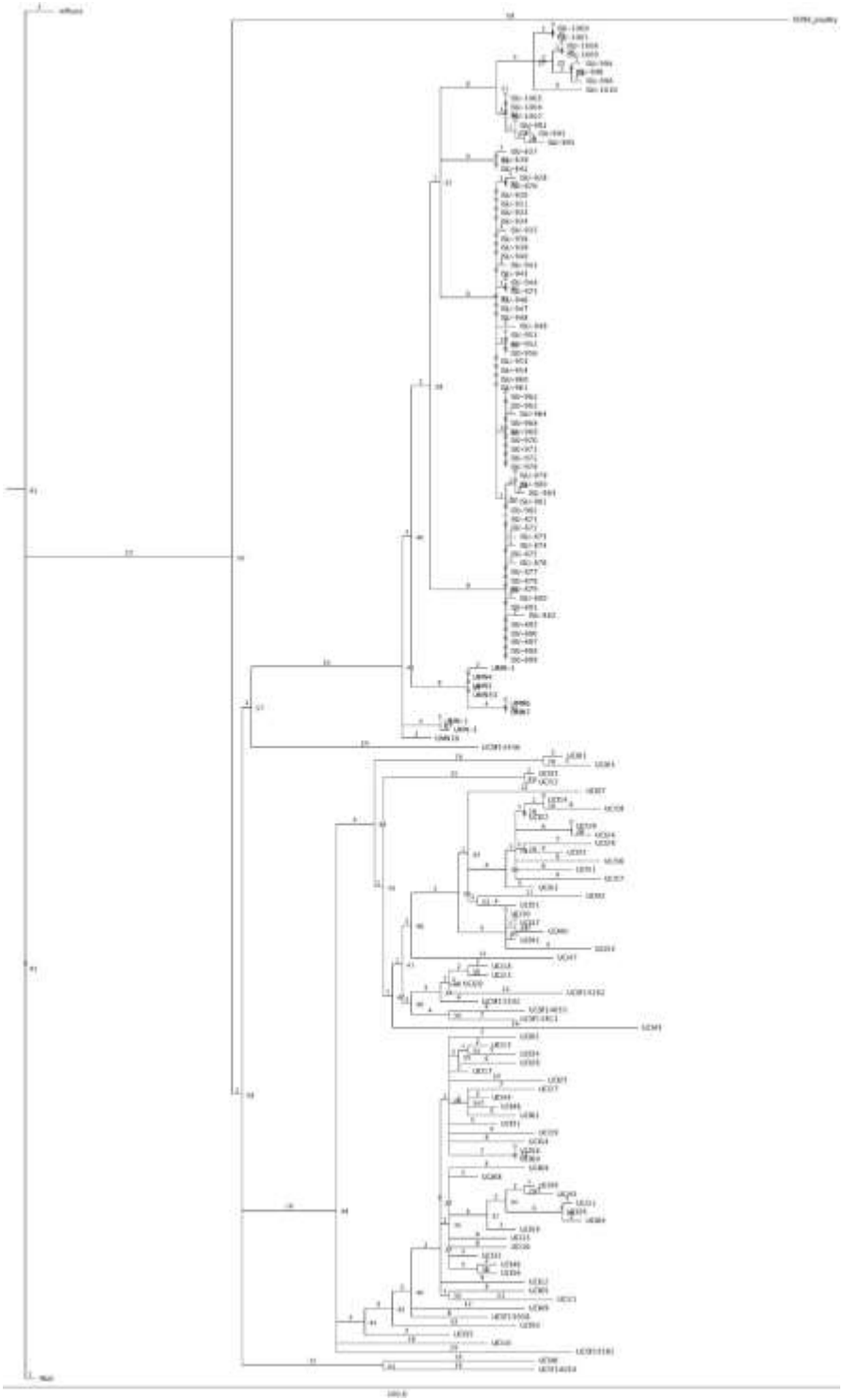

Figure 2. Adherence of ST5 strains to HEK cells expressed as the proportion of adherent bacteria to the original inoculum. Bars represent means of three independent experiments +/- the standard deviation.

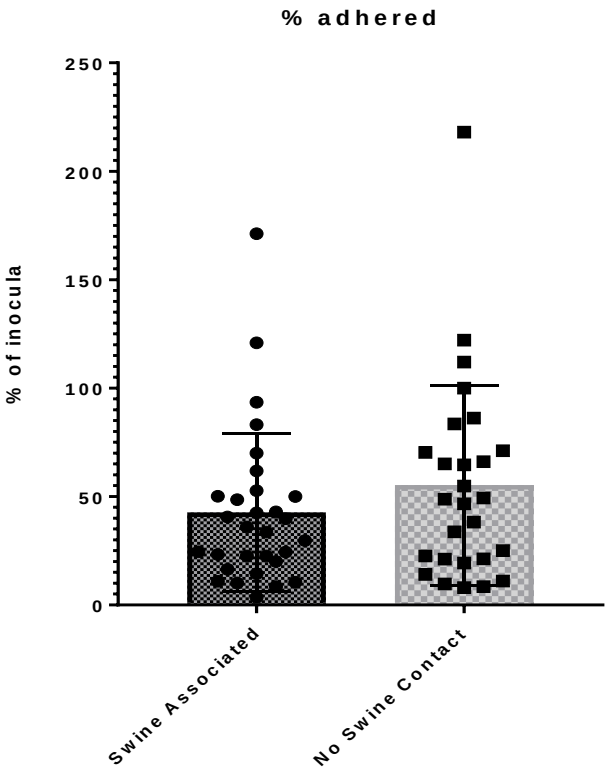
% adhered
% of inocula
250
200
150
100
50
0
Swine Associated
No Swine Contact

Figure 3. Adherence of ST5 strains to HEK cells expressed as the proportion of adherent bacteria to the number of HEK cells. Bars represent means of three independent experiments +/- the standard deviation.