Scientific Reports

https://doi.org/10.1038/s41598-026-52331-y

Article in Press

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Received: 25 December 2025

Accepted: 5 May 2026

Published online: 09 May 2026

Cite this article as: Hatrongjit R., Boueroy P., Kajeekul R. *et al.* Non-penicillin -susceptible and linezolid-non-susceptible *Streptococcus suis* clonal complex 233 from humans. *Sci Rep* (2026). https://doi.org/10.1038/s41598-026-52331-y

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Non-penicillin -susceptible and linezolid-non-susceptible *Streptococcus suis* clonal complex 233 from humans

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Keywords: *Streptococcus suis*, linezolid resistance, *optrA*, clonal complex

Running title: Human *optrA*-harbouring *Streptococcus suis* clonal complex 233

Abstract

Purpose

Streptococcus suis is an important swine pathogen and a zoonotic pathogen responsible for systemic diseases in humans. In 2021, an outbreak of non-

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penicillin-susceptible, *optrA*-harbouring *S. suis* serotype 2 ST1656, belonging to clonal complex 233 (CC233), associated with the consumption of raw pork dishes, was reported in northeastern Thailand. This study aimed to characterize the genetic relationship between non-penicillin-susceptible, *optrA*-harbouring *S. suis* CC233 isolates from the outbreak and post-outbreak periods in 2021.

Methods

We investigated the characteristics of 18 *S. suis* clonal complex 233 strains isolated post-outbreak in 2021 in Thailand using serotyping, multilocus sequence typing, and antimicrobial susceptibility testing. *S. suis* strains belonging to sequence type (ST) 1656 were subjected to whole-genome sequencing. Comparative genome analysis was conducted between the ST1656 outbreak and post-outbreak strains.

Results

The analysis revealed that 17 out of 18 strains showed intermediate resistance to penicillin, with three strains exhibiting non-susceptibility to linezolid. PCR serotyping and MLST analysis showed that among the three linezolid-non-susceptible CC233 post-outbreak strains, two were serotype 2 ST1656 and one was serotype 14 ST233. ST1656 post-outbreak strains clustered with ST1656 outbreak strains but formed a separate branch, indicating that the ST1656 outbreak and post-outbreak strains shared the same ancestor. Differences in the genetic organization of the *optrA* gene,

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which is associated with linezolid resistance, were observed between outbreak and post-outbreak strains, suggesting that *optrA* may have been acquired from distinct origins. In contrast, the amino acid sequences of *pbp1a*, *pbp2b*, *pbp2x*, and *mraY* were identical between the ST1656 outbreak and post-outbreak strains, and their PBP and MraY amino acid sequences were homologous to sequences from non-penicillin-susceptible *S. suis* ST104 or ST233 strains.

Conclusion

These findings highlight the zoonotic risk and emergence of antimicrobial-resistant *S. suis* CC233, underscoring the need for One Health surveillance and continued monitoring of penicillin and linezolid susceptibility in both swine and human populations.

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Introduction

Streptococcus suis is one of the most economically important swine pathogens worldwide, and it is regarded as a zoonotic pathogen responsible for systemic diseases in humans [1]. In recent years, resistance of *S. suis* to antimicrobial agents has been reported worldwide [2-3]. Among these, resistance to clinically important antimicrobials used for treatment, such as penicillin, ceftriaxone, and oxazolidinones, has been reported [4-8]. This situation raises concern regarding the risks posed by increasing antimicrobial-resistant *S. suis*, including acquisition and transmission of resistance, as well as its role as a reservoir for resistance genes [9].

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Oxazolidinones, including linezolid, tedizolid, and contezolid, are critically important antimicrobial agents because they are last-resort drugs for the treatment of human infections caused by multidrug-resistant Gram-positive bacteria such as vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* [10-11]. At least three types of acquired oxazolidinone resistance genes have been identified, including *cfr*, *optrA*, and *poxtA* [6,12]. The *cfr* gene family (*cfr*, *cfr(B)*, *cfr(C)*, *cfr(D)*, *cfr(E)*) encodes an rRNA methyltransferase that confers resistance to phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A, whereas *optrA* encodes an ABC-F protein that confers phenicol and oxazolidinone resistance [6,12]. Finally, *poxtA* (*poxtA* and *poxtA2*) encodes an ABC-F protein that confers resistance to phenicol, oxazolidinone, and tetracycline [12].

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Linezolid-resistant *S. suis* serotypes 2–5, 9, 11, 12, 16, 21, 23, 28–31, and nontypable strains have been reported in China, Thailand, and Italy [5,6,13-17]. Almost linezolid-resistant *S. suis* strains detected carried the *optrA* gene, and some also carried *cfr* [6,13-15,18]. Several studies show that the *optrA* gene is the major determinant of linezolid resistance in *S. suis* [5,6,13,15]. In China, surveillance of swine and human *S. suis* isolates has reported linezolid resistance rates ranging from approximately 8.3% to 28.7% in some studies, with *optrA* identified as the predominant driver of resistance [13–15]. In Europe, *optrA*-harboring *S. suis* was also identified in porcine isolates in Italy [6]. In Thailand, non-penicillin-susceptible and linezolid-resistant *S. suis* was detected during an outbreak in Nakhon Ratchasima

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Province, northeastern Thailand, in April 2021 [5]. The outbreak strain was serotype 2, sequence type (ST) 1656, and belonged to clonal complex (CC) 233 [5].

The genetic organization of the *optrA* gene in *S. suis* varies among strains and is often associated with mobile genetic elements, particularly insertion sequences from the *IS*1216 family, which facilitate its dissemination. A study conducted in China on *S. suis* isolates from diseased pigs identified 12 distinct genetic environments (types I–XII) associated with the *optrA* gene, demonstrating substantial diversity in the genetic contexts mediating phenicol–oxazolidinone resistance [15]. In that study, *optrA* was frequently flanked by *IS*1216E elements, which facilitate its integration into various mobile genetic elements (MGEs), including plasmids, integrative and conjugative elements (ICEs), and prophages [15]. However, the *optrA*-carrying fragment identified in Thai *S. suis* ST1656 outbreak strains differed from these previously described types, as it was associated with a transposon belonging to the *IS*1216 family that carried both the *optrA* and *ermA* resistance genes on the ICE [5].

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Notably, the diversity in the genetic context of the *optrA*-carrying fragment, remains poorly understood in Thai *S. suis* strains. In particular, it is unclear how these elements have evolved and disseminated following the outbreak, and whether post-outbreak strains share a common origin or represent independent acquisition events. To address this gap, we investigated the genetic relationships and characteristics of *optrA*-

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harbouring *S. suis* CC233 strains collected during the post-outbreak period in 2021 (May–December), and characterized the genetic organization of the *optrA* gene. This study provides insights into the dissemination dynamics and evolutionary relationships between outbreak and post-outbreak CC233 strains, particularly ST1656.

Materials and Methods

Bacterial strains

As shown in Table 1, a total of 86 *S. suis* strains were prospectively collected between May and December 2021 (post-outbreak) in Nakhon Ratchasima Province, northeastern Thailand. These isolates were initially identified as *S. suis* by the hospital laboratory. Among them, 18 belonged to CC233 and 68 to non-CC233 lineages. These strains were isolated in hospital laboratories from patient specimens as part of routine diagnostic services and were subsequently sent to us as pure cultures for further confirmation. Additionally, 14 representative ST1656 (CC233) outbreak strains (April 2021), two ST233 and one ST379 strains from a previous study, were used for comparative genome analysis [5]. The clinical data of post-outbreak strains were collected as described previously [5].

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Identification and antimicrobial susceptibility

S. suis strains were cultured on sheep blood agar (BioMedia, Thailand) for 18 hours at 37ºC under 5%CO_2. Pure culture was selected for DNA extraction

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using ZymoBIOMICS DNA Kits (Zymo Research, CA, USA) according to the manufacturer's instructions. Confirmation of species identity and serotyping of all *S. suis* post-outbreak strains were performed using a previously described multiplex PCR assay [19]. Briefly, the PCR mixture consisted of 1X PCRBIO HS Taq Mix red master mix (PCRBIO Taq DNA Polymerase, London, UK), 1.25 µM of each *S. suis*-specific primer, 0.125 µM of *cps1,14J* primers, 0.125 µM of *cps2,1/2J* primers, 0.5 µM of *cps2,14K* primers, and 1 µL (100 ng) of template DNA in a total reaction volume of 20 µL. The thermal cycling conditions were optimized for multiplex PCR, comprising an initial activation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 20 s and combined annealing/extension at 61 °C for 90 s, with a final extension at 72 °C for 5 min. *S. suis* reference serotypes 1/2 (strain 2651), 1 (NCTC10237), 2 (strain P1/7), and 14 (strain 13730) were used as positive control strains, while *Streptococcus pneumoniae* ATCC49619 served as a negative control in the PCR assay.

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PCR determination of CCs was conducted as described elsewhere [20]. Briefly, multiplex PCR was performed using 1× JumpStart REDTaq ReadyMix Reaction Mix (Sigma) and 0.4 µM of each primer pair targeting the *hp1*, *col*, *mp*, *pep*, and *srtBCD* genes. PCR conditions: initial activation at 95 °C for 3 min; 30 cycles of 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 2.5 min; followed by a final extension at 72 °C for 5 min. *S. suis* strains P1/7, 30190, 28739, 24525, 33329, and 40747 were used as positive controls for CC1, CC25, CC28, CC104, CC221/234, and CC233, respectively, whereas *S. pneumoniae*

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ATCC 49619 served as a negative control. Multilocus sequence typing, as described elsewhere [21], was performed for CC233 strains.

Susceptibility to penicillin, ceftriaxone, levofloxacin, vancomycin, and linezolid was determined by the minimum inhibitory concentration (MIC) using the Sensititre™ Complete Automated AST System according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). We followed the M100 (34th ed.) Clinical and Laboratory Standards Institute (CLSI-M100) guidelines [22] to classify penicillin and linezolid susceptibility. Since there are currently no breakpoints recommended for *S. suis* isolates from humans, the breakpoints established for viridans group streptococci were applied, as defined in the guidelines [22]. *Streptococcus pneumoniae* ATCC49619 was used as a quality control strain for antimicrobial susceptibility testing in accordance with the CLSI M100 guideline.

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Whole-genome sequencing

Only linezolid-resistant *S. suis* CC233 strains (n = 3; STC128, STC260, and STC315; Table 1) were chosen for whole-genome sequencing (WGS). These strains were cultured on sheep blood agar plates as described above. Only single colony was selected to propagate on sheep blood agar and DNA was extracted using ZymoBIOMICS DNA Kits (Zymo Research, CA, USA) according to the manufacturer's instructions. The extracted DNA concentration was measured using Qubit Fluorometer (Thermo Fisher Scientific, MA, USA), and its quality was assessed by 0.5% agarose gel

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electrophoresis. Complete genome sequencing was performed using Illumina HiSeq 2500 (Illumina, Inc., CA, USA) and GridION instrument (Oxford Nanopore Technologies, Oxford, United Kingdom) sequencing platforms as described elsewhere [23].

Briefly, Illumina sequencing libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, UK) according to the manufacturer's instructions. Genomic DNA was randomly fragmented to an average size of 350 bp, followed by end repair, A-tailing, and adapter ligation. The libraries were sequenced as 150 bp paired-end reads on a HiSeq 2500 platform. Adapter sequences were removed using Fastp v0.19.5, and the quality of the processed reads was assessed using FastQC v0.11.8. For the ONT platform, Libraries were prepared using the Rapid Barcoding DNA Sequencing protocol with the RBK114.96 kit from Oxford Nanopore Technologies, in which genomic DNA is rapidly fragmented and barcoded via a transposase-mediated reaction, enabling multiplexing and efficient library preparation for nanopore sequencing. Sequencing was then carried out on an R10.4.1 flow cell using a GridION instrument. Raw reads were basecalled and demultiplexed with Dorado v0.5.1 (ONT), and adapter sequences were trimmed using Porechop v0.2.4.

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Genome assembly and annotation

Hybrid assemblies with ONT and Illumina data were generated using Unicycler v0.4.8 [24], and the genome sequences were checked for quality

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using QUAST v5.0.2 [25]. Assembly quality was assessed using standard metrics, including total genome size, number of contigs, N50, and GC content. All assemblies were considered acceptable, with genome sizes ranging from 2.25 to 2.27 Mb, contig numbers ranging from 1 to 4, N50 values ranging from 1.58 to 2.27 Mb, and GC content of approximately 41%. Genome sequences were submitted to the NCBI Prokaryotic Genome Annotation Pipeline (PGAP v4.12) for annotation. The default parameters were used for all software unless otherwise specified. The genome sequences of three *S. suis* CC233 post-outbreak strains were deposited in the NCBI GenBank under Bioproject accession number PRJNA691075 (Submitted and accepted in September 2023).

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Bioinformatics analysis

Antimicrobial resistance genes were detected using ResFinder 4.7.2 with 80% identity and 60% coverage [26]. Sequence type (ST) was confirmed using the PubMLST database (https://pubmlst.org/organisms/streptococcus-suis). Virulence-associated genes (VAG) were analyzed using MyDBFinder 2.0 (https://cge.food.dtu.dk/services/MyDbFinder/) in three post-outbreak strains (Supplementary file 1). These results were then compared with those of the ST1656 outbreak strain and with the ST233 and ST379 strains of CC233 reported in the previous study [5]. Mobile genetic elements were analyzed using MobileElementFinder v1.0.3 and PlasmidFinder 2.1 [27,28]. Core genome-based phylogeny of the CC233 strains in this study (n = 17; 14

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outbreak and 3 post-outbreak strains) was determined as described elsewhere [29]. Briefly, the chromosomal sequences of post-outbreak and outbreak strains were aligned using Parsnp [30] generating core-genome single-nucleotide polymorphisms (SNPs). Phylogenetic tree based on core SNP was constructed using maximum-likelihood phylogenetic trees by FastTree embedded in the Parsnp. In sequence alignment, recombinant regions were filtered using Gubbins v2.4.0 with default parameters, based on alignment. The bootstrap value was set at 1000 times. The phylogenetic tree was visualized using the online website iTOL (http:// itol. embl. de/).

The genetic organization of *optrA* between outbreak and post-outbreak strains was compared. The *optrA* gene cluster was annotated using Prokka v1.14.6 [31], with default parameters, including a minimum contig length of 200 bp, *E*-value threshold of 1^{e-6} for BLAST searches, and annotation against the core Prokka databases. The protein sequences of the *optrA* clusters of our three strains were aligned with the reference strain STC78 (ON944186) using clinker with a sequence identity threshold of 30% for gene clustering [32]. Visualization was generated using clustermap.js v0.021 with default settings [32]. The *optrA* cluster was defined based on the presence of *optrA* and adjacent genes potentially involved in antimicrobial resistance genes and mobile genetic elements. A ~30 kb region (±15 kb flanking *optrA*) was analyzed to characterize its genetic context.

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Comparative genomic analysis

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Comparative genomic analyses between outbreak ST1656 and post-outbreak ST1656 strains were performed using the anvi'o v7 workflow [33]. This workflow identified gene clusters and single-copy genes in the study genomes, including 16 ST1656 strains (14 outbreak and 2 post-outbreak strains), one ST233 post-outbreak strain, and two serotype 2 genomes, the epidemic strain SC84 (accession no. FM252031) and the highly virulent strain P1/7. Pangenome analysis was performed using the "anvi-run-workflow" pipeline. Genes were annotated with "anvi-run-ncbi-cogs", and genomes were added to an anvi'o genome storage database using "anvi-gen-genomes-storage". The pangenome was constructed with "anvi-pan-genome", using DIAMOND for amino acid similarity searches and MCL (Markov Cluster algorithm) for gene clustering under the default anvi'o settings (minbit = 0.5, inflation = 2.0). Core genes were defined as gene clusters present in all genomes, while accessory genes were defined as those present in only a subset of genomes. Pangenome results were visualized with anvi-display-pan. UpSetR plots were generated using the UpSetR package [34] in R v4.5 to visualize gene overlaps among bacterial strains. Gene lists obtained from the pangenome analysis were used as input, and gene sets were sorted by frequency, with the top 40 gene sets retained for visualization.

Results

S. suis post-outbreak

Since April 2021, an outbreak due to *S. suis* serotype 2 ST1656 belonging to CC233 occurred in Nakhon Ratchasima Province, Thailand [5]. This was the

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first report of linezolid-resistant strains in serotype 2 CC233 isolated from humans, and these outbreak ST1656 strains harboured an *optrA* gene that contributed to resistance to linezolid. We continued monitoring *S. suis* between May and December 2021 (post-outbreak). A total of 86 *S. suis* post-outbreak strains were isolated from patients at Maharaj Nakhon Ratchasima Hospital. PCR characterization of these 86 post-outbreak strains revealed that 18 belonged to CC233, whereas 68 were non-CC233, including 48 CC1, 16 CC104, and 4 unknown or unidentified CCs. PCR serotyping demonstrated that the 86 post-outbreak strains comprised serotype 2 (n = 82) and serotype 14 (n = 4).

Of the 18 CC233 post-outbreak strains, the isolates were obtained from patients with a median age of 60.5 years (interquartile range [IQR] 16; range 29–73 years), whereas the 15 outbreak cases had a median age of 67 years (IQR 7; range 50–89 years). Of these, 13 post-outbreak cases were male (72.2%). Most of the 18 CC233 cases presented with septicemia (n = 14), with one case each of infective endocarditis and meningitis (Table 1). One ST1656 post-outbreak strain was isolated from a patient with infective endocarditis (Table 1).

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Antimicrobial susceptibility

Susceptibility to penicillin, ceftriaxone, levofloxacin, vancomycin, and linezolid was tested in 18 *S. suis* CC233 post-outbreak strains (17 serotype 2 and 1 serotype 14). Seventeen CC233 post-outbreak strains showed intermediate resistance to penicillin, whereas one strain (STC128) was

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resistant to ceftriaxone (Table 1). All CC233 post-outbreak strains were susceptible to levofloxacin and vancomycin. Most CC233 post-outbreak strains were susceptible to linezolid; three strains were non-susceptible (Table 1). MLST analysis showed that among the three linezolid-non-susceptible post-outbreak strains, two were serotype 2 ST1656, the same ST as the outbreak strains, whereas the remaining strain was serotype 14 ST233 (Table 1).

Antimicrobial-resistant genes

Whole-genome analysis revealed the presence of antimicrobial resistance genes in these three linezolid-non-susceptible post-outbreak strains (Table 1). No plasmids were identified in the post-outbreak strains. The genes *aph(3')-III, tet(O), erm(B)*, and *optrA*, which confer resistance to aminoglycosides, tetracycline, macrolides, and linezolid, respectively, were detected in all three post-outbreak strains. In the ST1656 outbreak strains*,* *erm*(*A*), *erm*(*B*), *tet*(*O*), and *optrA* were present (Table 1). Notably, two ST1656 post-outbreak strains (STC260 and STC315) contained additional antimicrobial resistance genes, *cat* and *tet*(*O/W/32/O*) (Table 1). In addition, the post-outbreak ST233 strain STC128 contained *erm*(*A*), *erm*(*B*), *tet*(*O*), and *optrA*, similar to the ST1656 outbreak strains, but it also harbored additional antimicrobial resistance genes, *ant(6)-Ia* and *aph(3')-III* (Table 1).

Analysis of the *optrA* genetic organization in these three post-outbreak strains and comparison with a representative outbreak ST1656 strain (STC78) are presented (Figure 1). The post-outbreak ST233 strain STC128

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shared the *optrA* genetic organization with the outbreak ST1656 strain STC78, whereas the two post-outbreak ST1656 strains (STC260 and STC315) had an *optrA* genetic organization that differed from both the outbreak ST1656 and the post-outbreak ST233 strains. BLASTN analysis revealed that the *optrA* cluster of the two post-outbreak ST1656 strains was highly similar to *S. suis* SC183, with 93% sequence coverage and 97% sequence identity. The strain SC183 was isolated from swine in China in 2011 and belonged to ST316.

As shown in Figure 1, the antimicrobial resistance genes located upstream and downstream of the *optrA* fragment in the two post-outbreak ST1656 strains were arranged as follows: *erm(B)*, *aph(3′)-III*, *cat*, *optrA*, *erm(A)*, *tet(W)*, and *erm(A)*. This fragment was flanked by an ISSsu7 element, a member of the IS110 family. In contrast, analysis of the post-outbreak ST233 strain revealed that only *emr(A)* was located downstream of *optrA*, while non-antimicrobial-resistance genes were present both upstream and downstream. The *optrA* fragment in the post-outbreak ST233 strain was also flanked by an ISSsu7 element, similar to that observed in the two post-outbreak ST1656 strains; however, the overall genetic structure differed.

Analysis of mutations in the translated amino acid sequences of *pbp1a*, *pbp2b*, *pbp2x*, and *mraY*, which are associated with β-lactam resistance, demonstrated that the two post-outbreak ST1656 strains had sequences that were 100% identical to those of the representative ST1656 outbreak strain STC78 (Figure 2 and the supplemental files 2-5). In contrast, the post-

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outbreak ST233 strain STC128 showed differences in these four genes compared with the other strains (Figure 2). PBP1A and PBP2B sequences of the ST1656 strains were 100% identical to the sequences of non-penicillin-susceptible ST233 strains 37647 and 40747 in a previous study [35]. PBP2X and MraY sequences were 100% identical to the sequence of the non-penicillin-susceptible ST104 strain 22715 [35].

Comparative genome analysis

Core genome-SNP phylogenetic analysis indicated that strain STC128 (ST233 post-outbreak strain) formed a distinct branch from the ST1656 strains, whereas the two ST1656 post-outbreak strains clustered with the ST1656 outbreak strains but formed a separate branch from the outbreak strains (Figure 3). However, both the ST1656 outbreak and post-outbreak strains shared the same ancestor. To investigate this in detail, we conducted comparative genomic analysis of ST1656 outbreak (n = 14) and post-outbreak (n = 2) strains. Comparative genome analysis revealed 244 coding sequences shared by both outbreak and post-outbreak strains, whereas 28 and 8 coding sequences were unique to outbreak and post-outbreak strains, respectively (Figure 4). These eight unique sequences in the ST1656 post-outbreak strains encoded a dihydrofolate reductase family protein, a 23S rRNA methyltransferase attenuation leader peptide, a replication initiation protein, aminoglycoside O-phosphotransferase APH(3')-IIIa, chloramphenicol O-acetyltransferase (*cat*), and three hypothetical proteins.

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Analysis of 153 virulence-associated genes (VAGs) showed that 121 genes were present in the post-outbreak strains, as well as in the ST1656 outbreak strain and the ST233 and ST379 strains reported in the previous study. In contrast, 32 genes were absent from the ST1656 outbreak and post-outbreak strains, as well as from the ST233 post-outbreak strains and those described in the previous study (Supplementary file 1). The absent genes included *1910HK, 1910HR, adcA,* autolysin (*atl*)*, cbp40, endoSS, endoD, epf, fhb-I, gh92, Hhly3, IdeS, IgdE, mrp, nadR, ofs, pnuC, revS, rfeA, rgg, sadP, salK, salR, SpyM3-0908, srtBCD cluster, SssP1, SSU05-0473, tran, virB4, virD4, zymC,* and *zur.* However, two VAG, *nisK* and *nisR*, were detected only in the ST1656 post-outbreak strains (Supplementary file 1). Another VAG, *virA*, was detected in the ST1656 and ST233 post-outbreak strains (Supplementary file 1).

Discussion

In the present study, we have continued to monitor *S. suis* post-outbreak strains until December 2021 and have detected three CC233 strains (one ST233 and two ST1656) exhibiting linezolid non-susceptibility and carrying *optrA*. Most post-outbreak cases occurred in older individuals and were predominantly male. This pattern is consistent with previous studies identifying male sex as a risk factor for *S. suis* infection [36,37]. Additionally, advanced age may increase susceptibility to infection due to age-related decline in immune function (immunosenescence), which can compromise host defense against pathogens [38,39]. The clinical manifestations observed

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among CC233 outbreak and post-outbreak strains were more frequently associated with non-meningitis presentations (e.g., sepsis, infective endocarditis, and septic arthritis) than with meningitis. This observation is consistent with a previous study indicating that CC233 is predominantly associated with non-meningitis cases [40], suggesting that certain key virulence factors related to meningitis may be absent. However, the virulence factors and pathogenic mechanisms of CC233 remain to be fully elucidated.

As *S. suis* is a zoonotic pathogen, pigs are strongly implicated as the primary reservoir [1,2]. It is therefore highly likely that swine populations play a central role in sustaining the circulation of CC233, as well as other clonal complexes identified in this study, including CC1 and CC104.This is supported by the presence of ST members within CCs 233, 1, and 104 that have been identified in swine (https://pubmlst.org/organisms/streptococcus-suis). clone has not yet been reported outside Thailand. Transmission to humans likely occurs through occupational exposure or the consumption of raw or undercooked pork [1,36]. Almost all CC233 isolates in the current study were serotype 2; however, serotype 14 was also identified within ST233. In addition, serotypes 2 and 14 are the most prevalent in human infections in Thailand [40]. As serotypes 2, 1/2, 1, and 14 share similar capsular loci and structural features, capsule switching may have occurred among these serotypes [41,42]. Phylogenetic analysis indicated that the ST1656 post-outbreak strains were genetically closely related to the ST1656 outbreak strains, as they clustered together. However, the formation of a

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distinct branch suggests that the post-outbreak strains have accumulated genetic differences and may represent a divergent sub-lineage that emerged after the outbreak or may not be directly descended from the outbreak strains. This may be attributable to the accumulation of genetic mutations, post-outbreak persistence in hidden reservoirs, reintroduction of closely related strains from external sources, or the role of environmental reservoirs in maintaining and reshaping population structure, thereby allowing the pathogen to persist. Continuous monitoring enables early detection of emerging sub-lineages, supports timely public health interventions, and enhances understanding of pathogen evolution and transmission pathways following an outbreak.

Comparative genomic analysis provides insight into the post-outbreak evolution of the ST1656 lineage. Although phylogeny indicates a shared ancestor with outbreak strains, the presence of a small set of unique genes in post-outbreak isolates suggests ongoing microevolution and adaptation. Notably, these include antimicrobial resistance–related genes such as APH(3')-IIIa, *cat*, a dihydrofolate reductase family protein, and a 23S rRNA methyltransferase-associated element, suggesting a potential expansion of the resistance profile. The detection of a replication initiation protein further suggests possible horizontal gene transfer via mobile elements, which may facilitate the spread of resistance determinants. The remaining hypothetical proteins, although of unknown function, may also play roles in fitness, host adaptation, or genome plasticity and warrant further investigation. Overall,

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these findings indicate that post-outbreak ST1656 strains have acquired distinct genetic features that may enhance survival and drive continued evolutionary divergence.

Analysis of VAGs showed that 32 VAGs were absent in the post-outbreak strains, the ST1656 outbreak strain, as well as in ST233 and ST379, which belong to CC233. Clinical manifestations observed in the current and previous studies indicate that CC233 members, including ST233, ST379, and ST1656, were more frequently associated with non-meningitis (septicemia or infective endocarditis) than with meningitis [5]. Several VAGs have been linked to the pathogenesis of meningitis in *S. suis*. Among these, *mrp, sly*, and *epf* are commonly linked to virulent *S. suis* strains associated with meningitis [43,44]. In addition, genes such as *adcA, atlA, dltA, fhb, eno, gapdh, hyl, lmb, nadR, pgdA, prsA, rss04, sadP, SsadS, sspA, Sssp1, SsPepO, srtA, STK/STP, vraSR,* and phospholipase C are involved in pathogenic processes, including bacterial adhesion, invasion of the blood–brain barrier, and cytotoxic effects on the central nervous system [44]. In this study, *epf, mrp, adcA, atlA, fhb, nadR, sadP,* and *SssP1* were absent among the CC233 members examined. The absence of these meningitis-associated VAGs may reduce the pathogenic potential for meningitis in the CC233 strains. However, further investigation should be done.

Interestingly, the ST1656 post-outbreak strains possessed the *nisK/nisR*, a two-component system, which is considered part of the regulatory network that controls pathogenic traits in *S. suis* [45]. It enhances

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S. suis virulence by promoting host colonization, survival against immune defenses, and successful infection [45]. This gene was not detected in the ST1656 outbreak strain, suggesting that the ST1656 post-outbreak strains may have acquired *nisK/nisR* through horizontal gene transfer, as these genes are often located within a pathogenicity island (e.g., the 89K island) present in highly virulent *S. suis* strains [46].

This study has demonstrated differences in the ST1656 *optrA* genetic features between outbreak and post-outbreak strains. This finding indicates that these strains may have acquired the *optrA* element from different origins. This observation is concordant with phylogenetic and comparative genomic analyses, which have revealed that the post-outbreak ST1656 strains differ from the outbreak ST1656 strains. In the ST1656 outbreak strains, the *optrA* cluster has been carried on integrative and conjugative elements, and clonal expansion has been the mechanism of *optrA* dissemination in ST1656 outbreak strains [5]. Similarly, in the two post-outbreak ST1656 strains, the *optrA* organizations are similar, indicating that clonal expansion may also be a dissemination mechanism, as observed for the outbreak ST1656 strains. Notably, the post-outbreak ST233 strain exhibits an *optrA* genetic organization similar to that of the ST1656 outbreak strain, suggesting that these distinct STs may have acquired the *optrA* cluster from a common source via horizontal gene transfer, or potentially through direct acquisition from the ST1656 outbreak lineage. In addition, a previous study has described 12 types of genetic environments of *optrA* in *S. suis* [15]. The

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optrA-carrying fragments of the ST1656 outbreak and post-outbreak strains differ from these 12 types. Collectively, the differences in *optrA* genetic environments observed in our study and the previous study suggest ongoing diversification of resistance-associated mobile elements in *S. suis*. Such variability may reflect active genetic recombination and horizontal gene transfer occurring among circulating strains. This genetic plasticity could facilitate the persistence and spread of oxazolidinone resistance across different lineages, highlighting the need for continued genomic surveillance to monitor emerging resistance structures.

Previous studies have shown that *IS1216E* (IS26 family) is frequently associated with *optrA* in *S. suis*. In addition, other insertion sequences—such as *ISSsu7* (IS110 family), *ISS1S* (IS6/IS26 family), or members of the *IS6* family—have been identified flanking *optrA* [15,47,48]. In our study, *ISSsu7* elements were found flanking the *optrA* fragments in all three post-outbreak strains. These insertion sequences may form part of mobile genetic structures, such as integrative and conjugative elements (ICEs), enabling movement between different genomic regions or plasmids and thereby facilitating the spread or evolution of antimicrobial resistance genes among bacterial strains. Notably, the *optrA* cluster in the two post-outbreak ST1656 strains was highly similar to the Chinese swine *S. suis* SC183 strain, suggesting a shared origin or horizontal gene transfer. The high identity (97%) indicates strong conservation, while lower coverage (93%) may reflect structural variation. This supports that *optrA* is mobilizable and spreading

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among *S. suis* lineages, rather than being restricted to a single clone. Its presence in post-outbreak ST1656 strains suggests ongoing evolution and acquisition of resistance, likely driven by antimicrobial selection pressure, and may reflect pig-to-human transmission or regional circulation of resistant strains.

Many previous studies have focused on β-lactam antibiotic susceptibility, particularly penicillin and cephalosporins [5,8,49,50]. Penicillin-non-susceptible *S. suis* human strains have been reported, and CC233 has shown higher non-susceptibility than other CCs [35]. In addition, human outbreak ST1656 strains belonging to CC233 have shown non-penicillin susceptibility [5]. Our study has demonstrated that almost all CC233 post-outbreak strains are non-penicillin-susceptible. The presence of non-penicillin-susceptible CC233 strains observed in this study suggests that this clonal complex may represent an emerging high-risk lineage associated with antimicrobial resistance in *S. suis*. The persistence of CC233 strains after the outbreak further indicates the potential for continued circulation and expansion of this lineage. These findings highlight that CC233 should receive particular attention in antimicrobial resistance surveillance, as the increasing occurrence of strains with reduced susceptibility to β-lactams may complicate treatment strategies. This is especially important because penicillin and ceftriaxone are commonly used as first-line therapies for severe *S. suis* infections. In addition, the detection of resistance to oxazolidinones raises further concern, as these antimicrobials are important options for

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treating infections caused by resistant Gram-positive pathogens. It is necessary to strengthen antimicrobial stewardship and update treatment guidelines to ensure the effective management of resistant infections. Enhanced diagnostic strategies, including molecular methods, are needed for accurate identification and early detection of resistance. Strengthened surveillance—particularly genomic and antimicrobial resistance monitoring—is essential to track transmission and emerging trends. Furthermore, susceptibility to penicillin should be tested routinely to monitor strains from humans and diseased pigs. Markers for the prediction of non-penicillin susceptibility may be useful for high-throughput screening of large numbers of isolates.

There are up to six *pbp* genes (*pbp1a*, *pbp1b*, *pbp2a*, *pbp2b*, *pbp2x*, *pbp3*) in *S. suis*, but mutations in *pbp1a*, *pbp2b*, *pbp2x*, and *mraY* have mainly been related to β-lactam resistance [51]. Our analysis of amino acid substitutions in these four genes has revealed that the *pbp1a* and *pbp2b* amino acid sequences of ST1656 outbreak and post-outbreak strains are identical to those of non-penicillin-susceptible ST233 strains in a previous study [35]. Similarly, the *pbp2x* and *mraY* amino acid sequences of ST1656 outbreak and post-outbreak strains are identical to the *pbp2x* and *mraY* sequences of the non-penicillin-susceptible ST104 strain reported previously [35]. The presence of similar amino acid sequences in these resistance-associated genes across different sequence types suggests that genetic exchange may occur among circulating *S. suis* lineages. Such recombination

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events may facilitate the spread of mutations associated with reduced susceptibility to β-lactam antibiotics and contribute to the emergence and persistence of non-penicillin-susceptible clones [51,52]. This process could contribute to the emergence and persistence of resistant clones in both human and animal populations, highlighting the importance of monitoring these genetic determinants in antimicrobial resistance surveillance.

This study has several limitations. First, the number of post-outbreak isolates analyzed was limited, which may not fully represent the genetic diversity and dissemination of *optrA*-carrying *S. suis* strains. Second, the isolates were obtained from a restricted geographic area and over a relatively short post-outbreak surveillance period. Third, functional experiments were not performed to confirm the mobility and transfer mechanisms of the *optrA* genetic elements identified in this study. Therefore, further large-scale and long-term surveillance, including isolates from both human and animal sources, is needed to better understand the persistence, spread, and evolution of ST1656 and CC233 strains.

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Conclusion

We identified non-penicillin-susceptible and linezolid-non-susceptible ST1656 post-outbreak strains that were genetically closely related to the ST1656 outbreak strains. We identified *nisK/nisR* in the ST1656 post-outbreak strains, whereas these genes were absent in the ST1656 outbreak strains. The genetic organization surrounding *optrA* differed between the ST1656 outbreak and post-outbreak strains, suggesting that these strains acquired

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optrA from distinct origins. In contrast, the amino acid sequences of *pbp1a*, *pbp2b*, *pbp2x*, and *mraY* were identical in both the outbreak and post-outbreak ST1656 strains. Moreover, the PBP and MraY sequences were homologous to those found in *S. suis* strains belonging to ST104 or ST233. These findings support the implementation of integrated genomic surveillance and antimicrobial resistance monitoring in both animal and human health sectors. Strengthening surveillance systems will be important for early detection of emerging resistant clones and resistance determinants such as *optrA*, which has significant implications for treatment options and public health, particularly in regions where *S. suis* infections are endemic.

Supplemental files.

Supplemental file 1. Virulence-associated genes analysis in the *Streptococcus suis* CC233 in this study

Supplemental file 2. PBP1A amino acid alignment of *Streptococcus suis* STs 1656, 233, and 104.

Supplemental file 3. PBP2B amino acid alignment of *Streptococcus suis* STs 1656, 233, and 104.

Supplemental file 4. PBP2X amino acid alignment of *Streptococcus suis* STs 1656, 233, and 104.

Supplemental file 5. MraY amino acid alignment of *Streptococcus suis* STs 1656, 233, and 104.

Author contributions

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R.H., S.N., C.S., and A.K. contributed to the conception and design of the study; R.H., P.B., R.K., N.B., P.T., P.J., T.W., P.C., J.B., S.N., and A.K. performed the experiments, analyzed, and interpreted the data; R.H., R.K., P.B., J.L., and J.B. collected and analyzed the clinical data; R.H., J.B., C.S., S.N., and A.K. drafted and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the Health Systems Research Institute (HSRI), under the Genomics Thailand Initiative, with grant number HSRI 67-129, and Kasetsart University Institute for Advanced Studies (KUIAS) through the Reinventing University Program.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgment

The Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand provided English-editing assistance.

Declaration

Ethics statement

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Ethical approval for this study was obtained from the Ethics Committee of Maharat Nakhon Ratchasima Hospital in Thailand. When evaluating the medical records, the authors followed a protocol approved by the Ethics Committee. This study was conducted following the Principles of the Declaration of Helsinki. The approval numbers are 087/2021 and 021/2022.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure legend

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Figure 1. Organization of optrA genes in *Streptococcus suis* outbreak strain (STC78) and post-outbreak strains (STC128, STC315, and STC260).

Figure 2. Dendrogram of amino acid sequences of PBP1A, PBP2B, PBP2X, and MraY of *Streptococcus suis* STs 1656, 233, 104, and reference strain P1/7.

Figure 3. Core genome-SNP-based phylogenetic tree of *Streptococcus suis* ST1656 outbreak strains (red), *S. suis* post-outbreak strains (blue), and *S. suis* type strain S735.

Figure 4. UpSetR showing the number of genes that are shared and unique between the three *S*treptococcus *suis* ST1656 post-outbreak and outbreak strains, ST1 strain P1/7, and ST7 strain SC84.

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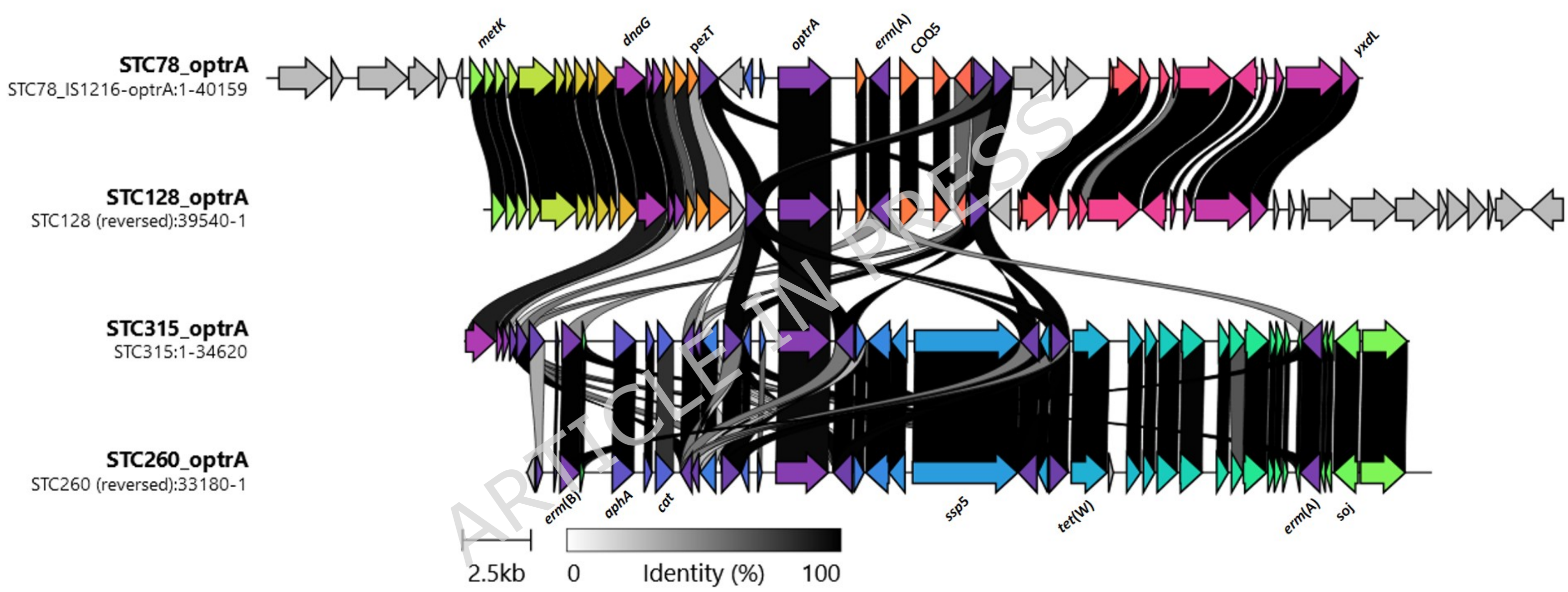

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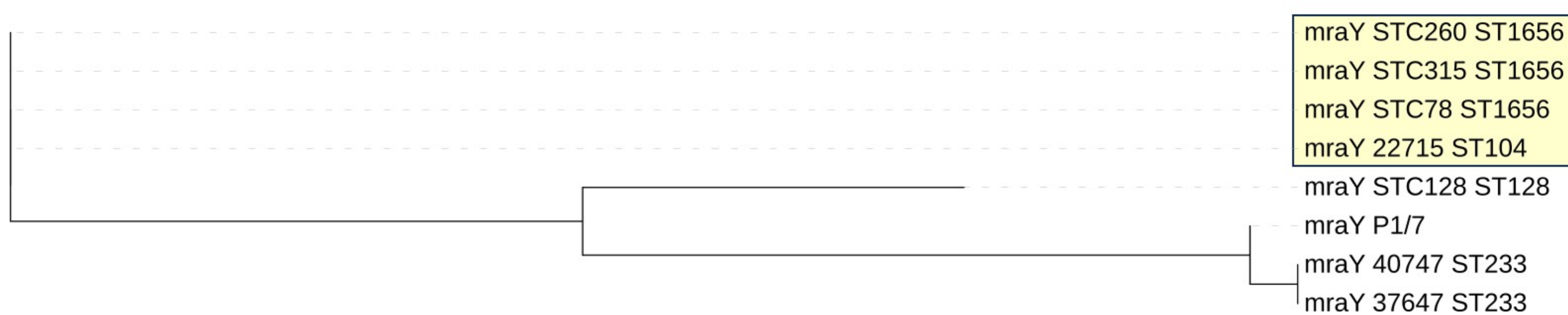

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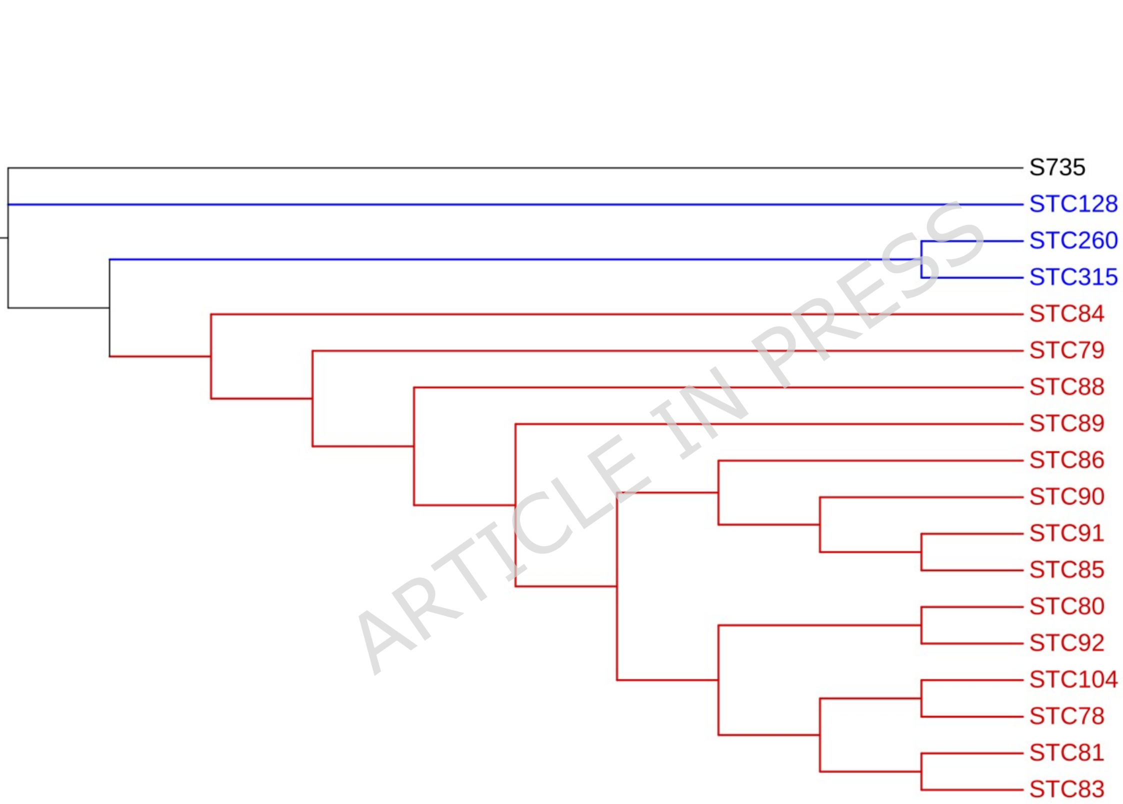

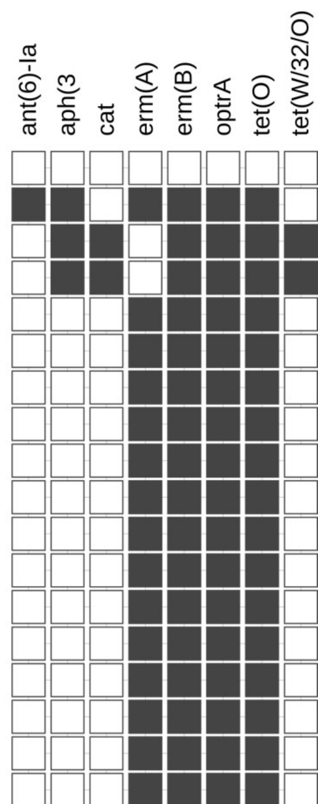

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1581

1500

Gene Intersections of ST1656

1000

500

244

164

38 28 11 8 6 5 4 2 2 2 2 2 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1

0

P1
SC84
STC260
STC315
STC86
STC85
STC104
STC81
STC80
STC90
STC78
STC83
STC84
STC92
STC91
STC89
STC88
STC79

1500 1000 500 0

Gene Per Genome

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Table 1. Characteristics of *Streptococcus suis* CC233 for 18 post-outbreak strains and 15 outbreak strains in this study

	ID no.	Sex	Age (year)	Serotype	ST	AMR genes*	Penicillin	Ceftriaxone	Linezolid[#]	Levofloxacin	Vancomycin
Post-outbreak	STC128	M	52	14	233	*ant(6)-Ia, aph(3')-III, erm*(A), *erm*(B), *optrA*, *tet*(O)	0.5 (I)	>4 (R)	4 (NS)	≤0.05 (S)	≤0.5
	STC260	M	67	2	1656	*aph(3')-III, erm*(B), *optrA*, *cat*, *tet*(O/W/32/O), *tet*(O)	0.25 (I)	0.125 (S)	4 (NS)	≤0.05 (S)	≤0.5
	STC315	M	46	2	1656	*aph(3')-III, erm*(B), *optrA*, *cat*, *tet*(O/W/32/O), *tet*(O)	0.5 (I)	0.5 (S)	6 (NS)	≤0.05 (S)	≤0.5
	STC130	M	55	2	379	ND	0.5 (I)	1 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC131	F	60	2	233	ND	0.5 (I)	1 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5
	STC132	M	61	2	233	ND	0.5 (I)	0.5 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5

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	STC135	M	69	2	379	ND	0.5 (I)	0.75 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC153	M	48	2	233	ND	0.5 (I)	0.75 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC173	M	29	2	233	ND	0.25 (I)	0.5 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC174	F	65	2	3019	ND	0.25 (I)	0.5 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5
	STC191	M	73	2	233	ND	0.25 (I)	0.5 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5
	STC212	M	44	2	233	ND	1 (I)	1 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC213	M	61	2	3019	ND	0.125 (S)	0.125 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5
	STC300	M	68	2	233	ND	0.5 (I)	0.5 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5
	STC318	ND	ND	2	233	ND	0.5 (I)	1 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC403	ND	ND	2	233	ND	1 (I)	0.75 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5
	STC405	F	60	2	233	ND	0.25 (I)	0.25 (S)	0.5 (S)	≤0.05 (S)	≤0.5
	STC407	M	62	2	1713	ND	0.5 (I)	0.5 (S)	≤0.25 (S)	≤0.05 (S)	≤0.5

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Outbreak	STC78	M	67	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1.5 (I)	3 (NS)	0.19 (S)	≤0.5
	STC79	M	73	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1 (S)	3 (NS)	0.25 (S)	≤0.5
	STC80	F	72	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1 (S)	6 (NS)	0.25 (S)	≤0.5
	STC81	M	68	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1 (S)	6 (NS)	0.25 (S)	≤0.5
	STC83	M	75	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.5 (I)	0.75 (S)	6 (NS)	0.19 (S)	≤0.5
	STC84	F	62	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	0.75 (S)	4 (NS)	0.125 (S)	≤0.5
	STC85	F	50	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1 (S)	6 (NS)	0.19 (S)	≤0.5
	STC86	M	64	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1 (S)	4 (NS)	0.19 (S)	≤0.5
	STC88	M	67	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	0.75 (S)	6 (NS)	0.19 (S)	≤0.5
	STC89	M	73	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.5 (I)	1 (S)	6 (NS)	0.25 (S)	≤0.5
	STC90	F	66	2	1656	*erm*(A), *erm*(B),	0.75 (I)	1 (S)	6 (NS)	0.25 (S)	≤0.5

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						optrA, *tet*(O)					
	STC91	F	66	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	0.75 (S)	4 (NS)	0.19 (S)	≤0.5
	STC92	F	72	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.75 (I)	1 (S)	8 (NS)	0.19 (S)	≤0.5
	STC93	M	89	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.5 (I)	0.75 (S)	4(NS)	0.19 (S)	≤0.5
	STC104	F	67	2	1656	*erm*(A), *erm*(B), *optrA*, *tet*(O)	0.5 (I)	0.75 (S)	6 (NS)	0.25 (S)	≤0.5

*Only STC128, STC260, STC315, and outbreak strains were conducted whole-genome analysis; ND = Not determine

Linezolid < 2 ug/ml = susceptible

Information on outbreak strains was retrieved from Brizuela et al., 2023 [5]

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