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Original Article

Identification, genotyping, and genomic comparison of *Streptococcus porcinus* isolated from diseased swine in Brazil

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Abstract

Introduction: *Streptococcus porcinus* is considered a zoonotic opportunistic pathogen for several animal species, including swine, and can cause systemic clinical conditions. There are morphological similarities between streptococcal species, leading to possible incorrect diagnosis and inappropriate treatment choice. Here, we describe the identification, and genotypic and genomic characterization of *S. porcinus* isolated from 5 adult sick pigs from Minas Gerais and São Paulo (Brazil) between 2010–2017.

Methodology: *Streptococcus* spp. strains were isolated from vaginal discharge, joint abscess, and brain tissue received for routine diagnosis. They were identified as *S. porcinus* by mass spectrometry and partial sequencing of the *groEL* gene. Genotypic characterization by amplified fragment length polymorphism was performed. In addition, the genome of one of the *S. porcinus* invasive strains was sequenced and comparative analyses were performed.

Results: Genotyping revealed that the nervous system and joint abscess invasive strains had higher genetic similarity and clustered separately from vaginal discharge strains. Genome sequencing of one of the invasive strains revealed the presence of genes conferring resistance to erythromycin, tetracycline, lincosamides, and macrolides. A high level of similarity of the Brazilian strain genome with British and American strains was found. However, these strains also presented higher variation in their accessory genomes.

Conclusions: The circulation of *S. porcinus* invasive and resistant strains, and the lack of its identification demands attention, posing a risk for animals and workers in the swine industry.

Key words: *Streptococcus porcinus*; swine; MALDI-TOF MS; resistance; genotyping.

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Introduction

Streptococcus porcinus is a Gram-positive, anaerobic, facultative, and hemolytic coccus belonging to the Lancefield E, P, U, and V groups. This species was first described in 1985 and isolated from swine [1]. Group E *Streptococcus* has already been associated with diseases of various animal species such as cows, horses, goats, canines, and rabbits. *S. porcinus* is considered an opportunistic pathogen in swine, and can cause systemic clinical conditions that may start as pyogenic infections, leading to the appearance of edematous lymph nodes and hemorrhagic abscesses, further progressing to pneumonia and sepsis. The pathogen has also been isolated from vaginal secretions of sows with endometritis [2–4]. In Brazil, this bacterium was described only once, so far, from an isolate of suppurative myositis in sow identified by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [5].

S. porcinus also has zoonotic potential, with reports of genitourinary infection in women and related neonatal death [6,7]. Moreover, other studies have indicated horizontal gene acquisition from other pathogenic streptococci [8], which can increase virulence or resistance characteristics and become strains with higher pathogenic potential for pigs or humans.

Biochemically, *S. porcinus* is characterized by positive reaction for mannitol, sorbitol, and the leucine aminopeptidase test, and negative reaction for hippurate

[9]. Due to the low specificity of biochemical tests as an identification method and the zoonotic potential that this bacterial species presents, identification with more specific and practical methods is necessary [10,11]. In addition, information at the genotypic, epidemiological, and genomic levels is required to better understand *S. porcinus* as a pathogen.

The aim of the present study was to characterize *S. porcinus* isolated from diseased pigs in Brazil, through genotyping and complete genome sequencing for epidemiological and antimicrobial resistance features assessment. Furthermore, we verified the applicability of MALDI-TOF mass spectrometry as a quick and practical tool for identifying this bacterium.

Methodology

Bacterial strains

Five strains with morphological characteristics suggestive of *Streptococcus* spp. were isolated from vaginal discharge (SP0816-4P, SP5117, SP5017), joint abscess (SP8310), and brain (SP0816-2) samples from distinct diseased pigs in the years of 2010, 2016, and 2017. The animals originated from 5 herds in the states of Minas Gerais (MG) and São Paulo (SP). Each sample was inoculated in 4 mL of brain-heart infusion (BHI) broth (Difco, Sparks, MD, USA) enriched with 5% fetal bovine serum, and in sheep blood agar (5%), and incubated in aerobic conditions for 24 hours at 37 °C. Two aliquots (1 mL) were separated from this culture for DNA and ribosomal protein extraction.

Identification by mass spectrometry

The ribosomal protein extraction, for bacterial identification by MALDI-TOF MS, was done following the protocol described by Hijazin *et al.* [12]. The α-cyano matrix (10 mg/mL α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonics, Inc. Billerica, MA, USA) was added to protein extracts and subjected to Microflex™ mass spectrophotometer (Bruker Daltonics, Inc. Billerica, MA, USA). Spectra were identified using the BioTyper™ 3.0 software (Bruker Daltonics, Inc. Billerica, MA, USA), following the manufacturer's recommendations. Log scores > 2.0 were considered reliable for identification at the species level.

DNA extraction

DNA extraction was performed by the protocol described by Boom *et al.* [13], following a 1-hour digestion at 37 °C with lysozyme (100 mg/mL) and proteinase K (20 mg/mL) (US Biological, Swampscott, MA, USA). The extracted DNA was stored at -20 °C until use.

Partial sequencing of the groEL gene

The partial amplification of the *groEL* gene was performed to confirm identification, using the primers published by Glazunova *et al*. [14]. The amplicons were purified with Illustra GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare do Brasil Ltd, São Paulo, Brazil) and sent to the Human Genome Research Center (University of São Paulo) for further sequencing. Phylogenetic analysis was performed with Mega X [15] using the maximum-likelihood method and 500 bootstrap replicates for branch support statistical inference.

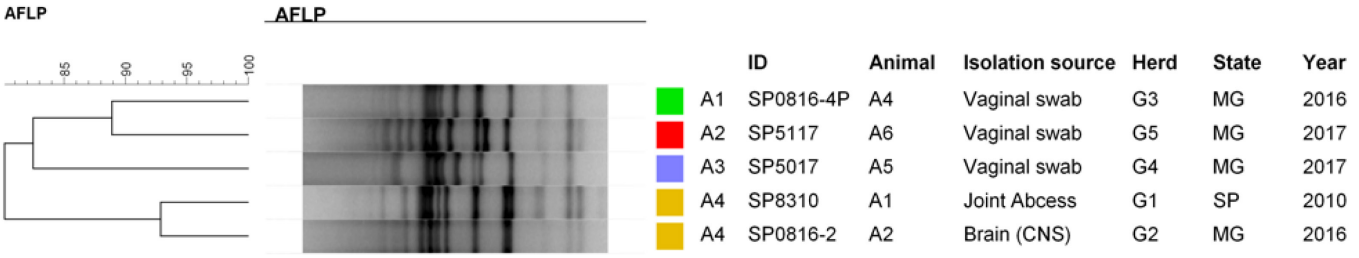
Genotyping by amplified fragment length polymorphism (AFLP)

The strains were genotyped by the AFLP technique with a single restriction enzyme (SE-AFLP) — *Hind*III (New England Biolabs Inc., Ipswich, MA, USA) — following the protocol reported by McLauchlin *et al.* [16]. The DNA fragments were detected by 2% agarose gel electrophoresis at 90 V for 3 hours, and stained with BlueGreen® (LGC Biotechnology, Cotia, SP, Brazil). The images were captured under UV illumination by the Gel Doc XR system (Bio-Rad Laboratories, Hercules, CA, USA). The amplified fragments were identified based on the molecular weight marker 100 bp DNA Ladder® (LGC Biotechnology, Cotia, SP, Brazil), and the cluster analysis of the restriction profiles was performed using BioNumerics 7.6 software (Applied Maths NV, Sint-Martens-Latem, Belgium). A dendrogram was constructed using the Dice coefficient and unweighted pair-group method using arithmetic average (UPGMA). A 90% genetic similarity cut-off was used to distinguish SE-AFLP profiles [17].

Genomic analysis

The invasive strain SP0816-2 (a pure isolate from brain tissue) was submitted for genome sequencing and comparative analysis. The genomic DNA was extracted with DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The library preparation with Nextera™ DNA Sample Prep Kit (Illumina®, San Diego, CA, USA) and the paired-end 150 bp Illumina® NextSeq sequencing was performed at the Core Facility for Scientific Research - University of São Paulo (CEFAP-USP).

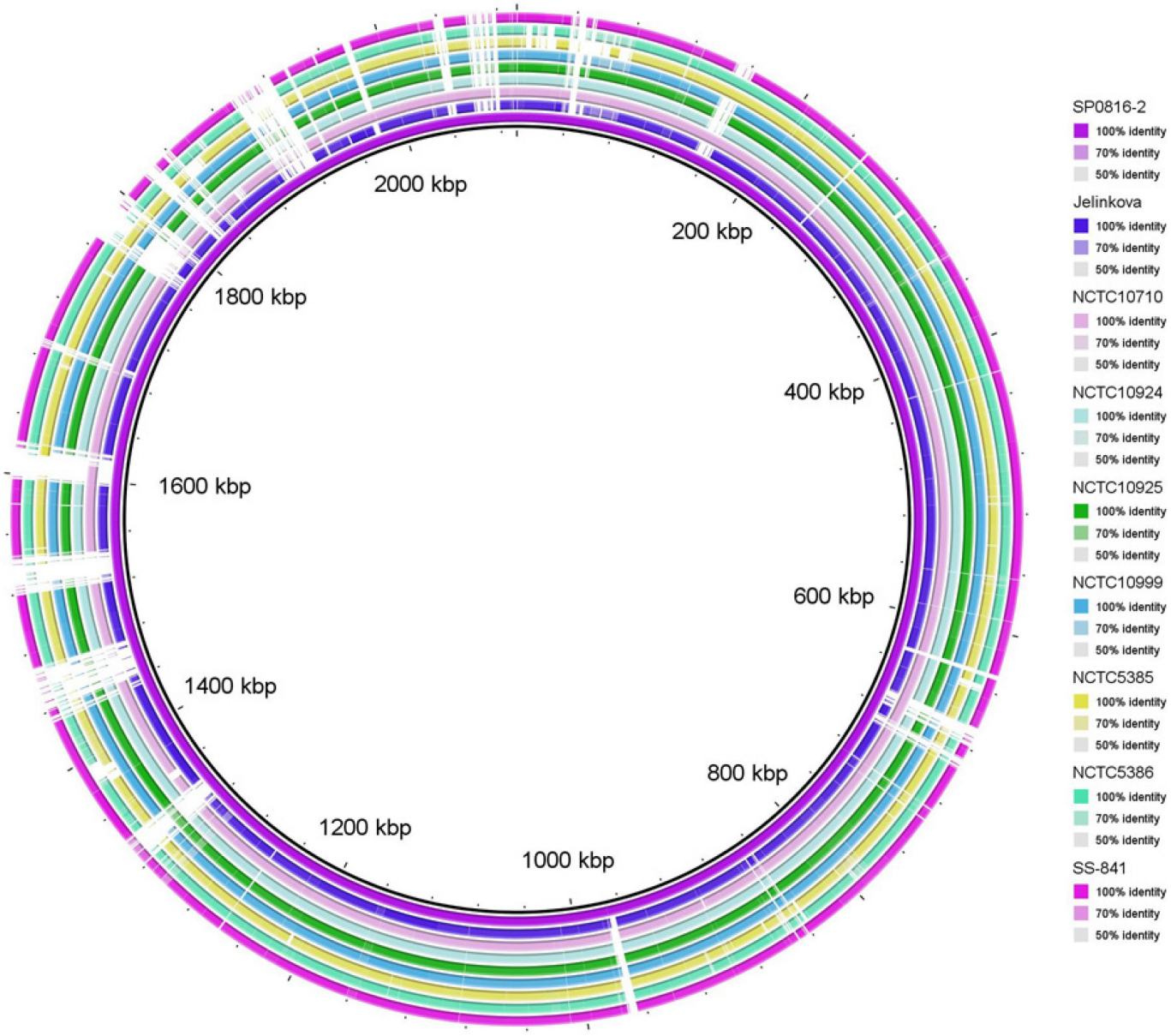
Figure 1. Dendrogram showing the relationship between *S. porcinus* SE-AFLP profiles.



	ID	Animal	Isolation source	Herd	State	Year
A1	SP0816-4P	A4	Vaginal swab	G3	MG	2016
A2	SP5117	A6	Vaginal swab	G5	MG	2017
A3	SP5017	A5	Vaginal swab	G4	MG	2017
A4	SP8310	A1	Joint Abcess	G1	SP	2010
A4	SP0816-2	A2	Brain (CNS)	G2	MG	2016

SE-AFLP: single restriction enzyme amplified fragment length polymorphism; CNS: central nervous system; MG: Minas Gerais; SP: São Paulo.

Figure 2. Whole-genome sequencing analysis of *S. porcinus* — BRIG plot displaying genomic similarities.



Base-calling, trimming, and *de novo* assembly was performed with CLC Main Workbench 7.5.1 (QIAGEN CLC Genomics Workbench - https://digitalinsights.qiagen.com). Automatic genome annotation was performed with Prokka [18]. The *in silico* detection of antibiotic resistance genes and plasmid markers were performed with ResFinder 3.2 and PlasmidFinder 2.1 [19,20]. Pan-genome analysis was performed with Roary [21] and Geneious tree builder (Geneious R10, https://www.geneious.com) was applied for core genes alignment phylogenetic analysis. Complementary, circular comparison images were generated by BRIG BLAST Ring Image Generator program [22] and the Interactive Tree of Life (iTOL) v4 [23] was used to draw an integrative datasets tree.

Results

Strains identification

The studied strains were identified as *S. porcinus* by MALDI-TOF MS. Phylogenetic analysis of partial *groEL* sequence corroborated the mass spectrometry identification, and clustered the studied isolates with the reference strain CIP 103218 (over 97% of identity). Furthermore, the SP5117 and SP5017 strains that were isolated from vaginal discharge in 2017 clustered separately from the remaining Brazilian *S. porcinus* strains (Supplementary Figure 1). The DNA sequences from this study were deposited in GenBank under accession numbers MT784753–MT784757.

SE-AFLP genotyping

SE-AFLP analysis resulted in 2 main clusters with over 80.0% genetic similarity and 4 distinct profiles (A1–A4) (Figure 1). The first cluster comprised the isolates from vaginal discharge distributed in three SE-AFLP profiles (A1–A3), while the second group comprised the SP8310 and SP0816-2 strains, which were isolated from the joint abscess and central nervous system (CNS) disorder over a 6-year interlude, clustering into the A4 profile. This suggests a higher similarity between invasive *S. porcinus* strains.

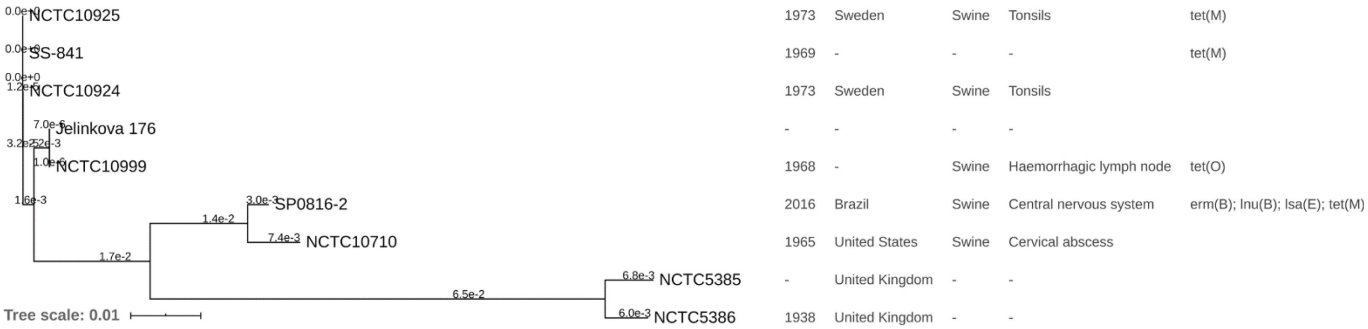
Comparative genomic analysis

The SP0816-2 strain sequencing resulted in a total of 7,538,880 reads, with over 200X coverage. The *de novo* assembly resulted in 43 scaffolds, with N_{50} of 115,372 bp and N_{75} of 49,378 bp, 36.34% of GC content, and an estimated genome length of 2,093,169 bp. In total, 2,053 genes were identified, including 2,020 coding DNA sequences (CDSs), 3 rRNAs, 29 tRNAs, and 1 tmRNA. The SP0816-2 draft was deposited in GenBank under the accession number JACEGE000000000.

The comparative analysis included the genome sequences of *S. porcinus* strains NCTC10999 (NZ_LS483388.1), NCTC10710 (NZ_LR594036.1), NCTC5385 (NZ_LR594035.1), NCTC10925 (NZ_LR594050.1), NCTC10924 (NZ_LR594052.1), NCTC5386 (CABEHT000000000.1), Jelinkova_176 (AEUU00000000.2), and SS-841 (FZQN00000000.1) (Figure 2). Regarding resistance genes, the *lsa*(E), *lnu*(B), and *erm*(B), associated with lincosamides and macrolides resistance; and *tet*(M), which is associated with tetracycline resistance; were identified in the SP0816-2 strain. The remaining *S. porcinus* genomes presented only *tet*(M) (NCTC10925 and SS-841) or *tet*(O) (NCTC10999) (Figure 3). No plasmid markers were detected among *S. porcinus* genomes.

S. porcinus core genome was comprised of 730 genes, out of a total of 4,566 evaluated genes. The SP0816-2 strain presented higher similarity with NCTC10710 reference strain, NCTC5386, and NCTC5385, based on core genes phylogenetic analysis (Figure 3). However, these 4 strains also presented higher variation in their accessory genomes (Supplementary Figure 2). The SP0816-2 strain presented 242 unique genes in its accessory genome, of which 83.9% codify hypothetical proteins. The remaining accessory genes codify toxin PezT; antitoxin PezA; lipoprotein-releasing system ATP-binding

Figure 3. Core-genome phylogenetic tree of studied *S. porcinus.*



protein; lipid A export ATP-binding/permease protein; streptomycin 3"-adenylyltransferase; cadmium, cobalt, and zinc/H(+)-K(+) antiporter; putative acetyltransferases; tyrosine recombinase; putative ABC transporter ATP-binding proteins, and some dispersed IS3 family transposases.

Discussion

Although *Streptococcus suis* is the most frequent cause of systemic diseases in pigs, the morphological similarity of *Streptococcus* spp. colonies can cause failures in the diagnosis, with strains of *S. porcinus* being misidentified or classified as *Streptococcus*-like. That is why fast and specific techniques such as mass spectrometry are a good choice to identify this pathogen. Recent descriptions have shown MALDI-TOF mass spectrometry as a great tool for the diagnosis of various *Streptococcus* species including *S. porcinus* [24], and our findings corroborate its effectiveness.

Few genotyping analyses with fingerprint techniques have been reported for *S. porcinus*. Abdulmawjood *et al*. [25] were the only ones to use a fingerprint technique on this species applying restriction fragment length polymorphism (RFLP) in 16S rDNA amplified products to differentiate it from other *Streptococcus* species. In our study, molecular typing was performed with the SE-AFLP technique that enabled the differentiation of *S. porcinus* into four profiles mainly distinguishing vaginal discharge isolates from invasive ones.

Weinert *et al.* [26] and Estrada *et al.* [27] described a *S. suis* pathotypes categorization system based on the isolation sites, in which strains were classified as "pathogenic" (invasive disease-causing), "possibly opportunistic" (non-invasive disease-causing strains), and "commensals" (isolates from the upper respiratory tract of asymptomatic animals). By applying this classification for our *S. porcinus* strains, the three SE-AFLP profiles of vaginal discharge isolates (A1–A3) would be identified as "possibly opportunistic", while the remaining A4 profile would be "pathogenic" comprising isolates from CNS and joint abscess.

Previous studies using the AFLP technique for genotyping *S. suis* affecting pigs on a large scale have also shown the tendency to cluster strains according to their invasiveness characteristics [28]. Considering these data, our results may initially propose a tendency of clustering *S. porcinus* strains due to their pathogenic potential. Furthermore, Monteiro *et al.* [5] isolated *S. porcinus* from suppurative myositis from deceased sows. This data corroborates the potential ability of *S. porcinus* to cause disease in swine. In spite of this, further studies are necessary with more strains to understand the species pathogenicity and spread dynamics.

Recently, Wang *et al.* [4] reported a multi-drug-resistant *S. porcinus* strain isolated from the vaginal discharge of a diseased sow. This presented resistance to aminoglycosides, quinolones, macrolides, and tetracyclines. Shewmaker *et al.* [29] also reported *S. porcinus* strains resistant to clindamycin and erythromycin, which presented the *erm(B)* gene, and in the same study, tetracycline-resistant strains were also detected but without associated resistance gene identification. Among the evaluated *S. porcinus* genomes, only tetracycline resistance genes *tet(M)* and *tet(O)* were detected on NCTC10925, SS-841, and NCTC10999 strains. The Brazilian SP0816-2 genome stands out from the remaining by containing not only the *erm(B)* and *tet(M)* genes, as previously described, but also the *lsa(E)* and *lnu(B)* genes, which are associated with pleuromutilin–lincosamide–streptogramin A and lincosamide resistance, respectively.

Interestingly, the *lsa(E)* and *lnu(B)* genes have already been reported in *S. suis* within a transposon integrative conjugative element (ICESsuNC286) [30]. This association with the dispersion of the identified resistance genes in distinct scaffolds and the presence of IS3 family transposases in the Brazilian *S. porcinus* strain SP0816-2 genome suggests the possibility of horizontal gene transfer between streptococcal species. Previous *S. suis* genomic studies have identified insertion sites in the genomes that present genetic information of other pathogenic streptococci, such as *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes*, evidencing a horizontal transmission between species [8]. This ability to transfer or receive genetic material from other bacteria is of great importance in species such as *S. porcinus* as it can increase virulence or resistance characteristics and as a result it can form strains with higher pathogenic potential for pigs or humans.

Previous studies have shown the zoonotic potential of *S. porcinus* causing urinary tract infections in women. There are also reports in which the bacteria were able to cause ruptures in the placental membranes and cervical insufficiency in pregnancy, causing premature births, neonatal death, and abortions [6,31,32]. These data are important for understanding the pathogenic potential of this bacterium, especially for technicians who work with pigs or pig products.

Conclusions

This was a preliminary study with a limited number of *S. porcinus* isolates. In spite of this, the genotypic profiles of Brazilian *S. porcinus* presented a relationship with pathotypes, separating groups between invasive and non-invasive strains. The Brazilian *S. porcinus* genome presented more resistance genes; notably genes associated with pleuromutilin, streptogramin A, and lincosamide resistance; in contrast to previously described genomes which only presented genes related to tetracycline resistance. The similarity of resistance genes context and the presence of dispersed transposases in the accessory genome of the Brazilian strain suggests the possibility of horizontal gene transfer between streptococcal species. Although *S. porcinus* is not frequently identified as a swine pathogen, the possibility of the emergence of invasive and resistant strains demands attention, posing a risk for animals and workers in the swine industry.

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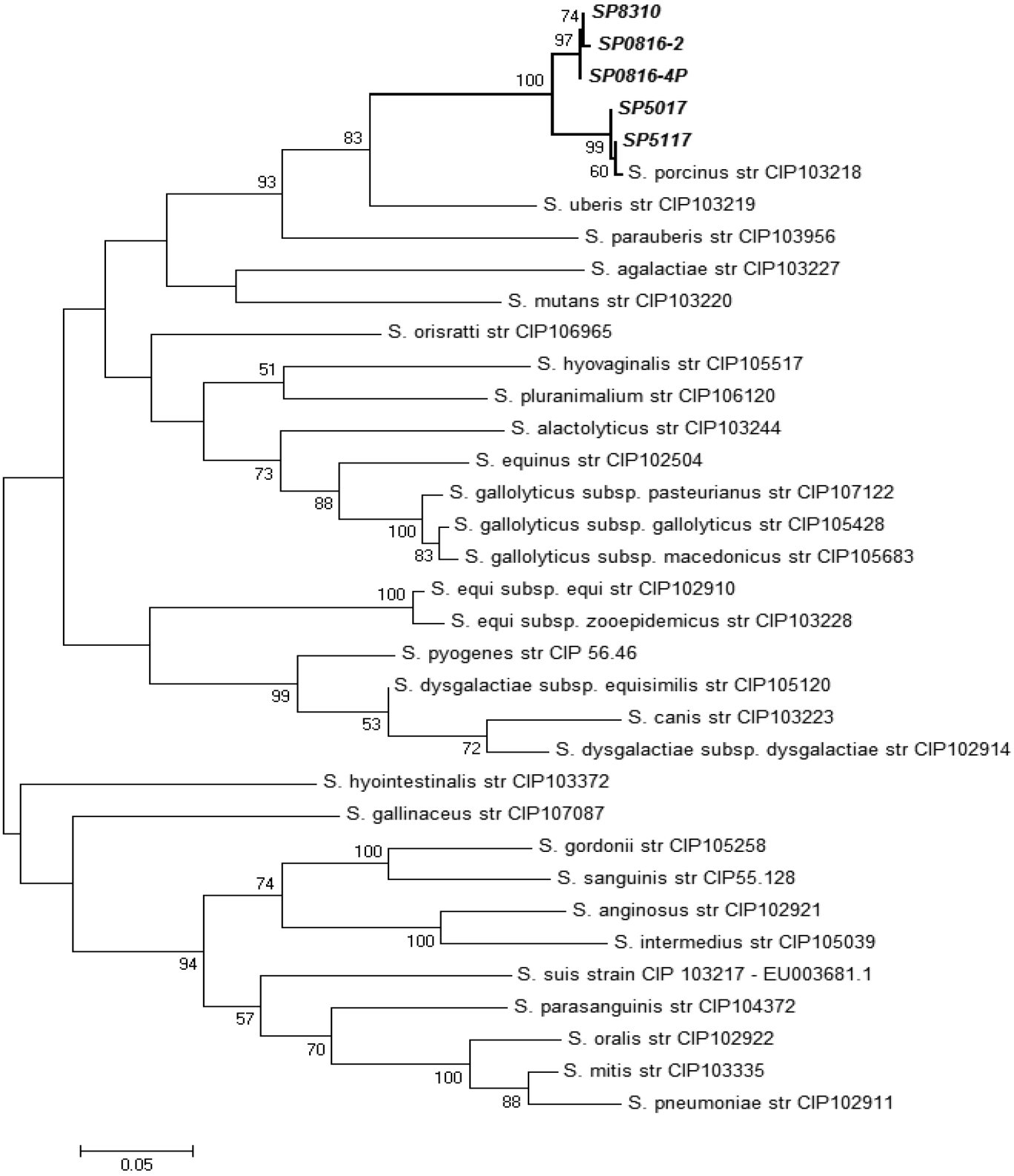
Annex – Supplementary Items

Supplementary Figure 1. Maximum likelihood tree of partial groEL sequences of *Streptococcus* spp.



Supplementary Figure 2. Pan-genome analyses of studied *S. porcinus*. Core-genome phylogenetic tree associated with Roary core and accessory genes matrix demonstrated by blue (present) and white (absent).