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Research paper

Molecular and antimicrobial susceptibility profiling of atypical *Streptococcus* species from porcine clinical specimens

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ABSTRACT

The *Streptococcus* species present broad phenotypic variation, making identification difficult using only traditional microbiological methods. Even though *Streptococcus suis* is the most important species for the worldwide swine industry, other *Streptococcus* species appear to be able to cause disease in swine and could represent a higher underestimated risk for porcine health. The aim of this study was to identify *Streptococcus*-like isolates by MALDI-TOF MS and 16S rRNA sequencing and further molecular and antibiotic susceptibility characterization of the atypical *Streptococcus* species capable of causing disease in swine. Fifty presumptive *Streptococcus* isolates from diseased pigs isolated from different Brazilian States between 2002 and 2014 were evaluated. Among the studied isolates, 26% were identified as *Streptococcus hyovaginalis*, 24% as *Streptococcus plurianimalium*, 12% as *Streptococcus alactolyticus*, 10% as *Streptococcus hyointestinalis*, and the remaining isolates belonged to *Streptococcus henryi* (6%), *Streptococcus thoraltensis* (6%), *Streptococcus gallolyticus* (6%), *Streptococcus gallinaceus* (4%), *Streptococcus sanguinis* (4%), and *Streptococcus mitis* (2%). The *Streptococcus* isolates were successfully identified by spectral cluster analysis and 16S rRNA sequencing with 96% of concordance between the techniques. The SE-AFLP analysis also supported *Streptococcus* species distinction and enabled further observation of higher genetic heterogeneity intra-species. The identified *Streptococcus* species presented variable MIC values to β-lactams, enrofloxacin and florfenicol, and high resistance rates to tetracyclines and macrolides, which appear to be directly related to the industry's antimicrobial usage and resistance selection.

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1. Introduction

The *Streptococcus* genus is composed of Gram-positive cocci that are characterized as non-motile, catalase negative and facultative anaerobes. Several pathogenic species comprise the genus and may cause serious impact on both human and animal health (Köhler, 2007). For the swine industry, however, the *Streptococcus suis* is the most important species worldwide (Goyette-Desjardins et al., 2014). Most veterinary diagnostic laboratories are limited to *S. suis* identification by species-specific PCR, while the non *S. suis* isolates are just classified as *Streptococcus*-like or *Streptococcus* sp.

The *Streptococcus* species present broad phenotypic variation, making identification difficult using only traditional microbiological methods. The application of molecular techniques, especially 16S rRNA sequencing, revolutionized the *Streptococcus* genus taxonomy with over 50 species and six species groups (Gao et al., 2014; Richards et al., 2014). However, the introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), as a rapid and accurate tool for microbiological diagnosis, appears to enable proper *Streptococcus* differentiation (Arinto-Garcia et al., 2015; Doern and Burnham, 2010; Wang et al., 2012) facilitating the identification of underestimated *Streptococcus* species that may represent a risk for animal health.

The aim of this study was to identify *Streptococcus*-like isolates, previously characterized as non *S. suis*, by MALDI-TOF MS and 16S rRNA sequencing and further molecular and antibiotic susceptibility characterization of the atypical *Streptococcus* species capable of causing disease in swine.

2. Material and methods

2.1. Samples and bacterial isolation

Fifty presumptive *Streptococcus* isolates were studied. The *Streptococcus*-like colonies were isolated from lung, heart, central nervous

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system (CNS), joint, urine, vaginal discharge, and skim of diseased pigs, from different Brazilian States between 2002 and 2014, presenting encephalitis, arthritis, pneumonia, metritis, urinary tract infection and septicemia. Porcine samples were plated on Columbia Blood Agar base (Oxoid Limited, Basingstone, Hants, England), containing 5% bovine sterile blood and the SR0126 supplement (Oxoid Limited, Basingstone, Hants, England), for *Streptococcus* selective isolation and incubated for 24 h at 37 °C. At first the isolates were characterized as *Streptococcus*-like by colony morphology and were screened for *S. suis* by PCR (Okwumabua et al., 2003).

2.2. MALDI-TOF MS bacterial identification

For MALDI-TOF MS sample preparation, ethanol/formic acid protocol (Kuhnert et al., 2012) was applied for bacterial protein extraction. The protein suspension (1 μL) was transferred to a polished steel MALDI target plate (Bruker Daltonik) and overlaid with 1 μL of matrix (10 mg α-cyano-4-hydroxy-cinnamic acid ml − 1 in 50% acetonitrile/ 2.5% trifluoroacetic acid). Bacterial mass spectra (2–20 kDa range) were acquired using Microflex™ mass spectrometer (Bruker Daltonik). Each sample was distributed over three spots and measured twice.

Primarily, for MALDI-TOF MS identification, the captured spectra were loaded into MALDI BioTyper™ 3.0 and compared with the manufacturer's library. Standard Bruker interpretative criteria were applied; scores ≥2.0 were accepted for species assignment and scores ≥1.7 but ≤2.0 for genus identification. For further analysis, the spectral replicates were used to generate a main spectrum for each isolate in BioNumerics 7.5 (Applied Maths). The cluster analysis was performed using the number of different peaks detected and UPGMA method to generate a dendrogram.

2.3. Species confirmation by 16S rRNA sequencing

For the species confirmation, 16S rRNA sequencing was performed on all studied isolates. DNA extraction was performed according to Boom et al. (1990) protocol with previous enzymatic treatment with lysozyme (100 mg) and proteinase K (20 mg) (US Biological) at 37 °C for 60 min. Twomey et al. (2012) primers were applied for partial gene amplification and sequencing was performed by the Human Genome Research Center (University of São Paulo, Brazil). A phylogenetic tree was constructed using the maximum-likelihood method by Mega 5.10 (Tamura et al., 2011). The DNA sequences from this study were deposited in GenBank under accession numbers KR819485, KR819488, KR819489, KR819493 - KR819502, KX485314 - KX485315, KX500122 - KX500156.

2.4. SE-AFLP genotyping

Single enzyme amplified fragments length polymorphism (SE-AFLP) was applied for *Streptococcus* genotyping according to McLauchlin et al. (2000) protocol. DNA fragments were detected through electrophoresis at 24 V for 26 h in 2% agarose gel stained with BlueGreen® (LGC Biotecnologia, São Paulo, Brazil). SE-AFLP fingerprints were analyzed using the Dice coefficient by means of Bionumerics 7.5 software (Applied Maths NV, Saint-Martens-Latem, Belgium) to generate the dendrogram. A cut-off value of 90% of genetic similarity was applied for clusters analysis (van Belkum et al., 2007).

2.5. Antimicrobial susceptibility profiling

The minimal inhibitory concentration (MIC) was determined by broth microdilution technique as recommended by the Clinical and Laboratory Standards Institute for fastidious organisms (CLSI, 2013) using Sensititre® Standard Susceptibility MIC Plates BOPO6F (TREK Diagnostic Systems/Thermo Fisher Scientific, Waltham, MA, USA). *S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 29213 were used as internal quality control. The MIC_{50} and MIC_{90} values for the respective antimicrobials were determined according to Schwarz et al. (2010) for the *Streptococcus* species with more than six isolates.

3. Results

A total of 50 presumptive *Streptococcus* isolates were identified as non *S. suis* and were selected for further phenotypic and molecular analysis. Initially, the isolates were grouped according to their isolation sites (Table 1). MALDI-TOF MS successfully identified all isolates with log (score) values > 2.0. Among the fifty studied isolates, 26% were identified as *Streptococcus hyovaginalis*, 24% as *Streptococcus plurianimalium*, 12% as *Streptococcus alactolyticus*, 10% as *Streptococcus hyointestinalis*, and the remaining isolates belonged to *Streptococcus henryi* (6%), *Streptococcus thoraltensis* (6%), *Streptococcus gallolyticus* (6%), *Streptococcus gallinaceus* (4%), *Streptococcus sanguinis* (4%), and *Streptococcus mitis* (2%) (Table 1).

The MALDI-TOF MS spectral cluster analysis enabled the distinction of the *Streptococcus* isolates according to the identified species (Fig. 1). The 16S rRNA sequencing presented 96% agreement with MALDI-TOF MS for species identification; only two *S. plurianimalium* isolates were misidentified as *S. hyovaginalis* by MALDI-TOF MS (SS224 and SS296). The topology of both spectral dendrogram and phylogeny tree was also maintained (Fig. S1), demonstrating the closest relationship between the clusters comprised by: *S. hyovaginalis*, *S. plurianimalium* and *S. thoraltensis*; *S. gallolyticus* and *S. alactolyticus*; *S. mitis* and *S. sanguinis*.

Table 1
Distribution of *Streptococcus* species according to isolation site and identified species.

Species Identification	Isolation site					Total N (%)
	CNS[a] N (%)	Respiratory N (%)	Genitourinary N (%)	Joints N (%)	Others[b] N (%)	
S. hyovaginalis	3 (37.5)	0 (0.0)	10 (45.5)	0 (0.0)	0 (0.0)	13 (26.0)
S. plurianimalium	3 (37.5)	0 (0.0)	5 (22.7)	1 (100.0)	3 (60.0)	12 (24.0)
S. alactolyticus	0 (0.0)	4 (28.6)	2 (9.1)	0 (0.0)	0 (0.0)	6 (12.0)
S. hyointestinalis	0 (0.0)	5 (35.7)	0 (0.0)	0 (0.0)	0 (0.0)	5 (10.0)
S. henryi	0 (0.0)	1 (7.1)	0 (0.0)	0 (0.0)	2 (40.0)	3 (6.0)
S. thoraltensis	0 (0.0)	0 (0.0)	3 (13.6)	0 (0.0)	0 (0.0)	3 (6.0)
S. gallolyticus	2 (25.0)	0 (0.0)	1 (4.5)	0 (0.0)	0 (0.0)	3 (6.0)
S. sanguinis	0 (0.0)	2 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	2 (4.0)
S. gallinaceus	0 (0.0)	1 (7.1)	1 (4.5)	0 (0.0)	0 (0.0)	2 (4.0)
S. mitis	0 (0.0)	1 (7.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)
Total	8 (100)	14 (100)	22 (100)	1 (100)	5 (100)	50 (100)

[a] CNS - central nervous system.
[b] Others – peritoneum, heart or skin lesion.

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