Group C Streptococcus dysgalactiae subsp. equisimilis in south-east Brazil: genetic diversity, resistance profile and the first report of human and equine isolates belonging to the same multilocus sequence typing lineage

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Streptococcus dysgalactiae subsp. equisimilis (SDSE) isolates are the most common group C streptococci in humans and reports of invasive infections associated with SDSE have been increasing. Molecular epidemiology studies are an important strategy to trace the emergence and spread of possible well-fit bacterial pathogens of humans and animals. In this work, we analysed the antimicrobial and clonal profiles of 115 SDSE infection and colonization isolates of human and equine origin. PFGE revealed the spread of two main clusters: clone A (57.4%) and clone A (26.1%). Remarkably, two isolates from clone B obtained from human colonization cases displayed identical PFGE patterns to those of three equine infection isolates. In addition, multilocus sequence typing allocated these isolates to ST129 (CC31). All of the SDSE isolates were susceptible to penicillin, vancomycin, gentamicin, levofloxacin and chloramphenicol. Tetracycline and erythromycin resistance rates were 65.2 and 13.9% respectively. Nevertheless, none of the isolates displaying sporadic PFGE patterns showed erythromycin resistance. The majority of erythromycin-resistant isolates from clone A had inducible resistance to macrolides, lincosamines and streptogramins B (iMLS B phenotype), which is associated with the presence of the ermA gene, whereas the resistant isolates from clone B showed the M phenotype, associated with the mefA gene. In conclusion, the data indicated that the analysed collection of SDSE isolates displayed a clonal structure and that the isolates found in human colonization cases could also be involved in equine infections.

INTRODUCTION

Streptococcus dysgalactiae subsp. equisimilis (SDSE) has increasingly been recognized as the aetiological agent of several human invasive infections worldwide (Bruun et al., 2013; Sunaoshi et al., 2010; Takahashi et al., 2010, 2011; Tsai et al., 2014). SDSE isolates are frequently classified as Lancefield group C or G. Comparative genomic analyses revealed that several commonly found group A streptococci virulence factors have also been detected in SDSE isolates, including C5a peptidase, M protein, glyceraldehyde 3-phosphate dehydrogenase, hyaluronidase, streptokinase, streptolysin O and streptolysin S, amongst others (Reissmann et al., 2012; Sunaoshi et al., 2010; Suzuki et al., 2011; Watanabe et al., 2013). Consequently, human infections related to SDSE resemble those associated with group A streptococci (McMillan et al., 2010). These infections include pharyngitis, septic arthritis, pneumonia, bacteraemia, endocarditis, meningitis, streptococcal toxic shock-like syndrome, cellulitis and necrotizing fasciitis (Brandt & Spellerberg, 2009). Similar to group A streptococci, SDSE has also been linked to post-infectious sequelae such as glomerulonephritis and rheumatic fever (Takahashi et al., 2011). Although SDSE isolates remain susceptible to penicillin, different studies have reported an increased prevalence of antimicrobial resistance to non-β-lactam agents clinically employed for streptococcal infections, including tetracycline, clindamycin and erythromycin (Kim & Uh, 2004; Rato et al., 2010; Sunaoshi et al., 2010; Takahashi et al., 2011).

It is well known that SDSE isolates can also be associated with equine and other animal diseases, including strangles,
mastitis, and wound and genital infections (Erol et al., 2012; Facklam, 2002; Fernández et al., 2004). However, zoonotic infections caused by group C or G streptococci are rare, often involving *Streptococcus equi* subsp. *zooepidemicus*, and generally associated with human-to-animal transmission or the consumption of unpasteurized dairy food (Beres et al., 2008; Brandt & Spellerberg, 2009; Facklam, 2002). To the best of our knowledge, zoonotic infections caused by SDSE have not yet been described. A recent study described the isolation of an identical strain colonizing the oropharynx of a child and dog from the same household in an aboriginal community in Australia. However, no infectious process was reported in either the child or pet (Schriebel et al., 2014). To better understand the epidemiological aspects of SDSE infections, it is important to trace the occurrence and spread of possible well-adapted SDSE clones. However, there is little data in the literature concerning the molecular epidemiology of SDSE. Indeed, the vast majority of these studies involve group G SDSE isolates (Davies et al., 2007; Pinho et al., 2006; Rantala et al., 2010; Takahashi et al., 2010).

The aim of this study was to analyse the genetic relationship and antimicrobial susceptibility patterns amongst SDSE isolates obtained from different human and equine clinical sites separated by a considerable geographical distance and a period of three decades.

**METHODS**

**Bacterial isolates.** In this study, a convenience collection of 115 SDSE isolates of group C streptococci obtained from 1979 to 2008 in different cities in south-east Brazil was analysed. Amongst the human isolates, 54 were collected from the throats of carriers, and the remaining 50 were obtained from symptomatic oropharynx infections (n=26), wound secretions (n=4), undetermined infection sites (n=3), blood (n=3), tracheal secretions (n=1), abscess drainage (n=1) and vaginal secretions (n=1); the clinical origin (colonization or infection) of an additional 11 isolates was unknown. The detection of anti-streptococcal antibody titres is not recommended in the routine diagnosis of acute pharyngitis, as they reflect past but not current events (Shulman et al., 2012), and therefore the criteria used to judge these SDSE infections was the isolation of SDSE as the only bacterial pathogen in blood cultures of symptomatic patients. However, this criteria does not rule out the presumption that some of these SDSE isolates were merely colonizers. Of the 104 isolates from humans, the majority were from Rio de Janeiro, RJ (n=63), followed by the cities of Angra dos Reis, RJ (n=17), Ribereiro Preto, SP (n=5), Guarapetes, SP (n=2) and Niterói, RJ (n=1), as well as 16 others from unknown regions. The equine isolates (n=6) were obtained from abscess drainage (n=1) and uterine secretions (n=5) in diseased animals from Seropédica, RJ. The host origin (animal or human) was not reported for isolates obtained from two vaginal secretions, two wound secretions and one unidentified clinical sample. Details of the isolates are provided in Table S1 (available in the online Supplementary Material). All of the experiments were performed as blind tests.

**Species identification.** The isolates had been sent previously to our laboratory as group C SDSE. The confirmation of the identification was initially performed by routine tests for colony morphology, *β*-haemolysis, Gram staining and catalase activity. Lancefield serogrouping was performed using a SlideX Strep Plus latex agglutination test (bioMérieux). The bacterial species identifications were done as recommended by the Centers for Disease Control and Prevention (http://www.cdc.gov/streplab/strep-doc/index.html) and with an API 20 Strep system (bioMérieux).

**Antimicrobial susceptibility tests.** Kirby–Bauer testing was performed according to the Clinical and Laboratory Standards Institute guidelines using Mueller–Hinton agar supplemented with 5 % defibrinated sheep blood (CLSI, 2014), and erythromycin (15 µg), clindamycin (2 µg), tetracycline (30 µg), levofloxacin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg) and vancomycin (30 µg) discs (Cecon), incubated at 37 ºC for 18 h. In addition, inducible clindamycin resistance was detected with a D test as described previously (Prabu & Menon, 2013). The MICs for the isolates that showed resistance to erythromycin and tetracycline were determined by the agar dilution method as recommended in the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014) for concentration ranges of 0.015–124 and 0.5–64 µg·ml⁻¹, respectively. In addition, the penicillin MIC was determined for all isolates for a concentration range of 0.0075–0.48 µg·ml⁻¹. MIC20 and MIC90 were the antimicrobial concentrations that inhibited 20 and 90 % of the isolates, respectively. *Streptococcus pneumoniae* ATCC 49619 was used as the reference strain for the antimicrobial susceptibility testing.

**Genotypic characterization of antimicrobial resistance.** Genes commonly associated with erythromycin (*ermB*, *ermA* and *mefA*) and tetracycline (*tetK*, *tetL*, *tetM*, *tetO* and *tetS*) resistance were assessed by PCR (Haenni et al., 2010) using DNA template obtained as described previously (Pacheco et al., 1997). The primer sequences and references are listed in Table S2. The PCR program for the erythromycin resistance genes was: initial denaturation for 2 min at 95 ºC; 30 cycles of 1 min at 95 ºC, 1 min 30 s at 55 ºC and 1 min 30 s at 72 ºC; and a final extension at 72 ºC for 7 min. The PCR program for the tetracycline resistance genes was: initial denaturation for 2 min at 95 ºC; 30 cycles of 1 min at 93 ºC, 1 min at 62 ºC and 3 min at 72 ºC; and a final extension at 72 ºC for 3 min. The strains used as positive in-house controls for the erythromycin-associated genes were 53157 (group C streptococci), 015195 (group G streptococci) and 06196 (group G streptococci); and those for the tetracycline-associated genes were 31 PSPT 81 (*Escherichia coli*), CL-5596 (group B streptococci), CL-5570 (group B streptococci) and CL-5592 (group B streptococci). In addition, a segment of the 16S rRNA gene was amplified as an internal control for the PCRs and the group C streptococci isolate 53157 was used as a positive control.

**PFGE.** Agarose plugs containing genomic DNA were prepared as described previously (Pinho et al., 2006), except that the lysis buffer contained 2.5 × 10⁻² U lysosome ml⁻¹ (Sigma-Aldrich), 5 U mutanolysin·ml⁻¹ (Sigma), 0.5 % (v/v) Brij 35 (Sigma) and 2.5 U RNase ml⁻¹ (Sigma) in Tris/EDTA buffer, pH 7.6. After Smal digestion, electrophoresis was performed in a CHEF-DRIII system (Bio-Rad) at 6.4 V·cm⁻¹ for 23 h at 11.3 ºC with the pulse time ranging from 1 to 30 s. After staining with ethidium bromide, the images were scanned with a Lumibis 1.4 Photodocumentation System (Scorpion SCOR-1450M; DNR Bio-Imaging Systems) under UV light. The PFGE patterns were evaluated with GelCompar II software (Applied Maths) to create dendrograms by the unweighted pair-group method using arithmetic averages (UPGMA). The Dice similarity coefficient was used, and, as suggested by Duck et al. (2003), the optimization and position tolerance settings were determined from the band pattern produced by running one isolate (SDSE 85-101) in all gels as a benchmark, resulting in optimization and position tolerance settings of 1.0 and 1.5 %, respectively, as the criteria. With these limitations, all of the patterns obtained for the 85-101 isolate in the different gels were aligned and considered identical. An 80 % genetic similarity cut-off was used to define the clones in the dendrogram (Pinho et al., 2006).

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Multilocus sequence typing (MLST). One representative SDSE isolate obtained from humans and another from equines displaying the same B PFGE patterns were submitted to MLST analyses. The DNA was extracted (Moore et al., 2004) and MLST was performed as described previously (Ahmad et al., 2009; McMillan et al., 2010). Further analyses included the construction of a global optimal eBURST diagram using goeBURST version 1.2.1 (http://goeburst.phyloviz.net/). In addition, a concatenation tree was constructed using PHYLIP 3.67 (http://mobyle.genouest.org/cgi-bin/Mobyle/portal.py#forms:neighbor). MLST was performed for one human isolate and one equine isolate that displayed identical PFGE patterns.

RESULTS

Population structure

The clonal structure analysis detected 15 clones (A–O) amongst the total of 115 SDSE isolates. However, most (83.5%) were grouped within two main clones: A (66 isolates) and B (30 isolates; Fig. 1). Clone A accounted for 62 of 104 (59.6%) human isolates and was detected in Rio de Janeiro, Angra dos Reis, Ribeirão Preto and Guarapes.
The remaining four clone A isolates were from undetermined hosts. Regarding the clinical origin, clone A was distributed amongst both patients (23/62; 37.1%) and carriers (30/62; 48.4%), and its distribution over the years was quite homogeneous. Before 1991, clone A accounted for 57.3% of the isolates; after 1992, for 57.7% (Table S1).

The second most prevalent PFGE pattern was clone B (n=30), which was largely detected in human isolates (n=27; 90%) collected in the cities of Rio de Janeiro and Angra dos Reis (Fig. 2). Clone B differed from the predominant clone A, which had a broader spatial distribution (Fig. 2). The vast majority of the clone B isolates were recovered before 1992 and of the 27 collected from humans, only five (18.5%) were from patients, with the majority 22 (81.5%) derived from carriers. The three remaining clone B isolates (10%) were collected from diseased equines.

Nineteen of the total 115 SDSE isolates tested were classified in 13 sporadic patterns (SpPs) named from C to O. Patterns C and D, and E and F included three and two isolates each, respectively, and only one isolate was classified in each of the nine remaining patterns (Fig. 1). SpP isolates were detected from human (n=15), animal (n=3) and unknown (n=1) hosts. Amongst the 15 human isolates grouped in SpP, only four were collected from carriers, and the majority were obtained from clinical samples such as blood (n=1), wound secretions (n=1), tracheal aspirate (n=1), vaginal secretions (n=1) and symptomatic oropharyngeal infections (n=7). In addition, the three SpP isolates from animals were collected from uterine infections.

**Comparative genotyping between human and equine isolates**

Three of the six equine isolates (50%) were classified in clone B. Interestingly, the isolates 83-060, 84-025 and 84-030 (collected from the same farm in Seropédica in 1983–1984) showed an identical PFGE band pattern as humans isolates 81-681 and 81-724 (obtained from the oropharynx of two healthy children attending the same public school in Rio de Janeiro in 1981). The MLST analyses were performed for representative human (81-681) and equine (84-030) isolates. To the best of our knowledge, there was no apparent epidemiological relationship between the human and equine cases. The MLST data also showed that these isolates were the same strain type (ST129) belonging to clonal complex CC31, which apparently has ST31 as the ancestral sequence type (Fig. 3).

**Antimicrobial resistance**

As expected, all 115 SDSE isolates (human and equine) were susceptible to penicillin, with MICs ranging from 0.0075 to 0.06 μg·mL⁻¹ (Table S1). The great majority of the isolates displayed MIC values ≤0.03 μg·mL⁻¹ (114 isolates; 99.1%). The MIC₉₀ and MIC₅₀ values for the isolates as a whole were 0.015 and 0.03 μg·mL⁻¹, respectively.

**DISCUSSION**

The 115 SDSE isolates examined in this study were distributed into 15 clones, although the vast majority were grouped into clones A and B. A study carried out in...
Portugal with 116 invasive and non-invasive SDSE isolates reported that half were allocated into three large clones (Pinho et al., 2006). In China, PFGE analyses performed with invasive SDSE isolates revealed considerable genetic diversity (Yin et al., 2012), yet three larger clones accounted for over half of the isolates and the prevalence of specific

![Fig. 3.](a) A global optimal eBURST diagram of the relationship between the SDSE isolates overall and the human and equine SDSE isolates clustered in PFGE clone B. Strain types assigned in the same clonal complex are linked by straight lines. Light green indicates the group founder and dark green signifies a subgroup founder. Light blue indicates common nodes. PFGE clone B was typed as ST129, which clustered in CC31. (b) Minimum evolutionary tree showing the position of the SDSE strains within CC31. The tree is based on the concatenated sequences of seven housekeeping genes.

Table 1. Molecular characterization of erythromycin and tetracycline resistance amongst SDSE isolates

<table>
<thead>
<tr>
<th>PFGE clone (n)</th>
<th>Erythromycin (%)</th>
<th>Tetracycline (%)</th>
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<tr>
<td></td>
<td>M*</td>
<td>iMLS₉</td>
</tr>
<tr>
<td>A (66)</td>
<td>7 (58.3)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>B (30)</td>
<td>4 (100)</td>
<td>4</td>
</tr>
<tr>
<td>SpP (19)</td>
<td>0</td>
<td>13 (92.9)</td>
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<tr>
<td>Total</td>
<td>4</td>
<td>7</td>
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*Constitutive macrolide resistance.
clones varied depending on the geographical region. In our study, the majority of the human SDSE isolates from Angra dos Reis clustered within clone B, whereas clone A predominated in Rio de Janeiro. It is possible that the increased incidence of clone B in Angra dos Reis was due to the low number of isolates collected and the fact that they were from a relatively closed community (military school). The clone A isolates were the most prevalent SDSE, corresponding to ~57% of the isolates and periodically detected throughout the 30 year study period. This contrasts with clone B, which was primarily detected before 1991. It is of note that the clone A isolates from this collection were not involved in outbreaks. Therefore, some genomic characteristics of this specific clone might be responsible for its predominance amongst the colonized and diseased humans in south-east Brazil. It is possible that the isolates of this clone have acquired specific traits and strategies associated with an improved fitness.

In 1996, *S. dysgalactiae* was divided into two subspecies (Vandamme et al., 1996). The species SDSE was proposed for human isolates with vaginal and respiratory tract habitats, whilst *Streptococcus dysgalactiae* subsp. *dysgalactiae* was suggested for the animal isolates. Nevertheless, an SDSE strain (group C; ST3) was detected colonizing both a dog and child in the same household in an aboriginal community in Australia, illustrating the potential for cross-transmission between humans and animals (Schriever et al., 2014). Analysing a small collection of SDSE isolates from equines at a farm located in Seropédica, we found that three of the isolates clustered in clone B. These animal isolates were collected from abscess and uterine secretions, and had a PFGE pattern identical to that of two isolates from human colonization cases.

Our data revealed a possible genetic link between the human and equine PFGE clone B isolates. The absence of an apparent epidemiological relationship between the children and infected equines excludes the possibility of cross-transmission of the ST129 isolates between them. In addition to causing animal diseases, the human clone B isolates were also collected from a wound infection and symptomatic pharyngitis, suggesting the potential of these isolates to infect both hosts.

Studies by Biedenbach et al. (2006) investigating β-haemolytic streptococci from Europe and the USA found two SDSE isolates with a high penicillin MIC of 0.25 μg·ml⁻¹, suggesting the importance of continuous monitoring of the penicillin MIC amongst SDSE isolates. However, in our collection the highest MIC detected was 0.06 μg·ml⁻¹. These isolates were also susceptible to gentamicin, vancomycin, chloramphenicol and levofloxacin. However, an increased resistance rate for levofloxacin (12%) was detected from 1998 to 2005 amongst SDSE isolates from Portugal (Pinho et al., 2010). Tetracycline no longer represents an option for the empirical treatment of SDSE-associated diseases due to the commonly observed high resistance rate (Brandt & Spellerberg, 2009). We also detected a high level of tetracycline resistance and all except one of the tetracycline-resistant isolates harboured tetM.

Regarding erythromycin, differences in the resistance levels were found amongst the PFGE clones. Despite the fact that isolates of the predominant clones, A and B, showed similar resistance rates of 18.2 and 13.7%, respectively, none of the isolates displaying sporadic PFGE patterns showed erythromycin resistance. This phenomenon may be the consequence of an increased selective pressure on the more adapted (i.e. more frequently detected) SDSE lineages. Macrolide antibiotics are important therapeutic drugs in treating streptococcal infections, especially for patients with β-lactam hypersensitivity. The overall resistance rate to erythromycin in our study was similar to that reported in south India (12.2%) for group C and G streptococci from humans (Prabu & Menon, 2013). However, a higher level of macrolide resistance (26.4%) was detected for SDSE in France from 2006 to 2010 (Loubinoux et al., 2013).

In our study, all of the erythromycin-resistant isolates in clone A showed the iMLSB phenotype and carried the ermA gene. As also found by others (Clancy et al., 1996; Weisblum, 1995), combinations of ermA and mefA genes were detected amongst the erythromycin-resistant isolates with the iMLSB phenotype. This inducible resistance was not found amongst the clone B isolates, which displayed the M phenotype and generally only carried the mefA gene. As reported previously, it has been verified that the M phenotype is linked to the mefA gene, which is involved in an active efflux pump system for 14- and 15-membered macrolides and lincosamines (Clancy et al., 1996), whilst the ermA gene is correlated with the iMLSB phenotype (Weisblum, 1995). A previous study (Prabu & Menon, 2013) reported rates of 9.2 and 3.1% for the iMLSB and M phenotypes, respectively, which are similar to those found for our SDSE collection. Although ermB has been detected in other SDSE collections (Van Leer Buter et al., 2010; Prabu & Menon, 2013; Takahashi et al., 2011), the isolates from Brazil only harboured ermA, probably due to the high clonal structure of the collection.

A considerable increase in human infections associated with SDSE has been reported since the end of the last decade and studies have shown that the incidence of these isolates is similar to or even exceeds that of *S. pyogenes* in some countries (Bramhachari et al., 2010; Broyles et al., 2009). Despite our having analysed a convenience collection, the data clearly revealed some new and important information about SDSE. Even though SDSE is considered a human-associated bacteria, for the first time in the literature, to our knowledge, we have reported isolates displaying identical PFGE clones well-adapted to both colonize humans and infect equines. Indeed, one representative of the PFGE clone B from each host tested belonged to the same MLST type. The fact that no connection could be found between the infected equines and colonized children negates the possibility of a direct...
cross-transmission between them. Further studies are needed to extend these preliminary findings. Furthermore, our data suggest that the SDSE isolates from clone A have some particular adaptive traits that allow them to predominate over the years. Finally, further studies are required using large and current collections from different hosts to gain a better understanding of the SDSE transmission dynamics, zoonotic potential, clonal structure and micro-evolutionary events.

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