**Abstract**

*Streptococcus tigurinus* is a new member of the *Streptococcus viridans* group and is closely related to *Streptococcus mitis*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus oralis*, and *Streptococcus infantis*. The type strain AZ_3a\(^T\) of *S. tigurinus* was originally isolated from a patient with infective endocarditis. Accurate identification of *S. tigurinus* is facilitated only by newer molecular methods like 16S rRNA gene analysis. During the course of study on bacteraemia and infective endocarditis with reference to periodontitis and viridians group of streptococci, a strain of *S. tigurinus* isolated from subgingival plaque of a patient with periodontitis identified by 16S rRNA gene analysis, which was originally identified as *Streptococcus pluranimalium* by Vitek 2. Confirmation by 16S rRNA gene analysis showed 99.39% similarity (1476/1485 bp) with *S. tigurinus* AZ_3a\(^T\) (AORU01000002). To the best of our knowledge, this is the first report of isolation of *S. tigurinus* from the oral cavity of a periodontitis patient.

**Introduction**

*Streptococcus tigurinus* has been recently described as a novel member of the *Streptococcus mitis* group causing invasive infections (Zbinden et al., 2012a, b). Based on phenotypic and molecular analysis, *S. tigurinus* is most closely related to *S. mitis*, *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae*, *Streptococcus oralis*, and *Streptococcus infantis*. This novel species probably was not recognized in the past due to the limitations of conventional phenotypic test methods and of commercial systems (API 20 Strept and Vitek 2; bioMérieux, Marcy l’Etoile, France; Facklam, 2002; Arbique et al., 2004; Bossard et al., 2004; Haanpera et al., 2007; Spellerberg & Brandt, 2011). More recently, accurate identification of *S. tigurinus* is facilitated exclusively by introduction of nucleic acid-based molecular methods like 16S rRNA gene sequencing (Zbinden et al., 2012a, b).

Oral streptococci form the part of normal flora of the oral cavity in humans and animals. Most of these strains are thought to be commensals, and a few of them are potential opportunistic pathogens. Nearly 500 bacterial strains have been recovered from the subgingival crevice (van Winkelhoff et al., 1996; Rams et al., 1997; Kroes et al., 1999). During our investigation on subgingival microbial communities of the oral cavity and its possible role in bacteraemia in patients (n = 80) undergoing tooth extraction with focus on viridians streptococci, automated Vitek 2 (bioMérieux) system revealed identification of six uncommon isolates of *Streptococcus pluranimalium*, five from subgingival plaque samples and one from blood sample, respectively.

All the six strains of *S. pluranimalium* were selected for 16S rRNA gene analysis for more accurate identification, as *S. pluranimalium* have been reported only from cases of subclinical mastitis, genital tract and tonsils of cattle, tonsils of goat and cat, and the crop and respiratory tract of canaries (Devriese et al., 1999); however, isolation of this species from human clinical samples has not been reported so far. The 16S rRNA gene sequence-based analysis is one of the gold standards for microbial identification in clinical settings and is a valuable tool to rule out possibility of probable misidentification by existing microbial identification systems. Isolation of *S. tigurinus*
from subgingival plaque of patients with periodontitis has not been reported so far. Here, we report for the first time the isolation of *S. tigurinus* from the oral cavity.

**Materials and methods**

This study is a prospective microbiological analysis of subgingival plaque samples of patients with periodontitis and blood samples after tooth extraction. In this study, 80 patients undergoing tooth extraction were screened.

**Microbiological analysis**

Subgingival plaque samples of the tooth were collected from the gingival area of buccal and lingual tooth surfaces of affected tooth using sterile curettes into sterile transport media. Five millilitre of blood for culture was collected before tooth extraction using proper aseptic precautions (Dajani *et al.*, 1997) and 3 min after dental extraction and inoculated aseptically into brain–heart infusion broth for aerobic culture. These specimens were processed using conventional techniques (Carroll & Sbor, 1980). Cultures with bacterial growth were Gram-stained and subcultured onto special media, such as Tryptone soya blood agar supplemented with Strept Supplement (Nalidixic acid 3.750 mg, Neomycin sulfate 1.060 mg, and Polymyxin B sulfate 8500 units for 500 mL media) and Mutans–Sanguis agar (HiMedia Laboratories, Mumbai, India). Cultures with growth were further subjected to standard biochemical identification and antimicrobial susceptibilities using automated Vitek 2 (bioMérieux) system.

**DNA extraction, PCR amplification, and sequencing of 16S rRNA gene**

Selected microbial strains (*n* = 6, identified as *S. pluranimalium* by Vitek 2 system) were processed for 16S rRNA gene-based sequencing to confirm the identification of these strains. Pure bacterial colonies directly from plate were taken for DNA extraction and downstream applications. Cells suspensions prepared in Tris buffer were lysed using CTAB and proteinase K at 37 °C for 1 h. Chromosomal DNA was isolated by the standard phenol/chloroform/isoamyl alcohol (25 : 24 : 1) extraction and isopropanol precipitation method (Sambrook *et al.*, 1989). Integrity of the extracted DNA was assessed by 0.7% agarose horizontal gel electrophoresis in 0.7% agarose horizontal gel electrophoresis in TAE buffer (40 mM Tris, 20 mM acetate, and 2 mM EDTA) and visualized by etidium bromide staining. The concentration of extracted DNA was checked on NanoDrop Lite spectrophotometer (NanoDrop Biotechnologies) and a range within 100 ng was taken for amplification. The 16S rRNA gene was amplified from DNA extracted from cultured isolates using universal bacteria-specific primers: 16F27 (5′CCAGAGTTTGATCMTGGCTCAG-3′) and 16R1525 (5′-TTCTGACGCTAGAAGGAGGTGWTCCAGGCC-3′) (Dharne *et al.*, 2008). The PCR was carried out in 50 µL of reaction mixture containing 10 nM (each) primer (Eurofins), 200 µM (each) deoxynucleoside triphosphate (dNTP) (Genei), 1 U of Taq polymerase (Genei) in the appropriate reaction buffer, and 100 ng of DNA extract as a template. PCR conditions were 35 cycles of 60 s at 95 °C, 60 s at 58 °C, and 1 min 30 s at 72 °C. PCR amplicons were purified using Exo-SAP (USB) and sequenced for complete 16S rRNA gene in ABI 3500xl genetic analyzer (Invitrogen/Life Technologies) using internal primers to get appropriate overlaps of contigs (Weisburg *et al.*, 1991).

**Sequence analysis for taxonomic and phylogenetic interpretations**

The 16S rRNA gene sequences were assembled and edited using CHROMASLITE version 1.5 (www.techneleyum.com.au). Edited sequences were cross-checked with BLASTN using default parameters (Altschul *et al.*, 1997) followed by comparison with closest cultured sequences retrieved from the database. Alternately, EzTaxon database of type strains with validly published prokaryotic names (Chun *et al.*, 2007) was used to validate the species level strain identities for all strains. Multiple sequence alignments were performed using CLUSTALW version 1.8 (Thompson *et al.*, 1994), and aligned sequences were edited and corrected manually using MEGA 5 software (Tamura *et al.*, 2011) to generate an unambiguous sequence alignment. Phylogenetic dendrograms were constructed by neighbor-joining method using MEGA 6 software (Tamura *et al.*, 2013) to determine the relationship of these isolates with known sequences in database. One thousand bootstrap iterations were generated to derive a consensus tree. The 16S rRNA gene sequences from strains obtained in this study have been deposited in the GenBank under accession numbers KJ575555–KJ575560.

**Results and discussion**

This study was designed to establish the relationship between periodontitis, tooth extraction, and transient bacteraemia; accordingly, 80 patients undergoing tooth extraction were screened for periodontal status and bacteraemia after tooth extraction.

Here, we report the results of 16S rRNA gene sequencing for the identification of a novel subgingival strain in patients undergoing tooth extraction. Isolation of *S. pluranimalium* has never been reported from human clinical
samples earlier. In this study, initially six strains of S. pluranimalium were identified by the Vitek 2 automated system. To confirm the identification of these strains, we initiated the 16S rRNA gene sequence analysis of these isolates. Of the total subgingival plaque samples \((n = 80)\), we had isolated S. pluranimalium from five (6.25%) samples. Similarly, of the total blood samples collected after tooth extraction \((n = 80)\), one strain of S. pluranimalium was isolated from one (1.25%) blood sample (Table 1).

Species identification of Gram-positive, catalase-negative, coccoc-shaped nonhemolytic streptococci is challenging with conventional phenotypic methods. Sequence analysis of the highly conserved 16S rRNA gene is required for closely related species such as the nonhemolytic streptococci. Various other studies in which culture-independent analysis of bacterial rRNA genes had provided insights into the composition of mixed microbial communities occurring in the environment or in animals (Jill & Clarridge, 2004). Isolation of communities occurring in the environment or in animals provided insights into the composition of mixed microbial communities with independent analysis of bacterial rRNA genes had pro-

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required for closely related species such as the nonhemo-

analysis of the highly conserved 16S rRNA gene is

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ative, coccoc-shaped nonhemolytic streptococci is challeng-

Table 1. Comparative results of Vitek 2 identification and 16S rRNA gene analysis of six isolates

<table>
<thead>
<tr>
<th>Sr. no.</th>
<th>Clinical sample and ID</th>
<th>Vitek 2 identification showing closest similarity</th>
<th>16S rRNA gene identification showing closest homology</th>
<th>GenBank accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blood isolate BB11</td>
<td>Streptococcus pluranimalium</td>
<td>Granulicatella adiacens ATCC 49175 (ACKZ010000002) 98.10% (418/422)</td>
<td>KJ575555</td>
</tr>
<tr>
<td>2</td>
<td>Subgingival plaque isolate DP15</td>
<td>Streptococcus pluranimalium</td>
<td>Streptococcus mitis NCTC 12261 (AEDX010000001) 99.33% (594/600)</td>
<td>KJ575556</td>
</tr>
<tr>
<td>3</td>
<td>Subgingival plaque isolate DP34</td>
<td>Streptococcus pluranimalium</td>
<td>Streptococcus mitis NCTC 12261 (AEDX010000001) 99.22% (637/642)</td>
<td>KJ575558</td>
</tr>
<tr>
<td>4</td>
<td>Subgingival plaque isolate DP40</td>
<td>Streptococcus pluranimalium</td>
<td>Streptococcus mitis NCTC 12261 (AEDX010000001) 98.67% (597/605)</td>
<td>KJ575559</td>
</tr>
<tr>
<td>5</td>
<td>Subgingival plaque isolate DP42</td>
<td>Streptococcus pluranimalium</td>
<td>Streptococcus tigurinus NCTC 12261 (AEDX010000001) 99.30% (860/868)</td>
<td>KJ575560</td>
</tr>
<tr>
<td>6</td>
<td>Subgingival plaque isolate DP23</td>
<td>Streptococcus pluranimalium</td>
<td>Streptococcus tigurinus AZ_3a (AORU010000002) 99.39% (1476/1485)</td>
<td>KJ575557</td>
</tr>
</tbody>
</table>

Underlined values indicate nucleotide differences of isolate DP23 when compared with S. tigurinus AZ_3a using EzTaxon identity option.
Table 2. Minimum inhibitory concentration of six isolates of Streptococcus pluranimalium by Vitek 2

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MIC (µg mL⁻¹)</th>
<th>S/VR</th>
<th>MIC (µg mL⁻¹)</th>
<th>S/VR</th>
<th>MIC (µg mL⁻¹)</th>
<th>S/VR</th>
<th>MIC (µg mL⁻¹)</th>
<th>S/VR</th>
<th>MIC (µg mL⁻¹)</th>
<th>S/VR</th>
<th>MIC (µg mL⁻¹)</th>
<th>S/VR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DP 15</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>≤ 0.06 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.06 S</td>
<td></td>
<td>≤ 0.06 S</td>
<td></td>
<td>≤ 0.06 S</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
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<tr>
<td>Levofloxacin</td>
<td>1 S</td>
<td></td>
<td>1 S</td>
<td></td>
<td>1 S</td>
<td></td>
<td>1 S</td>
<td></td>
<td>1 S</td>
<td></td>
<td>≤ 0.25 S</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
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<td>≤ 0.12 S</td>
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<tr>
<td>Clindamycin</td>
<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
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<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
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<td>≤ 0.25 S</td>
<td></td>
<td>≤ 0.25 S</td>
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<tr>
<td>Linezolid</td>
<td>≤ 2 S</td>
<td></td>
<td>≤ 2 S</td>
<td></td>
<td>≤ 2 S</td>
<td></td>
<td>≤ 2 S</td>
<td></td>
<td>≤ 2 S</td>
<td></td>
<td>≤ 2 S</td>
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</tr>
<tr>
<td>Vancomycin</td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>≤ 8 S</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>0.5 S</td>
<td></td>
<td>2 S</td>
<td></td>
</tr>
<tr>
<td>Trimethoprim/</td>
<td>≤ 10 S</td>
<td></td>
<td>≤ 10 S</td>
<td></td>
<td>≤ 10 S</td>
<td></td>
<td>≤ 10 S</td>
<td></td>
<td>≤ 10 S</td>
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<td>≤ 10 S</td>
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<tr>
<td>Sulfamethoxazole</td>
<td></td>
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<tr>
<td>Ceftriaxone</td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
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<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>≤ 0.12 S</td>
<td></td>
<td>4 R</td>
<td></td>
</tr>
</tbody>
</table>

MIC, minimum inhibitory concentration; DP, subgingival plaque sample; BB, blood sample; S, susceptible; I, intermediate; R, resistant.

*DP23 – Subgingival plaque sample from which Streptococcus tigurinus was identified by 16S rRNA gene analysis.

Isolation of S. tigurinus from the subgingival plaque of periodontitis patient has never been reported in literature. No single species could be identified as the decisive pathogen in periodontitis; instead, the disease is induced by the activity of a mixed bacterial biofilm growing under anaerobic conditions. The limitation of this study is the single instance of isolation of S. tigurinus from a periodontitis patient. Hence, it is hard to establish a specific risk factor profile for the development of invasive infection like periodontitis, because there is currently no data confirming the pathogenic role of this organism in periodontitis, but at the same time, it is important to know that isolation and identification of S. tigurinus was not possible in the past with conventional phenotypic test methods and of commercial systems due to the limitations of these methods (Facklam, 2002; Arbique et al., 2004; Bosshard et al., 2004; Haanpera et al., 2007; Spellerberg & Brandt, 2011). Hence, it is quite likely that there are no reports of isolation of this organism. The subgingival plaque and oral cavity could be the ecological niche of S. tigurinus, where this organism forms a part of biofilm, contributing to the disease process of periodontitis. Nevertheless, now this novel pathogen is reported to be isolated in multiple blood cultures and aortic valve specimens of a patient with infective endocarditis (Zbinden et al., 2012b). Recently, it has also been reported that S. tigurinus is an emerging pathogen causing invasive infections like infective endocarditis, spondylodiscitis, bacteraemia, meningitis, prostatic joint infection, and thoracic empyema (Zbinden et al., 2012b). Other members of the S. mitis group, such as S. mitis and S. oralis, which are known agents of infective endocarditis (Douglas et al., 1993; Spellerberg & Brandt, 2011) and sepsis in neutropenic cancer patients (Beighton et al., 1994; Lucas et al., 1997), are commensals of the oral flora. Similarly, the oral cavity could be an ecological niche of S. tigurinus as well; more studies are needed to confirm its habitat and virulence factors, as this organism seems to be an emerging pathogen.

Acknowledgements

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Statement

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of S. tigurinus AZ_3a(T) (AORU01000002) determined in this study is KJ575557.

References


Reference

Streptococcus tigurinus from periodontitis

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