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# Members of a new subgroup of *Streptococcus anginosus* harbor virulence related genes previously observed in *Streptococcus pyogenes*

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ABSTRACT

Conventionally categorized as commensals, the Streptococci of the species *S. anginosus* are facultative human pathogens that are difficult to diagnose and often overlooked. Furthermore, detailed investigation and diagnosis of *S. anginosus* infections is hampered by unexplored taxonomy and widely elusive molecular pathogenesis. To explore their pathogenic potential, *S. anginosus* isolates collected from patients of two geographical locations (Vellore, India and Leipzig, Germany) were subjected to multi-locus sequence analysis (MLSA). This analysis revealed the potential presence of a new distinct clade of the species *S. anginosus*, tentatively termed here as genomosubspecies *vellorensis*. A complementary PCR-based screening for *S. pyogenes* virulence factor as well as antibiotic resistance genes revealed not only the presence of superantigen- and extracellular DNase coding genes identical to corresponding genes of *S. pyogenes*, but also of erythromycin and tetracycline resistance genes in the genomes of the analyzed *S. anginosus* isolates, thus posing a matter of significant health concern. Identification of new pathogenic *S. anginosus* strains capable of causing difficult to treat infections may pose additional challenges to the diagnosis and treatment of *Streptococcus* based infections.



## 1. Introduction

Members of the gram positive species *Streptococcus anginosus* can currently be grouped into three subspecies: *S. anginosus* subsp. *anginosus*, *S. anginosus* subsp . *whileyi* and *S. anginosus* genomosubsp. *AJ1* (Whiley et al., 1999; Jensen et al., 2013). Such grouping is mainly based on the Multilocus Sequence Analysis (MLSA) of concatenated sequences of seven housekeeping genes (Bishop et al., 2009) that laid the foundation of modern taxonomic analysis of this group of organisms, thereby avoiding various taxonomic confusions of the past, which was due partially to inadequate phenotypic markers (Whiley et al., 1999; Jensen et al., 2013) and partially to dearth of consensus on the nomenclature (Facklam, 2002). Although *S. anginosus* is a commensal of the human oropharyngeal, gastrointestinal and genitourinary flora, members of this species harbor a considerable pathogenic potential. As compared to *Streptococcus pyogenes*, a significant streptococcal pathogen, human infections caused by *S. anginosus* are as frequent and have been seen to cause similar suppurative infections, including pharyngitis, bacteremia and serious deep tissue infections (Whiley et al., 1999; Junckerstorff et al., 2014; Siegman-Igra et al., 2012; Claridge et al., 2001; Laupland et al., 2006). Furthermore, *S. anginosus* can infect internal organs like lung, liver and brain (Junckerstorff et al., 2014; Siegman-Igra et al., 2012; Claridge et al., 2001) and have a proclivity to cause abscesses (Claridge et al., 2001; Whiley et al., 1992). Overall, *S. anginosus* infections exist in a broad variety of manifestations of pyogenic character.

Until now, only a few virulence factors of *S. anginosus* have been described (Asam and Spellerberg, 2014). For instance, a laminin binding protein (Allen and Höök, 2002) may serve as an adhesin during colonization of inner organs and anatomical cavities. Homologs of streptolysin S, a potent hemolytic exotoxin described in *S. pyogenes*, are known to occur in *S. anginosus* and have been described to cause cytolysis of erythrocytes (Tabata et al., 2013; Asam et al., 2013). However, *S. pyogenes* additionally holds a set of structurally related proteins referred as streptococcal pyrogenic exotoxins (SPEs), which include superantigens (Proft and Fraser, 2003), that stimulate a massive release of pro-inflammatory mediators and a polyclonal proliferation of T-cells independent of

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antigen presentation, which inhibits pathogen specific responses. Whereas some of the superantigens like SmeZ, SpeJ and SpeG are chromosomally encoded (Ferretti et al., 2001; Friães et al., 2013) others are prophage-encoded (Ferretti and McShan, 2006). As part of mobile genetic elements, they have been transmitted among *S. pyogenes* strains and to other streptococcal species (Igwe et al., 2003). To date, evidence for superantigens in *S. anginosus* is missing, except of potential SPE amplicons that were detected in *S. anginosus*, but not sequenced (Anand et al., 2012).

Thus, the insights into the molecular pathogenesis of *S. anginosus* infections remain superficial. In the current report, we characterized *S. anginosus* isolates obtained from pyogenic infections for their antibiotic resistance and pyogenic virulence factor profile.

## 2. Materials and methods

### 2.1. Bacterial isolates

The study comprised 76 *S. anginosus* isolates, out of which 17 isolates originated from clinical infections that were diagnosed at the University Hospital Leipzig, Germany and from cases of infective endocarditis and 59 β-hemolytic *S. anginosus* isolates originated from purulent infections in Vellore, India (Naveen Kumar et al., 2014; Reissmann et al., 2010). Details of the isolates are given in Supplementary Table 1. All isolates were stored at −80 °C in THY medium containing 25% glycerol. For further analysis, the isolates were cultivated over night at 37 °C, 5% $CO_2$ on Todd Hewitt Agar plates containing 0.5% yeast extract (THY-Agar). For liquid cultures THY medium was inoculated with bacteria and incubated over night at 37 °C, 5% $CO_2$.

### 2.2. DNA isolation

Genomic DNA was extracted from 15 ml overnight culture grown in THY. The bacterial cells were disrupted using zirconia beads and a FastPrep 24 device (MP Biomedical) at 4 m/s for 30 s. After removing bacterial debris by centrifugation (500*g*, 30 s), the DNeasy Blood and Tissue Kit (Qiagen) was used to extract the DNA from the lysate. The DNA was examined by 1% TAE agarose gel electrophoresis and stored at −20 °C.

### 2.3. Multilocus Sequence Analysis (MLSA)

Seven house-keeping genes *map*, *pfl*, *pyk*, *ppaC*, *rpoB*, *sodA* and *tuf* were amplified and sequenced by PCR as described previously (Bishop et al., 2009) but with slight modification (Naveen Kumar et al., 2014). Sequences for MLSA were determined as described above (76 isolates) or retrieved from GenBank database (68 isolates). In total, the analysis included sequences of 144 *S. anginosus* isolates, 68 of which were already available (Jensen et al., 2013; Naveen Kumar et al., 2014; Bishop et al., 2009). Sequences were aligned, trimmed, edited and concatenated using BioEdit 7.0.1 (Isis Pharmaceuticals) and Codon Code Aligner version 4.0 (Codon Code Corporation). Cluster analysis of the concatenated nucleotide sequences (3063 bp) of aforementioned seven house-keeping genes was conducted with MEGA 7.0 using the Maximum Likelihood method and tested by bootstrapping with 1000 replicates.

### 2.4. Screening of virulence related genes of S. pyogenes

The 76 *S. anginosus* isolates were screened for the presence of 13 streptococcal pyrogenic exotoxin encoding genes (*speA*, *speB*, *speC*, *speG*, *speF*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *smeZ*), four DNase encoding genes (*sdaB*, *sdaD*, *sdc*, *spd3*) and four immune-modulatory virulence related genes of *S. pyogenes* (*sic*, *mac*, *scpA*, *spyCEP*) by a multiplex PCR consisting of five independent reactions that was developed based on previous protocols (Friães et al., 2013; Borek et al., 2011). Primer sequences are given in Supplementary Table 2 together with specific amplicon sizes and the *S. pyogenes* strains used as controls. PCR conditions are described in Supplementary Table 3. Genomic DNA of the genome sequenced *Streptococcus gordonii* strain Challis GP204 was used as a negative control in all reactions. DNA samples negative in all reactions were checked for integrity by PCR amplification of *gyrA* (gyrase subunit A) as described in Supplementary Tables 2 and 3. The *emm* gene was amplified as per standard protocol provided by the Center of Disease Control (CDC) to eliminate the probability of contamination by *S. pyogenes*.

To amplify the complete gene sequence of detected *S. pyogenes* virulence factor genes, primers were designed either targeting the phage region surrounding *speC* or targeting 100 bp up- and downstream of the gene based on *S. pyogenes* genome information. The primer sequences are given in Supplementary Table 4 together with the sizes of specific amplicons, utilized *S. pyogenes* control strains and PCR conditions. The PCR mixtures consisted of 3 μl template DNA (500 ng – 1 μg), 2.5 μl of the appropriate 10× PCR Buffer, 1.5 mM $MgCl_2$, 10 mM dNTPs, 10 pmol of each primer and 1 unit of the indicated DNA polymerase filled with PCR grade water to a final volume of 25 μl.

### 2.5. DNA sequencing

Amplicons of the seven house-keeping and virulence genes were sequenced in both directions using the specific primers described above, ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM® 3730XL Analyzer (Applied Biosystems, USA). Sequencing was done at the Helmholtz Centre for Infection Research. New sequences were deposited in GenBank: House-keeping genes – *map* (KP214642 – KP214732); *pfl* (KP214733 – KP214823); *ppaC* (KP214824 – KP214914); *pyk* (KP214915 – KP215005); *rpoB* (KP215006 – KP215096); *sodA* (KP215097 – KP215187); *tuf* (KP215188 – KP215278), Virulence genes – *sdaD* (KP856881, KP856882), *sdc* (KP856883, KP856884), *speG* (KP856885, KP856886), *speC* (KP856887). Full gene sequences of virulence factors were deposited under the accession number: *speC* (phage sequence; KX181402), *sdaD* (KX181405, KX181406), *sdc* (KX181403, KX181404) and *speG* (KX181407, KX181408).

### 2.6. Similarity search using Basic Local Alignment Search Tool (BLAST)

A similarity search for *speA*, *speB*, *speC*, *speG*, *speF*, *speH*, *speI*, *speJ*, *speK*, *speL*, *speM*, *ssa*, *smeZ*, *sdaB*, *sdaD*, *sdc*, *spd3*, *sic*/*drs*, *mac* (*ideS*), *scpA* and *spyCEP* against the genome sequences of 12 *S. anginosus* strains (Supplementary Table 5) was carried out with BLAST (Altschul et al., 1990) using the megablast algorithm with a word size of 16, cut off of 50% similarity and other parameters in default settings.

### 2.7. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MIC) of benzylpenicillin, erythromycin and tetracycline were determined after 18 h of incubation at 37 °C by the agar dilution method with two fold dilution series of the agents in accordance with the protocol of the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org).

Isolates were classified as benzylpenicillin resistant (R >2 mg/l) or susceptible (S ≤0.25 mg/l) using the interpretive criteria given