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CASE REPORT

Severe Soft Tissue Infection Caused by a Non-Beta-Hemolytic *Streptococcus pyogenes* Strain Harboring a Premature Stop Mutation in the *sagC* Gene

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We recovered a non-beta-hemolytic *Streptococcus pyogenes* strain from a severe soft tissue infection. In this isolate, we detected a premature stop codon within the *sagC* gene of the streptolysin S (SLS) biosynthetic operon. Reintroduction of full-length *sagC* gene on a plasmid vector restored the beta-hemolytic phenotype to our clinical isolate, indicating that the point mutation in *sagC* accounted for loss of hemolytic activity. To the best of our knowledge, this is the first report to demonstrate that a severe soft tissue infection can be caused by a non-beta-hemolytic *S. pyogenes* strain lacking a functional SagC.

CASE REPORT

A 33-year-old man developed pain and swelling after an insect bite to his left forearm. Conservative therapy on an outpatient basis was initiated involving oral roxithromycin and splinting with a cast. The past medical history was uneventful except for a herniated intervertebral disc and arterial hypertension. Except for the prescribed antibiotic, the patient was on no other medication. After 1 week of treatment without any improvement, the patient was referred to the Department of Plastic and Hand Surgery with an abscess on the distal left forearm.

On examination, the obese patient (body mass index [BMI], 32 kg/m^2) was afebrile. His blood pressure was 140/80 mm Hg, and his pulse was 80 beats per minute. According to the patient, there had previously been pronounced lymphangitis involving the forearm that had improved by the time of presentation to our department. Around the distal forearm, there was an abscess with central soft tissue necrosis (Fig. 1A). The surrounding soft tissue was erythematous, swollen, and tender (Fig. 1A). The sensation of light touch was intact, and the patient was able to move his fingers. There was no crepitation. Lab tests revealed a leukocytosis (11.17/nl; normal range, 4 to 10/nl) and an elevated C-reactive protein level (52.4 mg/liter; normal range, <5 mg/liter).

Emergency surgery with abscess incision and drainage was performed. Necrotic tissue was removed. Upon further surgical exploration, the peritendineous area surrounding the flexor carpi radialis and the palmaris longus tendons showed extensive pannus formation extending to and involving the loose tissue surrounding the radial artery, suggesting a longer duration of the infectious process prior to surgery. Tenolysis and microsurgical arteriolysis of the radial artery were performed meticulously, and surgical débridement of the abscess was achieved. Tissue specimens were sent for Gram staining and bacterial culture, followed by placement of drains for irrigation and suction of the wound bed.

An antibiotic regimen with amoxicillin-clavulanic acid was initiated empirically and continued for 2 weeks. The patient fully recovered and was discharged. Since surgical débridement left only a small defect, no plastic surgery was necessary and the wound healed uneventfully.

Direct examination of the surgical tissue specimen revealed abundant neutrophils and Gram-positive cocci ordered in chains. Cultures of surgical specimens yielded many colonies of non-beta-hemolytic, catalase-negative, Gram-positive cocci. Testing for pyrrolidonyl arylamidase was positive. The strain expressed the group A Lancefield antigen (Streptex; bioMérieux) and was subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry using a Microflex mass spectrometer (Bruker Daltonik, Bremen, Germany). The retrieved spectra were analyzed with the BioTyper software (Bruker), yielding a definitive identification as *Streptococcus pyogenes*. In addition to *S. pyogenes*, the culture of the tissue specimen yielded a few white colonies that were identified as *Staphylococcus epidermidis* using the Microflex mass spectrometer; these colonies were considered to be contaminants. The *S. pyogenes* strain was serotyped as T-4. Molecular analysis of the *emm* genes was performed by PCR using "all M primers" (1) and resulted in the genotype *emm-4*. Profiling of the presence of streptococcal pyrogenic exotoxin genes (*spe*), the streptococcal mitogenic exotoxin Z gene (*smeZ*), and the streptococcal superantigen gene (*ssa*) was performed as described previously (2). This analysis revealed the coexistence of *speC*, *smeZ*, and *ssa*, in accordance with reports demonstrating the association of these superantigen genes with the *emm-4* lineage (3, 4). The strain was sensitive to penicillin G, amoxicillin, cefo-

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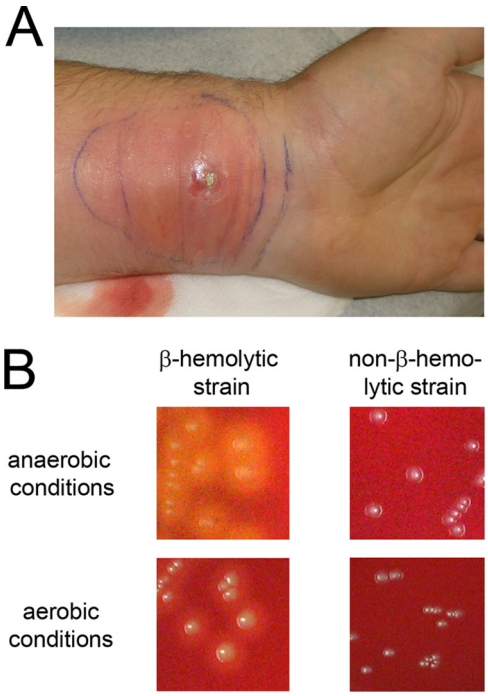

FIG 1 Clinical picture and *in vitro* growth of the non-beta-hemolytic *S. pyogenes* isolate. (A) Clinical presentation of the soft tissue infection prior to surgical intervention. (B) A beta-hemolytic *S. pyogenes* strain (control) and the non-beta-hemolytic *S. pyogenes* strain isolated from the patient were grown on sheep blood agar plates for 18 h at 37°C under aerobic or anaerobic conditions.

taxime, clarithromycin, clindamycin, tetracycline, and levofloxacin, as determined by agar disc diffusion and broth microdilution assay according to CLSI criteria.

After subculture on sheep blood agar under aerobic and anaerobic conditions, single colonies of the strain failed to produce a zone of beta-hemolysis (Fig. 1B). After prolonged incubation and in the bacterial lawn, we observed a muddy green zone of alpha-hemolysis. *S. pyogenes* usually possesses two hemolysins: the oxygen-dependent labile streptolysin O (SLO), encoded by the *slo* gene, and an oxygen-stable streptolysin S (SLS), which is responsible for the characteristic beta-hemolytic phenotype on blood agar. The SLS precursor is encoded by the *sagA* gene, which belongs to the nine-gene *sag* operon (*sagABCDEFGHI*). For conversion into SLS, SagA is posttranslationally modified by SagBCD, a trimeric oxazole/thiazole synthetase (5, 6). The role of the remaining Sag proteins is less clear. However, it is assumed that they may play an important role in the secretion of SLS (see reference 7 and the references therein). Recently, Yoshino et al. reported on 9 nonhemolytic *S. pyogenes* strains with various deletions in the *sag* operon associated with uncomplicated mucosal infections (8). Therefore, we decided to use next generation sequencing in order to analyze the integrity of the *slo* gene and the *sag* operon in our strain. An indexed Illumina sequencing library was constructed from 50 ng bacterial genomic DNA by tagmentation with the Nextera DNA sample prep kit (Illumina, San Diego, CA) and sequenced using the Illumina MiSeq personal sequencer (MiSeq Reagent Kit 300 cycle, Illumina). The obtained 2 × 150 bp paired reads were trimmed and aligned using the Genome Workbench 5 software suite (CLCbio, Aarhus, Denmark). A total of 1,896,767 of 2,105,948 reads mapped to the reference genome of *S. pyogenes* (GenBank accession no. AE004092), with an average coverage of 146.3-fold. The *slo* gene was intact and showed some minor conservative exchange polymorphisms (data not shown). Furthermore, we analyzed the integrity of the *covR* (also known as *csrR*) and *covS* (also known as *csrS*) genes, which encode a two-component regulatory system that is known to regulate *sagA* expression (9). These two genes were also intact and showed only minor conservative exchange polymorphisms (data not shown). Immediately upstream of the *sag* operon, we identified a 3.3-kb deletion in the *epf* gene (Fig. 2), which codes for a cell surface adhesion molecule (10). We confirmed this deletion by performing a long-range PCR (Fig. 2B) as described by Yoshino et al. (8). Most interestingly, our sequencing approach revealed a point mutation in the *sagC* gene, 478C (Gln)→478T (AMB), that resulted in a premature stop codon (Fig. 2A) and predicted size difference of 193 amino acids (aa) compared to the wild-type SagC (352 aa). We confirmed this point mutation by conventional Sanger sequencing of an ~300-bp fragment covering the putative point mutation at position 478 of the *sagC* gene (Fig. 2C). SagC is absolutely required for hemolysis (11). It provides a high-affinity substrate-binding site that allows the efficient modification of the structural SLS precursor SagA by the SagBCD complex (5, 6). In order to analyze whether the premature stop codon in the *sagC* gene caused the non-beta-hemolytic phenotype of our patient isolate, we introduced the full-length wild-type *sagC* gene on a plasmid vector (11) by electroporation into our patient isolate. This enabled our clinical isolate to produce a robust zone of beta-hemolysis (Fig. 2D). Therefore, we conclude that the premature stop mutation in the *sagC* gene alone accounts for the absence of a zone of beta-hemolysis in our clinical isolate.

A hallmark of *S. pyogenes* is the distinct zone of beta-hemolysis around gray colonies grown on blood agar plates, which is seen under both normoxic conditions (mediated by SLS) and anaerobic conditions (mediated by SLS and/or SLO) (12). Abundant experimental evidence from molecular mutagenesis studies and animal infection studies indicates that SLS and SLO can serve as virulence factors in the pathogenesis of streptococcal infection (11, 13, 14). However, our case report clearly demonstrates that neither a fully functional *sag* operon nor the ability of *S. pyogenes* to express SLS represents an absolute prerequisite for human virulence.

In line with our clinical finding, there are several previous case reports of infections caused by nonhemolytic *S. pyogenes* or *Streptococcus agalactiae* (15–21). Furthermore, in 1968 an outbreak of pharyngitis and acute rheumatic fever at Lowry Air Force Base was associated with nonhemolytic streptococci (22). However, in none of these reports was the molecular basis for the bacterial phenotype unraveled. Therefore, it remains unclear whether the *in vitro* conditions did not allow the SLO or SLS to be active or whether the genes necessary for the expression of SLS or SLO were mutated or deleted.

A study from New Zealand described nonhemolytic variants of *S. pyogenes* from clinical specimens with symptoms of pharyngitis and used biochemical methods in order to trace this observation back to a deficiency of SLS (23). Recently, Yoshino et al. screened for nonhemolytic *S. pyogenes* isolates within 1,690 specimens taken from patients with pharyngitis and otitis media, which led to

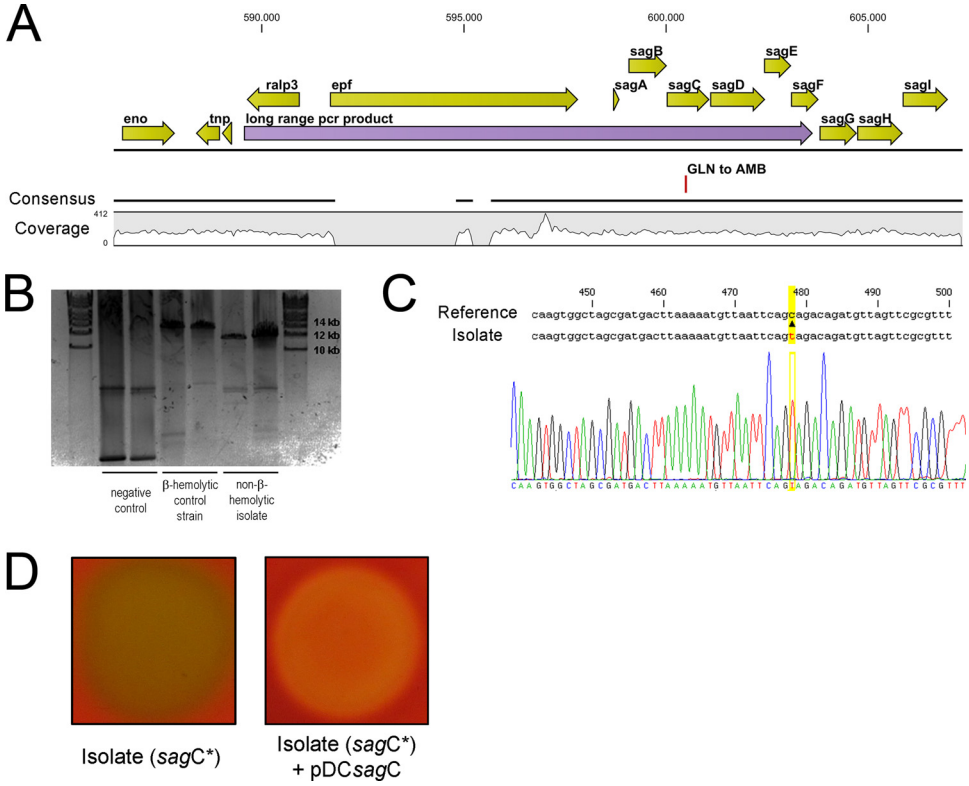

FIG 2 Analysis of *slo* gene and the *sag* operon of the non-beta-hemolytic *S. pyogenes* isolate. (A) DNA was extracted from the non-beta-hemolytic *S. pyogenes* strain (isolate) that we retrieved from the described patient with a severe soft tissue infection. Shown is the alignment of next generation sequencing reads with the *S. pyogenes* (GenBank accession no. AE004092.1) 21-kbp *sag* operon region (reference positions 586337 to 607336). The region corresponding to the long-range PCR product (according to reference 8) is indicated. The average coverage (excluding the zero-coverage region) is 159.6-fold. The AMB mutation in *sagC* is indicated. (B) DNA was extracted from a hemolytic *S. pyogenes* strain (beta-hemolytic control strain) and the non-beta-hemolytic *S. pyogenes* strain that we retrieved from a patient with a severe soft tissue infection (non-beta-hemolytic isolate). Duplicate long-range PCR for the genes comprising the *sag* operon was performed, resulting in a PCR product of 14 kb (long-range PCR product). The primers used for long-DNA-fragment amplification were as described in reference 8: sense primer 5′-TGTGGATGCCGTTTAGAACA-3′ and reverse primer 5′-GAATAGCGACACGCCTTAGC-3′. (C) DNA was extracted from the non-beta-hemolytic *S. pyogenes* strain (isolate). In order to amplify an ~300-bp fragment around the putative point mutation at position 478 in the *sagC* gene, we used the sense primer 5′-GCTGATGCTTGAGGATGACTAC-3′ and the antisense primer 5′-GTAATCGCACAAGGCTTCTG-3′. The resulting fragment was subjected to conventional Sanger sequencing. Alignment of the Sanger sequencing reads to the *sagC* gene (GenBank accession no. AF067649) was performed. In the chromatogram, the point mutation at position 478 is highlighted in yellow. (D) pDC*sag*C was electroporated into the non-beta-hemolytic isolate that harbored a premature stop codon in the *sagC* gene (*sag*C*). pDC*sag*C is a pDCerm plasmid derived from pAD*sag*C, which harbors a wild-type full-length *sagC* gene (11). Twenty-five microliters of overnight cultures (*sag*C* and *sag*C* + pDC*sag*C) was plated on blood agar plates.

the identification of 9 nonhemolytic *S. pyogenes* strains with deletions in the *sag* operon (8).

In summary, to the best of our knowledge this is the first report carefully analyzing the integrity of the *slo* gene and *sag* operon in a non-beta-hemolytic *S. pyogenes* strain that had caused a severe soft tissue infection. Furthermore, this is the first report that traced the lack of beta-hemolysis back to a premature stop mutation in the *sagC* gene, which encodes a cyclodehydratase that generates thiazoline and (methyl)-oxazoline heterocycles in the SLS propeptide essential for toxin activity (5, 6).

Our case dramatically illustrates that a beta-hemolysis zone on sheep blood agar plates may be absent in *S. pyogenes*, rendering the diagnosis initially difficult. Therefore, a careful analysis of colonies that resemble viridans streptococci is warranted, especially if they are grown from specimens taken from otherwise sterile compartments.

Nucleotide sequence accession numbers. The sequence determined in this study is available from the European Nucleotide Archive (ENA) Short Read Archive (SRA), Study ERP002284: samples ERS217631 and ERS217535, experiments ERX207843 to ERX207848.

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