ARTICLE

Evaluation of genotypic and phenotypic methods for differentiation of the members of the Anginosus group streptococci

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Abstract The terminology and classification of the Anginosus group streptococci has been inconsistent. We tested the utility of 16S rRNA gene and *tuf* gene sequencing and conventional biochemical tests for the reliable differentiation of the Anginosus group streptococci. Biochemical testing included Rapid ID 32 Strep, API Strep, Fluo-Card Milleri, Wee-tabs, and Lancefield antigen typing. Altogether, 61 Anginosus group isolates from skin and soft tissue infections and four reference strains were included. Our results showed a good agreement between 16S rRNA gene and *tuf* gene sequencing. Using the full sequence was less discriminatory than using the first part of the 16S rRNA gene. The three species could not be separated with the API 20 Strep test.

Streptococcus intermedius could be differentiated from the other two species by β-galactosidase (ONPG) and β-N-acetyl-glucosaminidase reactions. Rapid ID 32 Strep β-glucosidase reaction was useful in separating *S. anginosus* strains from *S. constellatus*. In conclusion, both 16S rRNA gene and *tuf* gene sequencing can be used for the reliable identification of the Anginosus group streptococci. *S. intermedius* can be readily differentiated from the other two species by phenotypic tests; however, 16S rRNA gene or *tuf* gene sequencing may be needed for separating some strains of *S. constellatus* from *S. anginosus*.

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Introduction

The Anginosus group streptococci (AGS) consist of three species, designated *Streptococcus anginosus*, *Streptococcus constellatus*, and *Streptococcus intermedius* [1, 2]. Clinical review and our studies have shown that, while the AGS are found as normal flora in humans, these bacteria are pathogens recognized for their role in pyogenic infections and are strongly associated with abscess formation [3–5]. In a recent study of 400 traumatic and surgical wound infections, we found that species of the Anginosus group were the predominant streptococci recovered; 71 AGS strains were isolated from 60 specimens [6]. The AGS has been described as a heterogenous group, and the terminology and classification of the AGS has always been a source of confusion [7]. An identification scheme proposed by Whiley et al. is considered to be a standard for phenotypic identification of the AGS [1, 8]. The 16S rRNA gene sequencing and commercial identification kits commonly used to identify bacteria have been inconsistent in identifying the AGS [7, 9–13]. As a result, AGS strains may often be reported out in the clinical microbiology laboratory

as 'viridans streptococci.' Picard et al. described a polymerase chain reaction (PCR)-based assay targeting the *tuf* gene encoding elongation factor Tu for the specific detection of streptococcal sequences [14]. However, only a few AGS strains were included [14]. We tested the utility of *tuf* gene sequencing identification and a battery of biochemical tests for the identification of AGS strains to the species level using 16S rRNA gene sequencing as a reference method. Biochemical testing included Rapid ID 32 Strep, API Strep, Fluo-Card Milleri, Wee-tabs, and Lancefield antigen typing.

Methods

Strains and growth conditions

Sixty-one strains isolated at the VA Wadsworth Medical Center from patients at Olive View—UCLA Medical Center were included. The reference strains for *S. anginosus* (ATCC 33397^T), *S. constellatus* (ATCC 27823^T and ATCC 24889), and *S. intermedius* (ATCC 27335^T) were included in the biochemical and molecular characterization. Strains were maintained at −70°C in double-strength skim milk before characterization. The strains were grown at 37°C on trypticase soy blood agar (TSA, Becton Dickinson Microbiology Systems, Sparks, MD) under microaerophilic conditions (5% CO_2). All testing was done with 24 to 48-h-old pure cultures.

Genotypic testing

Genomic DNA was extracted and purified from bacterial cells in the mid-logarithmic growth phase by using a QIAamp DNA Mini Kit (Qiagen, Inc., Chatsworth, CA). The 16S rRNA gene fragments were amplified by standard methods. Two subregions of the 16S rRNA gene were amplified by using two pairs of primers. The first part of the 16S rRNA gene was defined as an approximately 800-bp region between primers 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907B (5′-CCGTCAATTCMTTTAGTTT-3′). The second part of the 16S rRNA gene, defined as approximately 700-bp sequences between primers 774A (5′-GTAGTCCACG CTGTAAACGATG-3′) and 1485B (5′-TACGGTTACCT TGTTACGAC-3′), was sequenced to obtain the complete 16S rRNA gene sequence on 22 strains (eight *S. anginosus*, nine *S. constellatus*, and five *S. intermedius*). PCR was performed for 35 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min at 72°C, with a final extension at 72°C for 5 min. The *tuf* gene fragments were amplified with universal primers Tseq271 (5′-AAYATGATIACIGGIG CIGCICARATGG-3′) and Tseq1138 (5′-CCIACIGTICK

ICCRCCYTCRCG-3′) to obtain an approximately 760-bp portion of the gene. The PCR conditions were as follows: 95°C for 3 min; 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 5 min. The PCR products were excised from a 1% agarose gel after electrophoresis and purified using a QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were sequenced directly with BigDye Terminator Cycle Sequencing Kits (Biotech Diagnostics, Tustin, CA) on an ABI 3100 *Avant* Genetic System (Applied Bio-systems, Foster City, CA). The sequencing data was analyzed by comparison of the consensus sequences with GenBank sequences by using the Ribosomal Database Project (RDP-II) and the Basic Local Alignment Search Tool software (BLAST), and the percentage similarity to other sequences was determined. Closely related sequences were retrieved from GenBank and were aligned with the newly determined sequences by using the program CLUSTAL W.

Phenotypic testing

The Lancefield antigen was detected using the Streptex* Lancefield Streptococi Latex Test (Remel, Lenexa, KS) according to the manufacturer's instructions. Preformed enzyme profiles were characterized by API Strep and Rapid ID Strep (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions using API Suspension Medium (bioMérieux) to prepare inocula. Fluo-Card Milleri tests were performed according to the manufacturer's instructions (Key Scientific Products Co., Stamford, TX). Additionally, Wee-tabs α-glucosidase, β-fucosidase, β-glucosidase, β-N-acetyl-glucosaminidase (β-NAG), esculin, and ONPG (Key Scientific Products Co.), were included in the characterization. TSA blood agar was used for the detection of hemolysis.

Results

Strains

Next to *Staphylococcus aureus*, the AGS were the second most common isolates (71 isolates from 60 specimens; 31 *S. anginosus*, 28 *S. constellatus*, 11 *S. intermedius*, and one AGS) found during our comprehensive microbiological evaluation of 400 skin and soft tissue infections. Of the 61 strains included in this study, 29 were *S. anginosus* (sources: perirectal and torso below the diaphragm [17], lower extremity [4], upper extremity [7], and breast [1]), 21 *S. constellatus* (perirectal and torso below the diaphragm [11], lower extremity [3], upper extremity [1], and head and neck [6]), and 11 *S. intermedius* (perirectal and

torso below the diaphragm [3], upper extremity [6], and head and neck [2]).

Genotypic testing

The reference strains' 16S rRNA gene sequences matched those deposited in GenBank with 100% sequence similarity. Similarly, for all of the clinical strains tested, the 16S rRNA gene sequencing identified the correct species as the first choice with ≥98% sequence similarity to the reference strain sequences (Table 1). Twenty-eight of 29 (97%) of *S. anginosus* were named with ≤2% distance from the next named species using the first part of the 16S rRNA gene, whereas only 4/8 (50%) *S. anginosus* tested were named with ≤2% distance using the full 16S rRNA gene. Similarly, 6/21 (29%) of *S. constellatus* and 10/11 (91%) of *S. intermedius* were named with ≤2% distance from the next named species using the first part of the 16S rRNA gene, but only 1/9 (11%) and 2/5 (40%), respectively, using the full 16S rRNA gene. Further, the full 16S rRNA gene sequence did not give discriminatory results for three *S. constellatus* and two *S. intermedius* strains. For all of the strains tested, the *tuf* gene sequencing identified the correct species at 100–98% sequence similarity to the reference species (Table 1). Nine of 29 (31%) of *S. anginosus*, 20/21 (95%) of *S. constellatus*, and 11/11 (100%) of *S. intermedius* were named with ≤2% distance from the next named species by *tuf* gene sequencing.

Phenotypic testing

Of the 29 *S. anginosus* strains, two were β-hemolytic and the rest were α-hemolytic. Most *S. anginosus* strains possessed a Lancefield antigen: 16 were Lancefield group F, six Lancefield group C, three Lancefield group G, and four were non-typable. Of the 21 *S. constellatus* strains, seven were β-hemolytic and the rest were α-hemolytic; eight strains possessed Lancefield group F antigen, whereas the rest had no antigen. All 11 *S. intermedius* isolates were α-hemolytic, three belonged to Lancefield group F, and eight had no antigen. The key phenotypic characteristics obtained by the Rapid ID 32 Strep, API Strep, and Wee-tab systems are given in Table 2. The other results are given below. All strains were positive for arginine dihydrolase, alkaline phosphatase, saccharose, Voges-Proskauer, alanyl-phenylalanyl-proline arylamidase, and maltose, and all strains were negative for β-glucuronidase, ribose, mannitol, sorbitol, arabinose, arabitol, cyclodextrin, glycogen, mele-zitose, and urease using the Rapid ID 32 Strep test. In addition, *S. constellatus* and *S. intermedius* were negative for α-galactosidase, raffinose, pyroglutamic acid arylami-dase, hippurate, melibiose, and tagatose, whereas *S. anginosus* reactions were variable. All of the strains were positive for Voges-Proskauer, alkaline phosphatase, leucine aminopeptidase, arginine dihydrolase, and trehalose acidi-fication, and variable for lactose and amidon acidification using the API Strep system. At 24 h, *S. constellatus* esculin was variable. In addition, *S. anginosus* were occasionally positive for α-galactosidase and ribose, arabinose, manni-tol, inulin, and raffinose acidification. Wee-tabs α-glucosidase, β-glucosidase, and esculin reactions were variable with all of the strains tested.

Using the Fluo-Card Milleri, all *S. intermedius* strains were β-fucosidase-positive and, thus, correctly identified (Table 3). Only 45% of the *S. anginosus* strains were correctly identified as *S. anginosus*; 55% of the *S.*

Table 1 16S rRNA and *tuf* gene sequencing identification of the *Streptococcus anginosus* group

	% sequence similarity[1] (next named species)	
	16S rRNA sequencing ID	*tuf* gene ID
S. anginosus		
ATCC 33397[T]	100% *S. anginosus* (96% *S. intermedius*)	100% *S. anginosus* (97% *S. constellatus*)
Clinical isolates (*n*=29)	100–98% *S. anginosus* (96–94% *S. intermedius/constellatus*)	99–98% *S. anginosus* (98–97% *S. intermedius/constellatus*)
S. constellatus		
ATCC 27823[T]	100% *S. constellatus* (98% *S. intermedius*)	99% *S. constellatus* (97% *S. intermedius*)
ATCC 24889	100% *S. constellatus* (98% *S. intermedius*)	100% *S. constellatus* (98% *S. intermedius*)
Clinical isolates (*n*=21)	100–99% *S. constellatus* (98–97% *S. intermedius*)	100–99% *S. constellatus* (98–97% *S. anginosus/intermedius*)
S. intermedius		
ATCC 27335[T]	100% *S. intermedius* (98% *S. constellatus/anginosus*)	100% *S. intermedius* (96% *S. constellatus/anginosus*)
Clinical isolates (*n*=11)	100% *S. intermedius* (98% *S. constellatus*)	99–98% *S. intermedius* (97–96% *S. anginosus/constellatus*)

[1] For all of the strains tested, both the 16S rRNA gene sequencing and the *tuf* gene sequencing identified the correct species as the first choice

Table 2 Key differential phenotypic characteristics of the *S. anginosus* group[1]

	S. anginosus		*S. constellatus*			*S. intermedius*	
	ATCC 33397	Clinical strains, *n*=29	ATCC 27823	ATCC 24889	Clinical strains, *n*=21	ATCC 27335	Clinical strains, *n*=11
Lancefield antigen	G	F, C, G, N	N	N	N, F	N	N, F
Hemolysis	β	α, β	α	α	α, β	α	α
Rapid ID 32 STREP							
β-glucosidase	+	+	-	-	-$^+$	+	+
β-galactosidase	-	-	-	-	-	+	+
β-N-acetyl-glucosaminidase	-	-	-	-	-	+	+
Pullulan	+	+$^-$	-	-	-	+	v
β-mannosidase	+	v	-	-	-	+	+
API Strep							
Esculin (4 h)	+	+	-	-	-	-	v
Wee-tabs							
β-fucosidase	-	-	-	-	-	+	+
β-N-acetyl-glucosaminidase	-	-	-	-	-	+	+
ONPG	-	-	-	-	-	+	+

[1] N = no antigen; + = >90% of strains are positive; - = >90% of strains are negative; +$^-$ = 80–90% of strains are positive; -$^+$ = 80–90% of strains are negative; v = variable reaction

anginosus strains were β-fucosidase-positive (but β-glucosidase-negative). Fluo-Card Milleri identified 76% of *S. constellatus* strains correctly (Table 3).

Discussion

It has been reported that there is an association between clinical sites of infection and the different species of AGS where *S. anginosus* is predominantly associated with gastrointestinal sources, *S. constellatus* with all types of abscesses, and *S. intermedius* with intracerebral abscesses [3, 5, 15–17]. Our study included isolates from a skin and soft tissue infection study only, but some general trends can be observed from our results: *S. anginosus* were predom-

inantly isolated from below the diaphragm specimens, whereas *S. intermedius* were mostly isolated from above the diaphragm specimens. Also, similar to previous reports, all of the isolates from head and neck sources were either *S. constellatus* or *S. intermedius* [17]. Our results on the distribution of hemolysis and Lancefield group were similar to those previously described (Table 2) [1, 13, 17]. However, none of our β-hemolytic *S. constellatus* isolates carried the Lancefield group C antigen associated with *S. constellatus* subsp. *pharyngis* and the human throat [2].

Our results showed good agreement between the 16S rRNA gene sequencing and *tuf* gene sequencing. However, GenBank has a lot of sequences of poorly characterized (misnamed) species. Therefore, for identification, the 16S rRNA gene sequences must be compared only to those of

Table 3 Fluo-Card Milleri identification of the *S. anginosus* group[1]

16S ID	Fluo-Card reaction			Fluo-Card ID
	β-fucosidase	β-glucosidase	α-glucosidase	
S. anginosus				
(*n*=14)[2]	-	+	-$^+$	*S. anginosus* (45%)
(*n*=16)	+	-	+	*S. intermedius* (55%)
S. constellatus				
(*n*=18)[2]	-	-	+$^-$	*S. constellatus* (76%)
(*n*=5)	-	+	-$^+$	*S. anginosus* (24%)
S. intermedius				
(*n*=11)[2]	+	+	+$^-$	*S. intermedius* (100%)

[1] + = >90% of strains are positive; - = >90% of strains are negative; +$^-$ = 80–90% of strains are positive; -$^+$ = 80–90% of strains are negative

[2] Includes reference strain(s)

well characterized reference strains in GenBank: accession numbers AB 355609 and AF 10467 for *S. anginosus*; AB 355605 and AF 104676 for *S. constellatus*; and AF104671 for *S. intermedius*. Furthermore, the last part of the 16S rRNA gene seems to be less discriminatory, and, therefore, using the first part of the 16S rRNA gene (~700–800 bp) gave better results than the full 16S rRNA gene sequence (~1,400 bp). Picard et al. performed an extensive sequence analysis of 28 streptococcal species and showed that *tuf* gene sequencing generally resulted in better discrimination of the species than 16S rRNA gene sequencing [14]. More specifically, they observed that *tuf* gene sequencing performed well in discriminating the AGS; however, their study included only the type strains of the three species and three *S. anginosus* clinical strains [14]. Our results expand this observation: *tuf* gene sequencing correctly identified all of the reference strains and 61 clinical isolates included in the present study.

We studied the utility of Rapid ID 32 Strep, API Strep, Fluo-Card Milleri, and Wee-tabs for the identification of the AGS. Our biochemical test results were generally in agreement with those reported earlier [1, 7, 13]. The Rapid ID 32 Strep proved to be most useful in separating the three AGS species (Table 2). *S. intermedius* could readily be differentiated from the other two species by Rapid ID 32 Strep β-galactosidase and β-NAG reactions; this result is unlike that reported by Limia et al. l [11], who found that only 57% of the *S. intermedius* strains were correctly identified by the Rapid ID 32 Strep test. Three of the *S. anginosus* strains tested gave red-orange (positive) reaction in the Rapid ID 32 Strep resorufin-βD-galactopyranoside test (well 1.2), whereas the 2-naphthyl-βD-galactopyrano-side test (well 0.2) yielded a pale purple color, which would be interpreted as negative. Wee-tabs β-fucosidase, β-NAG, and ONPG readily separated *S. intermedius* (all positive) from the other two species (all negative). The Rapid ID 32 Strep β-glucosidase reaction could be used to separate *S. anginosus* strains from *S. constellatus*; all β-glucosidase-negative strains were *S. constellatus*. Our Fluo-Card Milleri results are not in complete agreement with previous reports [10, 13]. All *S. intermedius* strains were correctly identi-fied; however, 55% of the *S. anginosus* strains also showed β-fucosidase activity with this method, and would, there-fore, be identified as *S. intermedius* by Fluo-Card (Table 3). Unlike *S. intermedius*, all β-fucosidase-positive *S. anginosus* strains were β-glucosidase-negative, which could be used as a distinguishing characteristic between these two species. Our results also indicate that 24% of *S. constellatus* are β-glucosidase-positive in the Fluo-Card Milleri test and, therefore, could be misidentified.

In conclusion, both 16S rRNA gene and *tuf* gene sequencing can be used for the reliable identification of the AGS. If using 16S rRNA gene sequencing for the identification of the AGS, care must be taken to compare the sequences only to those of well characterized strains in GenBank. *S. intermedius* could readily be differentiated from the other two species by biochemical tests, such as the Rapid ID 32 Strep and Wee-tab β-NAG and ONPG tests. Biochemical tests, such as the Rapid ID 32 Strep β-glucosidase reaction, are useful in the differentiation of *S. anginosus* from *S. constellatus*; however, in some cases, 16S rRNA gene or *tuf* gene sequencing is needed to separate these two species.

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