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Species-Level Molecular Identification of Invasive "*Streptococcus milleri*" Group Clinical Isolates by Nucleic Acid Sequencing in a Centralized Regional Microbiology Laboratory

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Organisms belonging to the "*Streptococcus milleri*" group are important invasive human pathogens. A detailed understanding of their pathogenesis in human infection has only recently been facilitated by the use of molecular methods to study this group of organisms.

Since their first description over four decades ago as nonhemolytic streptococcal mouth flora (5), the "*Streptococcus milleri*" group (SMG) has undergone significant taxonomic change. Although initially considered a single species, DNA-DNA hybridization studies have resulted in the taxonomic recategorization of the SMG group into three distinct species, *S. anginosus*, *S. constellatus*, and *S. intermedius* (12, 13). These organisms are found as part of the normal bacterial flora of the human respiratory, gastrointestinal, and genitourinary tracts, although they are also known for their propensity to cause serious invasive infections, including liver, lung, and brain abscesses; bacteremia; endocarditis; and intra-abdominal infections (9). Microbiologically, members of the SMG form tiny (<0.5 mm in diameter) colonies that may demonstrate alpha- or beta-hemolysis or no hemolysis on blood agar media (9). Strains may possess Lancefield group A, C, F, or G antigens or be nongroupable (6, 9). Phenotypic methods that may be used to differentiate members of this group from other streptococci include colony characteristics, Lancefield grouping, caramel smell, and various biochemical traits such as the ability to hydrolyze arginine, acetoin production (positive Voges-Proskauer test), and the inability to ferment sorbitol (2, 6, 9). While multiple methods to distinguish between the three SMG species have emerged (4, 7, 11), the biochemical scheme proposed by Whiley and coworkers (11) initially became the "gold standard" to which other phenotypic methods are still compared. However, definitive identification methods such as 16S rRNA gene sequencing have only recently been employed to study this group of organisms. Here, we report our nucleic acid sequence-based anatomic site-specific analysis of 98 presumptive SMG invasive clinical isolates recovered at a centralized microbiology laboratory over a period of two and a half years.

Testing was performed at Calgary Laboratory Services, an integrated medical laboratory that provides centralized diagnostic microbiology services for the entire Calgary Health Region (population, >1.25 million), including ambulatory, long-term care, and hospitalized patients. Frozen, non-species-identified, sequential, nonduplicate sterile-site presumptive SMG clinical isolates recovered at Calgary Laboratory Services during a 21-month period (April 1999 to December 2000) underwent partial 16S rRNA gene (first 500 base pairs) sequencing. Isolates had previously been phenotypically identified as SMG based on Gram stain, colony characteristics, and tests for catalase, arginine hydrolysis, and acetoin production. Data on the anatomic source of each isolate were recorded. After two consecutive overnight subcultures onto 5% sheep blood agar, genomic DNA was extracted from growing bacterial colonies using QIAGEN DNA minikits (QIAGEN, Inc., Alameda, CA). PCR amplification and cycle sequencing reactions were performed using MicroSeq 500 kits (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, with sequenced products analyzed on an ABI Prism 3100 genetic analyzer. Sequence data were compared with the GenBank database using BLAST (available at http://www.ncbi.nlm.nih.gov/BLAST/). Isolates were identified to the species level based on a best sequence match with respective species-specific16S rRNA sequences in the GenBank database, with a minimum of 97% sequence identity and 400-nucleotide alignment lengths. A total of 98 presumptive SMG isolates were tested after excluding duplicate same-source isolates from the same patient. Of these, three failed to grow while another three failed to sequence, despite multiple attempts. In eight cases, organisms not belonging to the SMG were identified, including five streptococcal species (two strains of *S. sanguinis* and one strain each of *S. cristatus*, *S. australis*, and *S. gordonii*) and three nonstreptococcal species (one strain each of *Staphylococcus* sp., *Corynebacterium* sp., and *Actinomyces* sp.). Three isolates had 16S rRNA gene sequence patterns suggesting an SMG but with a best-match species-specific sequence identity below the 97% threshold level. Of the 81 true SMG isolates sequenced, 25 (30.8%) were recovered from blood cultures while 56 (67.1%) were obtained from other sterile body sites. *Streptococcus intermedius* was the most commonly isolated species. Overall, 32/81 (39.5%) SMG isolates were found to be *S. intermedius* and 28/81 (34.6%) were *S. anginosus*, while 21/81 (25.9%) were *S. constellatus*. Blood culture isolates showed a relatively equal predominance between the three SMG species. However, intra-abdominal source

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TABLE 1. Species-specific distribution of SMG organisms by anatomic site

Anatomic site	No. (%) of isolates		
	S. anginosus (n = 28)	*S. intermedius* (n = 32)	*S. constellatus* (n = 21)
Blood	10	7	8
Central nervous system	0	2	1
Head and neck	1	0	0
Intrathoracic	1	17	3
Intra-abdominal	10	1	3
Pelvic	1	2	0
Bone/deep soft tissue	4	1	3
Unknown	1	2	3

isolates were predominantly *S. anginosus* (10/14 [71.4%]; χ^2 = 9.57 using Monte Carlo simulation; $P = 0.008$), while respiratory and intrathoracic isolates were predominantly *S. intermedius* (17/21 [80.6%], $\chi^2 = 21.71$; $P < 0.0001$). In two patients, SMG strains were recovered from two anatomic sites, including blood and either liver abscess (*S. intermedius*) or cerebrospinal fluid (*S. constellatus*). Table 1 summarizes the species-specific distribution of SMG organisms by anatomic site.

Sequencing of the eubacterial 16S rRNA gene offers an extremely discriminatory tool for identification to the species level of SMG strains and other microorganisms. Although the superiority of DNA sequencing over phenotypic methods was not specifically addressed in our study, the results obtained allowed us to make some general conclusions regarding the site-specific predilection of the member species. *Streptococcus intermedius* was the most commonly isolated species in our study, in contrast to previous reports noting a predominance of *S. anginosus* (1, 3). In agreement with previous studies (1, 3), *S. anginosus* was well represented from intra-abdominal sources. However, intrathoracic infections were overwhelmingly caused by *S. intermedius*, in contrast to previous reports of *S. constellatus* predominance (3, 6). When bloodstream isolates alone were considered, a relatively equal distribution of the three species was observed, reflecting the capability of all members of this group to invade and disseminate throughout the body. No studies prior to ours have examined this group of organisms in the setting of a regionalized clinical microbiology laboratory, which may partially account for the uniqueness of our observations. Some of the differences in the results observed between our study and those of other investigators may be related to different methods (phenotypic versus genotypic) used to characterize this group of organisms. Nevertheless, further study of the clinical utility of species identification for SMG strains is required. Identification to the species level is not likely to be important in guiding medical therapy for infections caused by these organisms, since the antimicrobial susceptibility profiles of genetically characterized SMG strains among the three species appear to be very similar (10). Species-level identification of blood culture isolates in patients without an obvious focus of infection may help point to the likely source of the bacteremia. Whether sterile-site infections caused by *S. anginosus* represent an occult gastrointestinal disorder, as is often the case for *Streptococcus bovis*, requires further investigation.

The findings of our study using 16S rRNA gene sequencing have facilitated our understanding of the pathogenesis of these organisms, allowing us to conclude that the pathogenic nature of the individual species is distinctive. Other genetic targets with species-specific signature sequences, such as *sodA* (encoding a manganese-dependent superoxide dismutase), have also been shown to be reliable for the characterization of these and other streptococcal strains (8).

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