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Phylogenetic Analysis of Viridans Group Streptococci Causing Endocarditis[▽]

Keith E. Simmon,[1] Lori Hall,[2] Christopher W. Woods,[2,3] Francesc Marco,[4] Jose M. Miro,[4] Christopher Cabell,[5] Bruno Hoen,[6] Mercedes Marin,[7] Riccardo Utili,[8] Efthymia Giannitsioti,[9] Thanh Doco-Lecompte,[10] Suzanne Bradley,[11] Stanley Mirrett,[3] Arjana Tambic,[12] Suzanne Ryan,[13] David Gordon,[14] Phillip Jones,[15] Tony Korman,[16] Dannah Wray,[17] L. Barth Reller,[3] Marie-Francoise Tripodi,[8] Patrick Plesiat,[6] Arthur J. Morris,[18] Selwyn Lang,[19] David R. Murdoch,[20] Cathy A. Petti,[1,21]* and the International Collaboration on Endocarditis Microbiology (ICE-Micro) Investigators

ARUP Laboratories, Salt Lake City, Utah[1]; Duke University Medical Center[2] and Veterans Administration Medical Center,[3] Durham, North Carolina; Hospital Clinic Institut d'Investigacions Biomediques August Pi i Sunyer-University of Barcelona, Barcelona, Spain[4]; Quintiles Transnational[5]-University Medical Center of Besançon,[6] Besançon France; Hospital General Universitario Gregorio Maranon, Madrid, Spain[7]; Second University of Naples, Naples, Italy[8]; Attikon University General Hospital, Athens, Greece[9]; CHU Nancy-Brabois, Nancy, France[10]; Veterans Administration Medical Center, Ann Arbor, Michigan[11]; University Hospital for Infectious Diseases, Zagreb, Croatia[12]; South Eastern Sydney and Illawarra Area Health Service, Wollongong, Australia[13]; Flinders Medical Centre, Adelaide, Australia[14]; The University of New South Wales, Sydney, Australia[15]; Southern Health, Clayton, Australia[16]; Medical University of South Carolina, Charleston, South Carolina[17]; Auckland City Hospital, Auckland, New Zealand[18]; Middlemore Hospital, Auckland, New Zealand[19]; University of Otago, Christchurch, New Zealand[20]; and Departments of Pathology and Medicine, University of Utah School of Medicine, Salt Lake City, Utah[21]

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Identification of viridans group streptococci (VGS) to the species level is difficult because VGS exchange genetic material. We performed multilocus DNA target sequencing to assess phylogenetic concordance of VGS for a well-defined clinical syndrome. The hierarchy of sequence data was often discordant, underscoring the importance of establishing biological relevance for finer phylogenetic distinctions.

Identification of viridans group streptococci (VGS) to the species level by genotypic and phenotypic methods is challenging since biologic or biochemical profiles may be ambiguous because of natural competence, i.e., the ability of streptococci to take up free DNA from the surrounding environment (5), and other genetic transfer events. Clinical laboratories can encounter "atypical pneumococci" that are optochin resistant, bile insoluble, or unencapsulated (15). Similarly, some species, such as *Streptococcus mitis*, an oral commensal commonly associated with infective endocarditis, may carry antimicrobial resistance genes or pneumolysin genes that are commonly associated with *Streptococcus pneumoniae* (2, 15). Hence, taxonomical classifications for members within VGS are not well defined and for simplicity are divided into groups such as the *S. anginosus* group (*S. anginosus*, *S. constellatus*, and *S. intermedius*), the *S. bovis* group (*S. equinus*, *S. gallolyticus* subsp. *gallolyticus*, *S. gallolyticus* subsp. *macedonicus*, *S. gallolyticus* subsp. *pasteurianus*, *S. infantarius*, and *S. alactolyticus*), *S. gordonii* (*S. gordonii*), the *S. mitis* group (*S. cristatus*, *S. infantis*, *S. mitis*, *S. oralis*, and *S. pneumoniae*), the *S. mutans* group (*S. mutans* and *S. sobrinus*), the *S. salivarius* group (*S. salivarius*, *S. thermophilus*, and *S. vestibularis*), and the *S. sanguinis* group (*S. sanguinis* and *S. parasanguinis*).

Since taxonomy of VGS is still evolving, our specific aim was to better understand the phylogenetic and phenotypic relationships among VGS by studying a large collection of isolates from a medically important, well-defined clinical syndrome. Endocarditis is an important infectious disease that is commonly caused by VGS. The International Collaboration on Endocarditis Microbiology, a large, multicenter, multinational consortium, provided a unique opportunity to perform DNA target sequencing with 16S rRNA, *tuf* (encoding elongation factor Tu), and *rpoB* (beta subunit of RNA polymerase) genes to evaluate the degree of phylogenetic concordance for VGS isolates and to assess our ability to definitively assign a species designation for patients with endocarditis.

VGS isolates from patients with definite endocarditis were submitted by ICE investigators representing a collection from 18 medical centers in 12 countries. All patients were defined as having definite infective endocarditis using the modified Duke criteria (8). Conventional identification and susceptibility testing were performed at a central laboratory using a commercially available panel processed on a Microscan Walkaway instrument (PC-21; Dade Behring, Deerfield, IL) with a standard laboratory protocol. Template DNA preparation and amplification were performed directly on frozen stocks as previously described (12). Amplification of the 16S rRNA, *tuf*, and *rpoB* genes was achieved with the following primer pairs: 16S rRNA 5F (5′-TTGGAGAGTTTGATCCTGGCTC-3′) and 534R (5′-TACCGCGGCTGCTGGCAC-3′); *tuf* Str1 (5′-GTA CAGTTGCTTCAGGACGTATC-3′) and Str2 (5′ ACGTTC GATTTCATCACGTTG-3′) (9); and *rpoB* 31F (5′-GCCTTA GGACCTGGTGGTTT-3′) and 830R (5′-GTTGTAACCTTC

* Corresponding author. Mailing address: University of Utah School of Medicine, Department of Pathology, ARUP Laboratories, 500 Chipeta Way, Salt Lake City, UT 84108. Phone: (801) 583-2787. Fax: (801) 584-5207. E-mail: cathy.petti@aruplab.com.

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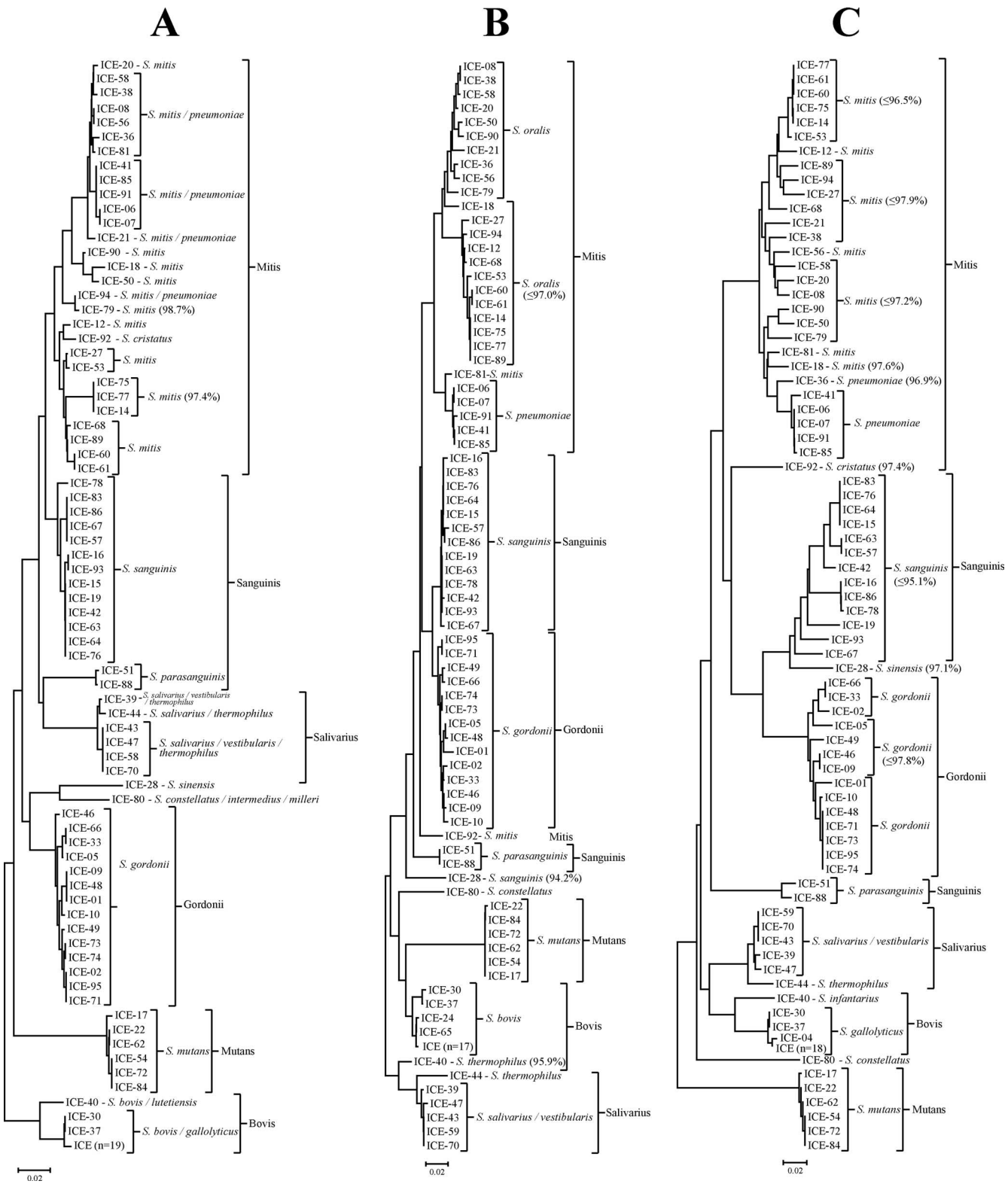

FIG. 1. Neighbor-joining dendrograms of the 16S rRNA (A), *tuf* (B), or *rpoB* (C) gene for viridans group streptococci from patients with endocarditis. Each entry represents a unique sequence among study isolates. Key: *S. bovis* group (isolates 37 and 90; *S. gallolyticus* subsp. *gallolyticus* isolates 03, 04, 11, 13, 23, 24, 25, 26, 29, 31, 32, 34, 35, 45, 52, 55, 65, 69, and 82; and *S. infantarius* isolate 40); *S. constellatus* (isolate 80); *S. gordonii* (isolates 01, 02, 05, 09, 10, 33, 46, 48, 49, 66, 71, 73, 74, and 95); *S. mutans* group (isolates 17, 22, 54, 62, 72, and 84); *S. mitis* group (isolates 08, 12, 14, 18, 20, 21, 27, 36, 38, 44, 53, 56, 58, 60, 61, 68, 75, 77, 79, 81, 89, 90, and 94; *S. pneumoniae* isolates 06, 07, 41, 85, and 91; *S. cristatus*, isolate 92); *S. salivarius* group (isolates 39, 43, 47, 59, and 70; *S. thermophilus* isolate 44); *S. sanguinis group* (isolates 15, 16, 19, 42, 57, 63, 64, 67, 76, 78, 83, 86, and 93; *S. parasanguinis* isolates 51 and 88); and *S. sinensis* isolate 28.

CAWGTCAT-3′) (3). PCR products were bidirectionally sequenced with original amplification primers, and sequences were compared to related sequences in the SmartGene IDNS-Bacteria software program [SmartGene Inc., Raleigh, NC] using a standard laboratory protocol (12). Nucleotide alignments and phylogenetic trees were constructed with the neighbor-joining method using Kimura's two-parameter distance correction model and 1,000 bootstrap replications in the MEGA version 3.1 software package (7).

Ninety-four VGS isolates from 94 patients with definite endocarditis were examined. Characterization of these isolates by three genetic targets is summarized in Fig. 1. For purposes of comparison with conventional methods, final identification for DNA target sequencing was arbitrarily defined as phylogenetic concordance with two of three DNA targets. Using this definition, DNA target sequencing identified the *S. mitis* group (n = 29) as the most common VGS, followed by the *S. bovis* group (n = 22), the *S. sanguinis* group (n = 15), *S. gordonii* (n = 14), 6 isolates each of the *S. mutans* and *S. salivarius* groups, and 1 isolate each of the *S. anginosus* group and *S. sinensis*. Conventional methods identified 17 isolates to the *Streptococcus* genus level only, 14 as VGS, 6 as belonging to the *S. anginosus* group, 24 as belonging to the *S. mitis* group, 16 as *S. bovis*, 2 as *S. mutans*, 1 as *S. salivarius*, and 14 as *S. sanguinis*. Identification by conventional methods disagreed with gene sequencing results for 25 (27%) of 94 isolates. Neighbor-joining dendrograms for all gene targets did not always demonstrate phylogenetic concordance (Fig. 1). Although intraspecies and interspecies variability differed with each gene target, phylogenetic concordance was uniformly observed with all 3 gene targets for isolates identified as *S. gordonii* (n = 14), *S. mutans* (n = 6), *S. parasanguinis* (n = 2), *S. salivarius* (n = 6), or *S. sanguinis* (n = 13). Within the *S. mitis* group, five *S. pneumoniae* isolates (no. 6, 7, 41, 85, and 91) demonstrated concordance among all three targets but two isolates (no. 36 and 81) had no distinguishing genotypic or phenotypic characteristics to enable definitive identification as pneumococcus. For the S. *bovis* group, 19 isolates were identified as *S. gallolyticus* subsp. *gallolyticus*, of which 15 isolates demonstrated concordance among all 3 targets. Isolates 3, 24, and 65 clustered with *S. gallolyticus* subsp. *gallolyticus* for 16S rRNA and *rpoB* genes, but with the *tuf* gene they diverged on a unique branch. Isolate no. 40, identified as *S. infantarius* by the *rpoB* gene, had a unique divergent branch with each gene target. *S. constellatus* (no. 80) had concordance with all three gene targets. *S. cristatus* (no. 92) and *S. sinensis* (no. 28) were discordant among three gene targets.

To our knowledge, this study is the first to evaluate the phylogenetic relationships of VGS with 16S rRNA, *tuf*, and *rpoB* gene targets for patients with endocarditis. Since causality is an important criterion when comprehending the significance of finer phylogenetic distinctions by different gene targets, we specifically studied a well-defined clinical syndrome, namely endocarditis. Overall, VGS isolates demonstrated a high degree of variability for all three targets, which was not a surprising observation since transfer of genetic material among microorganisms has been well described for streptococci (2, 5, 15). In our study, classifications within groups were not always predictable or correlated with phenotype or phylogeny, an observation also noted by Hoshino et al. for isolates from patients with bacteremia and meningitis (6). By the current and published standard, our unusual sequence variants could support several new species designations. In fact, prior investigators have proposed the assignment of new species based on isolated case reports or have claimed a greater accuracy of identification with sequencing of the 16S rRNA, *rnpB*, *rpoB*, *sodA*, and/or 16S-23S rRNA spacer targets (1, 3, 4, 6, 13, 14). We have shown that both phenotype and hierarchy-of-sequence data are often discordant, and the relative ability of a particular DNA target to draw finer phylogenetic distinctions does not necessarily warrant the description of a new species or support greater accuracy of identification. Each DNA target has its own evolutionary time clock, and reliance on both single- and multilocus sequencing, including concatenated sequence analysis, may result in taxonomical misclassification or overclassification.

Microbiologists and clinicians need a common lexicon to convey information about microorganisms that is biologically meaningful (10, 11). Population-based genomic and proteomic approaches most likely are necessary to facilitate taxonomical classifications that encompass the microbial diversity of VGS from diverse environments and their unique features (i.e., virulence factors). Phenotypic and genotypic repositories are invaluable for the community of microbial systematics. Until we better define VGS and more fully understand the added value of new species designations, clinical microbiologists should classify VGS with ambiguous phenotypic and genotypic profiles in their respective taxonomical clusters regardless of the method of identification.

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