Comparasion of five gene loci (*rnpB*, 16S rRNA, 16S-23S rRNA, *sodA* and *dnaJ*) to aid the molecular identification of viridans-group streptococci and pneumococci

Y. MAEDA[*†], C. E. GOLDSMITH[*], W. A. COULTER[‡], C. MASON[‡], J. S. G. DOOLEY[†], C. J. LOWERY[†], B. C. MILLAR[*] and J. E. MOORE[*†]

[*]*Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast;* [†]*School of Biomedical Sciences, University of Ulster, Coleraine; and* [‡]*School of Dentistry, Queen's University of Belfast, Royal Group of Hospitals, Belfast, Northern Ireland, UK*

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ABSTRACT

Viridans-group streptococci (VGS) consist of several taxa which historically have been highly diverse. However, at times it may become necessary to have a reliable scheme for the identification of these organisms to the species level. The aim of this study is to compare the ability of five gene loci, namely *rnpB*, 16S rRNA, 16S–23S rRNA, *sodA* and *dnaJ*, to speciate such organisms through a sequence typing-based approach. Reference organisms consisting of six VGS species were compared based on sequence typing, followed by comparison of 31 wild-type respiratory isolates, and showed that employment of sequence typing using the *rnpB* gene locus was the most specific and reliable. Therefore, the use of *rnpB* sequencing for the identification of VGS to species level is a reliable and feasible option, based on a single gene target.

KEY WORDS: Molecular typing.
Streptococcus pneumoniae.
Viridans streptococci.

Introduction

Oral streptococci are largely composed of members of the viridans-group streptococci (VGS), which currently encompasses 20 species, which are commensal inhabitants of the oropharyngeal cavity and the gastrointestinal and genital tracts of mammals.[1] On the basis of 16S rRNA sequence homology, these bacteria are categorised into four groups: the salivarius rRNA homology group, including *Streptococcus thermophilus*, *S. vestibularis* and *S. salivarius*; the mitis group, including *S. cristatus*, *S. gordonii*, *S. oralis*, *S. mitis*, *S. pneumoniae*, *S. sanguinis* and *S. parasanguinis*; the anginosus group, including *S. anginosus*, *S. constellatus* and *S. intermedius*; and the mutans group, including *S. mutans*, *S. criceti*, *S. downei*, *S. ferus*, *S. macacae*, *S. ratti* and *S. sobrinus*.[2]

Correct identification of VGS is difficult due to their phenotypic variability coupled with high similarity of their gene sequences.[3] Traditionally, biochemical tests have been used to identify these organisms and commercially available kits have become available to help clinical microbiology laboratories with this task whenever a definitive identification is required; for example, in the identification of a blood culture isolate causing infective endocarditis. However, there are limitations to successful identification of VGS by biochemical tests, due mainly to variable phenotypic characteristics as well as their limited coverage in commercial databases provided by manufacturers. To overcome this problem, many molecular methods have been developed including species-specific polymerase chain reaction (PCR),[4,5] restriction fragment length polymorphism (RFLP),[6] random amplified polymorphic DNA (RAPD),[7] DNA hybridisation[8,9] and target gene sequence analysis using a variety of genes.[10–13] However, there is only one report on a comparative study of those target gene sequence analyses.

This study aims to speciate clinical isolates of VGS using five previously published gene sequencing methods, and evaluate the robustness of each to help guide clinical microbiology laboratories towards the most successful single gene sequence typing assay to adopt for VGS identification purposes.

Materials and methods

Five reference strains of VGS and *S. pneumoniae* were employed in this study: *S. anginosus* NCTC 10713, *S. mutans* NCTC 10449, *S. oralis* NCTC 11427, *S. pneumoniae* NCTC 7465, *S. pneumoniae* ATCC 49619, *S. salivarius* NCTC 8618 and *S. sanguinis* NCTC 7863. In order to construct a comprehensive phylogenetic tree, sequences of the following reference strains were obtained from the GenBank: *S. australis* NCTC 13166, *S. cristatus* NCTC 12479, *S. gordonii* NCTC 7865, *S. infantis* ATCC 700779, *S. mitis* NCTC 12261, *S. parasanguinis* ATCC 15912 and *S. pneumoniae* R6. For *S. pneumoniae* and

Correspondence to: Professor John E. Moore
Northern Ireland Public Health Laboratory, Department of Bacteriology, Belfast City Hospital, Belfast BT9 7AD, Northern Ireland, UK
Email: jemoore@niphl.dnet.co.uk

Table 1. PCR primer sequences, amplicon sizes and thermocycling conditions used.

Target	Primer name	Primer sequence (5' to 3')	Product size (bp)	Annealing temperature
rnpB	rnpBF	TGC AAT TTT YGG ATA ATC GC	390	54˚C
	rnpBR	TAT GCA ATT TTC TRT AAG CC		
16S rRNA gene	27F	AGT GTT TGA TCM TGG CTC AG	1490	58˚C
	1492R	ACG GYT ACC TTG TTA CGA CTT		
16S-23S rRNA gene ITS	13BF	GTG AAT ACG TTC CCG GGC CT	550-700	63˚C
	6R	GGG TTY CCC CRT TCR GAA AT		
sodA	sodAF	ATG GCW ATT ATY YTW CCW GA	550	47˚C
	sodAR	GCY TKG ATR TAR TYH GGA CG		
dnaJ	dJ2	ACA ATA CHG AAT WTT AYG AYC GTC	720	51˚C
	dJ3	TTR AAR CCW GCT TCH CCT TG		

Table 2. BLASTn search results for the 31 clinical isolates examined with the five gene loci showing the closest species and percentage similarity in parenthesis.

	Identity by gene sequence				
Strain	***rnpB***	**16S–23S rDNA ITS**	**16S rDNA**	***sodA***	***dnaJ***
C61	*S. anginosus* (99)[a]	*S. anginosus* (99)	*S. anginosus* (99)	*S. anginosus* (100)	*S. gordonii* (95)
C143	*S. anginosus* (99)	*S. anginosus* (98)	*S. anginosus* (99)	*S. anginosus* (97)	*S. anginosus* (90)
C210	*S. anginosus* (99)	*S. anginosus* (100)	*S. anginosus* (99)	*S. anginosus* (100)	*S. anginosus* (91)
C89	*S. mitis* (96)[b]	*S. australis* (99)	*S. sanguinis* (99)	*S. australis* (98)	*S. peroris* (83)[d]
C51	*S. cristatus* (99)	*S. cristatus* (98)	*S. cristatus* (99)	*S. cristatus* (96)	*S. sanguinis* (84)
C122	*S. cristatus* (99)	*S. cristatus* (98)	*S. cristatus* (99)	*S. cristatus* (100)	*S. mitis* (82)
C107	*S. gordonii* (98)	*S. gordonii* (98)	*S. gordonii* (99)	*S. gordonii* (100)	*S. gordonii* (94)
C146	*S. gordonii* (98)	*S. gordonii* (100)	*S. gordonii* (99)	*S. gordonii* (100)	*S. gordonii* (97)
C163	*S. gordonii* (98)	*S. gordonii* (100)	*S. gordonii* (99)	*S. gordonii* (97)	*S. gordonii* (96)
C49	*S. infantis* (94)	*S. infantis* (98)	*S. infantis* (99)	*S. infantis* (99)	*S. infantis* (98)
C50	*S. infantis* (99)	*S. infantis* (100)	*S. infantis* (99)	*S. infantis* (99)	*S. infantis* (98)
C131	*S. infantis* (100)	*S. infantis* (98)	*S. infantis* (98)	*S. peroris* (94)	*S. infantis* (99)
C31	*S. mitis* (96)	*S. pneumoniae* (100)	*S. mitis* (99)	*S. pneumoniae* (97)	*S. mitis* (95)
C42	*S. mitis* (97)	*S. mitis* (99)	*S. mitis* (99)	*S. mitis* (97)	*S. mitis* (99)
C44	*S. mitis* (97)	*S. pneumoniae* (99)	*S. pneumoniae* (99)	*S. pneumoniae* (96)	*S. mitis* (98)
C169	*S. mutans* (99)	*S. mutans* (100)	*S. mutans* (100)	*S. mutans* (99)	*S. mutans* (99)
C3	*S. oralis* (99)	*S. oralis* (100)	*S. mitis* (99)	*S. oralis* (98)	*S. oralis* (99)
C12	*S. oralis* (98)	*S. oralis* (100)	*S. mitis* (99)	*S. oralis* (97)	*S. oralis* (96)
C56	*S. oralis* (99)	*S. oralis* (99)	*S. oralis* (99)	*S. oralis* (95)	*S. oralis* (100)
C27	*S. parasanguinis* (96)	*S. parasanguinis* (97)	*S. oralis* (99)	*S. oralis* (99)	*S. peroris* (85)
C68	*S. mitis* (100)[c]	*S. parasanguinis* (98)	*S. oralis* (99)	*S. parasanguinis* (100)	*S. peroris* (85)
C86	*S. mitis* (99)[c]	*S. parasanguinis* (98)	*S. parasanguinis* (99)	*S. parasanguinis* (100)	*S. peroris* (83)
C10	*S. pneumoniae* (99)	*S. mitis* (99)	*S. mitis* (99)	*S. mitis* (100)	*S. mitis* (96)
C30	*S. pneumoniae* (99)	*S. pneumoniae* (100)	*S. mitis* (99)	*S. oligofermentans* (97)	*S. mitis* (97)
C60	*S. pneumoniae* (99)	*S. pneumoniae* (100)	*S. pneumoniae* (100)	*S. pneumoniae* (99)	*S. pneumoniae* (100)
C4	*S. salivarius* (99)	*S. salivarius* (99)	*S. salivarius* (99)	*S. salivarius* (99)	*S. thermophilus* (91)[e]
C5	*S. salivarius* (99)	*S. salivarius* (100)	*S. salivarius* (100)	*S. salivarius* (99)	*S. thermophilus* (87)
C9	*S. salivarius* (97)	*S. salivarius* (98)	*S. salivarius* (99)	*S. vestibularis* (94)	*S. thermophilus* (90)
C62	*S. sanguinis* (99)	*S. sanguinis* (100)	*S. sanguinis* (100)	*S. sanguinis* (97)	*S. sanguinis* (97)
C72	*S. sanguinis* (99)	*S. sanguinis* (100)	*S. sanguinis* (99)	*S. sanguinis* (100)	*S. sanguinis* (96)
C83	*S. sanguinis* (99)	*S. sanguinis* (100)	*S. sanguinis* (99)	*S. sanguinis* (98)	*S. sanguinis* (97)

[a] percentage of homology by BLAST search

[b] *S. australis rnpB* gene sequence is not available in GenBank

[c] *S. mitis* ATCC 903 has recently been revised as *S. parasanguinis*

[d] *S. australis dnaJ* gene sequence is not available in GenBank

[e] *S. salivarius dnaJ* gene sequence is not available in GenBank

S. mitis, optochin susceptibility tests and bile solubility tests were conducted in order to support the molecular identification results.

In a proof-of-concept experiment to evaluate the ability of each of the five loci to speciate unknown VGS isolates into distinct species, streptococcal isolates (n=31) were obtained from the culture collection of the Northern Ireland Public Health Laboratory, Belfast City Hospital. These isolates had originally been obtained from respiratory specimens and had been obtained by conventional microbiological culture techniques for the presence of VGS organisms and pneumococci. All isolates were recovered on mitis-salivarius agar for 48 h at 37˚C under microaerophilic conditions.

Purified isolates were subcultured on Columbia blood agar supplemented with 5% (v/v) defibrinated horse blood for 24 h at 37˚C under microaerophilic conditions. All DNA isolation procedures were carried out in a Class II biological safety cabinet in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the 'post-PCR' room, in accordance with the Good Molecular Diagnostic Procedures (GMDP) guidelines of Millar *et al.*,[14] in order to minimise contamination and hence the possibility of false-positive results. Bacterial genomic DNA was extracted from a few colonies of each isolate using the Roche High Purity PCR Template Preparation Kit, in accordance with the manufacturer's instructions. Extracted DNA was stored at –20˚C prior to PCR amplification.

The *rnpB* gene,[15] 16S–23S rRNA ITS,[16] 16S rRNA gene,[17] *sodA* gene[18] and *dnaJ* gene[19] were amplified by PCR. Primer sequences and annealing temperature are summarised in Table 1. Reaction mixes (25 µL) contained 1 µL DNA template containing 25 ng DNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 µmol each dNTP, 0.6 units *Thermus aquaticus* (*Taq*) DNA polymerase and 10 µmol each primer. Following a 'hot start', the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermocycler: 95˚C for 5 min followed by 35 cycles of 95˚C for 30 sec, annealing for 30 sec, 72˚C for 45 sec, followed by a final extension at 72˚C for 5 min. Following amplification, PCR products were visualised on 1–1.5% (w/v) agarose gels in 0.5 x TBE buffer followed by staining with ethidium bromide (0.5 µg/100 mL) and ultraviolet (UV) illumination with a gel image analysis system.

For DNA sequencing, amplicons were purified using the QIA Quick PCR purification kit, following the manufacturer's instructions. Sequences of the partial regions of all five gene loci were determined using the BigDye Terminator Cycle Sequencing Kit and ABI 3100 genetic analyser. Sequence analysis was carried out using Genetyx. For phylogenetic analysis, sequences of the type strains *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis* and *S. parasanguinis* were obtained from GenBank. Phylogenetic trees were constructed by the neighbour-joining method using MEGA 4.0.2.[20] To evaluate robustness, 1000 bootstrap replicates were performed. In addition, sequences were subjected to BLASTn searches so that assigned species could be compared with all five gene loci.

Results

Confirmed sequences were obtained for each of the five gene loci examined for all isolates examined (i.e., five reference strains and 31 wild-type strains). In the majority of permutations, all five gene loci correctly identified the species for the five references strains examined following correct speciation of the reference strains, with the exception of *S. australis* and *S. infantis* (no *rnpB* sequence deposited in GenBank [*S. australis*], and *rnpB* sequences of *S. infantis* and *S. peroris* share 100% homology). Subsequently, species were assigned to each of the wild-type isolates based on a consensus between the species given following BLASTn analyses of the five gene loci (Table 2).

One representative sequence from each species for each respective gene was deposited in GenBank under the accession numbers (Table 3). Overall, 12 VGS species were identified from the 31 wild-type respiratory isolates, including *S. anginosus*, *S. australis*, *S. cristatus*, *S. gordonii*, *S. infantis*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguinis*, *S. pneumoniae*, *S. salivarius* and *S. sanguinis*. The similarity score of each gene sequence among 12 species were *rnpB*

Table 3. The GenBank accession numbers for five gene sequences of representative strains

		GenBank Accession Number				
Species	Strain	*rnpB*	16S-23S rRNA ITS	16S	*sodA*	*dnaJ*
S. anginosus	C61	GU907491	GU907504	GU907516	GU907528	GU907540
S. australis	C89	GU907492	GU907505	GU907517	GU907529	GU907541
S. cristatus	C122	GU907493	GU907506	GU907518	GU907530	GU907542
S. gordonii	C163	GU907494	GU907507	GU907519	GU907531	GU907543
S. infantis	C50	GU907495	GU907508	GU907520	GU907532	GU907544
S. mitis	C42	GU907496	GU907509	GU907521	GU907533	GU907545
S. mutans	C169	GU907497	GU907510	GU907522	GU907534	GU907546
S. oralis	C12	GU907498	GU907511	GU907523	GU907535	GU907547
S. parasanguinis	C86	GU907499	GU907512	GU907524	GU907536	GU907548
S. pneumoniae	C30	GU907500	GU907513	GU907525	GU907537	GU907549
S. salivarius	C5	GU907501	GU907514	GU907526	GU907538	GU907550
S. sanguinis	C62	GU907502	GU907515	GU907527	GU907539	GU907551

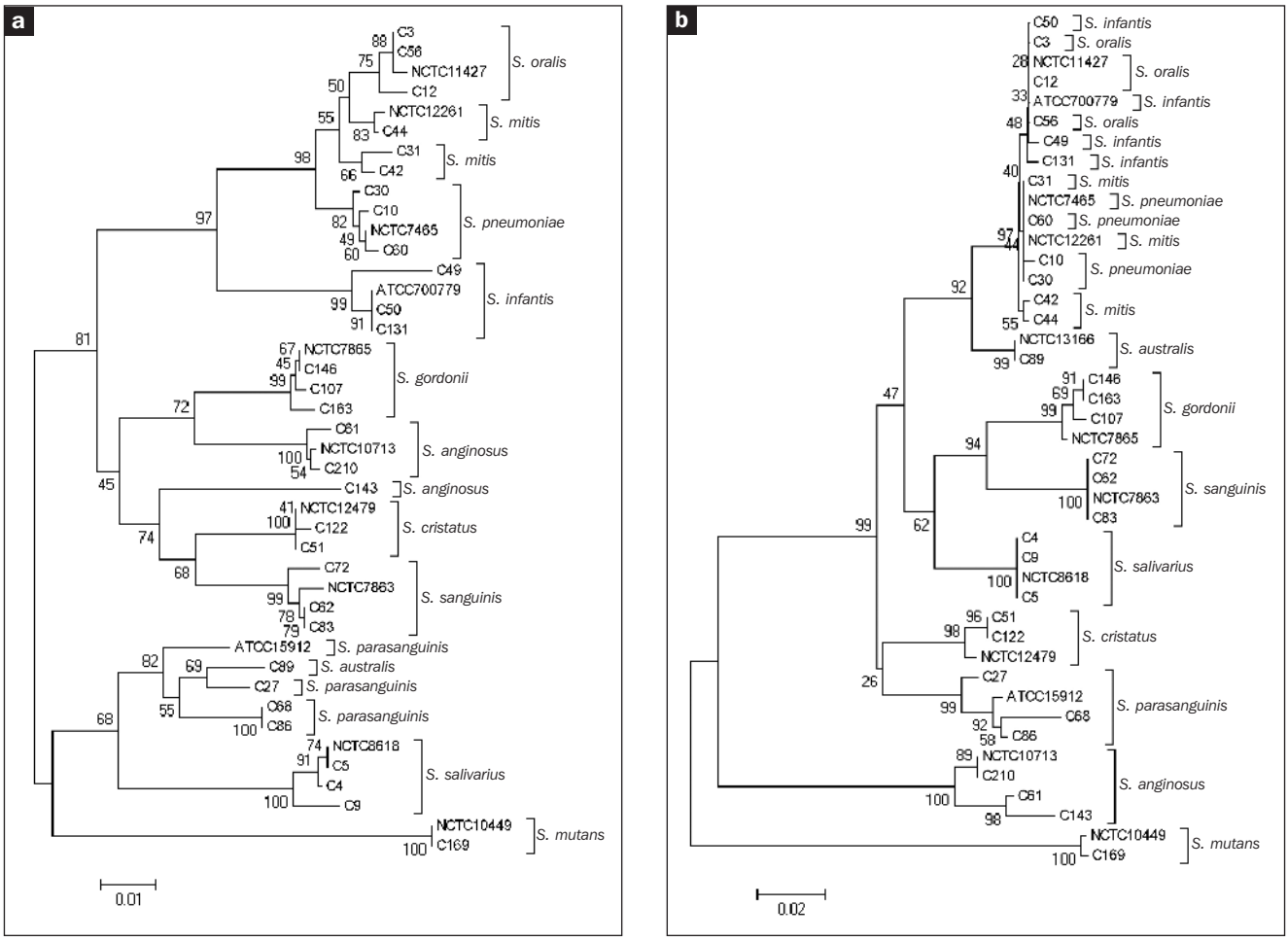


Fig. 1. Phylogenetic tree constructed from (a) partial *rnpB* gene sequences (b) complete 16S-23S ITS sequences (c) partial 16S rRNA sequences (d) partial *sodA* sequences (e) partial *dnaJ* sequences (f) concatenated five gene sequences using the neighbour-joining method. The numbers on nodes show 1000 bootstrap percentages.

71–100%, 16S–23S rRNA ITS 32–100%, 16S rRNA 90–100%, *sodA* 71–100% and *dnaJ* 67–100%.

A phylogenetic tree was constructed for each of the five gene loci (Fig. 1a–e) which generally showed good concordance. *S. pneumoniae*, *S. mitis* and *S. oralis* made a mosaic cluster with four genes, while the *rnpB* gene could clearly distinguish these three species (Fig.1a–e). In addition, all five gene sequences were combined into a single composite sequence by connecting trimmed sequences end to end and a phylogenetic tree was constructed (Fig. 1f).

All resulting sequences were subjected to BLASTn searches. This showed good correlation among four gene loci but not with the *dnaJ* gene. Where available, additional phenotypic tests were performed to confirm the molecular results, whereby those isolates identified as *S. pneumoniae* were confirmed as optochin susceptible and bile soluble, and the opposite for *S. mitis*.

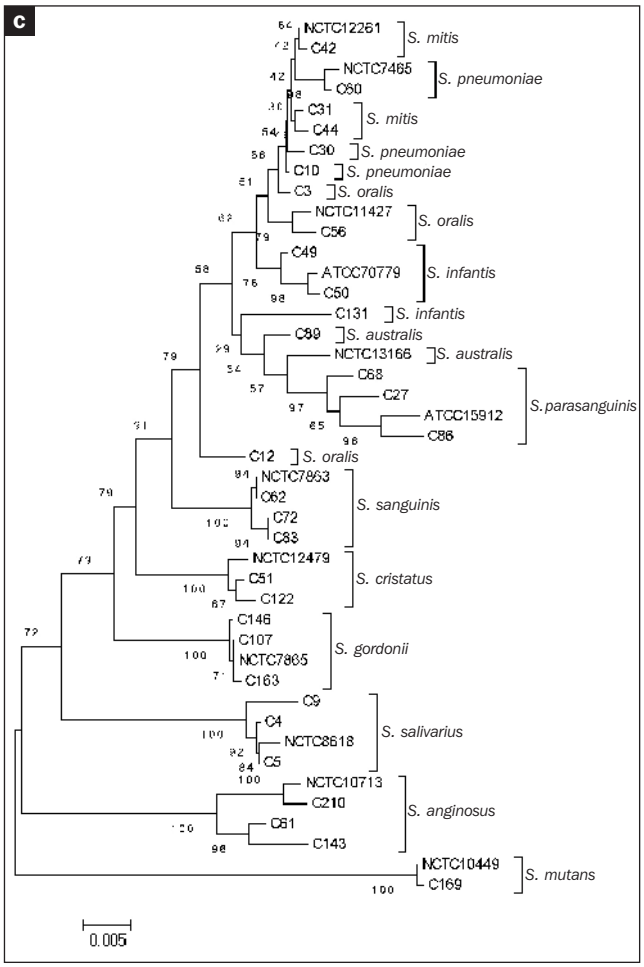
In relation to the BLAST search result (Table 2, isolate C86) which showed *S. parasanguinis* as *S. mitis* (*rnpB* gene), this *S. mitis* sequence was obtained from an ATCC 903 strain, which has recently been revised to *S. parasanguinis*. *S. australis* was not identified correctly in this study because no *rnpB* sequence is deposited for this taxon in GenBank; however, results from the other genes support its identification as *S. australis*.

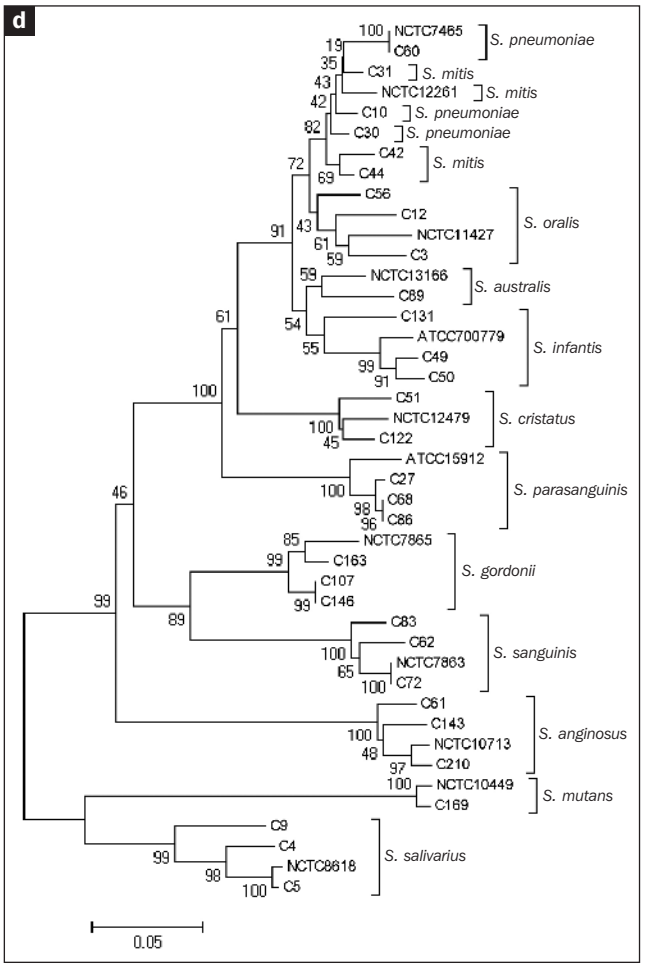
Only one problematic species, *S. infantis*, was identified, and this was due to the fact that the *rnpB* sequence of *S. infantis* and *S. peroris* share 100% homology. Therefore, to identify these two species correctly would require additional gene sequencing (e.g., 16S rRNA gene sequencing).

Discussion

This study aimed to compare five gene loci that could identify VGS to the species level, without the need to use a combination of phylogenetic gene targets. All investigations involved identification of VGS to the species level; however, due to the limitation of biochemical tests to identify VGS to the individual species level, numerous efforts have been made to develop efficient molecular identification methods. As a result, about 20 molecular identification tools have been developed to date through a sequence-based approach. Interestingly, there is no report that compares those various molecular tools to date, whereas comparisons of commercial identification kits and molecular identification have been documented.[5,8]

The 16S rRNA gene sequencing method has gained widespread uptake and application to identify many bacterial species,[21] and studies have showed that it is a useful tool to separate species in the genus *Streptococcus*, although genetic similarities among streptococci make it impossible to





distinguish some species (more than 99% homology).[22,23] Therefore, new molecular identification tools are required to achieve discrimination.

The *dnaJ* gene is one of the latest gene targets for sequencing analysis, and it encodes a heat shock protein belonging to the Hsp 70 family.[19] It has been shown that the *dnaJ* sequence of *S. mitis* and *S. pneumoniae* are 90.9% and 89.6% similar, respectively, to *S. oralis*. However, the disadvantage of this sequencing method is a lack of compatibility to the salivarius- and mutans-group streptococci. Therefore, a novel primer set was constructed to achieve universal amplification of the *dnaJ* gene; however, it showed an excessive level of intraspecies sequence diversity.

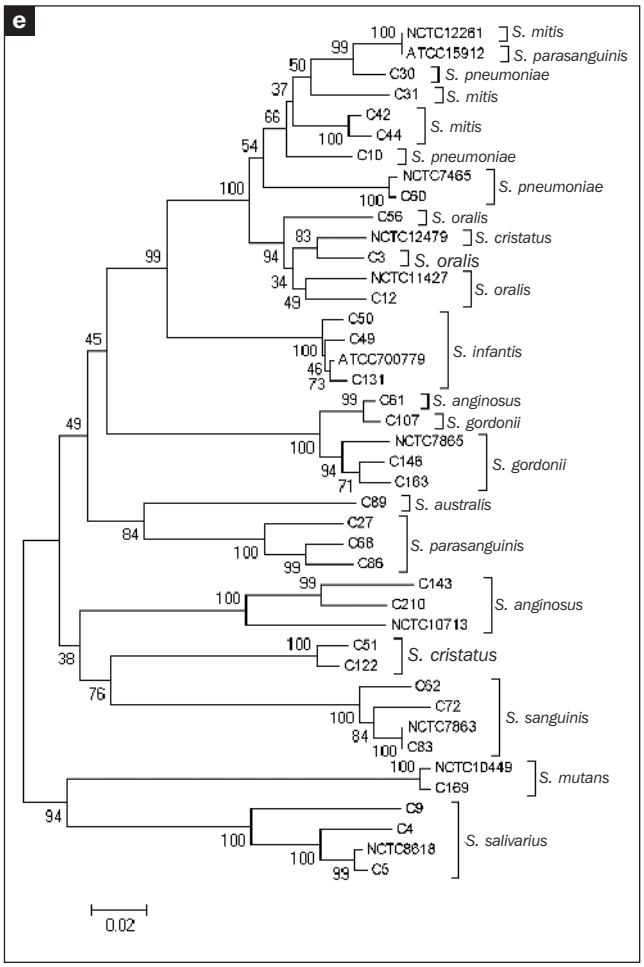
16S rRNA–23S rRNA ITS sequence analysis provided more discriminative power than the single 16S rRNA gene sequence, despite the length of the ITS being short at 240–390 bp.[16] Although an inability to distinguish *S. mitis*, *S. oralis* and *S. pneumoniae* was noted, a follow-up study demonstrated the existence of molecular signatures and species-specific sequences, which allow these species to be distinguished.[24] However, in the present study, the molecular signatures were found to have an irregular presence, depending on strain type. Therefore, without the molecular signatures it would be difficult to apply this target gene sequence analysis to the identification of VGS.

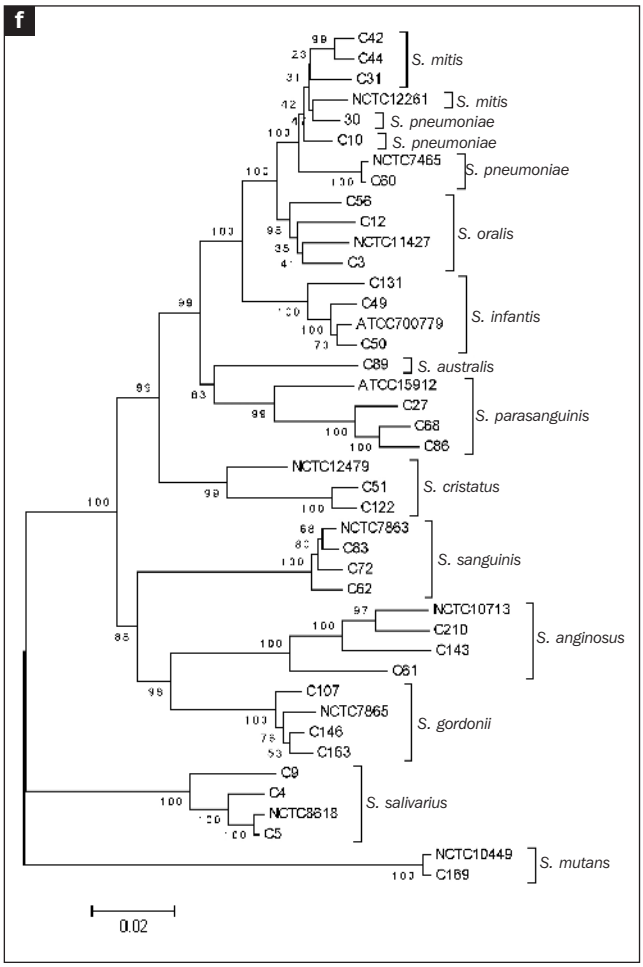
The *sodA* gene is one of the most frequently used sequencing targets. It requires sequencing of an approximate 430 bp PCR amplicon, which is over three times shorter when compared to 16S rRNA gene sequence analysis.[18] It proved difficult to separate *S. pneumoniae* and its close relatives, and intraspecies sequence diversity is greater than interspecies sequence diversity in some species (e.g., *S. sanguinis* [91–100% identity]). Similar results have been reported by other workers.[2]

Using *rnpB* sequence analysis, *S. oralis* was identified easily. It can differentiate *S. pneumoniae* and *S. mitis*, which are close relatives. The *rpnB* gene encodes part of a single protein subunit, which together with an RNA subunit makes RNase P in Gram-positive organisms. The gene comprises approximately 400 nucleotides and has a structure that allows variation between species, making it an attractive option on which to base a molecular speciation tool. Although there are only a limited number of *rnpB* sequences deposited in GenBank, this actually is an advantage as it improves this identification method. As 16S rRNA gene sequences are common, there are many incorrectly labelled sequences and this reduces the accuracy of speciation.[25]

Three studies of the *rnpB* gene sequence are from Sweden where there appears to be a local *rnpB* gene sequence database, which unfortunately is not an open resource.[15,26,27] Therefore, to increase *rnpB* gene sequences in the GenBank, researchers should undertake sequencing of VGS under controlled conditions to ensure that only accurate data are made available for identification purposes.

Recently, a multilocus sequence analysis (MLSA) scheme for VGS has been introduced. It appears to be a powerful tool and provides accurate results even with closely related species.[28] However, it is a labour-intensive procedure and





would not fit into routine bacterial identification, especially in laboratories that have no (or only limited) high-throughput sequencing facilities.

In conclusion, this study compares the ability of five gene loci to speciate VGS organisms. It shows that sequence typing using the *rnpB* gene locus is the most specific and reliable, and is thus a reliable and feasible option, based on a single gene target. □

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