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ORIGINAL RESEARCH

Capsular-type prediction by phylogenetic tree of glycosyltransferase gene polymorphism in *Streptococcus pneumoniae*

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Abstract: *Streptococcus pneumoniae* can cause severe infections among children and the elderly. Molecular capsule typing is being investigated extensively as a replacement of conventional serotyping using antisera. We focused on the glycosyltransferase (GT) genes in the capsular polysaccharide synthesis (*cps*) gene cluster of *S. pneumoniae* for classification of capsular types. The Sanger Institute provided sequences of the *cps* loci of 90 serotypes of *S. pneumoniae*. Each *cps* locus contained 1–6 putative GT genes per strain, for a total of 352 GT genes. Phylogenetic analysis of GT gene polymorphisms distinguished 90 serotypes into 64 phylogenetic groups. However, the sequence data contained only one sample from each serotype. Therefore, we selected six clinical isolates belonging to serogroup 6 and seven clinical isolates belonging to serotype 19F by antisera and sequenced GT genes. From phylogenetic analysis, these sequences were very similar to those of the Sanger Institute, and we can use GT genes as serotype-specific genes.

Keywords: *Streptococcus pneumoniae*, phylogenetic tree, glycosyltransferase gene

Introduction

Streptococcus pneumoniae is a common Gram-positive pathogen that colonizes the upper respiratory tract. The bacterium can cause severe infections, such as otitis media and sinusitis, and more life-threatening diseases, such as pneumonia, bacteremia, and meningitis if it gains access to the lower respiratory tract or the bloodstream.[1] *S. pneumoniae* can be divided into >90 serotypes based on differences in the composition of the capsular polysaccharides.[2,3] However, only seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) are responsible for 65% of all cases of pneumococcal disease[4] and 23 serotypes for 90% of cases.[5] The emergence of antibiotic resistance and the spread of resistant strains have increased the importance of vaccines as a primary prevention. The serotypes of *S. pneumoniae* most commonly isolated from patients with invasive pneumococcal disease vary in different age groups and geographic locations.[6] Therefore, continued surveillance is critical in order to monitor vaccine efficacy and changes in incidence and distribution of colonizing and invasive serotypes. Any increase in disease caused by previously uncommon nonvaccine serotypes could necessitate a change in vaccine composition. Various methods are currently used to identify pneumococcal serogroup and serotype using large panels of expensive antisera. These methods include the capsular swelling (Quellung) reaction, latex agglutination, and coagglutination.[7–9] Cross-reactions between serotypes and discrepancies between methods can occur and some strains are nonserotypable.[7,10,11]

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Molecular typing has the potential to improve discrimination and provide additional information.

With the exception of serotypes 3 and 37, which are produced by the synthase pathway, pneumococcal capsular polysaccharides (CPSs) are generally synthesized by the Wzx/Wzy-dependent pathway.[12] The genes for the latter pathway are located at the same chromosomal locus (*cps*), between *dexB* and *aliA*.[13] The DNA sequences of the 90 pneumococcal *cps* loci have been determined by the Sanger Institute.[12] There are four conserved genes (*wzg, wzh, wzd,* and *wze*) at the 5′ end of all *S. pneumoniae cps* loci that use the Wzy pathway. The *cps* loci also include genes whose products are involved in the biosynthesis of nonhousekeeping components (*cps*-specific biosynthesis pathway genes), initiation of capsule biosynthesis (initial sugar transferase genes), and transfer of sugar moieties and their assembly in the repeat unit (glycosyltransferase [GT], acetyltransferase, sugar phosphate transferase, and pyruvyltransferase genes).[14] GT proteins catalyze the formation of glycosidic bonds between the lipid-linked glycan precursor (acceptor) and a nucleotide-activated sugar (donor). Therefore, GT proteins determine the sequence of components in the repeating polysaccharide units that comprise pneumococcal capsules.

The GT genes in the *cps* loci were examined to determine their utility in using phylogenetic analysis to classify the serotypes of *S. pneumoniae*.

Materials and methods

Phylogenetic analysis

The nucleotide sequences of the GT genes in *S. pneumoniae cps* loci were retrieved from the database of the Sanger Institute (accession numbers CR931632-CR931722; see http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/). A phylogenetic tree was made by the neighbor-joining method using program Clustal_X[15] and visualized with Njplot.[16]

Clinical isolates and growth conditions

Clinical specimens were selected from isolates submitted to hospital laboratories in Japan from 1998 to 2007. The isolates were frozen at −80°C in brain–heart infusion broth (Eiken, Tokyo, Japan) supplemented with 0.3% yeast extract (Becton Dickinson, Boston, MA) (BHI-Y) with 80% glycerol. Frozen isolates were subcultured on blood agar medium containing sheep erythrocytes (Denka Seiken, Tokyo, Japan) or grown in BHI-Y for 24 h at 37°C in 5% CO_2.

The isolates were identified as *S. pneumoniae* by colony morphology, alpha hemolysis, and optochin susceptibility in the clinical laboratories that isolated each strain. Six pneumococcal strains representing serogroup 6 (D11, D12, D13, D14, D19, and D25) and seven representing serotype 19F (D5, D15, D20, D28, D33, D50, and D53) were chosen for study.

Serotyping was performed by a slide agglutination test (Denka Seiken, Tokyo, Japan) or by the Quellung reaction (Statens Serum Institut, Copenhagen, Denmark).

Genomic DNA extraction

S. pneumoniae isolates were grown in BHI-Y at 37°C in the presence of 5% CO_2 for 24 hours. Following sedimentation, the cells were resuspended in 450 µL of 50 mM EDTA and 12 µL of lysozyme (100 mg/mL). The cells were incubated for 1 hour at 37°C before genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

Gene amplification, sequencing, and alignment

The primers were designed to target two GT genes: *wciP* of serotype 6B and *wchQ* of serotype 19F. Sequences of the targeted genes were retrieved from the website of the Sanger Institute. All primers were synthesized by Invitrogen (Tokyo, Japan). The primer designations, sequences, product sizes, and numbered base positions are shown in Table 1.

Thermal cycling was performed in the GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 94°C for 5 minutes followed by 30 amplification cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, then a final extension at 72°C for 7 minutes. The PCR amplicons were extracted after agarose

Table 1 Oligonucleotide primers used in this study

Serotype	GT gene	Primer name	Primer sequence (5′→3′)	Nucleotide position[a]	Product size (bp)
6B	*wciP*	6B-*wciP*-F	aat act ata aaa ata ctg gc	8021	1233
		6B-*wciP*-R	ccc tca aat aat ata aat gt	9253	
19F	*wchQ*	19F-*wchQ*-F	ara aag tat gat tgg aaa aa	9752	1196
		19F-*wchQ*-R	wtr aaa gca aar aaa tag aa	10947	

Note: [a]Start position of each primer are represented.
Abbreviation: GT, glycosyltransferase.

gel electrophoresis and purified with the QIAprep Spin Miniprep Kit (250) (Qiagen, Tokyo, Japan).

The PCR products were sequenced using dye terminator cycle sequencing with the CEQ8000 DNA Analysis System (Beckman Coulter, Fullerton, CA). The corresponding amplification primers or inner primers were used as sequencing primers.

DNA sequences were aligned and edited using Sequencher software (Gene Codes, Ann Arbor, MI).

Multiple-sequence alignments were performed with the Genetyx program (Genetyx, Tokyo, Japan).

Results

Phylogenetic analysis of the GT gene sequences

The assignment of gene functions predicted by the Sanger Institute found 352 putative GT genes (including pseudogenes) in the *cps* loci of 90 *S. pneumoniae*. Each

Table 2 Glycosyltransferase genes of each serotype

Serotype	GT genes included in *cps* locus					Serotype	GT genes included in *cps* locus					
1	*wchB*	*wchD*				19C	*wchO*	*wchQ*	*wchS*	*wchU*		
2	*wchF*	*wchG*	*wchH*	*wchI*		20	*wciB*	*whaJ*	*wciL*	*wcwK*	*wciD*	*whaF*
3	*wchE*					21	*wchF*	*wcwA*	*wcwK*	*wcyT*	*wcyU*	
4	*wciJ*	*wciK*	*wciL*			22F	*wchF*	*wcwA*	*wcwV*	*whaB*		
5	*wciJ*	*whaC*	*whaD*			22A	*wchF*	*wcwA*	*wcwV*	*whaB*		
6A	*wciN*	*wciP*				23F	*wchF*	*wchV*	*wchW*			
6B	*wciN*	*wciP*				23A	*wchF*	*wchV*	*wchW*			
7F	*wchF*	*wcwA*	*wcwF*	*wcwG*	*wcwH*	23B	*wchF*	*wchV*	*wchW*			
7A	*wchF*	*wcwA*	*wcwF*	*wcwG*	*wcwH*	24F	*wchF*	*wcxI*	*wcxJ*			
7B	*wchF*	*wcwI*	*wcwL*	*wcwK*	*wcxU*	24A	*wchF*	*wcxI*	*wcxJ*			
7C	*wchF*	*wcwI*	*wcwL*	*wcwK*	*wcxU*	24B	*wchF*	*wcxI*	*wcxJ*			
8	*wciO*	*wciR*	*wciS*	*wciT*		25F	*wcyA*	*wcyB*	*wcyC*	*wcyD*	*wcyE*	
9A	*wchO*	*wcjA*	*wcjB*	*wcjC*		25A	*wcyA*	*wcyB*	*wcyC*	*wcyD*	*wcyE*	
9V	*wchO*	*wcjA*	*wcjB*	*wcjC*		27	*wchF*	*whaK*	*wcyS*			
9L	*wchO*	*wcjA*	*wcjB*	*wcjC*		28F	*wchF*	*wciU*	*wcxN*			
9N	*wchO*	*wcjA*	*wcjB*	*wcjC*		28A	*wchF*	*wciU*	*wcxN*			
10F	*wciB*	*wcrC*	*wcrD*	*wciF*	*wcrH*	29	*wciB*	*wcrM*	*wcrH*			
10A	*wciB*	*wcrC*	*wcrD*	*wciF*	*wcrG*	31	*wciB*	*wcrP*	*wcrR*	*wcrW*	*wcrX*	
10B	*wciB*	*wcrC*	*wcrD*	*wciF*	*wcrG*	32F	*wchF*	*wchQ*	*wcyS*			
10C	*wciB*	*wcrC*	*wcrD*	*wciF*	*wcrH*	32A	*wchF*	*wchQ*	*wcyS*			
11F	*wchK*	*wcyK*	*wcrL*			33F	*wciB*	*wciC*	*wciD*	*wciE*	*wciF*	
11A	*wchK*	*wcyK*	*wcrL*			33A	*wciB*	*wciC*	*wciD*	*wciE*	*wciF*	
11D	*wchK*	*wcyK*	*wcrL*			33B	*wciN*	*wcrC*	*wciD*	*wciE*	*wciF*	
11B	*wchK*	*wcyK*	*wcrL*			33C	*wciN*	*wcrC*	*wcrD*	*wciF*		
11C	*wchK*	*wcyK*	*wcrL*			33D	*wciN*	*wcrC*	*wciD*	*wciE*	*wciF*	
12F	*wciJ*	*wcxB*	*wcxD*	*wcxE*	*wcxF*	34	*wciB*	*wcrC*	*wcrD*			
12A	*wciJ*	*wcxB*	*wcxD*	*wcxE*	*wcxF*	35F	*wciB*	*wcrC*	*wcrD*			
12B	*wciJ*	*wcxB*	*wcxD*	*wcxE*	*wcxF*	35A	*wciB*	*wcrI*	*wcrK*	*wcrH*		
13	*wchK*	*wciF*	*wcrD*			35B	*wciB*	*wcrM*	*wcrH*			
14	*wchK*	*wchL*	*wchM*	*wchN*		35C	*wciB*	*wcrI*	*wcrK*	*wcrH*		
15F	*wchK*	*wchL*	*wchM*	*wchN*		36	*wchO*	*wcjA*	*wciF*	*wcrH*		
15A	*wchK*	*wchL*	*wchM*	*wchN*		37	*wciB*	*wciC*	*wciD*	*wciE*	*wciF*	
15B	*wchK*	*wchL*	*wchM*	*wchN*		38	*wcyA*	*wcyB*	*wcyC*	*wcyD*	*wcyV*	
15C	*wchK*	*wchL*	*wchM*	*wchN*		39	*wciB*	*wciE*	*wcrC*	*wcrD*	*wciF*	*wcrG*
16F	*wchF*	*wciU*	*wcxN*			40	*wchF*	*wcwI*	*wcwL*	*wcwK*	*wcxU*	
16A	*wchK*	*wcyK*	*wcxS*	*wciB*		41F	*wciB*	*wcrP*	*wcrQ*	*wcrR*	*wcrX*	
17F	*wchF*	*wciP*	*wcrV*			41A	*wciB*	*wcrP*	*wcrQ*	*wcrR*	*wcrX*	
17A	*wciB*	*wcrP*	*wcrQ*	*wcrR*	*wcrV*	42	*wciB*	*wcrI*	*wcrK*	*wcrH*		
18F	*wchF*	*wciU*	*wciV*	*wciW*		43	*wciB*	*wciE*	*wcrC*	*wcyM*	*wcyN*	*wcrH*
18A	*wchF*	*wciU*	*wciV*	*wciW*		44	*wciJ*	*wcxB*	*wcxD*	*wcxE*	*wcxF*	
18B	*wchF*	*wciU*	*wciV*	*wciW*		45	*wciJ*	*wcxB*	*wciL*	*wcxS*		
18C	*wchF*	*wciU*	*wciV*	*wciW*		46	*wciJ*	*wcxB*	*wcxD*	*wcxE*	*wcxF*	
19F	*wchO*	*wchQ*				47F	*wciB*	*wcrC*	*wcrD*			
19A	*wchO*	*wchQ*				47A	*wciB*	*wcrC*	*wcyM*	*wcyN*	*whaM*	
19B	*wchO*	*wchQ*	*wchS*			48	*wchF*	*wcyS*				

Abbreviations: *cps*, capsular polysaccharide synthesis; GT, glycosyltransferase.

cps contained 1 to 6 GT genes (Table 2). A phylogenetic tree was constructed to explore the sequence diversity and relatedness of the GT genes in each *cps* locus. The nucleotide sequences from the Sanger Institute inserted into Clustal_X produced a phylogenetic tree showing that GT genes are highly variable and are therefore suitable targets for serotype/serogroup identification (Figure 1). A comparison of the sequences of neighboring GT genes showed that some were highly similar while others shared partial similarity. For example, according to the Sanger database, two serotypes in serogroup 6 (6A and 6B) have two GT genes, *wciN* and *wciP*. The phylogenetic tree and sequence alignment showed that while the *wciN* nucleotide sequences of the two serotypes were almost identical, they shared only partial similarity with *wciN* from serotype 33D (Figure 2A). Therefore, serogroup 6 and serotype 33D were distinguishable based on the nucleotide sequence of *wciN*. Another GT gene in serogroup 6, *wciP,* shared a minor similarity with *wciP* in serotype 17F, therefore, the nucleotide sequence of *wciP* could be used to separate serogroup 6 and serotype 17F (Figure 2B). Further analysis of GT gene sequences revealed that 90 *S. pneumoniae* serotypes

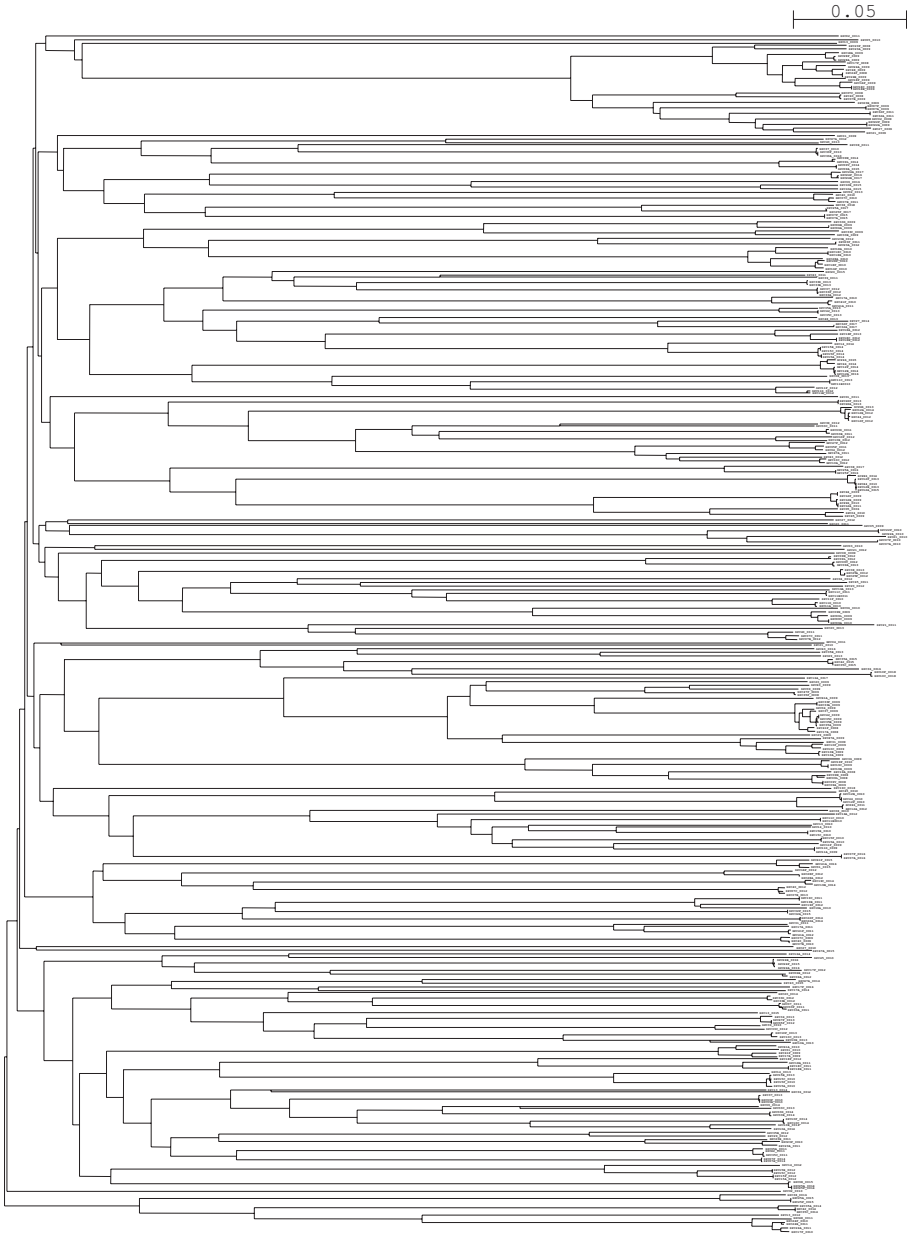

Figure 1 Phylogenetic tree generated from glycosyltransferase gene sequences in the capsular polysaccharide synthesis locus of 90 *S. pneumoniae* serotypes from the Sanger Institute database. All nucleotide sites were used to construct the tree using the neighbor-joining method. The sequence names are given as SPC-serotype-Sanger Institute database gene number.

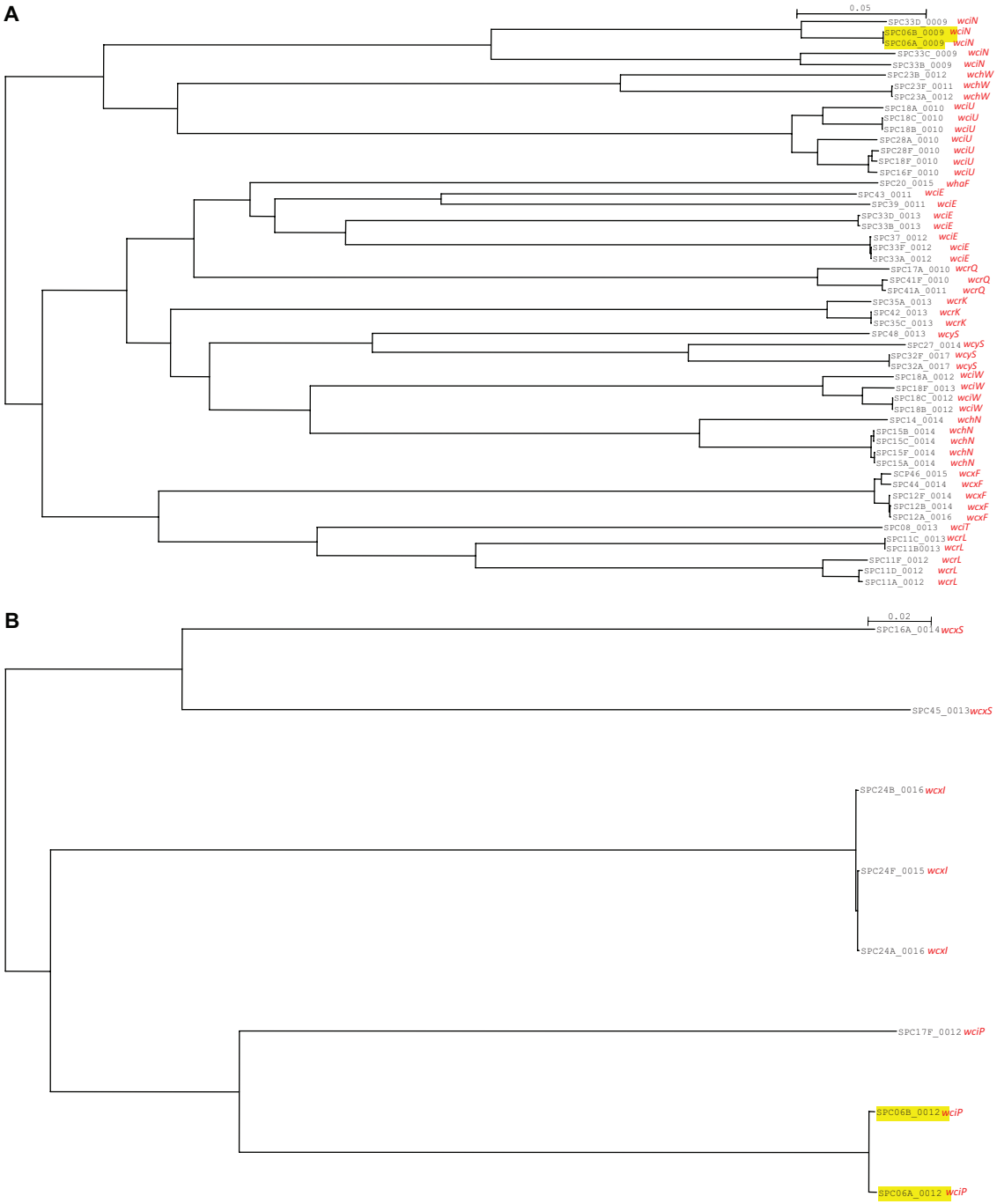

Figure 2 Partial view of the glycosyltransferase (GT) phylogenetic tree. The sequence names are given as SPC-serotype-Sanger Institute database gene number. The GT gene name given by the Sanger Institute is shown in red.

were separated into 64 phylogenetic groups (Table 3). For example, strains belonging to serogroup 19 were divided into 4 groups (serotype 19F, 19A, 19B, and 19C) since these groups had different GT gene number and the sequences were distinguishable. On the other hand, strains belonging to serogroup 6 (serotype 6A and 6B) had very similar GT genes, so that they were indistinguishable and put into same group.

Sequencing GT genes of clinical isolates and phylogenetic analysis

Because the Sanger Institute used a single strain for each serotype when sequencing the *cps* locus, we assessed sequence conservation among the GT gene of several representative strains of two serogroups. Six clinical isolates were selected from serogroup 6 and seven from serotype 19F by antiserum testing. The *wciP* of serogroup 6 and *wchQ* of serotype 19F

Table 3 Grouping of 90 serotypes into 64 groups using glycosyltransferase gene sequence polymorphisms

1	10C	18B/18C	16F/28F/28A	41F
2	11F	19F	31	41A
3	11A/11D	19A	32F/32A	42
4	11B/11C	19B	33F/33A/37	43
5	12F/12A/12B/44/46	19C	33B	45
6A/6B	13	20	33C	47F
7F/7A	14	21	33D	47A
7B/7C/40	15F/15A	22F/22A	34	48
8	15B/15C	23F/23A	35F	
9A/9V	16A	23B	35A/35C	
9L/9N	17F	24F/24A/24B	35B	
10F	17A	25F/25A	36	
10A	18F	27	38	
10B	18A	29	39	

were sequenced. All of the *wciP* sequences from clinical samples were similar to those taken from the Sanger database (Figure 3A). Likewise, the *wchQ* sequences perfectly matched the web data for serotype 19F (Figure 3B).

Discussion

Several molecular capsular-typing methods of *S. pneumoniae* have been developed based on serotype-specific sequences.[17–26] In this study, we focused on the GT genes due to their role in forming capsular polysaccharides. Each serotype contains various sets of GT genes in the *cps* locus. A phylogenetic tree based on nucleotide sequences was made to explore the sequence diversity and relatedness of the GT genes in each *cps* locus (Figure 1). The structure of the tree showed that 90 serotypes used for this study could be divided into 64 phylogenetic groups on the basis of GT gene sequence, and that these sequences can be used to differentiate serotype. The management of pneumococcal disease has become more difficult because of the rapid increase of antimicrobial resistance. It is generally agreed that the use of an effective pneumococcal vaccine during infancy could significantly reduce the morbidity and mortality associated with pneumococcal infections among young children. A 7-valent anti-pneumococcal vaccine is already licensed in several countries and has shown promising results.[27–30] Thus, clinical monitoring of the disease preventive effects of the anti-pneumococcal vaccine, is increasingly important. In particular, surveillance of the emergence of new capsular types following vaccination aids the development of new vaccines. Our bioinformatic approach will help survey the emergence of new *S. pneumoniae* capsular types. Sequencing the GT genes of a clinical sample and placing that data into our phylogenetic tree will reveal if this sample has any of the known GT genes of a particular serogroup or serotype. If GT gene sequence differs from that of known GT genes, the sample could contain an emergent *S. pneumoniae* CPS.

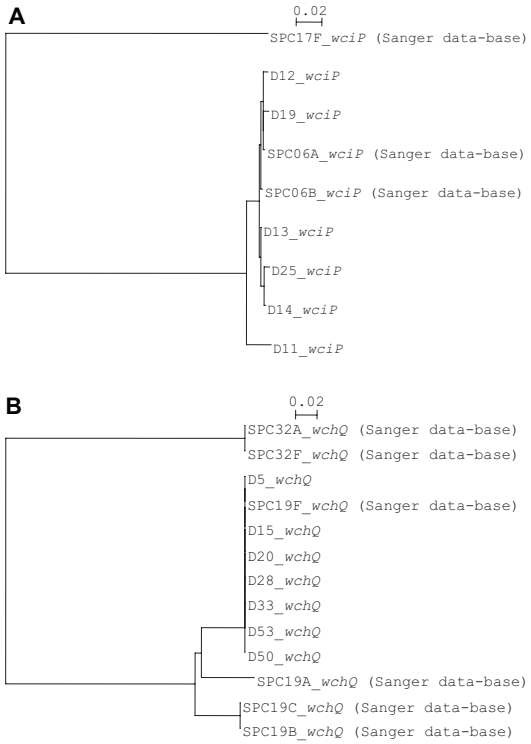

Figure 3 Phylogenetic trees of glycosyltransferase gene sequences using web data and clinical isolates. **A**) Phylogenetic tree of *wciP*. **B**) Phylogenetic tree of *wchQ*.

The clinical samples that were classified as serogroup 6 also grouped with the serogroup 6 in the Sanger Institute database. However, serological assays further divide serogroup 6 into serotype 6A, 6B, and 6C.[31] The *cps* loci of serotypes 6A and 6B are almost identical, except for a single nucleotide polymorphism in *wciP*.[32] Serotype 6C appears to have originated from a single recombination event in which the 6A *wciN* gene was replaced by a different *wciN* gene of unknown origin.[33] These results indicate that sequencing the GT genes of clinical isolates of *S. pneumoniae* and knowing the differences in these sequences by phylogenetic analysis will help to identify new capsular type of *S. pneumoniae.*

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Disclosure

The authors declared no conflicts of interest in relation to this paper.

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