JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 2004, p. 250–256 Vol. 42, No. 1
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.1.250–256.2004
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Serotype 14 Variants of the France $9V^{-3}$ Clone from Baltimore, Maryland, Can Be Differentiated by the *cpsB* Gene

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Received 6 April 2003/Returned for modification 19 July 2003/Accepted 22 September 2003

European serotype 14 variants of the France $9V^{-3}$ clone, which have arisen through recombination events involving the penicillin binding protein 1a (*pbp1a*) gene, have *cpsB* sequences distinct from those of the $9V^{-3}$ clone. Serotype 14 variants of the $9V^{-3}$ clone have not been compared to genetically diverse serotype 14 strains isolated from an entire metropolitan area in the United States. All serotype 14 non-penicillin-susceptible *Streptococcus pneumoniae* strains causing invasive disease in Baltimore, Md., from 1995 to 1996 were compared by using pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *pbp1a* PCR restriction profiles, and *cpsB* and *pbp1a* sequences. The *cpsB* genes from strains of 13 serotypes also were analyzed to assess the correlation with serotype. Twenty-seven percent (3 of 11) of the serotype 14 strains were related by PFGE and MLST to the $9V^{-3}$ clone. The serotype 14 variants from Baltimore, unlike the European variants, were related neither to the $9V^{-3}$ clone nor to the R6 strain from positions 1498 to 1710 of the *pbp1a* gene. All serotype 14 strains had *cpsB* sequences that differed by ≤1% (0 to 5 of 476 bp) from each other and that were ≥16% (78 to 83 of 476 bp) divergent from that of the $9V^{-3}$ clone. Allowing for a 2-bp difference in the *cpsB* sequence resulted in the highest correlation between the *cpsB* gene and serotype. Overall, 95% (84 of 88) of the strains were classified correctly by serotype with the *cpsB* sequence. The distal recombination site of the Baltimore serotype 14 variants of the $9V^{-3}$ clone was not identical to that of the European serotype 14 variants. The *cpsB* gene was serotype specific regardless of whether capsular switching occurred. Although the correlation between serotype and the *cpsB* sequence was high, the overall diversity of the *cpsB* gene within a serotype likely will limit the role of this gene in a sequence-based serotyping method.

Pneumococci are naturally transformable, with recombination rates 10-fold higher than mutation rates (5). Griffith et al. (8a) first described pneumococcal transformation in 1928, while Avery et al. determined that the transforming factor was DNA (1). Strains which are found to be highly related by pulsed-field gel electrophoresis (PFGE) and multilocus sequencing typing (MLST) may have different serotypes, indicating serotype capsular recombination (2, 3, 18, 24). The capsular locus is comprised of a series of alphabetically named capsular genes, which are flanked by the conserved genes *dexB* and *aliA*; approximately 5.8 kb downstream from *aliA* is the penicillin binding protein 1a (*pbp1a*) gene (2). Since a capsular locus has been identified for a variety of serotypes, the capsular genes are attractive targets for a sequence-based serotyping method. An association between some capsular gene sequences and serotype has been noted in the literature, even for strains which have undergone capsular transformation (2, 3).

The France $9V^{-3}$ pneumococcal clone is 1 of approximately 20 international clones and has been detected as serotype 14, serotype 9A, and serogroup 19 variants (18). These international clones account for a significant proportion of non-penicillin-susceptible *Streptococcus pneumoniae* (PNSP) strains in the United States (26). The proximal recombination site for the serotype 14 variants of the $9V^{-3}$ clone from Uruguay, Denmark, and Spain, but not Poland, was detected within the *cpsA* gene (2). The distal site of recombination occurred within the *pbp1a* gene, which has an orientation divergent from that of the capsular genes. The serotype 14 variants had a *pbp1a* sequence that was a combination of that of the non-penicillin-susceptible $9V^{-3}$ clone and that of the nonencapsulated penicillin-susceptible reference strain R6. The *pbp1a* gene sequence of the variants was identical to that of the $9V^{-3}$ clone until position 1854 or 1922. Thereafter, the gene sequence diverged from that of clone $9V^{-3}$ but was identical to that of strain R6.

Among all of the available non-penicillin-susceptible pneumococcal strains collected over a 2-year period from the Baltimore, Md., metropolitan area, 7.7% (11 of 143) were serotype 14 strains; 27.2% (3 of 11) of the serotype 14 strains were highly genetically related to the France $9V^{-3}$ clone rather than to the other serotype 14 strains (17). In this study, we characterized all serotype 14 PNSP strains and compared them to the France $9V^{-3}$ clone and to a genetically related serotype 9V strain by using PFGE, MLST, and phylogenetic trees of the concatenated housekeeping gene sequences. We also compared the PCR *pbp1a* restriction profiles and sequenced inter-

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TABLE 1. *cpsB* sequences of 88 strains of selected serotypes

Serotype	GenBank accession no.	Reference
1[a]	Z83335	22
2[a]	AF026471	11
4[a]	AF316639	12
6B	AF316640	
8[a]	AF316641	
18C[a]	AF316642	
8[a]	AJ239004	23
9V[a]	AF402095	29
14	X85787	13
19A[a]	AF094575	20
19F	U09239	9
19F	U09239	19
19F-NCTC11906[a]	AF030367	3
19F-PO-329 (19F variant of 23F-1 clone)	AF030371	
19F-SP-GA71 (19F variant of 23F-1 clone)[a]	AF030370	
19F-SP-496 (19F variant of 23F-1 clone)[a]	AF030368	
19F-SP-VA92 (19F variant of 23F-1 clone)[a]	AF030369	
19F-SP-VA96 (19F variant of 23F-1 clone)[a]	AF030372	
23F-SP-264[a]	AF030373	
23F-UK-577[a]	AF030374	
23F[a]	AF057294	25
33F[a]	AJ006986	14
37[a]	AJ131984	15
International clones		
Spain 23F^{-1}[a]	AY359448	
Spain 6B^{-2}[a]	AY359449	
France 9V^{-3}[a]	AY359450	
Tennessee 23F^{-4}[a]	AY359451	
Spain 14^{-5}[a]	AY359452	
Hungary 19A^{-6}	AY359453	
South Africa 19A^{-7}[a]	AY359454	
South Africa 6B^{-8}	AY359455	
England 14^{-9}	AY359456	
Slovakia 14^{-10}	AY359457	
Slovakia 19A^{-11}	AY359458	
Finland 6B^{-12}[a]	AY359459	
South Africa 19A^{-13}[a]	AY359460	
Taiwan 19F^{-14}	AY359461	
Taiwan 23F^{-15}	AY359462	

[a] Reference sequence for each serotype.

nal fragments of the *pbp1a* and *cpsB* genes from all serotype 14 strains to determine whether serotype 14 variants of the 9V^{-3} clone detected in Baltimore were similar to the European variants. Finally, we compared the internal fragments of the *cpsB* genes from all serotype 9V and 14 strains from Baltimore, 15 pneumococcal clones, and 23 strains from GenBank to assess the correlation between serotype and the *cpsB* sequence.

MATERIALS AND METHODS

Strains. Eleven serotype 14 strains and 39 serotype 9V strains from a previous study were included (17). In addition, the *cpsB* genes from 15 international pneumococcal clones (18) and 23 strains listed in GenBank (3, 9, 11–15, 19, 20, 22, 23, 25, 29) were analyzed (Table 1).

PFGE and susceptibility patterns. PFGE initially classified the 11 serotype 14 strains into five clonal groups (17). PFGE-based clonal groups had six or fewer band differences from each other and ≥80% relatedness on the dendrogram. Due to an error that occurred while making plugs for PFGE, strain VIII was represented twice, while strain XII was not included. The correction of this error creates six, not five, clonal groups (16, 17). The MIC for penicillin-intermediate strains is 0.12 to 1 μg/ml, while that for penicillin-resistant strains is ≥2 μg/ml.

PCR. Pneumococcal strains were incubated overnight on Trypticase soy agar containing 5% sheep blood. Genomic DNA was isolated by using Prepman Ultra in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, Calif.). PCR primers for restriction profiles were *pbp1a* F (GGCATTCG ATTTGATTCGCTTCTATCAT) and *pbp1a* R (CTGAGAAGATGTCTTCTC AGGCTTTTG) (8). The 30-μl reaction mixture contained 1.5 mM $MgCl_2$, 0.33 μM each primer, 25 μM each deoxyribonucleotide, 1.5 U of the thermostable DNA *Taq* polymerase mixture, 3 μl of 10× buffer (Invitrogen Corporation, Carlsbad, Calif.) and 20 ng of DNA template. PCR was performed with a model 9700 thermal cycler (Perkin-Elmer, Wellesley, Mass.). The samples were subjected to an initial denaturation at 95°C for 1 min, followed by 10 cycles of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min 50 s. This procedure was followed by 20 cycles with the same parameters but with sequential 10-s increments in the elongation cycle. A 7-min extension at 72°C followed the final cycle (8). PCR products were purified with Multiscreen PCR plates (Millipore, Bedford, Mass.). Five microliters of purified PCR products were restricted with 3 U each of *Mse*I and *Dde*I for 1 to 2 h at 37°C (24). The restriction digests were electrophoresed for 2.5 to 3.0 h at 125 to 150 V through 4% Nusieve 3:1 agarose gels and captured with GelDoc 2000 (Bio-Rad, Hercules, Calif.). Restriction patterns of bands between 300 to 10,000 bp were classified as patterns 1 to 4.

DNA sequencing. (i) MLST. MLST was performed as described at http://www.mlst.net with internal fragments of the following seven housekeeping genes (protein products are shown in parentheses): *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (D-alanine-D-alanine ligase). The primer sets were elongated for all except *gki* as described previously (7). Identical sequence types (ST), single-locus variants (SLV), and double-locus variants were determined, and strains with ≥5 of 7 alleles were denoted as a single complex.

(ii) Phylogenetic analysis. Phylogenetic analysis was performed on the nucleotide and amino acid sequences obtained by concatenating the seven genes in order of occurrence in a serotype 4 strain: *spi*, *gki*, *gdh*, *aroE*, *recP*, *ddl*, and *xpt* (28). The 3,199 nucleotides were translated to 1,064 amino acids with Sequencher. The sequences were edited with Sequencher and aligned with ClustalX. Neighbor-joining (NJ), maximum-likelihood (ML), and maximum-parsimony (MP) trees were created with Paup 4.0, version beta10 (Sinauer Associates, Sunderland, Mass) (10, 27). Five thousand bootstrap replicates were done to assess the statistical significance of the phylogenetic tree generated by the NJ algorithm. Analysis based upon a related ST (BURST analysis) was performed with the START program (http://outbreak.ceid.ox.ac.uk/software.shtml) to determine the MLST lineages.

(iii) *pbp1a* and *cpsB*. A 485-bp fragment from positions 1498 to 1982 of the *pbp1a* gene was sequenced with the following primers: *pbp1a* (F), 5′-GCAAGT AGTGAAAARATGGCTGCTGC, and *pbp1a* (R), 5′-GACTGTGAAGTTGA ACTWTCTGATG-3′. A 476-bp fragment from positions 72 to 547 of the *cpsB* gene was analyzed with published primers (3). The *cpsB* and *pbp1a* genes were amplified from the genomic DNA with the following PCR parameters: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and elongation at 72°C for 1 min. A 7-min extension at 72°C followed the final cycle. PCR products were sequenced with a Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems) and run on a model 3700 DNA sequencer (Applied Biosystems). Both the forward and the reverse strands were sequenced. Raw sequences were aligned with Sequencher and ClustalX. The *cpsB* genes from 23 strains representing 13 serotypes, obtained from GenBank, also were analyzed. For the serotype 37 strain, a base-pair insertion was present at position 212; thus, a 477-bp fragment was analyzed.

RESULTS

Serotype 14 strains were classified into six clonal groups, five ST complexes or lineages, and eight ST (Fig. 1 and Table 2). The *pbp1a* restriction profiles correlated with the *pbp1a* DNA sequences and classified strains into four groups (Table 2). Three serotype 14 variants (strains I to III) and the 9V^{-3} clone were in PFGE-based clonal group A (Fig. 1). The serotype 14 variants were isolated over an 8-month period from two children and one adult who lived in three different counties within the Baltimore metropolitan area. The 9V^{-3} clone, strain I, and strain II were ST 156, while strain III was an SLV of ST 156. Strain III had a *ddl* allele which differed from that of the 9V^{-3} clone by 32 bp and was penicillin intermediate rather than

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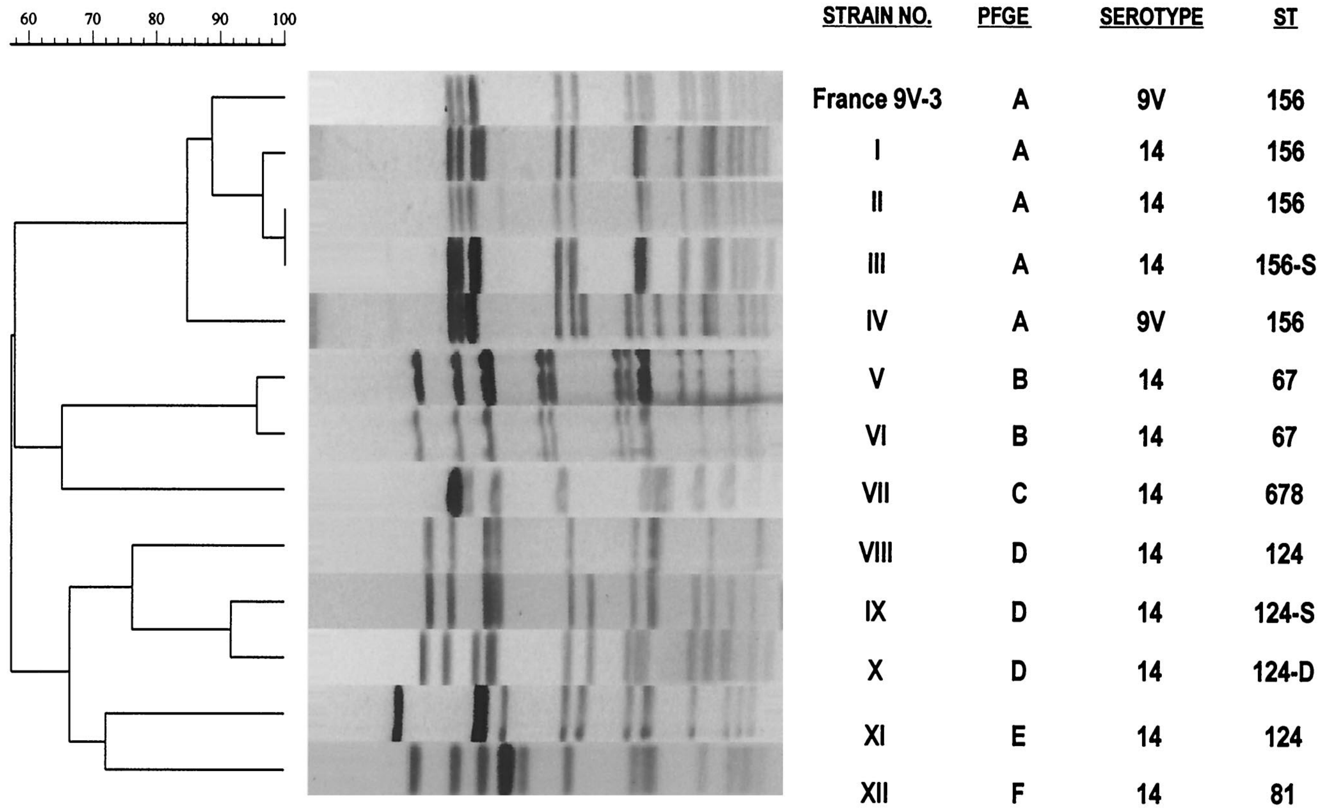

FIG. 1. PFGE and MLST for serotype 9V and 14 strains and the $9V^{-3}$ clone.

resistant. The serotype 14 variants had identical *pbp1a* restriction profiles and *pbp1a* DNA sequences. However, unlike the European serotype 14 variants, strains I to III were related neither to the $9V^{-3}$ clone nor to strain R6 from positions 1498 to 1710 (Fig. 2). From positions 1711 to 1871, the serotype 14 variants were identical to the $9V^{-3}$ clone; thereafter, they were identical to strain R6. In contrast, strains that were found to be 60 to 70% related by PFGE and for which six of seven alleles were found by MLST to be different from those of the serotype 14 variants had *pbp1a* sequences which were highly related to that of either the $9V^{-3}$ clone or strain R6. Strain XII had a *pbp1a* restriction profile and a DNA sequence identical to

TABLE 2. PFGE-based clonal groups, MLST alleles, Pbp1a profiles, and penicillin susceptibility patterns for serotype 14 and 9V strains and the $9V^{-3}$ clone

Strain	PFGE-based clonal group	Serotype	No. of strains with the following seven housekeeping genes, as determined by MLST							ST	Pbp1a profile	Penicillin susceptibility pattern[a]
			aroE	gdh	gki	recP	spi	xpt	ddl			
R6			7	5	1	5	10	7	15	128		S
France $9V^{-3}$	A	9V	7	11	10	1	6	8	1	156	3	R
I	A	14	7	11	10	1	6	8	1	156	2	R
II	A	14	7	11	10	1	6	8	1	156	2	R
III	A	14	7	11	10	1	6	8	98	930[b] and 156-S[c]	2	I
IV	A	9V	7	11	10	1	6	8	1	156	3	R
V	B	14	2	8	7	4	6	1	1	67	1	R
VI	B	14	2	8	7	4	6	1	1	67	1	R
VII	C	14	7	6	1	17	66[b]	1	104[b]	678[b]	4	I
VIII	D	14	7	5	1	8	14	11	14	124	4	R
IX	D	14	7	7	1	8	14	11	14	689[b] and 124-S[c]	4	I
X	D	14	7	6	1	8	14	110[b]	14	679[b] and 124-D[d]	4	I
XI	E	14	7	5	1	8	14	11	14	124	4	R
XII	F	14	4	4	2	4	4	1	1	81	3	R

[a] S, susceptible; R, resistant; I, intermediate.
[b] New designation by MLST.
[c] S, single-locus variant has six of seven identical alleles.
[d] D, double-locus variant has five of seven identical alleles.

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                    11111111111111111111111111111111111111111111111111111
                    55555555555555555555566666666666666666666666677777777777
                    01223344445556667788891112233333344555556778899011111112222
                    72170425894703628147918903256787804679217095820345678 90123
R6                  CATTGGGCTACATTCAGACTCAAGTTAAAATTGTCCCTGAACTCGAC........C.T
VII-XI              CATTGGGCTACATTCGGACTCAACTTAAAATTGTCCCTGAACTCGAC........C.T
I-III               AGCCTAATAGATAATAATTCATGCACTTGGACCATAGGAGTTCTTAA........A.A
XII                 CACTAAGCTACATTCGGACTCAACTTGTGGATCTCTCTAAACTTGGC........A.A
9V-3                CACTAAGCTACATTCGGACTCAACTTGTGGATCTCTCTAAACTTGGC........A.A


                    11111111111111111111111111111111111111111111111111111
                    77777777777777888888888888888888888888888888888888888
                    22233444556779000011111222333333344566677777788888888888999
                    67807079484064037925681450134569254069245678901234567890 12
R6                  CAATATCACCGAACTCCGAACTACACCTACGCTCCTGCC.G..T....GA.GCA....
VII-XI              CAATATCACCGAACTCCGAACTACACCTACGCTCCTGCT.G..T....GA.GCA....
I-III               GGCATAAGTTTGTTGTTATTACTAGTTCCTATATTAAGT.G..T....GA.GCA....
XII                 GGCATAAGTTTGTTGTTATTACTAGTTCCTATATTAAGT.A..A....AT.CTC....
9V-3                GGCATAAGTTTGTTGTTATTACTAGTTCCTATATTAAGT.A..A....AT.CTC....
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FIG. 2. Polymorphic sites within the *pbp1a* genes of strains R6, VII to IX, I to III, and XII and the $9V^{-3}$ clone. Identical sites are represented by dots. The polymorphic sites are numbered in vertical format. From positions 1498 to 1710, the serotype 14 variants had 37-bp differences from the $9V^{-3}$ clone and 45-bp differences from strain R6. From positions 1711 to 1874, the serotype 14 variants were identical to the $9V^{-3}$ clone and had 41-bp differences from strain R6. From positions 1875 to 1982, the serotype 14 variants had 33-bp differences from the $9V^{-3}$ clone and were identical to strain R6. Strain XII was identical to the $9V^{-3}$ clone. Strains VII to IX were nearly identical to strain R6.

those of the $9V^{-3}$ clone, and strains VII to XI had a *pbp1a* DNA sequence identical to that of the R6 strain except for four single-nucleotide polymorphisms at positions 1572, 1619, 1872, and 1970.

The other serotype 14 strains were <80% related to the $9V^{-3}$ clone and were classified into PFGE-based clonal groups B to F. These strains belonged to either ST 67, ST 81, ST 672, or ST 124, while strain R6 was ST 128. Our data generated three new alleles (spi66, ddl104, xpt110) and four new ST (ST 930, ST 78, ST 679, and ST 689) for the MLST website.

The NJ, ML, and MP trees of the concatenated MLST gene sequences produced clusters similar to those produced by BURST analysis. The ML and NJ methods each produced one tree, while the MP method produced two trees, both with the same structure but slightly different branch lengths. NJ tree bootstrapping with 5,000 replications revealed that strains I to III clustered with the $9V^{-3}$ clone 65% of the time. The remainder of the time, strain III clustered with either strain VII (18%) or both strain VII and the $9V^{-3}$ clone (17%). When amino acid sequences were used to create an NJ tree with 5,000 bootstrapping replications, strain III always clustered with the $9V^{-3}$ clone.

All 41 serotype 9V strains had identical *cpsB* gene sequences. The *cpsB* sequences of all of the serotype 14 strains, including the Maryland strains, GenBank strains, and international clones, differed by ≤5 bp. All serotype 14 strains were ≥16% (78 to 83 of 476 bp) divergent from the $9V^{-3}$ clone. The serotype 14 variants could be differentiated from the $9V^{-3}$ clone with the *cpsB* and the *pbp1a* genes but not by PFGE or MLST.

A bootstrapped NJ tree of the 15 international clones and the 23 strains from GenBank further demonstrated a correlation between serotype and the *cpsB* sequence. Moreover, the tree graphically displayed the most common sequence for each serotype (Fig. 3). The sensitivity and specificity of using the *cpsB* sequence as a surrogate for serotype designation were determined. For each serotype, the most common *cpsB* sequence was chosen as the reference sequence. For example, the identical *cpsB* sequence among serotype 6B strains occurred in 75% (three of four) of the strains. The sensitivity was defined as the ability of this *cpsB* sequence to correctly classify 6B strains as belonging to serotype 6B. The specificity was defined as the ability of this *cpsB* sequence to correctly classify strains from other serotypes as not belonging to serotype 6B. Among serotypes represented by multiple strains, allowing for a ≤2-bp difference in the *cpsB* gene resulted in the highest specificity, and allowing for a >2-bp difference resulted in the highest sensitivity overall. The *cpsB* sequence had a sensitivity of 75 to 100% and a specificity of 94 to 100% for the following serotypes: 6B, 8, 9V, 14, 19A, 19F, and 23F (Table 3). Overall, 95% (84 of 88) of the strains were classified correctly by serotype with the *cpsB* sequence.

DISCUSSION

We found that serotype 14 variants of the $9V^{-3}$ clone accounted for a substantial proportion of PNSP serotype 14 strains in Baltimore, Md., over a 2-year period. To our knowledge, we are the first to include all PNSP serotype 14 strains from a defined population base. Overall, we found a high correlation between PFGE and MLST. The one exception was strain XI, which was classified as a unique PFGE-based clonal group but belonged to the same MLST lineage and ST complex as three other strains.

Unlike the European strains, all three serotype 14 variants of the $9V^{-3}$ clone had a unique *pbp1a* restriction profile and *pbp1a* DNA sequence from positions 1498 to 1710 compared to all other serotype 14 strains, strain R6, and the $9V^{-3}$ clone. Thereafter, the Baltimore variants were related to the $9V^{-3}$ clone and then to strain R6. Although the exact crossover point between $9V^{-3}$ and R6 was different from that in the European variants, the pattern was conserved. These data suggest two

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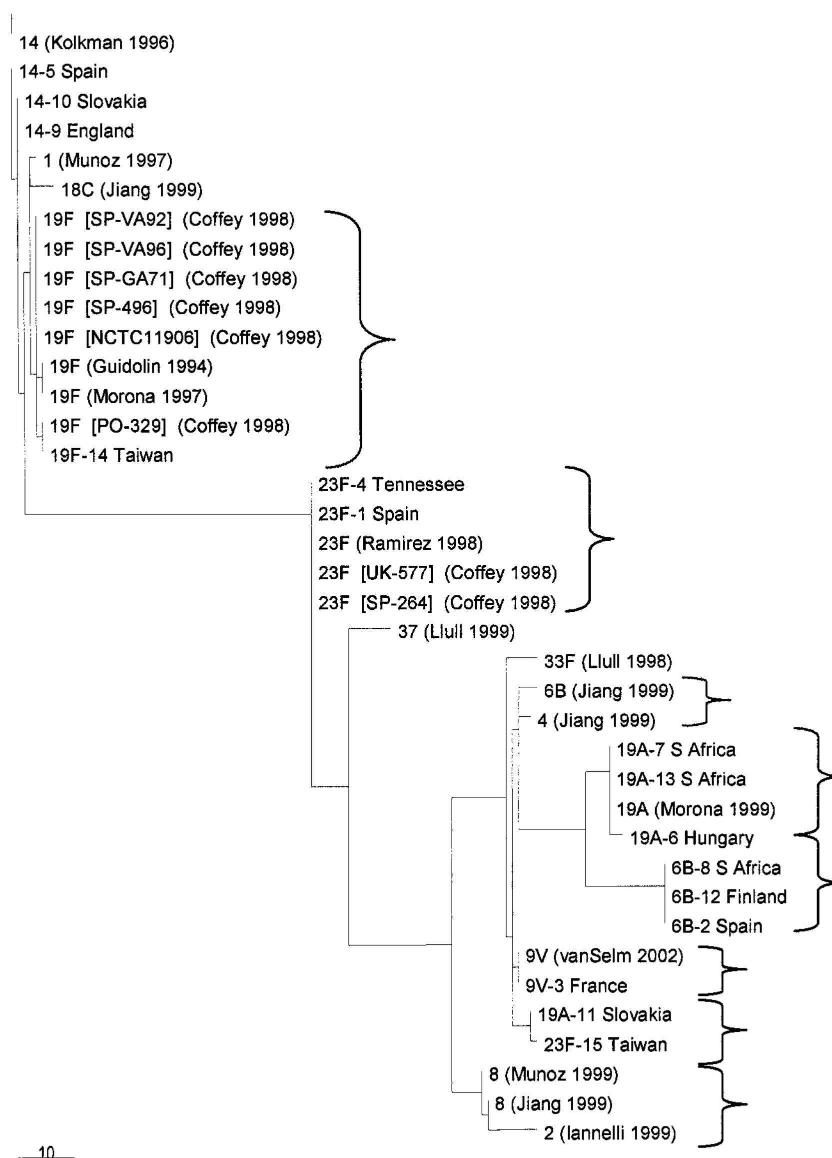

FIG. 3. NJ tree of 15 international clones and 23 GenBank strains. Strains which clustered ≥50% of the time with bootstrapping are indicated.

possibilities. Perhaps additional recombination events in the *pbp1a* gene occurred after capsular transformation with the $9V^{-3}$ clone, causing increased diversity in our strains compared to the European strains. Second, perhaps our variants arose through a recombination event different from that of the European strains. Previous studies demonstrated that variants have arisen on multiple occasions through unique recombination events (2, 3).

The variability that we noted within the *pbp1a* gene simply may reflect the mosaic patterns associated with the penicillin binding proteins. For example, some of the genetically unrelated serotype 14 strains had *pbp1a* sequences which were identical to or nearly identical to those of the $9V^{-3}$ clone or strain R6, respectively. Compared to the Spanish 23F-1 clone, strain XII has the same ST and a highly related PFGE pattern. The 23F-1 clone has the same *pbp1a* DNA sequence as the $9V^{-3}$ clone (21). Therefore, it is not unexpected that strain XII has the same *pbp1a* sequence as the $9V^{-3}$ clone. Strain R6 was not related to any of our serotype 14 strains by MLST but had a *pbp1a* sequence highly related to those of five strains. A search of the *pbp1a* sequences of the serotype 14 variants in GenBank demonstrated that this sequence was 99% related to that of a previously described 23F penicillin-resistant strain (6).

An inherent limitation of all studies focusing on recombination is the uncertainty of the original donor and recipient strains. An earlier study assumed that the *pbp1a* sequence of the original donor was identical to that of strain R6 and that the original recipient had a sequence identical to that of the $9V^{-3}$ clone (2). In contrast, we used all serotype 14 PNSP strains from a population base over a 2-year period and the $9V^{-3}$ clone as the donors and the recipient, respectively. Perhaps another suitable comparison group would be serotype 14

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TABLE 3. Sensitivity and specificity for *cpsB* sequences at various thresholds of base-pair differences

Serotype	% Sensitivity or specificity at the following threshold:												Total no. of strains
	0 bp		1 bp		2 bp[a]		3 bp		4 bp		5 bp		
	Sensitivity[b]	Specificity[c]	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	
1	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	94 (82/87)	100 (1/1)	84 (73/87)	100 (1/1)	74 (64/87)	100 (1/1)	71 (62/87)	1
2	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	1
4	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	53 (46/87)	100 (1/1)	52 (45/87)	1
6B[d]	75 (3/4)	100 (84/84)	75 (3/4)	100 (84/84)	75 (3/4)	100 (84/84)	75 (3/4)	100 (84/84)	75 (3/4)	100 (84/84)	75 (3/4)	100 (84/84)	4
8	50 (1/2)	100 (86/86)	100 (2/2)	100 (86/86)	100 (2/2)	100 (86/86)	100 (2/2)	100 (86/86)	100 (2/2)	100 (86/86)	100 (2/2)	100 (86/86)	2
9V	100 (41/41)	100 (47/47)	100 (41/41)	100 (47/47)	100 (41/41)	100 (47/47)	100 (41/41)	100 (47/47)	100 (41/41)	96 (45/47)	100 (41/41)	91 (43/47)	41
14	60 (9/15)	100 (73/73)	93 (14/15)	100 (73/73)	93 (14/15)	100 (73/73)	93 (14/15)	100 (73/73)	93 (14/15)	92 (67/73)	100 (15/15)	86 (63/73)	15
18C	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	93 (81/87)	1
19A	60 (3/5)	100 (83/83)	60 (3/5)	100 (83/83)	80 (4/5)	100 (83/83)	80 (4/5)	100 (83/83)	80 (4/5)	100 (83/83)	80 (4/5)	100 (83/83)	5
19F	22 (2/9)	100 (79/79)	78 (7/9)	100 (79/79)	100 (9/9)	99 (78/79)	100 (9/9)	97 (77/79)	100 (9/9)	92 (73/79)	100 (9/9)	81 (64/79)	9
23F	83 (5/6)	100 (82/82)	83 (5/6)	100 (82/82)	83 (5/6)	100 (82/82)	83 (5/6)	100 (82/82)	83 (5/6)	100 (82/82)	83 (5/6)	100 (82/82)	6
33F	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	1
37	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	100 (1/1)	100 (87/87)	1

[a] A threshold of 2 bp means that sequences with two or fewer base-pair differences from the reference sequence were classified as members of the serotype.
[b] Sensitivity is the percentage of sequences correctly identified as a member of the serotype. The values in parentheses indicate the number of sequences classified as a member of the serotype divided by the number of sequences that actually belong to that serotype.
[c] Specificity is the percentage of sequences correctly identified as not being a member of the serotype. The values in parentheses indicate the number of sequences classified as not being a member of the serotype divided by the number of sequences that actually do not belong to that serotype.
[d] The reference sequence for each serotype is the most common sequence.

penicillin-susceptible strains, since the *pbp1a* gene may have undergone further recombination events after the capsular recombination event.

MLST assigns equal weights to one nucleotide difference and multiple nucleotide differences within the same locus, thereby minimizing differences within a single locus. However, since recombination events are typically several kilobases in size, this approach appears to be an accurate means for assessing the genetic relatedness of strains (4). In our study, the concatenated gene sequence analysis provided results different from those provided by MLST in only one instance. Strain III, found to be an SLV of the 9V^{-3} clone by MLST, had 32 base-pair differences within the *ddl* gene. While the ML and MP trees clustered strains I to III with the 9V^{-3} clone, the bootstrapped NJ tree of the DNA sequence revealed that these strains clustered together only 65% of the time. On the surface, these data suggest that strain III may not be a serotype 14 variant of the 9V^{-3} clone. However, evidence clearly indicates that the variation seen within the *ddl* gene can be due to a hitchhiking phenomenon related to a single recombination event associated with the penicillin binding protein 2b gene (4). Our data support this theory, since strain III is the only penicillin-intermediate serotype 14 variant. In contrast, strains I and II and the 9V^{-3} clone are penicillin resistant.

We found that nearly a third of the serotype 14 PNSP strains collected over a 2-year period from the Baltimore metropolitan area were variants of the 9V^{-3} clone that differed from the European variants. Since they were detected over an 8-month period from persons residing in three different counties, it appears that this clone is widespread within the Baltimore metropolitan area. As shown previously, MLST and PFGE had a high degree of correlation and concurred with the concatenated gene sequence the majority of the time. The recombination history for the serotype 14 variants from Baltimore was different from that for the European variants.

As noted previously (2), the *cpsB* genes of all serotype 14 strains were highly related but markedly different from that of the 9V^{-3} clone, even among strains that had undergone capsular transformation. Allowing for a 2-bp difference, the *cpsB* sequence usually correlated with the serotype designation among this small sample of strains. The key limitation of this study was the choice of the reference sequence for each serotype; the reference sequence for each serotype was the most common sequence. Since the compositions of strains in other databases may differ markedly, the validity of the *cpsB* sequence as a surrogate for serotype could decrease dramatically. Thus, although the *cpsB* sequence was correlated with the serotype among this small sample of strains, the role of this gene as part of a sequence-based serotyping method may be limited.

ACKNOWLEDGMENTS

We thank the participating hospital infection control practitioners and microbiology laboratory personnel in the Baltimore metropolitan area for identifying the pneumococcal cases and providing the bacterial isolates; Yvonne Dean Hibbert and Jackie Hunter for assistance in conducting surveillance; Kim Holmes for assistance with data collection; and Althea Glenn, Laboratories Administration, Maryland Department of Health and Mental Hygiene, for processing the isolates. We thank Jim Jorgensen and his staff, University of Texas Health Sciences Center, for performing susceptibility testing and Richard R.

Facklam, Centers for Disease Control and Prevention, for performing serotyping. We gratefully acknowledge Bernard Beall for thoughtful review of the manuscript.

This work was supported in part by the State of Maryland, the Centers for Disease Control and Prevention, and career development awards from the National Institutes of Health to M. C. McEllistrem (K23 AI01788-03) and to L. H. Harrison (K24 AI52788).

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