Evaluation of Semiautomated Multiplex PCR Assay for Determination of *Streptococcus pneumoniae* Serotypes and Serogroups

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A semiautomated method for the determination of five serotypes and three serogroups in *Streptococcus pneumoniae* was developed. Primers specific for serotypes 1, 3, 14, 19F, and 23F and serogroups 6, 19, and 23 were combined in three multiplex PCRs. Products were separated by capillary electrophoresis with a 7-min run time, and a serotype or serogroup was assigned on the basis of fragment size. The method was used to test 93 clinical isolates, and all isolates of the serotypes concerned were correctly detected. The strategy would allow the detection of multiple serotypes in a single sample. Detection of additional serotypes could be included as capsule locus sequences become available.

Respiratory disease has overtaken diarrheal disease as the major cause of death in children under the age of 5 years worldwide. One of the most important pathogens involved is *Streptococcus pneumoniae*. At the same time, rates of resistance to penicillin and other antimicrobials have dramatically increased, leading to serious problems in the treatment of pneumococcal disease. The case for primary prevention of pneumococcal infection by vaccination is now compelling.

New pneumococcal conjugate vaccines, which include up to 11 of the 90 known capsule types, are undergoing extensive trials. A seven-valent conjugate vaccine covering serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F has been shown to be safe, immunogenic, and efficacious in the prevention of invasive pneumococcal disease in American children (4) and is licensed for use in the United States and for use for European infants and children between the ages of 2 months and 2 years. Conjugate vaccines are being recommended for routine use to reduce pneumococcal disease in early childhood (9). With the limited coverage of conjugate vaccines, it is important to maintain regular monitoring of serotype prevalence in case widespread vaccination programs lead to a selective increase in serotypes not covered by the conjugate vaccines (20).

Serotype prevalence is currently monitored by culture of the organism, followed by serology. We have previously demonstrated that evaluation of the polymorphisms within the *cpsA* and *cpsB* genes common to all capsule loci can be used as a DNA-based method to predict pneumococcal serotype (14). However, a limitation of both these methods is that the presence of multiple serotypes may not be detected (10). A potential solution is the use of PCR to detect DNA from regions of

the capsule locus that directly determine serotype and serogroup. The sequences of the genes responsible for capsule production in several serotypes have been published, and serotype-specific genes have been identified. Unfortunately, this approach requires multiple PCRs for each sample, and manual analysis would barely be feasible in laboratories typing large numbers of isolates.

To make a serotype-specific PCR method amenable for use for routine testing, we have investigated the use of a multiplex PCR procedure coupled with capillary electrophoresis for automated analysis. This was applied to the detection of five commonly occurring serotypes for which capsule locus sequence data are available, namely, serotypes 1, 3, 14, 19F, and 23F, and also to the detection of three serogroups, serogroups 6, 19, and 23. The potential of the system to determine serogroups and serotypes was examined by using pneumococcal cultures submitted to the major reference center in the United Kingdom.

MATERIALS AND METHODS

Bacterial isolates. Ten pneumococci each of serotypes 1, 3, 6A, 6B, 14, 19A, 19B, 19C, 19F, 23A, 23B, and 23F were selected from among clinical isolates submitted to the reference laboratory from September to December 1997, as was a reference strain of each of the 23 vaccine serotypes and other serotypes within serogroups 6, 19, and 23. These comprised serotypes 1 (NCTC 7465), 2 (NCTC 7466), 3 (NCTC 7978), 4 (NCTC 11886), 5 (NCTC 11887), 6A* (SSISP 6A/1), 6B (NCTC 11888), 7F (NCTC 11889), 8 (NCTC 11893), 9N (NCTC 11896), 9V (NCTC 11897), 10A (NCTC 11899), 11A (NCTC 11900), 12F (NCTC 11901), 14 (NCTC 11902), 15B (NCTC 11903), 17F (NCTC 11904), 18C (NCTC 11905), 19A (NCTC 11907), 19B* (SSISP 19B/1), 19C* (SSISP 19C/1), 19F (NCTC 11906), 20 (NCTC 11908), 22F (NCTC 11909), 23A* (2584/39), 23B* (SSISP 23B/1), 23F (NCTC 11910), and 33F (NCTC 11911). Isolates marked with an asterisk were obtained from the Statens Serum Institut, Copenhagen, Denmark. Uncharacterized pneumococci used for prediction of serotype were submitted to the reference laboratory over a 2-week period in March 1998 and were described previously (14).

Other streptococci used to test the specificity of the primers were *Streptococcus dysgalactiae* (NCTC 4669), *S. gordonii* (NCTC 7865), *S. bovis* (NCTC 8133), group G streptococcus (NCTC 9603), group K streptococcus (NCTC 10232), *S. anginosus* (NCTC 10713), *S. mutans* (NCTC 10832), *S. sanguis* (NCTC 11085), *S. oralis* (NCTC 11427), *S. pyogenes* (NCTC 12068), *S. vestibularis* (NCTC 12166),

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TABLE 1. Serotype- and serogroup-specific primers

Serotype or serogroup	Gene	Primer sequences	Nucleotide position ^a	Predicted size of PCR product (bp)
1	<i>cps1G</i>	6-FAM-5'-GTCGTTATGAGAAGGTGGA-3' 5'-TGACCAAATAGAACCTGATG-3'	9091-9110 9174-9194	103
3	<i>cps3B-cps3S</i>	5'-ATGTGGATTTCGAGAGTG-3' 6-FAM-5'-GATTACGCTCAGGGTCAA-3'	7511-7529 7646-7664	152
14	<i>cps14H</i>	6-FAM-5'-TGTATGGTGATATGGACTATT-3' 5'-AACCGACAAAAACAATAAG-3'	7488-7509 7688-7708	220
19F	<i>cps19fI</i>	6-FAM-5'-GTTCAACGACTAGGACGC-3' 5'-TAGGCACCAATGTTTCACT-3'	8351-8369 8462-8481	130
23F	<i>cps23fI</i>	6-FAM-5'-TGGTAGTGACAGCAACGA-3' 5'-CAAAGGCTAATTCAGCATC-3'	9660-9678 9818-9837	177
GP-6	<i>cps6bS</i>	6-FAM-5'-TATAGATCCGATACGACGTAAC-3' 5'-ATACCAATTACACCAAAGTCTG-3'	9407-9428 9580-9592	200
GP-19	<i>cps19fF-cps19fG</i>	6-FAM-5'-CTAATGAGCCTAAACGTCTCT-3' 5'-TTGACTGCACCAAGTACACT-3'	5777-5798 5979-5999	222
GP-23	<i>cps23fH</i>	6-FAM-5'-GATGCAAGAAATGTCGTA-3' 5'-TCTGCCTCATTTGTTCTCC-3'	10379-10398 10486-10505	126
IPC		5'-CGGCGTTTCGTGTCTCT-3' HEX-5'-GCCTCCCGTTATTGCAT-3'	7533-7549 7592-7608	75
<i>cpsA</i>		5'-TCAGCTCTGTGTCGCTCT-3' HEX-5'-TCATTATCAGTCCCAGTCG-3'	422-439 575-593	171

^a Serotypes 1, 3, 14, 19F, and 23F, Genbank accession no. Z83335, Z47210, X85787, U09239, and AF030373, respectively; IPC (bacteriophage lambda genomic DNA), Genbank accession no. J02459, *cpsA* (serotype 19F), Genbank accession no. U09239.

S. mitis (NCTC 12261), *S. parasanguis* (NCTC 12854), *S. agalactiae* (NCTC 12906), *S. viridans* (NCTC 11189), *S. intermedius* (NCTC 11324), *S. constellatus* (NCTC 11325), and *S. equisimilis* (NCTC 11564).

Serotyping of isolates. Preliminary serotyping was performed by slide agglutination (6) with capsular typing sera and factor sera (Statens Serum Institut). Any discrepancies or equivocal results were rechecked by the Quellung reaction.

DNA extraction. *S. pneumoniae* reference strains and streptococci of other species were cultured overnight in 20 ml of Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom). After sedimentation the cells were resuspended in 0.1 ml of TE (10 mM Tris HCl, 1 mM EDTA [pH 8.0]). Lysozyme (10 µl at 50 mg/ml) was added, and the cells incubated for 30 min at 37°C, followed by addition of 0.5 ml GES (5 M guanidine thiocyanate, 0.1 M EDTA [pH 8.0], 0.5% Sarkosyl) and incubation at room temperature for 10 min. Ammonium acetate (0.25 ml at 7.5 M) was added, and the mixture was placed on ice for 10 min. Chloroform-isoamyl alcohol (24:1) was added, the contents were mixed and separated by centrifugation, and 0.7 ml of the aqueous phase was recovered. DNA was precipitated with 0.54 volume of isopropanol and then redissolved and reprecipitated in 70% ethanol. The DNA was finally resuspended in 50 to 100 µl of TE.

Pneumococcal DNA from clinical isolates was prepared as a crude extract. A loopful of growth from a fresh plate was suspended in 100 µl of TE, and the mixture was heated to 100°C for 10 min and then placed on ice until it was used.

PCR. Standard PCR was performed with 50 µl of 50 mM KCl, 15 mM Tris-HCl (pH 8.0), 2.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 0.2 M, 20 pmol of each primer (Table 1), 1 µl of template DNA (purified or crude extract), and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Warrington, United Kingdom). The conditions for PCR were 1 cycle at 94°C for 10 min and then 32 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 60 s in a Perkin-Elmer GeneAmp 2400 instrument (Applied Biosystems). Alternative conditions used during optimization are described in Results. PCRs were set up in a UV-treated cabinet (Template Tamer; Oncor Appligene, Chester-le-Street, United Kingdom) and performed and analyzed in separate rooms.

PCR product detection. The PCR products from the primer specificity experiments were analyzed on 10-cm 2% agarose gels, stained with ethidium bromide, and viewed over UV illumination. Subsequently, the PCR products were visualized on an automated ABI Prism 310 genetic analyzer (Applied Biosystems) by laser-induced fluorescence of the fluorophores on the 5' terminus of the forward or reverse primer (Table 1); 1 µl of a PCR product was added to 10 µl of deionized formamide and 1 µl of the GeneScan 500 internal band size standard

TABLE 2. Standardized serotype-specific PCR conditions for three thermocyclers^a

Step ^b	GeneAmp 2400 instrument		Omni Gene instrument		GeneAmp 9700 instrument	
	Temp (°C)	Time	Temp (°C)	Time	Temp (°C)	Time
Denaturation	94	10 min	94	10 min	94	10 min
Denaturation	94	30 s	94	40 s	94	30 s
Annealing	61	30 s	62	15 s	61	30 s
Elongation	72	60 s	72	60 s	72	60 s
Elongation	72	5 min	72	5 min	72	5 min
Stop	4	∞	4	∞	4	∞

^a The ramp rates for the GeneAmp 2400 instrument and the Omni Gene instrument were 100% for all temperature changes; the ramp rates for the GeneAmp 9700 instrument were 80% from 94 to 61°C, 100% from 61 to 72°C, and 100% from 72 to 94°C. The total reaction times for the three instruments were 120, 200, and 120 min, respectively.

^b Denaturation was done for 1 cycle; denaturation, annealing, and elongation were done for 32 cycles; and elongation was done for 1 cycle.

FIG. 1. Agarose gel (2%) electrophoresis showing the multiplex PCR assay results for four clinical isolates (isolates PN1010, PN1008, PN1001, and PN1106). Lane M, 50-bp DNA ladder (Gibco-BRL); lanes 1 to 4 are multiplex PCRs: 3M (serotypes 3, 14, and 23F), 19M (serotypes 1 and 19F), GPM (serogroups 6, 19, and 23), and CPSA (pneumococcal capsule gene *cpsA*), respectively.

(Applied Biosystems), and the mixture was then heated at 95°C for 5 min and placed on ice until it was required.

A green flash capillary (47 cm by 50 µm [inner diameter]) cut down to 41 cm was used with 0.6× 310 genetic analyzer buffer (containing EDTA) and 2% 310 GeneScan polymer made up in 0.6× 310 genetic analyzer buffer containing EDTA. The separation conditions were 15 kV for 7 min at 60°C with filter set D. The samples were injected at 15 kV for 7 s.

The electropherograms were analyzed with the Genotyper program (version 2.1; Applied Biosystems). Peaks representing the specific PCR products were automatically identified according to their size, as described in Results.

RESULTS

Selection of primers and optimization of PCR. For each of serotypes 1, 3, 14, 19F, and 23F and serogroups 19 and 23, genes likely to be serotype or serogroup specific were selected on the basis of published hybridization and sequence results (2, 8, 13, 16–19) and searches of the National Center for Biotech-

nology Information database (<http://www.ncbi.nlm.nih.gov/blast/>) with the BLAST program (1). In addition, capsule locus sequences were available for both subtypes of serogroup 6 (11; D. B. Griffiths and L. M. C. Hall, unpublished data); no genes were sufficiently different between serotypes 6A and 6B for design of primers, but serogroup 6-specific genes were identified.

Primers were designed for each selected serotype and serogroup on the basis of the criteria of a melting temperature of $57 \pm 1.5^\circ\text{C}$ and the production of products of different sizes. A size range of 100 to 500 bp was chosen to minimize the likelihood of amplification of nonspecific products and increase the thermocycling speed. In the case of primers specific for serotype 19F, which has a high degree of similarity to serotype 19A, primers were designed on the basis of the *cps19fI* sequence, with the forward and reverse primers positioned so that the last

TABLE 3. Genotyper group, category, and detection size ranges for PCR products

Group ^a	Categories ^b	Peak range and fluorescent color
Multiplex	1	All peaks from 103.00 to 106.00 bp in blue
	3	All peaks from 152.00 to 156.00 bp in blue
	14	All peaks from 222.00 to 227.00 bp in blue
	19F	All peaks from 130.00 to 133.00 bp in blue
	23F	All peaks from 177.00 to 183.00 bp in blue
GPM	GP-6	All peaks from 220.00 to 222.00 bp in blue
	GP-19	All peaks from 129.00 to 131.00 bp in blue
	GP-23	All peaks from 197.00 to 200.00 bp in blue
IPC-CPSA	CPSA	All peaks from 171.00 to 176.50 bp in green
	IPC	All peaks from 74.00 to 77.00 bp in green

^a Primers labeled with a different fluorescent color are not allowed to be within the same group. GPM, serogroup multiplex PCR; CPSA, pneumococcal capsule gene *cpsA*.

^b Named after the serotype, serogroup (GP), gene, or purpose of the PCR product.

TABLE 4. An example of a Genotyper results table produced by the macro for blind trial isolate 2

File name	PCR group ^d	Peak1	Peak 2 ^e
A5 ^a n2 ^b -3M ^c	Multiplex	14 ^f	— ^g
A5n2-3M	IPC-CPSA	— ^h	—
A6n2-19M	Multiplex	—	—
A6n2-19M	IPC-CPSA	IPC	—
A7n2-GPM	GPM	—	—
A7n2-GPM	IPC-CPSA	IPC	—
A8n2-CPSA	Multiplex	—	—
A8n2-CPSA	IPC-CPSA	CPSA	—

^a Microtiter plate well number automatically given.

^b Number manually appointed to isolate when tested; i.e., this was the second isolate to be tested.

^c PCR type.

^d Group names.

^e Overflow column, in case a second peak is identified.

^f The category peak 14 (equating to the serotype 14 PCR product) of the multiplex group has been found in the 3M PCR.

^g A second category peak of the multiplex group was not found in the 3M PCR.

^h A category peak of the IPC-CPSA group was not found in the 3M PCR.

TABLE 5. Genotyper results, predicted serotype, and actual serotype for 93 clinical isolates^a

Isolate PN code	Multiplex PCR result													Predicted serogroup and serotype	Actual serotype
	3M				19M			GPM				CPSA			
	3	14	23F	IPC	1	19F	IPC	6	19	23	IPC	<i>cpsA</i>	IPC		
1044	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1124	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1012	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1019	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1090	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1094	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	5
1055	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1127	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1057	—	—	+	—	—	—	+	—	—	+	—	+	—	GP-23, 23F	23F
1017	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1099	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1104	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1039	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1035	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1120	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	12
1103	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1061	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1074	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1109	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	35F
1112	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	4
1095	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	12
1043	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1020	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9N
1122	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6A
1081	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1010	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1001	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1016	—	—	—	+	+	—	—	—	—	—	+	+	—	1	1
1079	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1013	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1062	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1014	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1091	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1018	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1071	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	8
1111	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	20
1031	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	20
1101	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	18C
1125	+	—	—	—	—	—	+	—	—	—	+	+	—	3	3
1108	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1075	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1023	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	16F
1085	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1022	—	—	—	—	—	—	+	—	—	—	+	+	—	NP	15
1050	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1042	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1106	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1092	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	20
1076	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	29
1105	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1033	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1053	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1021	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1078	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1097	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1041	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	8
1045	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1080	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6A
1003	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1009	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1054	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1060	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1040	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1065	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1011	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	8

Continued on following page

TABLE 5—Continued

Isolate PN code	Multiplex PCR result												Predicted serogroup and serotype	Actual serotype	
	3M				19M			GPM				CPSA			
	3	14	23F	IPC	1	19F	IPC	6	19	23	IPC	<i>cpsA</i>			IPC
1100	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1107	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1059	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1015	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	18C
1093	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1077	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	9V
1028	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1025	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1129	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1052	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1032	+	—	—	—	—	—	+	—	—	—	+	+	—	3	3
1066	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1072	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1130	—	—	+	—	—	—	+	—	—	+	—	+	—	GP-23, 23F	23F
1046	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1132	—	—	—	+	—	—	+	+	—	—	—	+	—	GP-6	6B
1008	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	8
1047	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1068	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1128	—	—	+	—	—	—	+	—	—	+	—	+	—	GP-23, 23F	23F
1088	—	+	—	—	—	—	+	—	—	—	+	+	—	14	14
1049	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	35F
1126	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1083	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	9V
1026	—	—	—	+	—	+	—	—	+	—	—	+	—	GP-19, 19F	19F
1064	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	18C
1067	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	12
1038	—	—	—	+	—	—	+	—	—	—	+	+	—	NP	16F

^a In the text, each isolate is identified by a code consisting of the two-letter prefix "PN" followed by a four-digit number; here, each isolate is identified solely by the four-digit number. +, positive PCR result; —, negative PCR result; GP, serogroup; NP, serogroup and serotype not predicted.

two bases at the 3' terminus were specific for serotype 19F. The genes and product sizes chosen are summarized in Table 1.

Reference laboratories require robust PCRs that are specific and efficient and that can be performed under standardized conditions, necessitating empirical optimization of the reaction conditions. For each serotype-specific primer pair the annealing temperature was tested between 58 and 64°C in 2°C steps on a Perkin-Elmer GeneAmp 2400 instrument. The best compromise among the optimum annealing temperatures for the various primer pairs was identified as 61°C, and that temperature was adopted in all subsequent PCRs. Similarly, for each pair a series of PCRs containing 1 to 3.5 mM MgCl₂ in 0.5 nM increments was performed, and 2.5 mM was selected as the optimal concentration. Amplifications were also optimized on alternative thermocyclers, the Perkin-Elmer GeneAmp 9700 instrument and the Hybaid Omni Gene instrument. The Omni Gene instrument was found to have a slow ramping time, so shorter annealing and elongation times of 15 and 60 s, respectively, could be used. Conversely, the 9700 instrument has a very fast ramping time. The difference in ramping between the 9700 instrument and the 2400 instrument was timed, and the ramping rate for the 9700 instrument was adjusted accordingly to 80% for the denaturation to annealing step. These adjustments allowed comparable results to be obtained between the three machines (Table 2).

For identification of pneumococci with serotypes not covered by the type- or group-specific PCRs, a species-specific

PCR (pneumococcus control) was developed with primers whose sequences are from conserved sequences within the common capsule gene *cpsA*. The primers were designed on the basis of the same criteria used to design the primers for the serotype-specific PCR.

To identify PCRs that are falsely negative due to inhibitors or human error, imperative in a clinical situation, an internal positive control (IPC) that could be used in all PCRs was required (3, 7). This was adapted from a 500-bp bacteriophage lambda internal positive control from the GeneAmp Gold PCR kit (Applied Biosystems), but it amplified from bacteriophage lambda DNA a product of 75 bp, well below the size range of the pneumococcal products. The concentrations of the bacteriophage lambda DNA and primers were limited so that amplification of serotype- and serogroup-specific and pneumococcus control products would not be impaired. A consequence of limiting the IPC and IPC primers, as described by Ke et al. (12), is that in the presence of target DNA the IPC is not amplified due to competitive inhibition. This provides a visual advantage during interpretation of the results (Fig. 1).

Primer specificities. The species specificities of all the primer pairs were tested with purified genomic DNA from 18 streptococcal species. No products from other species were visually detectable on ethidium bromide-stained agarose gels for any of the primer pairs.

The serotype and serogroup specificities of the primers were evaluated with purified genomic DNA from type strains of the

23 most prevalent serotypes (5), corresponding to those contained in current polysaccharide vaccines: serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9A, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. The primers for serotypes 19F and 23F and serogroups 6, 19, and 23 were also tested with all subtypes of the same serogroups. No serotype-specific primer pair yielded a product with DNA from any of the other 23 serotypes tested; the primers specific for serotypes 19F and 23F were also negative in assays with isolates of serotypes 19A, 19B, and 19C and serotypes 23A and 23B, respectively. The serogroup-specific primers yielded products from all subtypes of their respective serogroups and were negative with isolates of other serogroups and serotypes.

To ensure that the primers would detect all pneumococcal isolates of the intended serogroup or serotype, each primer pair was tested with crude colony preparations of 9 to 10 recent United Kingdom clinical isolates of the respective serogroup or serotype. Each primer pair yielded products for all isolates of the target serogroup or serotype.

Design of multiplex reactions. Primers were tested in combination, since multiplexing of the reactions would reduce labor, time, and cost. Three multiplex reactions were developed: one for serotypes 3, 14, and 23F, one for serotypes 1 and 19F, and one for serogroups 6, 19, and 23. The concentrations of the primers and the reaction conditions were adjusted to allow similar levels of amplification for each product when a mixture of DNA from all target serotypes and serogroups was tested.

Capillary electrophoresis and automated detection of products. Capillary electrophoresis was chosen for automated detection of PCR products. This allows detection on the basis of both the product size and the color of the fluorescent label, but it is less cumbersome than gel-based methods (15). The system used was an ABI Prism 310 genetic analyzer and GeneScan analysis software (the software assigns size labels to peaks). Analysis (serotype assignment and detection of control products) was automated by using ABI Prism Genotyper software.

For fluorescence detection of the PCR products, one primer from each pair was labeled at its 5' terminus with 6-carboxy-fluorescein (6-FAM) or hexachlorofluorescein (HEX) during synthesis. When they are excited by the argon laser (488 and 514.5 nm), these dyes fluoresce blue and green, respectively. Serotype- and serogroup-specific primers were labeled with 6-FAM, while the pneumococcus control and IPC primers were labeled with HEX; thus, the products were easily distinguished. The denatured PCR products were coinjected with a GeneScan 500 size standard (35 to 500 bp) labeled with 6-carboxy-X'-rhodamine, which fluoresces red, to enable accurate sizing. Peak height thresholds were set at 40, 500, and 200 fluorescent units for red, blue, and green, respectively, to prevent background noise and nonspecific peaks from being detected.

Initially, the manufacturer's recommended conditions of a 47-cm capillary, 4% polymer (POP4), and the GS STR POP4 (1 ml) D module were used. The module contains the voltage and time setting used during a run. Although the products were correctly detected and sized, each run was 24 min long. Thus, analysis of one isolate involving four multiplex reactions would be completed every 96 min. The protocol was therefore modified to reduce the run time to 7 min, and the products were still found to be correctly sized within the 5-bp resolution

of the method. The detection times of the size standards and the sizes of PCR products varied slightly between runs, depending on the temperature of the room.

For analysis of the results, a macro was programmed to scan the electropherograms for peaks of the expected sizes and colors, as defined in Table 3, and to apply a name to the corresponding peak. The results are presented in a tabular format, with each PCR generating two table rows, one for blue peaks (multiplexed serotype- and serogroup-specific PCRs) and one for green peaks (pneumococcus control and IPC). An example of the output for one isolate is given in Table 4. PCR products could also be separated by agarose gel electrophoresis for comparison with the result obtained with the genetic analyzer (Fig. 1). Interpretation of the results was always consistent between the two methods, although the small IPC product was only weakly stained with ethidium bromide.

Determination of serotypes of clinical isolates. To evaluate the ability of the multiplex PCR assay to assign serogroups and serotypes, 93 consecutive isolates submitted to the reference laboratory were tested. The four electropherograms obtained with the genetic analyzer for each isolate were automatically compared to the peak size database, and the results were tabulated as described above. On this basis, isolates positive for the pneumococcus control reaction, which detects *cpsA*, either were assigned to one of the serotypes and/or serogroups tested or were designated "other serotype." Isolates that were negative for the species-specific reaction but that had a positive result in PCRs with IPC would not be considered pneumococci.

The results of the prediction of pneumococcal species and serotypes by the multiplex PCR assay are summarized in Table 5. All 93 isolates were correctly predicted to be pneumococci. Thirty-two isolates with serotypes among the five vaccine types covered by the PCR were correctly assigned. Of these, the nine serotype 19F isolates and the three serotype 23F isolates were also correctly assigned to serogroups 19 and 23, respectively. Thirteen serotype 6B isolates and six serotype 6A isolates were correctly predicted to be serogroup 6, while one serotype 9V isolate was incorrectly predicted to be serogroup 6. Forty-one isolates with serotypes not covered by the PCRs were correctly predicted to have other serotypes. The IPC was present and detected in all PCRs with negative results. Overall, these results demonstrate that the multiplex PCR has a sensitivity of 100% and a specificity of 99% for the detection of the serotypes and serogroups covered.

The serotype 9V isolate (isolate PN1077) incorrectly typed as a serogroup 6 was investigated further. Ten individual colonies were selected from an overnight blood agar plate culture of PN1077 and subcultured onto separate plates. The multiplex PCR assay was performed with crude DNA preparations from each plate including the original. Colonies from all subculture plates were serogroup 6 negative, while the original culture was positive. This suggests that the stock culture of PN1077 had a low-level contamination with a serogroup 6 isolate.

DISCUSSION

The results of this study demonstrate that PCR amplification and detection of serotype- or serogroup-specific sequences within pneumococcal capsule loci can accurately predict the

serotype of an organism. Furthermore, the use of fluorescently labeled primers and capillary electrophoresis provides a more rapid and partially automated alternative to conventional gel electrophoresis. Although the present study covered a limited range of serotypes and serogroups, the multiplex PCR can readily be extended as further capsule locus sequences become available. The use of multicapillary instruments greatly improves the speed of analysis, even allowing additional reactions to cover more serotypes. Such instruments are becoming more commonplace in both reference laboratories and research institutes as applications for their use in diagnostic microbiology and for typing expand.

It is envisaged that the present method, like conventional pneumococcal serotyping, would be performed largely in reference laboratories in the first instance. With an appropriate infrastructure in place, the multiplex PCR described here is estimated to have running costs similar to those of conventional serotyping, although a lower proportion of isolates would be assigned a type at present.

We have previously described a method for serotype prediction in which a fragment from *cpsA-cpsB* common to the capsule loci of all serotypes was amplified and restriction polymorphisms were detected (14). Although there was a strong linkage between the *cpsA-cpsB* polymorphism and serotype, this was not absolute. The method required only one PCR for each isolate, and a correlation to the serotype did not necessitate knowledge of the capsule locus sequence for each serotype. In comparison to the earlier method, the method described here involves a larger number of PCRs, but interpretation of the results is simpler and the procedure is readily amenable to further automation. Perhaps most significant is the fact that the present method will allow the detection of more than one serotype in a single sample.

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