JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2007, p. 3411–3415 Vol. 45, No. 10
0095-1137/07/$08.00+0 doi:10.1128/JCM.01076-07

Identification of Less-Common *Streptococcus pneumoniae* Serotypes by a Multiplex PCR-Based Reverse Line Blot Hybridization Assay▽

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Received 26 May 2007/Accepted 29 July 2007

We developed a multiplex PCR-based reverse line blot (mPCR/RLB) assay to identify 50 uncommon pneumococcal serotypes. In combination with a previously described mPCR/RLB assay (3), all 90 pneumococcal serotypes can be identified individually (32 serotypes) or, because of predictable cross-reactions, to within small groups of two to five related serotypes (58 serotypes), which can be distinguished using serotype-specific antisera.

Recently, we described a multiplex PCR-based reverse line blot (mPCR/RLB) hybridization assay to identify 40 *Streptococcus pneumoniae* serotypes, including the 23 represented in the polysaccharide vaccine and 17 others that show reproducible cross-reactions (3). In this study, we designed one pair of specific primers and one probe for each of the remaining 50 serotypes to allow provisional identification of all 90 known serotypes by mPCR/RLB (5). The primer and probe sequences were based on recently published full *cps* gene cluster sequences of all 90 pneumococcal serotypes (1) and on other sequences available in GenBank (4, 7).

Twenty sets of serotype-specific primer pairs and probes were designed; the remaining 30 serotypes shared identical or very similar *wzy* sequences with one or two others in the same or closely related serogroups, as follows: 7B/7C/40, 10F/10C, 11B/11C, 15F/15A, 19B/19C, 24F/24A/24B, 25F/25A/38, 28F/28A, 32F/32A, 33B/33D, 35F/47F, 35A/35C/42, and 41F/41A. The primers and probes used in this assay are shown in Table 1.

Probes and primers were designed with similar physical characteristics to allow simultaneous amplification and hybridization in a multiplex reaction (5) and were synthesized by Sigma-Aldrich (Sydney, Australia). Primers were biotinylated at the 5′ end, and probes had a 5′ amine group (5). DNA extraction (6) and RLB hybridization were performed as previously described (5).

mPCR was performed according to the QIAGEN Hotstar *Taq* polymerase kit instructions (5) under the following conditions: 95°C for 15 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; 72°C for 10 min; and a hold at 22°C. The 34-primer-pair mPCR mixture was prepared to contain the following: 2 μl template DNA, 0.075 μl of each forward (100 pmol μl^{-1}) and reverse (100 pmol μl^{-1}) primer, 2.4 μl deoxynucleoside triphosphates (2.5 mM of each deoxynucleoside triphosphate), 3 μl 10× PCR buffer, 4.2 μl 25 mM $MgCl_2$ (final concentration, 5 mM), 0.2 μl QIAGEN Hotstar *Taq* polymerase (5 units μl^{-1}), and water to 30 μl.

Preliminary testing of reference strains of all 90 serotypes (Statens Serum Institut, Copenhagen, Denmark) showed that all 50 target serotypes (and none of the other 40) were amplified and hybridized by the mPCR/RLB system; 20 serotypes reacted only with the corresponding primers and probe, and 30 exhibited the predicted cross-reactions (Table 1; Fig. 1).

The method was further evaluated using 173 previously studied (4, 7) clinical isolates from China, Australia, and Canada, which included one to four isolates of all pneumococcal serotypes except for 10C, 11F, 23B, 25A, and 33D (for which clinical isolates were not available). They were tested by mPCR/RLB without knowledge of the serotype. Sixty-eight isolates belonging to 1 of 40 serotypes identified by our original mPCR/RLB assay were not amplified. One nonserotypable isolate was also not amplified by either the previous (3) or new mPCR, although it was confirmed to be *S. pneumoniae* by species-specific PCR and phenotypic characteristics. This result suggests that this isolate either contains a significant *cps* deletion or mutation (8) or belongs to a novel serotype (10).

Forty-four isolates were identified as being of individual serotypes corresponding with those identified by conventional serotyping, and 59 were identified as being one of a group of two or three related serotypes which, with one exception, included the serotype identified by conventional serotyping (Table 2).

The exception was an isolate from China, which was identified as serotype 33B/33D by mPCR/RLB and as serotype 33C by conventional serotyping. Both methods were repeated and the initial (discrepant) results confirmed. Single PCR was positive with 33B/33D-specific and negative with 33C-specific primers. Sequencing of a portion of *wzy* of this isolate showed that it was different from that of any known serotype (1, 7) but more closely related to that of 33C than to that of 33B/33D. Although the discrepancy between molecular and immunological serotype identifications remained unresolved, our results suggest that *wzy* is not the only determinant of antigenic specificity (3). Further investigation of this isolate is in progress.

Finally, the mPCR/RLB assay was used to test 152 clinical

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▽ Published ahead of print on 8 August 2007.

TABLE 1. Oligonucleotide primers and probes for mPCR/RLB assay used in this study

Primer[a]	Specificity[b]	T_m (°C)[c]	GenBank accession no.	Sequence (5′–3′)[d]
lytASb[e]	*S. pneumoniae*	66.46	M13812	681CAA CCG TAC AGA ATG AAG CGG701
lytAAp	*S. pneumoniae*	60.50	M13812	721GTC TTT CCG CCA GTG ATA AT702
lytAAn[e]	*S. pneumoniae*	66.68	M13812	999TTA TTC GTG CAA TAC TCG TGC G978
7B7C40Sb	Serotypes 7B, 7C, and 40	60.84	CR931641	11915AAA ACT CAA GTA TCT GTG C(T)CA CCT T11939
7B7C40Ap	Serotypes 7B, 7C, and 40	60.47	CR931641	11967TCC AAA TTT SAG TAA ACC AAC CTA A11943
7B7C40An	Serotypes 7B, 7C, and 40	63.99	CR931641	11990CAT CTC TAT TCG ACC TTG CGT TA11968
10F10CSb	Serotypes 10F and 10C	60.73	CR931652	6883TAG TTT TGG TTA CGT AGT TGT TGA CT6908
10F10CAp	Serotypes 10F and 10C	61.73	CR931652	6933GAA AAC TTG CCC AAA TCC TT6914
10F10CAn	Serotypes 10F and 10C	60.53	CR931652	6963GCA ATA(/G) AAT ACT GTA GCA TAC GAT AGT T6936
11F11A11DSb	Serotypes 11F, 11A, and 11D	59.48	CR931657	10665GAA ATA TCG CCA TTC ATC AG10684
11FAp	Serotype 11F	60.70	CR931657	10752ATT GAC CCA CTT AAC ATA AAA GTT AAA10726
11FAn	Serotype 11F	61.24	CR931657	10770GAT TGT ACC CCA TCA CCG10753
11B11CSb	Serotypes 11B and 11C	64.44	CR931654	11092TCT GGT GCT AAG GGG ATC AA11111
11B11CAp	Serotypes 11B and 11C	61.93	CR931654	11135TGC ATA AGC TGA TTA TGA GCA TAG11112
11B11CAn	Serotypes 11B and 11C	60.34	CR931654	11162CCA ATT ACT CCA TTA TCT ATT GCT AAT11136
13Sb	Serotype 13	61.04	CR931661	12845GAT GGG AAA ATA CGA TAT GCT C12866
13Ap	Serotype 13	61.06	CR931661	12895TGA GCT AAA TGT TGA ATA TTT ATA CCC12869
13An	Serotype 13	60.91	CR931661	12922GAA AAT CGT AAC ATG GAA AAA GTA A12898
15F15ASb	Serotypes 15F and 15A	60.57	CR931666	8304TAT TTC CTT CCT ATG GGA CAA C8325
15F15AAp	Serotypes 15F and 15A	60.73	CR931666	8369AGT CCT TTC CCA AAT ATA GCA CT8347
15F15AAn	Serotypes 15F and 15A	63.72	CR931666	8417GCA AGC ATT TTA CCA AGT TCA TAA A8393
16FSb	Serotype 16F	60.91	CR931668	11925TTG TTC TTA CAT TTA GCC GTA GTG11948
16FAp	Serotype 16F	60.73	CR931668	11987GTT GAA AGA ATA CGA TTC CTA CAA G11963
16FAn	Serotype 16F	62.84	CR931668	20011TCG TCG TTG AAA ACA ATT TCT TAC11988
16ASb	Serotype 16A	63.65	CR931667	10908CCG CTC ACG GTA TGG ACT A10926
16AAp	Serotype 16A	62.44	CR931667	10952GGA GTA AAT GAT GTG TAG TGA AAA CC10927
16AAn	Serotype 16A	61.61	CR931667	10978CCA GCA ATA TAC TCA GGA AAT AAT TC10953
17ASb	Serotype 17A	60.22	CR931669	13764GTA GAC TTC TTA GAG CCT ATT GTG G13788
17AAp	Serotype 17A	61.79	CR931669	13821TGC TAA ATG TCA TTT TTT TAC CAA G13797
17AAn	Serotype 17A	61.77	CR931669	13845 CAT TCG ACC AGA TAT AGG TAC GAT13822
19B19CSb	Serotypes 19B and 19C	60.22	CR931676	10179AGA ATT CGG AGA TTT GTG GTA T10200
19B19CAp	Serotypes 19B and 19C	60.49	CR931676	10222TTC GTA CTG AAA ATT CAT TTC G10201
19B19CAn	Serotypes 19B and 19C	62.26	CR931676	10479CAA TCC ACC TCC ATA AAC GA10460
21Sn	Serotype 21	60.16	CR931680	12674CAA TTC TAC TGA GTC CAT ATT ATG AAA12700
21Sp	Serotype 21	60.51	CR931680	12709GAT AGT TTC TCT GTA TCA AAT AGC GA12734
21Ab	Serotype 21	63.98	CR931680	12755ACC ATC GTA CCT GCA CCA TAA12735
23ASn	Serotype 23A	60.54	CR931683	8847TTT ACT TTA ATT TAT AGC TTT TTG GCT AA8875
23ASp	Serotype 23A	61.54	CR931683	8876TGC CTT TTT TAA CGA GGT TG8895
23AAb	Serotype 23A	63.69	CR931683	8918GGT GCA TGA GTT AGG AGA AAG TG8896
23BSb	Serotype 23B	63.02	CR931684	9692GGA TCG TTG TTC ATA GCG G9710
23BAp	Serotype 23B	60.09	CR931684	9752ATA ATT ACT GGT CTG TGA TTT TTC TTT9726
23BAn	Serotype 23B	60.58	CR931684	9782GAT AAT AAA GAA ATT ACT AAC CAT GTC GT9754
24F24A24BSb	Serotypes 24F, 24A, and 24B	62.46	CR931688	12110TCA ACA CTT ATG ATG G(A)TG CCT G12131
24F24A24BAp	Serotypes 24F, 24A, and 24B	61.44	CR931688	12175CAC AAT CCA AAA CTT AAG TTG TTT C12151
24F24A24BAn	Serotypes 24F, 24A, and 24B	60.37	CR931688	12210GCA GAA ACA AAA(/G) GTA AGA ATT ATA GAT ATC12181
25F25A38Sn	Serotypes 25F, 25A, and 38	62.77	CR931690	12994GAC TAC AAA CTG CGG TAG TAG AAA TG13019
25F25A38Sp	Serotypes 25F, 25A, and 38	60.18	CR931690	13020ATA GGA ACT CTA GGG TTT AGT TTT TTC13046
25F25A38Ab	Serotypes 25F, 25A, and 38	61.68	CR931690	13076TGG AAC AAT TCT AAT CGT TAA TAC G13052
27Sb	Serotype 27	63.88	CR931691	8672GCA GCC ACC TCT TCT CAT TC8691
27Ap	Serotype 27	60.18	CR931691	8733CGC CAA ATT CTA TAC CAA CTA GTA T8709
27An	Serotype 27	62.49	CR931691	9047GGA AGG AAC AAC CCA ACA AT9028
28F28ASb	Serotypes 28F and 28A	64.23	CR931693	10688CAG AGT TTG GTC GAG GTT CCT A10709
28F28AAp	Serotypes 28F and 28A	60.20	CR931693	10739AGA ACT AAA TAC AGT GCA ATA ATT GG10714
28F28AAn	Serotypes 28F and 28A	60.11	CR931693	10767GCT CAA CTT TAT TTC TCT AGA ATA AAC A10740
29Sb	Serotype 29	62.01	CR931694	6342CCG AAA ATT GTT CAC AGG ATA C6363
29Ap	Serotype 29	61.37	CR931694	6393TAA CAA GCA GAA TAA GCA AAA TAG C6369
29An	Serotype 29	60.34	CR931694	6418AGC TTT CTT TTG TAC GAC TCT TTT A6394
31Sb	Serotype 31	61.77	CR931695	9419TGA AAA TCC CTT AGT GAC ATC TG9441
31Ap	Serotype 31	60.48	CR931695	9489GAG CCT TCT CAA TAG TCA TAA AAA A9465
31An	Serotype 31	60.36	CR931695	9535GCC ATA ATC AAA AAT AAG TTA GAC ATA A9508
32F32ASb	Serotypes 32F and 32A	61.08	CR931697	12671GGT ATG CTT ACA ATG AGA CGC12691
32F32AAp	Serotypes 32F and 32A	60.46	CR931697	12711CCA CTT CCC AGA GGA AAA TA12692
32F32AAn	Serotypes 32F and 32A	63.63	CR931697	13019AAT TCG TTC CCG GAT AAG ATG12999
33B33D33CSb[f]	Serotypes 33B, 33D, and 33C	61.80	CR931699	13115TCG TTG GAT GAC AAA ACT CTT AC13137
33B33D33CAp[f]	Serotypes 33B, 33D, and 33C	62.88	CR931699	13157GCT CAA TGT GAC AGG GAG AA13138
33B33D33CAn[f]	Serotypes 33B, 33D, and 33C	62.28	CR931699	13378CCT CCC TGA GCC AAA ATA AC13359
33CSb	Serotype 33C	62.44	CR931700	11186CGA ATC GTC ATA AGG CAA AA11205
33CAp	Serotype 33C	60.68	CR931700	11232ACC TAC TGT GAC AGG GAA TAG TAA A11208
33CAn	Serotype 33C	60.51	CR931700	11258AAT AGG AGT AAC AAA GAG AAG CCT AA11233
34Sn	Serotype 34	60.67	CR931703	8296TTA AAA GTA TTA TTG GTA GTG ATT CTT TTG8325
34Sp	Serotype 34	61.14	CR931703	8322TTT GTG AAG AGT ACC AAT GGA TT 8344
34Ab	Serotype 34	61.79	CR931703	8366TGT AAA GAC ATT CCC TGT AGG C8345
35F47FSb	Serotypes 35F and 47F	60.62	CR931707	6904ATA AAA AGA AAG TCT TTG CCA GAG6927
35F47FAp	Serotypes 35F and 47F	60.89	CR931707	6986AAA GTC ACA TC(T)T AAA ATT GAC ACA AC6961
35F47FAn	Serotypes 35F and 47F	61.07	CR931707	7012CAA CTT TTG GAA GAT ACT GAA CAT AA6987
35A35C42Sn	Serotypes 35A, 35C, and 42	62.11	CR931704	7662GGA GAC TA(/G)T TAA AAC TTT TTT CGT TC7687
35A35C42Sp	Serotypes 35A, 35C, and 42	60.72	CR931704	7689CCT ACT TTA TTA ATG CCT GTT TGA G7713
35A35C42Ab	Serotypes 35A, 35C, and 42	61.13	CR931704	7739TTA AGT AAG TCT TCG CAA TCC AG7717
35BSb	Serotype 35B	63.57	CR931705	7623CTA ATT TGG CTA TGA AGC TAA TCC C7647
35BAp	Serotype 35B	60.57	CR931705	7693AAG CGT GAA AAA TTT TAA TAA AAG AC7668

Continued on following page

TABLE 1—*Continued*

Primer[a]	Specificity[b]	T_m (°C)[c]	GenBank accession no.	Sequence (5′–3′)[d]
35BAn	Serotype 35B	60.29	CR931705	7722TAA CTT AAA TAG GCA TTA ACA AAA TAG GT7694
36Sb	Serotype 36	60.58	CR931708	13687CAA TTT CCC CTT ATT CTG TAG TTC13710
36Ap	Serotype 36	60.38	CR931708	13739AGA TAA ATA CAT CAT TAT TGA CGA ACA13713
36An	Serotype 36	63.39	CR931708	13763TGG AGA TCC CCA AGA GAA AAT A13742
39Sb	Serotype 39	63.67	CR931711	11410ATT GGT TTG GGA ACT TGA TGT C11431
39Ap	Serotype 39	62.70	CR931711	11464TAA TAA CCA TAC TCT TCC GTC GG11442
39An	Serotype 39	62.76	CR931711	11490GCA ATA AGG CAA ATA AGG GAT AAT TA11465
41F41ASn	Serotypes 41F and 41A	60.08	CR931714	11239TGA CAC TAT TTA TAA TTG CTT TAT CCT T11266
41F41ASp	Serotypes 41F and 41A	60.11	CR931714	11272GGG TGC AAG GTG ATT ATG TAT11292
41F41AAb	Serotypes 41F and 41A	63.37	CR931714	11348TAG CGA GAA ACT ATC TGC ATC TTG11325
43Sb	Serotype 43	60.10	CR931716	10493AGA TCA AAT GGT GGT ATT AGG AA10515
43Ap	Serotype 43	61.11	CR931716	10538TCG GGT GTA CAA ATC CTA AAC TA10516
43An	Serotype 43	60.19	CR931716	10564GGA ATA GAT CAT TAA CCC TAA TGA AT10539
45Sb	Serotype 45	64.85	CR931718	13236TAT GCA GGA AAT ATC CGA GAA GG13258
45Ap	Serotype 45	64.64	CR931718	13296CAG CAT ATC TTG CAC GAT AAT GAA13273
45An	Serotype 45	64.31	CR931718	13618CCG TGA AAC AGA AAC GCT ATG13598
47ASb	Serotype 47A	63.03	CR931720	10554TAT TTG CCA TAA CGG ACT CTA GAA C10578
47AAp	Serotype 47A	59.71	CR931720	10659TTT TTA ACA ACC TTG TAT AGA ATA CCT C10632
47AAn	Serotype 47A	60.23	CR931720	10710GCT AAA ATA ATA AAT AGC GAA CTT ACT ACA10681
48Sb	Serotype 48	63.24	CR931722	12838GCA TTT GGA GTT ATT GCC CTA C12859
48Ap	Serotype 48	60.13	CR931722	12884CCT ATA AAC ACA CTC AAA ACT AGC A12860
48An	Serotype 48	61.76	CR931722	12927CGA CGG AAT CAA TAT AAA TAA GTG ATA12901

[a] S, sense; A, antisense; b, biotin-labeled primer (primers were biotin labeled at the 5′ end); p, probe (probes were 5′ end labeled with a C-6 amine); n, non-biotin-labeled primer.

[b] Based on published sequence data for the whole *cps* gene cluster, it was not possible to design primers/probes that could distinguish some individual serotypes from one to three closely related ones, usually (but not always) belonging to the same serogroup.

[c] T_m, melting temperature. Values were provided by the primer synthesizer (Sigma-Aldrich).

[d] Numbers represent the base positions at which primer/probe sequences start and finish (starting at position 1 of the corresponding GenBank sequence).

[e] One pair of previously published primers (11) were used as species-specific primers.

[f] Some 33C strains may have positive results with the primers/probe (see the text for further explanation).

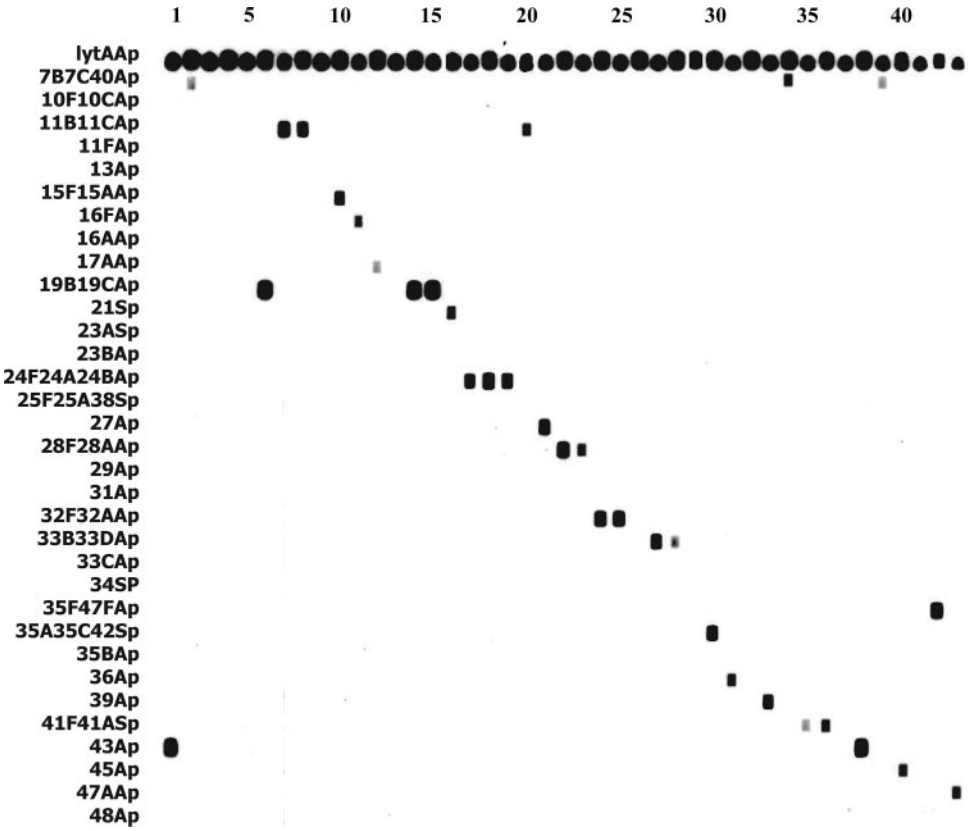

FIG. 1. mPCR/RLB results for a representative sample of 43 clinical isolates (see Table 1 for descriptions and specificities of the probes listed on the left). Conventional serotype-mPCR/RLB results for the isolates shown, from left to right, are 43, 7B, 9A,* 9L,* 9V,* 19B, 11B, 11C, 12A,* 15F, 16F, 17A, 18F,* 19B, 19C, 21, 24F, 24A, 24B, 11B, 27, 28F, 28A, 32F, 32A, 33A,* 33B, 33B, 33F,* 35C, 36, 37,* 39, 40, 41F, 41A, 44,* 43, 7B, 45, 46,* 47F, and 47A. Isolates marked with asterisks belong to one of 40 serotypes identified by our original mPCR/RLB assay (3) and were not amplified in this assay.

TABLE 2. mPCR/RLB and serotype identification results for 173 previously studied isolates from China, Australia, and Canada

Serotype(s)	No. of isolates	mPCR/RLB result
Forty serotypes represented in previously described mPCR/RLB assay (3)	68	Negative
7B	2	7B/7C/40
7C	2	7B/7C/40
40	1	7B/7C/40
10F	2	10F/10C
10C	0	
11B	3	11B/11C
11C	2	11B/11C
11F	0	
13	4	13
15F	3	15F/15A
15A	3	15F/15A
16F	3	16F
16A	1	16A
17A	2	17A
19B	2	19B/19C
19C	3	19B/19C
21	3	21
23A	3	23A
23B	0	
24F	3	24F/24A/24B
24A	1	24F/24A/24B
24B	1	24F/24A/24B
25F	2	25F/25A/38
25A	0	
38	4	25F/25A/38
27	4	27
28F	4	28F/28A
28A	3	28F/28A
29	1	29
31	4	31
32F	1	32F/32A
32A	2	32F/32A
33B	3	33B/33D
33D	0	
33C[a]	1	33B/33D
34	4	34
35F	3	35F/47F
47F	1	35F/47F
35A	1	35A/35C/42
35C	3	35A/35C/42
42	1	35A/35C/42
35B	3	35B
36	3	36
39	2	39
41F	2	41F/41A
41A	1	41F/41A
43	2	43
45	1	45
47A	1	47A
48	3	48
Nonserotypable[b]	1	Negative

[a] One isolate from China, which was identified as 33B/33D by single PCR and mPCR/RLB, was shown to be 33C by using serotype-specific antiserum.
[b] This isolate was amplified and hybridized by *S. pneumoniae*-specific primers and probes (targeting *lytA*) and had optochin susceptibility and bile solubility.

isolates collected during 2000–2007 at the Centre for Infectious Diseases and Microbiology and selected as belonging to one of the 50 uncommon serotypes identified by the new mPCR/RLB system.

Thirteen isolates were not initially amplified by mPCR. Of these, 11 were successfully identified after modification of the DNA extraction method (5) by adding 2 μl of proteinase K (20 mg/ml; Sigma, Australia) to tubes before heating them at 100°C for 10 min. Two isolates that still were not amplified had been identified, using antisera, as serotypes 11F and 16A. Conventional serotyping was repeated and showed that they belonged to serotypes 11A and 18C, respectively (not represented in the current mPCR/RLB system). Two isolates, previously identified as serotypes 35A and 29, were identified by mPCR/RLB as 35F and 35B, respectively. Retesting with antisera confirmed the mPCR/RLB results.

TABLE 3. mPCR/RLB and conventional serotyping results for 152 clinical isolates from Centre for Infectious Diseases and Microbiology

Serotype	No. of isolates	mPCR/RLB result
7C	9	7B/7C/40
10F	1	10F/10C
11F	1	Negative (11A)[a]
13	4	13
15A	5	15F/15A
16F	41	16F
16A	1	Negative (18C)[a]
21	2	21
24F	1	24F/24A/24B
25A	2	25F/25A/38
38	24	25F/25A/38
28F	1	28F/28A
28A	1	28F/28A
31	3	31
34	4	34
35F[b]	30	35F/47F
35C	1	35A/35C/42
35B[c]	20	35B
36	1	36

[a] Serotypes in parentheses are corrected serotypes after retesting by conventional serotyping; these serotypes are not included in the new mPCR/RLB assay.
[b] One isolate initially identified as serotype 35A was found to be 35F upon being retested.
[c] One isolate initially identified as serotype 29 was found to be 35B upon being retested.

Seventy-three isolates were identified by mPCR/RLB as single serotypes corresponding with those of conventional serotyping; 75 isolates gave positive results with probes cross-reacting with two or three related serotypes, which included the serotypes identified by conventional serotyping. The mPCR/RLB-predicted serotypes for these 152 isolates are shown in Table 3.

Because serotype-specific targets within *cps* gene clusters are not always available, this mPCR/RLB assay is less discriminatory—although more objective and reproducible—than conventional serotyping (2, 4). In combination with our previous mPCR/RLB assay (3), it can identify 32 serotypes precisely and 58 as belonging to one of two to five related serotypes. This is the first report of a practical molecular assay that can predict all 90 serotypes without sequencing. Potentially, it can be used to identify multiple serotypes directly in clinical (including culture-negative) specimens. It could be adapted to other platforms, such as "liquid" molecular beacons (9) and solid microarrays (12).

For routine serotyping of pure pneumococcal cultures, our first mPCR/RLB assay (identification of 40 serotypes) would be used initially to identify the most prevalent serotypes

(>95% of invasive isolates, based on unpublished data from the NSW Pneumococcal Reference Laboratory) (3). The current assay would then be needed, infrequently, to identify the small proportion of isolates belonging to less-common serotypes.

Fanrong Kong and Fei Zhou had similar contributions to the work and should be seen as co-first authors.

We thank Mitchell Brown and Shahin Oftadeh for conventional serotyping and for selecting appropriate clinical isolates.

This study was funded, in part, by National Health and Medical Research Council project grant 358351 (to G. L. Gilbert).

REFERENCES

1. **Bentley, S. D., D. M. Aanensen, A. Mavroidi, D. Saunders, E. Rabbinowitsch, M. Collins, K. Donohoe, D. Harris, L. Murphy, M. A. Quail, G. Samuel, I. C. Skovsted, M. S. Kaltoft, B. Barrell, P. R. Reeves, J. Parkhill, and B. G. Spratt.** 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet. **2:**e31.
2. **Henrichsen, J.** 1995. Six newly recognized types of *Streptococcus pneumoniae*. J. Clin. Microbiol. **33:**2759–2762.
3. **Kong, F., M. Brown, A. Sabananthan, X. Zeng, and G. L. Gilbert.** 2006. Multiplex PCR-based reverse line blot hybridization assay to identify 23 *Streptococcus pneumoniae* polysaccharide vaccine serotypes. J. Clin. Microbiol. **44:**1887–1891.
4. **Kong, F., and G. L. Gilbert.** 2003. Using *cpsA-cpsB* sequence polymorphisms and serotype-/group-specific PCR to predict 51 *Streptococcus pneumoniae* capsular serotypes. J. Med. Microbiol. **52:**1047–1058.
5. **Kong, F., and G. L. Gilbert.** 2006. Multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB)—a practical epidemiological and diagnostic tool. Nat. Protoc. **1:**2668–2680.
6. **Kong, F., S. Gowan, D. Martin, G. James, and G. L. Gilbert.** 2002. Serotype identification of group B streptococci by PCR and sequencing. J. Clin. Microbiol. **40:**216–226.
7. **Kong, F., W. Wang, J. Tao, L. Wang, Q. Wang, A. Sabananthan, and G. L. Gilbert.** 2005. A molecular-capsular-type prediction system for 90 *Streptococcus pneumoniae* serotypes using partial *cpsA-cpsB* sequencing and *wzy*- or *wzx*-specific PCR. J. Med. Microbiol. **54:**351–356.
8. **Llull, D., P. Veiga, J. Tremblay, and S. Kulakauskas.** 2005. Immobilization-based isolation of capsule-negative mutants of *Streptococcus pneumoniae*. Microbiology **151:**1911–1917.
9. **Marras, S. A., S. Tyagi, and F. R. Kramer.** 2006. Real-time assays with molecular beacons and other fluorescent nucleic acid hybridization probes. Clin. Chim. Acta **363:**48–60.
10. **Park, I., D. G. Pritchard, R. Cartee, A. Brandao, M. C. Brandileone, and M. H. Nahm.** 2007. Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J. Clin. Microbiol. **45:**1225–1233.
11. **Suzuki, N., M. Yuyama, S. Maeda, H. Ogawa, K. Mashiko, and Y. Kiyoura.** 2006. Genotypic identification of presumptive *Streptococcus pneumoniae* by PCR using four genes highly specific for S. pneumoniae. J. Med. Microbiol. **55:**709–714.
12. **Wen, L., Q. Wang, Y. Li, F. Kong, G. L. Gilbert, B. Cao, L. Wang, and L. Feng.** 2006. Use of a serotype-specific DNA microarray for identification of group B streptococcus (*Streptococcus agalactiae*). J. Clin. Microbiol. **44:**1447–1452.