

# Development of an Automated and Multiplexed Serotyping Assay for *Streptococcus pneumoniae*<sup>∇†</sup>

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***Streptococcus pneumoniae* expresses more than 90 capsule types, and currently available pneumococcal vaccines are designed to provide serotype-specific protection. Consequently, serotyping of pneumococcal isolates is important for determining the serotypes to be included in pneumococcal vaccines and to monitor their efficacy. Yet serotyping of pneumococcal isolates has remained a significant technical challenge. By multiplexing many assays, we have now developed a simple yet comprehensive serotyping assay system that can not only identify all known pneumococcal serotypes but also subdivide nontypeable (NT) isolates into those with or without the conventional capsule locus. We have developed this assay system to require only six key reagents: two are used in one multiplex inhibition-type immunoassay, and four are required in two multiplex PCR-based assays. The assay system is largely automated by a seamless combination of monoclonal antibody-based and PCR-based multiplex assays using the flow cytometric bead array technology from Luminex. The assay system has been validated with a panel of pneumococci expressing all known pneumococcal serotypes and was found to be easily transferable to another laboratory.**

*Streptococcus pneumoniae* is an important human pathogen expressing more than 90 serologically distinct polysaccharide (PS) capsules (5, 12, 21). Currently available pneumococcal vaccines provide serotype-specific protection (1, 23). Consequently, in the wake of the unqualified success of the 7-valent conjugate vaccine, the serotypes covered by the vaccine have become uncommon, whereas nonvaccine serotypes have become more common than before (13, 28). Also, the prevalence of a pneumococcal serotype differs for different locations and times (9, 11). Consequently, it is important to survey the serotypes of pneumococcal isolates circulating in many populations in order to monitor the efficacy of current vaccines and to select serotypes for future vaccines.

Classically, pneumococci are serotyped by the quellung reaction (12, 17). However, the quellung reaction is very labor-intensive, and several new approaches have appeared. One approach utilizes PCR-based systems that analyze DNA sequences of the capsular polysaccharide synthesis gene locus (*cps*), which contains the genes for capsule biosynthesis (3, 4, 15, 16, 19, 24). Another approach is a multiplex immunoassay for capsular polysaccharides using a set of monoclonal antibodies (MAbs) to identify the vaccine-related serotypes (30). While these two approaches overcome some of the limitations associated with the quellung reaction, they still have significant limitations. For instance, PCR products have commonly been identified by manual electrophoresis (20) or line blot hybridization (15). Also, due to vaccine-caused shifts in serotype prevalence, the multiplex immunoassay with limited coverage

can no longer identify the serotypes of a large fraction of contemporary isolates. Consequently, pneumococcal serotyping still remains a challenge to most laboratories.

An ideal serotyping system should not only be simple to perform but should also cover all pneumococcal serotypes. The ability to test for all known serotypes is important, because the serotype prevalence not only differs due to vaccine-induced serotype shifts but also differs among different regions (11) or over time (9). Also, vaccine usage may increase the prevalence of nontypeable (NT) pneumococci (25), which, in turn, are heterogeneous. A study (10) divided them into two groups: group I isolates have conventional but defective *cps*, whereas group II has nonconventional *cps* with novel genes (10). Within NT group II isolates, clade 1 has *pspK* (encoding pneumococcal surface protein K); clade 2 has *aliB*-like ORF1 (referred to here as *aliC*, for simplicity) and *aliB*-like ORF2 (named *aliD*); and clade 3 has *aliC* (10; also I. H. Park et al. unpublished data). To develop a simple yet comprehensive pneumococcal serotyping system, we have seamlessly combined the PCR- and MAb-based approaches to produce an automated and multiplexed typing system that is simple to perform and can easily be transferred to another laboratory.

## MATERIALS AND METHODS

**Bacterial strains.** Our study used a panel of bacteria that consisted of 290 isolates. The panel included 90 isolates purchased from the Statens Serum Institute (Copenhagen, Denmark), representing the 90 different pneumococcal serotypes described previously (12). In addition, pneumococci expressing the three newly described serotypes (6C, 6D, and 11E), a laboratory strain with no capsule (R36A), and three clades of NT clinical isolates with null capsule genes, recently described by us (Park et al., unpublished), were also included. The panel had five clinical isolates for each of serotypes 1, 3, 4, 5, 6A, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 11E, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F, four isolates expressing serotype 2, and three 6D isolates. The panel included 53 clinical isolates of pneumococci expressing the serotypes not included in the 23-valent PS vaccine. The panel also included 3 NT pneumococci obtained from the Centers for Disease Control and Prevention (CDC) (see Table 4). In addi-

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tion to pneumococci, the panel also contained isolates from several closely related species: one *Streptococcus mutans*, three *Streptococcus mitis*, and three *Streptococcus oralis* isolates. It also had one isolate each from *S. pyogenes* and *S. agalactiae*.

All bacterial isolates were recovered from frozen vials on blood agar plates, and isolated colonies were cultured in Todd-Hewitt broth with 0.5 yeast extract (THY) medium overnight. The pneumococci in the liquid cultures were lysed as described previously (30), and the lysates were stored at  $-20^{\circ}\text{C}$  until needed. These lysates were used for our serotyping system with 3 reactions: reaction A consists of 26 PSs detected with MABs, and reactions B and C are PCR based as described below.

**Coupling of polysaccharides to microspheres.** Each of 26 pneumococcal capsular PSs (serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 11E, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) was conjugated to one of 26 types of MicroPlex microspheres (Luminex, San Antonio, TX). All of the PSs were purchased from the ATCC (Manassas, VA) except for those of serotypes 6A, 6C, and 11E. Serotype 6A PS was obtained from G. Schiffman (Brooklyn, NY), but serotype 6C and 11E PSs were purified in our laboratory (21, 32). For each of the PSs, 12 million carboxyl MicroPlex microspheres (Luminex, San Antonio, TX) were washed twice in morpholineethanesulfonic acid (MES) buffer (100 mM MES in water [pH 6]), resuspended in 1 ml of adipic acid dihydrazide (ADH) solution (35 mg/ml of ADH in MES buffer), and mixed with 200  $\mu\text{l}$  of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) solution (200 mg/ml of EDC in MES buffer). The reaction mixture was briefly sonicated using a Branson 2200 sonicator (Branson Ultrasonics Corporation, Danbury, CT) and was vortexed gently before being incubated for 1 h at room temperature (RT) in the dark. The beads were washed with water and were resuspended in 400  $\mu\text{l}$  of water. The resuspended beads were first mixed with 320  $\mu\text{l}$  of a PS solution and 80  $\mu\text{l}$  of an EDC solution (100 mg/ml of EDC in water) and then incubated with shaking at  $37^{\circ}\text{C}$  for various periods. PS concentrations (10 to 250  $\mu\text{g}/\text{ml}$ ) and incubation periods (1 to 18 h) differed for different serotypes. After the incubation, 80  $\mu\text{l}$  of 500 mM glucose and 80  $\mu\text{l}$  of 500 mM glycine (pH 7) were added to the reaction mixture. After a 30-min incubation at RT with shaking, the beads were washed with the wash buffer (phosphate-buffered saline [PBS] with 0.1% Tween 20 and 0.02% sodium azide) and were resuspended in 1 ml of storage buffer (PBS with 0.01% Tween 20, 1% bovine serum albumin [BSA], 0.1% glucose, and 0.02% sodium azide). After the bead was aged for 10 days at  $4^{\circ}\text{C}$ , the stock bead mixture was made by mixing all 26 bead suspensions together with 54 ml of storage buffer, and this mixture was stored frozen in 1-ml aliquots.

**Multibead serotyping assay with MABs (reaction A).** One frozen vial of PS-coated stock bead mixture was thawed, diluted 3-fold with 2 ml of blocking buffer (PBS with 0.1% Tween 20, 0.02% sodium azide, and 1% BSA), and resuspended by sonication. Twenty-five microliters of the bead mixture was added to each well of a 96-well plate with a filter bottom (Thermo Fisher, Waltham, MA), and the beads were washed twice with wash buffer using vacuum suctioning. After resuspension of the beads with 25  $\mu\text{l}$  of blocking buffer, 25  $\mu\text{l}$  of one diluted bacterial lysate and 25  $\mu\text{l}$  of the diluted MAB pool were added to each well. The diluted bacterial lysates were prepared by diluting the bacterial lysates 10- or 30-fold in blocking buffer. The MAB pool was prepared by mixing 26 different hybridoma culture supernatants (see Table 1), stored frozen in 1-ml aliquots, and then diluted 3-fold with blocking buffer before use. After a 30-min incubation at RT in the dark with shaking (700 rpm), the plate was washed four times with the wash buffer. To detect bound MAB, 50  $\mu\text{l}$  of phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulin (BD Pharmingen, Franklin Lakes, NJ) was diluted 1:200 in blocking buffer and was added to each well. The plates were incubated for 30 min in the dark with shaking. After 4 washes, the beads were resuspended in 75  $\mu\text{l}$  of wash buffer, and the 96-well plate was then incubated for 2 h in the dark at RT before being placed in a Bio-Plex 200 analyzer (Bio-Rad, Hercules, CA) for the acquisition of fluorescence data. The fluorescence data of each sample for each serotype were converted to a normalized signal by the following equation: normalized signal =  $100 \times (\text{sample signal} - \text{background signal}) / (\text{blank-control signal} - \text{background signal})$ . For an unknown sample, the serotype with a normalized signal of  $<33\%$  was selected. To facilitate data analysis, a computer program generated a table containing the normalized signals of all serotypes for each sample. The normalized signals were displayed in red when they were less than 33%, in blue when they were between 33 and 67%, and in black when they were above 67%.

**Coupling of DNA probes to microspheres.** To prepare beads for conjugation, 2 million carboxyl MicroPlex microspheres (Luminex, San Antonio, TX) were washed with water before being suspended in 50  $\mu\text{l}$  of 0.1 M MES (pH 4.5) by sonication. Two microliters of the oligonucleotide probe stock (100  $\mu\text{M}$  in water, kept at  $-20^{\circ}\text{C}$ ) and 2.5  $\mu\text{l}$  of fresh EDC solution (10 mg/ml in MES) were added

to the bead suspension, and the reaction mixture was mixed well and was incubated for 30 min at RT in the dark with shaking. After the first incubation, 2.5  $\mu\text{l}$  of fresh EDC solution (10 mg/ml) was added to the reaction mixture before another 30-min incubation at RT in the dark with shaking. The beads were washed first with 1 ml of 0.02% Tween 20 and then with 1 ml of 0.1% sodium dodecyl sulfate (SDS) and were resuspended in 400  $\mu\text{l}$  of TE buffer (10 mM Tris-1 mM EDTA [pH 8.0]) by vortexing and sonication. All of the beads were mixed together, and the resulting bead pool (stock bead mixture) was stored frozen in 0.4-ml aliquots.

**Multibead serotyping assay with multiplex PCR (reactions B and C).** For serotypes not identified by MABs, two multiplex PCRs were performed in 25- $\mu\text{l}$  volumes containing 5  $\mu\text{l}$  of the bacterial lysate (diluted 1:50) and 20  $\mu\text{l}$  of the PCR mixture. The PCR mixture was made by mixing 990  $\mu\text{l}$  of 2 $\times$  primer pool, 990  $\mu\text{l}$  of water, and 20  $\mu\text{l}$  (100 U) of Takara Ex Taq DNA polymerase (Takara Bio, Madison, WI). One aliquot (990  $\mu\text{l}$ ) of 2 $\times$  primer pool is made by mixing 250  $\mu\text{l}$  of 10 $\times$  Ex Taq buffer (Takara Bio, Madison, WI), 200  $\mu\text{l}$  of 2.5 mM deoxynucleoside triphosphates (dNTP), and 6.25  $\mu\text{l}$  of each stock primer (except the *hlyA* primers) in Table 2, followed by the addition of enough water to make the final volume 990  $\mu\text{l}$ . Instead of 6.25  $\mu\text{l}$ , only 5.0  $\mu\text{l}$  of the *hlyA* primer stocks was added. One of each primer pair has a biotin label at the 5' end for detection by streptavidin. Thermal cycling was performed in a Mastercycler gradient system (Eppendorf, Westbury, NY) using the following conditions:  $94^{\circ}\text{C}$  for 15 min; 35 amplification cycles of  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 1 min; and finally  $72^{\circ}\text{C}$  for 10 min for extension. The PCR products were kept at  $4^{\circ}\text{C}$  until further analysis.

To identify the PCR amplicons, 20  $\mu\text{l}$  of the 1:100-diluted PCR mixture was transferred to a 96-well PCR plate and was heated for 10 min at  $95^{\circ}\text{C}$ . One aliquot of the DNA-coupled stock bead mixture corresponding to the PCR was thawed and washed with 1 $\times$  tetramethylammonium chloride (TMAC) buffer (3 M TMAC, 1 g/liter SDS, 50 mM Tris-HCl, 4 mM EDTA [pH 8.0]) by centrifugation. The beads were then suspended in 4 ml of 1.5 $\times$  TMAC at  $48^{\circ}\text{C}$  and were briefly sonicated. Forty microliters of the bead mixture was quickly transferred to each well and was mixed with the PCR product by brief vortexing. The plate was incubated at  $48^{\circ}\text{C}$  for 30 min in the dark. A 135- $\mu\text{l}$  volume of 1 $\times$  TMAC ( $48^{\circ}\text{C}$ ) was added to each well of the PCR plate and was mixed before the entire contents of each well were transferred to a filter plate (Millipore, Billerica, MA). After the plate was washed with 1 $\times$  TMAC, 50  $\mu\text{l}$  of PE-streptavidin (BD Pharmingen, Franklin Lakes, NJ) was added to each well of the plate. After a 20-min incubation at  $48^{\circ}\text{C}$ , the plate was washed once with 1 $\times$  TMAC and once with PBS-0.1% Tween 20. The beads were resuspended in 75  $\mu\text{l}$  of PBS-0.1% Tween 20, and the filter plate was inserted into a Bio-Plex 200 analyzer (Bio-Rad, Hercules, CA) for the acquisition of fluorescence data. Fluorescence signals were converted to signal-to-noise (S/N) ratios by dividing the fluorescence signal of a sample by the background signal for each serotype. All the control samples produced S/N ratios less than 4 for incorrect serotypes and greater than 30 for the correct serotypes. For an unknown sample, the serotype with an S/N ratio greater than 10 was chosen. To facilitate data analysis, a computer program generated a table containing the S/N ratios of all serotypes for each sample. The S/N ratios were displayed in red when they were greater than 10, in blue when they were between 3 and 10, and in black when they were below 3.

## RESULTS

**Characterization of MAB-based multibead serotyping assays (reaction A).** Although we have previously described a multiplexed immunoassay for 26 vaccine-related serotypes using MABs (30), it had a low degree of multiplexing and required several separate assays. We have now overcome these limitations by developing a 26-fold multiplexed assay with a new set of 26 different MABs (Table 1). While 16 MABs were monospecific, 10 MABs were multispecific: they reacted with related serotypes (Table 1). The multispecificity of cross-reactive MABs would not cause difficulties in serotype surveillance, however, since the cross-reactive serotypes, such as 7A or 9A, are rare and epidemiologically insignificant.

In the assay configuration used here, the multispecific antibodies were fully cross-reactive, as illustrated with a 9A/9V-specific antibody (Fig. 1B), except for MABs Hyp11AM1 and Hyp15BG5. Hyp11AM1 preferentially bound to serotype 11E

TABLE 1. Specificity and sensitivity of the MAb-based serotyping assay

MAb no.	MAb designation	Serotype specificity <sup>a</sup>	IC <sub>50</sub> (ng/ml) <sup>b</sup>
1	Hyp1G4	1	8
2	Hyp2M2	2	10
3	Hyp3M6	3	6
4	Hyp4M3	4	6
5	Hyp5M3	5	12
6	Hyp6AM3	6A	3
7	Hyp6BM1	6B	77
8	Hyp6DM5	6C/6D	14
9	Hyp7FM15	7F/7A	52
10	Hyp8M2	8	10
11	Hyp9NM3	9N	8
12	Hyp9VM7	9V/9A	1
13	Hyp10AG3	10A/39	58
14	Hyp11AM2	11A/11D/11F	13
15	Hyp11AM1	11E/(11A/11D/11F)	15
16	Hyp12FM3	12F	26
17	Hyp14M11	14	5
18	Hyp15BG5	15B/(15C)	68
19	Hyp17FM6	17F/17A	6
20	Hyp18CM1	18C	5
21	Hyp19AG1	19A	5
22	Hyp19FM3	19F	5
23	Hyp20G5	20	29
24	Hyp22FM2	22F/22A	4
25	Hyp23FG3	23F	16
26	Hyp33FG1	33F/33A	18

<sup>a</sup> Serotypes in parentheses are those with partial cross-reaction. Hyp10AG3 does not cross-react with 10B, unlike Danish factor serum 10d (12).

<sup>b</sup> The PS concentration required to inhibit 50% of MAb binding to the enzyme-linked immunosorbent assay plate.

and reacted weakly with serotypes 11A, 11D, and 11F. Since Hyp11AM1 is used to identify 11E isolates only, and another MAb (Hyp11AM2) is used to identify serotypes 11A, 11D, and 11F, the unequal binding pattern of Hyp11AM1 caused no difficulties. In the case of serotypes 15B and 15C, Hyp15BG5 reacted with 15C less efficiently, but the unequal cross-reactivity is not relevant, since serotypes 15B and 15C interconvert *in vitro* (26, 27).

The specificity of the tests included in this MAb-based multiplex assay, which is named reaction A, was then examined with a panel of pneumococcal isolates representing all 93 known pneumococcal serotypes, as well as both groups of NT pneumococci and some nonpneumococcal streptococcal species (Fig. 1A and B). Nonpneumococcal isolates produced normalized (fluorescence) signals of >67%. All pneumococcal isolates produced normalized signals less than 33% for the tests of their own or cross-reactive serotypes but more than 67% for other, unrelated serotypes. Figure 1 shows the normalized signals obtained by two tests (serotypes 14 and 9V) with all the isolates in the test panel, and Table 1 shows the summary of the specificity for all the tests. A normalized signal of 33% was used as the cutoff for unknown samples. The sensitivities of the tests included in reaction A were determined using purified capsular PS. Although the sensitivities differed more than 100-fold among the tests, all the tests in reaction A showed significant inhibition (i.e., <50% of the normalized signal) at 80 ng/ml of capsular PS (Fig. 2; Table 1); the concentrations of PS inhibiting 50% of MAb binding (50% inhibitory concentrations [IC<sub>50</sub>]) are shown in Table 1. These

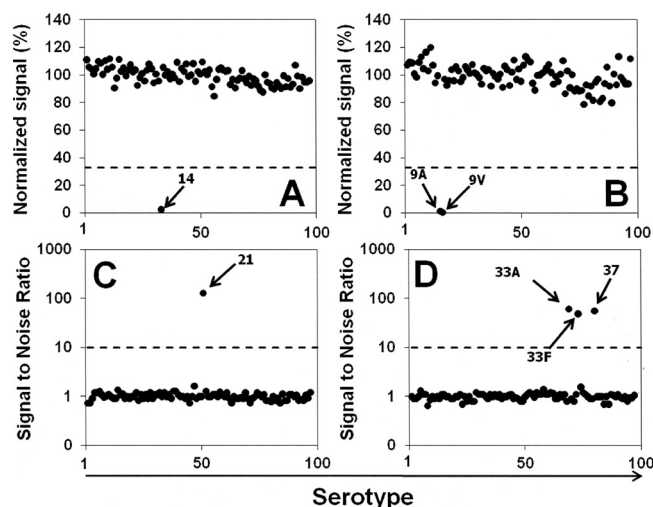


FIG. 1. (A and B) Normalized fluorescence signals of the beads coated with serotype 14 PS (A) or serotype 9V PS (B) obtained with the panel of test samples (x axis) by use of the multiplexed inhibition type immunoassay with MAbs. The numbers on the X axes represent the numbers assigned to the isolates in the test panel that includes all pneumococcal serotypes and NT isolates. (C and D) Signal-to-noise ratios of the beads coated with probes for serotype 21 (C) and serotype 33F (D) obtained with the panel of test samples by use of the PCR-based multiplexed assay. The panel includes all 93 pneumococcal serotypes that have been identified so far and 4 isolates of NT pneumococci representing both group I and group II. Dashed lines indicate cutoffs and separate the positively reacting serotypes from the nonreacting serotypes.

sensitivities were sufficient to identify serotypes even when a pneumococcal lysate was diluted 100-fold (data not shown).

**Characterization of the PCR-based multibead serotyping assays (reactions B and C).** Although multiplexed PCR methods for typing pneumococci are reported in the literature (15, 20, 33), generally, cumbersome methods are used to identify PCR products (15, 20), and the degree of multiplexing is low (20). We used high degrees (21- and 22-fold) of multiplexing

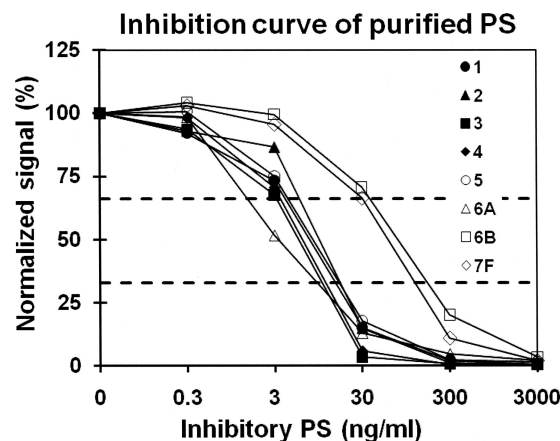


FIG. 2. Normalized fluorescence signals of the PS-coated latex particles in the presence of varying concentrations of homologous free inhibitory PS in solution. The serotypes of inhibitory PS are given in the key. Two horizontal dashed lines indicate 33% and 67% of normalized signals, the values that were used as cutoffs.

and simplified the identification of PCR products by using Luminex technology. Two PCR-based assays, designated reactions B and C, include tests for the serotypes that were not covered by reaction A, except for tests for 6C/6D and 15B/15C. There are tests for 6C/6D in both reactions A and B, since the 6C/6D test was developed for reaction B before a suitable MAb was developed for reaction A. The test for 15B/15C was included in order to independently monitor the performance of the 15B-specific MAb, which is unequally cross-reactive for 15C.

In addition to serotype-specific tests, tests for *lytA* and *cpsA* were added to reactions B and C to confirm the species identification as *S. pneumoniae* (Tables 2 and 3). Two *cpsA* tests were included, because the first *cpsA* test does not detect the *cpsA* of serotypes 25A, 25F, and 38 (20), but the second *cpsA* test does. Reaction C has tests for genes such as *aliC*, *aliD*, and *pspK* to classify group II NT pneumococci, which lack *cpsA*. In contrast, group I NT pneumococci are *cpsA*<sup>+</sup> (10). Even with these additional and overlapping tests, all 43 target genes could be detected easily and quickly with only two PCRs and the Luminex detection assays.

The specificities of reactions B and C were examined with a panel of pneumococci, including all 93 pneumococcal serotypes and both groups of NT pneumococci (Fig. 1C and D). All pneumococcal isolates produced S/N ratios greater than 30 for the tests of their own serotypes but less than 4 for other serotypes. Nonpneumococcal isolates produced S/N ratios less than 2. Figure 1 shows the results obtained by a monospecific test (serotype 21) and a multispecific test (serotypes 33F, 33A, and 37) with the bacterial isolates in the test panel. An S/N ratio of 10 was chosen as the cutoff for unknown samples, and the specificities of all assays are summarized in Tables 2 and 3. The assay is sensitive enough to detect 5 pg of DNA, which corresponds to ~2,000 copies of pneumococcal DNA. In practical terms, most of the lysates could be diluted an additional 50-fold, and therefore, the assay has sufficient sensitivity for practical uses.

**Analysis of archived clinical isolates with MAb and PCR tests.** Although all the tests in our assay system were tested with a test panel, including all known serotypes, reaction A was reevaluated with a panel of clinical isolates that included three 6D isolates, four serotype 2 isolates, and five isolates each of serotypes 1, 3, 4, 5, 6A, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 11E, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. All clinical isolates were correctly identified (see Table S1 in the supplemental material).

Testing reactions B and C with clinical isolates was difficult, because the serotypes detected by the two reactions are not common. Nevertheless, we tested our assay with 53 clinical isolates expressing the serotypes relevant to reactions B and C. Concordant results were obtained with all isolates (Table 4). Three samples that were originally identified as NT were *cpsA*<sup>+</sup> and lacked *pspK*, *aliC*, and *aliD*. These 3 isolates, therefore, belong to NT group I, with defective *cps* loci (10).

**Testing of mixtures of two different serotypes.** Although our assay was designed for individual pneumococcal isolates, it is inherently capable of detecting multiple serotypes in one sample. To investigate this capability, we prepared three pneumococcal lysates spiked with a secondary serotype at a 1% or 10% level. One was a type 3 lysate spiked with serotype 6B; another

was a type 5 lysate spiked with type 12F; and the third was type 19F mixed with 33F. The serotypes used for spiking were chosen because the test was least sensitive of all serotypes (serotype 6B) or the lysates had a low titer, and therefore, these spiked samples provided stringent testing conditions. When the samples were tested with reaction A, the primary serotype was identified without difficulty in all samples. The secondary serotypes could be identified easily (<33%) in all 3 samples containing 10% (Fig. 3), but the samples containing the secondary serotype at 1% produced normalized signals that were substantially reduced (<67%) but not below 33%. Although additional studies are needed, reaction A should identify all secondary serotypes present at a 10% abundance level but may not identify some secondary serotypes at a 1% abundance level. Analogous studies of reactions B and C suggest that they should detect the secondary serotypes present at a 0.1 to 1% abundance.

**Portability of the assay system.** We found that the six key reagents (1 MAb pool, 2 primer pools, and 3 bead mixtures) have long shelf lives. To investigate whether our system is simple and robust enough to be transferred to another laboratory with minimal experience in serotyping, we sent the six key reagents, our assay protocol, and 75 test samples to K. H. Kim's laboratory in Seoul, South Korea, which has equivalent equipment (Bio-Plex). The test samples were blinded and included 72 samples representing all 69 serotyping reactions performed by the assay system. Seven samples were mixtures of two serotypes. The laboratory was able to set up all three reactions with ease and correctly identified all of the samples.

## DISCUSSION

Since currently available pneumococcal vaccines provide serotype-specific protection and alter the distribution of pneumococcal serotypes, continued surveillance of serotypes of pneumococcal isolates is critical in monitoring pneumococcal vaccine efficacy and in improving vaccine formulations. However, the classical typing method used by many laboratories is labor-intensive, and therefore, serotyping surveillance is often limited to a small number of isolates and a few commonly found serotypes. By modifying and combining various approaches developed by us and others (8, 15, 20, 30), we have now developed a simple yet comprehensive serotyping assay system that can test pneumococcal isolates for all the known serotypes with only three multiplexed assays performing 69 different tests with only 6 key reagents (1 MAb pool, 2 primer pools, and 3 bead mixtures). The assay system is rapid and largely automated due to the use of flow cytometric bead array technology. Although the assay uses microtiter plates to accommodate many isolates, it can be used with individual tubes (e.g., Eppendorf tubes) for handling a few samples. In addition, the assay system is robust enough to be transferred to a distant laboratory with a suitable instrument but little experience in serotyping pneumococci. In summary, our assay system has overcome current limitations in serotyping pneumococci and should vastly simplify pneumococcal serotype surveillance.

Our assay system differs from other assays in several ways. First, although there are automated assay systems similar to ours using Luminex technology, these systems, unlike ours, can detect only a limited number of common serotypes (e.g., 40

TABLE 2. Sequences of primers and probes for reaction B

Specificity	Oligonucleotide code <sup>a</sup>	Sequence (5'-3') <sup>b</sup>	Source or reference
<i>lytA</i>	<b>5322</b>	<b>CAA CCG TAC AGA ATG AAG CGG</b>	33
	1006	GTC TTT CCG CCA GTG ATA AT	
	3322	TTA TTC GTG CAA TAC TCG TGC G	
<i>cpsA</i> (except 25A, 25F, 38)	<b>5202</b>	<b>GCA GTA CAG CAG TTT GTT GGA CTG ACC</b>	20 This study
	1039	GG TGC TGT CAC ACT CGT CAG	
	3202	GAA TAT TTT CAT TAT CAG TCC CAG TC	
7B, 7C, 40	<b>5317</b>	<b>AAA ACT CAA GTA TCT GTG C(T)CA CCT T</b>	33
	1001	TCC AAA TTT SAG TAA ACC AAC CTA A	
	3317	CAT CTC TAT TCG ACC TTG CGT TA	
21	5352	CAA TTC TAC TGA GTC CAT ATT ATG AAA	33
	1017	GAT AGT TTC TCT GTA TCA AAT AGC GA	
	<b>3352</b>	<b>ACC ATC GTA CCT GCA CCA TAA</b>	
33F, 33A, 37	<b>5351</b>	<b>TCA ACT AGT CAA GGA TTT GAT GG</b>	14
	1016	TGA TAC CAC AAG TAA CAG AGT CG	
	3351	CGT ATC AGA TTT GCG ATT TC	
15B, 15C	5346	TAA TAA GCG GAT GAT TGT AGC G	14
	1011	GAG CAG GAA TCA GAA CAC AAT C	
	<b>3346</b>	<b>TAT ACT GAT TAA CTT TCC AGA TGG G</b>	
16A	<b>5361</b>	<b>CCG CTC ACG GTA TGG ACT A</b>	33
	1026	GGA GTA AAT GAT GTG TAG TGA AAA CC	
	3361	CCA GCA ATA TAC TCA GGA AAT AAT TC	
16F	<b>5342</b>	<b>TTG TTC TTA CAT TTA GCC GTA GTG</b>	33
	1007	GTT GAA AGA ATA CGA TTC CTA CAA G	
	3342	TCG TCG TTG AAA ACA ATT TCT TAC	
18	5607	AAT TGT TCT TTT CCT GTA CTC AGT C	14
	1039	GGA GGA CTT AGT CAA TTT ATC TTG	
	<b>3607</b>	<b>CGA ACC ATT GAA ACT ATC ATC TG</b>	
23A	5518	TTT ACT TTA ATT TAT AGC TTT TTG GCT AA	33
	1002	TGC CTT TTT TAA CGA GGT TG	
	<b>3318</b>	<b>GGT GCA TGA GTT AGG AGA AAG TG</b>	
23B	<b>5319</b>	<b>GGA TCG TTG TTC ATA GCG G</b>	33
	1003	ATA ATT ACT GGT CTG TGA TTT TTC TTT	
	3319	GAT AAT AAA GAA ATT ACT AAC CAT GTC GT	
25F, 25A, 38	5343	GAC TAC AAA CTG CGG TAG TAG AAA GT	33
	1008	ATA GGA ACT CTA GGG TTT AGT TTT TC	
	<b>3343</b>	<b>TGG AAC AAT TCT AAT CGT TAA TAC G</b>	
10A, 10B	<b>5373</b>	<b>TGA GCT ATT TAA GGA CCT GGG</b>	14
	1024	GTT TAG AAA CCT TGC CCA GG	
	3373	GCA AGC GTC ACT TTC TTG A	
43	<b>5369</b>	<b>AGA TCA AAT GGT GGT ATT AGG AA</b>	33
	1034	TCG GGT GTA CAA ATC CTA AAC TA	
	3369	GGA ATA GAT CAT TAA CCC TAA TGA AT	
36	<b>5368</b>	<b>CAA TTT CCC CTT ATT CTG TAG TTC</b>	33
	1033	AGA TAA ATA CAT CAT TAT TGA CGA ACA	
	3368	TGG AGA TCC CCA AGA GAA AAT A	
48	<b>5372</b>	<b>GCA TTT GGA GTT ATT GCC CTA C</b>	33
	1037	CCT ATA AAC ACA CTC AAA ACT AGC A	
	3372	CGA CGG AAT CAA TAT AAA TAA GTG ATA	
34	<b>5608</b>	<b>TTA AAA GTA TTA TTG GTA GTG ATT CTT TTG</b>	This study
	1040	AA TCC ATT GGT ACT CTT CAC AAA	
	3608	TGT AAA GAC ATT CCC TGT AGG C	
35A, 35C, 42	5367	GGA GAC TA(/G)T TAA AAC TTT TTT CGT TC	33
	1032	CCT ACT TTA TTA ATG CCT GTT TGA G	
	<b>3367</b>	<b>TTA AGT AAG TCT TCG CAA TCC AG</b>	
35F, 47F	<b>5521</b>	<b>ATA AAA AGA AAG TCT TTG CCA GAG</b>	33
	1005	AAA GTC ACA TC(T)T AAA ATT GAC ACA AC	
	3321	CAA CTT TTG GAA GAT ACT GAA CAT AA	
35B	<b>5345</b>	<b>CTA ATT TGG CTA TGA AGC TAA TCC C</b>	33
	1010	AAG CGT GAA AAA TTT TAA TAA AAG AC	
	3345	TAA CTT AAA TAG GCA TTA ACA AAA TAG GT	
6C, 6D	5325	CATTTTAGTGAAGTTGGCGGTGGAGTT	This study
	1042	TTTGCAGGTGTAGTAGGCAGG	
	<b>3645</b>	<b>ACTTTACACGACAAACCCATAGAAGC</b>	

<sup>a</sup> Oligonucleotide codes beginning with 1, 3, or 5 designate probes, reverse primers, or forward primers, respectively.

<sup>b</sup> One primer in each primer pair (in boldface) has a biotin label at the 5' end for detection with avidin. All the probes have an amine group at the 5' end. Stock primer solutions have 100 μM oligonucleotides in water.

TABLE 3. Sequences of primers and probes for reaction C

Specificity	Oligonucleotide code <sup>a</sup>	Sequence (5'-3') <sup>b</sup>	Source or reference
<i>cpsA</i> for 25A, 25F, 38	5694	GGGAACGACTATCCTGTGGAAATC	This study
	1046	GTCAGCACTTGGTTTCGTTTCGAG	
9N, 9L	<b>3694</b>	<b>CTTCTGTGATCCGTCCTCGATC</b>	14
	<b>5358</b>	<b>TCA ATG GCG ACT TTA TTT GC</b>	
28F, 28A	1023	GAA CTT TGG GAA TAT AAT CAA AAG	33
	3358	AGT CTA TTA TCT CCT GTA GGG TGC	
10F, 10C	<b>5354</b>	<b>CAG AGT TTG GTC GAG GTT CCT A</b>	33
	1019	AGA ACT AAA TAC AGT GCA ATA ATT GG	
11B, 11C	3354	GCT CAA CTT TAT TTC TCT AGA ATA AAC A	33
	<b>5355</b>	<b>TAG TTT TGG TTA CGT AGT TGT TGA CT</b>	
13	1020	GAA AAC TTG CCC AAA TCC TT	33
	3355	GCA ATA(/G) AAT ACT GTA GCA TAC GAT AGT T	
24F, 24A, 24B	<b>5356</b>	<b>TCT GGT GCT AAG GGG ATC AA</b>	33
	1021	TGC ATA AGC TGA TTA TGA GCA TAG	
12, 44, 46	3356	CCA ATT ACT CCA TTA TCT ATT GCT AAT	33
	<b>5348</b>	<b>GAT GGG AAA ATA CGA TAT GCT C</b>	
19B, 19C	1013	TGA GCT AAA TGT TGA ATA TTT ATA CCC	33
	3348	GAA AAT CGT AAC ATG GAA AAA GTA A	
27	<b>5363</b>	<b>TCA ACA CTT ATG ATG G(A)TG CCT G</b>	33
	1028	CAC AAT CCA AAA CTT AAG TTG TTT C	
32F, 32A	3363	GCA GAA ACA AAA(/G) GTA AGA ATT ATA GAT ATC	14
	<b>5374</b>	<b>TGA ATA TGG ACG GTG GAG</b>	
33B, 33D	1025	GAA GAA GTT CAA CAA TCG CT	33
	3374	AGC AAA GAA AGC CGA AAG	
41F, 41A	<b>5362</b>	<b>AGA ATT CGG AGA TTT GTG GTA T</b>	33
	1027	TTC GTA CTG AAA ATT CAT TTC G	
47A	3362	CAA TCC ACC TCC ATA AAC GA	33
	<b>5364</b>	<b>GCA GCC ACC TCT TCT CAT TC</b>	
45	1029	CGC CAA ATT CTA TAC CAA CTA GTA T	33
	3364	GGA AGG AAC AAC CCA ACA AT	
31	<b>5365</b>	<b>GGT ATG CTT ACA ATG AGA CGC</b>	33
	1030	CCA CTT CCC AGA GGA AAA TA	
33C	3365	AAT TCG TTC CCG GAT AAG ATG	33
	<b>5347</b>	<b>TAT TTC CTT CCT ATG GGA CAA C</b>	
NCC2	1012	AGT CCT TTC CCA AAT ATA GCA CT	33
	3347	GCA AGC ATT TTA CCA AGT TCA TAA A	
NCC2 and NCC3	<b>5366</b>	<b>TCG TTG GAT GAC AAA ACT CTT AC</b>	33
	1031	GCT CAA TGT GAC AGG GAG AA	
47A	3366	CCT CCC TGA GCC AAA ATA AC	33
	<b>5344</b>	<b>TGA AAA TCC CTT AGT GAC ATC TG</b>	
NCC1	1009	GAG CCT TCT CAA TAG TCA TAA AAA A	33
	3344	GCC ATA ATC AAA AAT AAG TTA GAC ATA A	
47A	5357	TGA CAC TAT TTA TAA TTG CTT TAT CCT T	33
	1022	GGG TGC AAG GTG ATT ATG TAT	
33C	<b>3357</b>	<b>TAG CGA GAA ACT ATC TGC ATC TTG</b>	33
	<b>5353</b>	<b>CCG AAA ATT GTT CAC AGG ATA C</b>	
47A	1018	TAA CAA GCA GAA TAA GCA AAA TAG C	33
	3353	AGC TTT CTT TTG TAC GAC TCT TTT A	
NCC2	<b>5370</b>	<b>TAT GCA GGA AAT ATC CGA GAA GG</b>	33
	1035	CAG CAT ATC TTG CAC GAT AAT GAA	
NCC2	3370	CCG TGA AAC AGA AAC GCT ATG	33
	<b>5371</b>	<b>TAT TTG CCA TAA CGG ACT CTA GAA C</b>	
NCC2	1036	TTT TTA ACA ACC TTG TAT AGA ATA CCT C	33
	3371	GCT AAA ATA ATA AAT AGC GAA CTT ACT ACA	
NCC2	<b>5375</b>	<b>CGA ATC GTC ATA AGG CAA AA</b>	33
	1038	ACC TAC TGT GAC AGG GAA TAG TAA A	
NCC2	3375	AAT AGG AGT AAC AAA GAG AAG CCT AA	This study
	5433	AACACTTGAACGGAGAATG	
NCC2 and NCC3	1044	GACCAGATTACCAAGATCCAGCAAC	This study
	<b>3433</b>	<b>GCCCTTTGTTATACCTAGATGTTTC</b>	
NCC1	5666	ATGCCAAATGGTTCACGGCTG	This study
	1045	GTGACAGGTATCAAGTACGCAGTG	
NCC1	<b>3666</b>	<b>CTGGTCGTCGATTGCTTTCACAC</b>	This study
	5658	GCAAATCAGCCAGTAACTGTGA	
NCC1	1043	CTGTAGAACAGGCAAAGAGCAAGG	This study
	<b>3658</b>	<b>CAAGATAAGCTTCTGCACCTCT</b>	

<sup>a</sup> Oligonucleotide codes beginning with 1, 3, or 5 designate probes, reverse primers, or forward primers, respectively.

<sup>b</sup> One primer in each primer pair (in boldface) has a biotin label at the 5' end for detection with avidin. All the probes have an amine group at the 5' end. Stock primer solutions have 100 μM oligonucleotides in water.

TABLE 4. Testing of clinical samples with the PCR-based multibead assay

Assay	Serotype	No. of isolates detected	
Quellung	13	3	
	29	1	
	31	3	
	34	4	
	15A	3	
	15C	8	
	16F	3	
	18A	2	
	23A	3	
	23B	2	
	38	3	
	28A/28F	3	
	33A	2	
	35A/35C	2	
	35B	4	
	35F	1	
	7C	3	
	NT <sup>a</sup>	3	
	PCR based	13	3
		29	1
31		3	
34		4	
15A/15F		3	
15B/15C		8	
16F		3	
18A/18B/18C/18F		2	
23A		3	
23B		2	
25F/25A/38		3	
28A/28F		3	
33F/33A/37		2	
35A/35C/42		2	
35B		4	
35F/47F		1	
7B/7C/40		3	
NT ( <i>cpsA</i> <sup>+</sup> )		3	

<sup>a</sup> NT, nontypeable.

serotypes). Second, our system is highly flexible due to the seamless integration of immunoassays and DNA assays that share the sample types and the assay platform. Consequently, our system can be readily expanded by adding either an immunoassay or DNA assays, which have complementary strengths and weaknesses (8, 18). This expandability allowed us to add several additional tests for identifying *S. pneumoniae* species in reactions B and C. Finally, our assay system is easy to standardize because it uses well-defined and extensively characterized reagents, such as oligonucleotides and MAbs.

Despite its strengths, our assay system does have some limitations. Our system does not distinguish some serotypes (e.g., 9A and 9V; serotypes 33A, 33F, and 37), and resolution of such serotyping ambiguities would require conventional manual methods based on polyclonal factor sera or specific DNA tests. Although we anticipate that these serotyping ambiguities should not cause practical difficulties in serotype surveillance, additional tests may be incorporated into our assay system, if necessary. Another limitation may be that our system has been developed for testing individual pneumococcal isolates. This restriction may be undesirable in studies of nasopharyngeal carriage of pneumococci, because the nasopharynx frequently

harbors multiple serotypes (2), and identifying all the serotypes in the nasopharynx would require testing many isolates from a single swab. We imposed this restriction because pneumococcal and nonpneumococcal *cps* genes can be very similar to each other (29), and nonpneumococcal organisms may be the sources of the targets detected by any DNA detection system. Such false-positive detection may also occur with MAb-based immunoassays. However, our assay system is intrinsically capable of detecting multiple serotypes in a sample, and the genetic similarity may not cause practical problems. Consequently, the restriction of using isolates should be reevaluated in the future by studying nasopharyngeal swabs or their broth enrichment cultures (7), along with pneumococcal isolates from the swabs.

The vaccine-related serotypes that were included in reaction A could have been tested by PCR, as we have done for other serotypes. Nevertheless, the use of MAbs for reaction A provides several advantages. First, serotype surveillance often requires testing of pneumococcal isolates only for the vaccine-related serotypes, and the MAb-based reaction A would satisfy this need simply, without PCR. Also, the immunoassay provides more-reliable serotyping information, since it tests the capsule structure directly, unlike DNA tests, which provide indirect information. This was illustrated with a 19F strain that was genetically more similar to 19A (22) and with serotype 11A isolates that could not be genetically distinguished from serotype 11D (6). Also, the ability of the immunoassay to quantitatively measure capsular PS in samples can be valuable. For instance, with the immunoassay, pleural fluids were found to contain enough capsular PS to neutralize the anti-PS antibodies present in the host (31). Reaction A should be useful in vaccine production as well, since it can be used to quantify capsular PS during its purification and conjugation.

Because of difficulties in typing pneumococci, our knowledge of pneumococcal serotype prevalence is limited to select geographical areas and is deficient in a large part of the world. The areas with limited studies may have different serotype distributions or may harbor unusual serotypes. Because our assay can identify all serotypes and can be placed in laboratories with little experience in serotyping, the system should facilitate surveys of pneumococcal serotypes in different parts of the world. Since our assay can serotype pneumococcal isolates completely, including different clades of group II NT

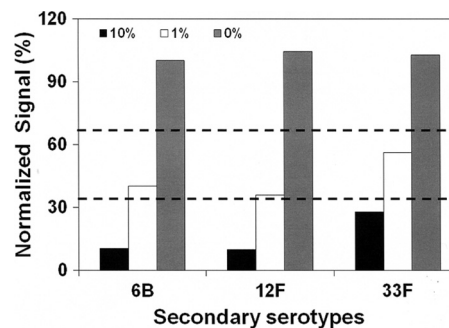


FIG. 3. Normalized signals for the secondary serotypes in three samples. The abundances of the secondary serotypes are 10%, 1%, and 0%. Two dashed horizontal lines at 33% and 67% are used for decision making.

pneumococci, we may find new capsule types. Perhaps our assay system may help us to discover new serotypes and new clades among NT pneumococci as well as to accomplish complete surveillance in different parts of the world.

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