A rapid pneumococcal serotyping system based on monoclonal antibodies and PCR

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Streptococcus pneumoniae expresses at least 91 different polysaccharide (PS) capsules and the currently available serotyping methods are tedious to perform. We have been developing a rapid pneumococcal serotyping assay (named the 'multibead assay') based on the capacity of pneumococcal lysates to inhibit the ability of 24 different anti-capsule antibodies to bind to latex beads coated with 24 different PSs (serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9N, 9V, 14, 18C, 19A, 19F, 23F, 2, 8, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F). Because the polyclonal antibodies used for 10 serotypes (2, 8, 10A, 11A, 12F, 15B, 17F, 20, 22F and 33F) had limited serotype specificity, we replaced them with monoclonal antibodies for the 10 serotypes. To extend the serotype coverage beyond the 24 serotypes, we have adapted multiplexed PCR for five additional serotypes (15A, 15C, 16F, 35B and 38) to be useful with the pneumococcal lysates prepared for the multibead assay. We then validated the combined assay with 157 clinical isolates from the Centers for Disease Control and Prevention and found that the new combined assay produced results that are concordant with the quellung reaction. The combined assay is robust and could be used to rapidly identify the serotypes of the majority of pneumococci (~90%). In addition, the assay validation study suggests the presence of serological subtypes within serotype 11A.

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INTRODUCTION

Because *Streptococcus pneumoniae* is a significant human pathogen, sustained efforts are under way to improve currently available pneumococcal vaccines (Wuorimaa & Kayhty, 2002). Since all the currently available pneumococcal vaccines provide protection in a serotype-specific manner, an effective vaccine is predicted to reduce the prevalence of pneumococci expressing the serotypes included in the vaccine without reducing the prevalence of the non-vaccine serotypes (Whitney *et al.*, 2003). Therefore, the efficacy of a vaccine given to a population can be monitored by serotyping pneumococcal isolates from the vaccinated population. Thus an efficient method for serotyping pneumococcal isolates is greatly needed.

Classically, pneumococci are serotyped by the quellung reaction (Henrichsen, 1995; Lund, 1960). Since the quellung reaction is very labour-intensive and requires a complex array of expensive typing sera, new typing systems

have recently been developed to meet the large serotyping needs of vaccine studies. Several groups developed DNA-based typing systems following the determination of the DNA sequence of the capsular biosynthetic gene (*cps*) loci from strains representing 90 pneumococcal serotypes (Batt *et al.*, 2005; Brito *et al.*, 2003; Kong *et al.*, 2005; Lawrence *et al.*, 2003; O'Halloran & Cafferkey, 2005; Rubin & Rizvi, 2004). Using extracted DNA, Pai *et al.* (2006) developed a multiplex PCR for 29 of the pneumococcal serotypes. Other groups developed improved serological assays such as latex agglutination assays (Slotved *et al.*, 2004) and dot-blot assays using intact pneumococci (Fenoll *et al.*, 1997). We developed a multiplex immunoassay for capsular polysaccharides (PSs) using pneumococcal lysates and described an assay employing monoclonal antibodies (mAbs) for 14 common serotypes and polyclonal antibodies for 10 additional serotypes (Yu *et al.*, 2005).

Experience with our multiplex immunoassay (named the 'multibead assay') showed that PS-coated latex particles are unstable and polyclonal antibodies have limitations in serotype specificity. To extend the usefulness of our multibead assay, we have covalently coupled PS to latex particles and have now developed mAbs to 10 additional serotypes and have characterized them. Additional serotype

Abbreviations: CDC, Centers for Disease Control and Prevention; DMTMM, 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PLL, poly-L-lysine; PS, polysaccharide; RT, room temperature; UAB, University of Alabama at Birmingham.

coverage is important for more detailed coverage, and is potentially of critical importance for serotype surveillance in developing countries. Compared with developing a mAb, developing a PCR for a new serotype is simple. We therefore investigated the new mAbs for the multibead assay method and also investigated combining a PCR method with the multibead method to identify additional serotypes.

METHODS

Bacterial isolates. To determine the assay specificity, we used 147 pneumococcal isolates kept frozen in our laboratory. The laboratory panel includes 90 isolates purchased from the Staten Serum Institut (Copenhagen, Denmark) representing all 90 known pneumococcal serotypes (Henrichsen, 1995). Also, our laboratory's strain collection includes isolates expressing the 6C serotype, a new serotype that we recently discovered (Park *et al.*, 2007). For clinical validation, a panel of 157 frozen clinical isolates of pneumococci was obtained from the Centers for Disease Control and Prevention (CDC). The CDC isolates were selected to primarily include the serotypes detected by the new mAbs (Tables 1 and 2).

Bacteria were recovered from the frozen isolates by plating scrapings of frozen samples on blood agar plates. After overnight incubation at 37 °C in a candle jar, microtubes containing 500 µl Todd-Hewitt broth with yeast extract (0.5 %) and 0.001 % bromocresol purple per tube were inoculated with a colony of bacteria. After overnight culture at 37 °C in a candle jar, 38 µl lysing solution (0.2 % sodium deoxycholate, 0.02 % sodium dodecyl sulfate, 0.1 % sodium azide, 0.3 M sodium citrate; pH 7.8) was added to each microtube. The microtubes were then incubated at 37 °C for about 1 h to induce lysis of the pneumococci. The resulting bacterial lysates were stored at -20 °C until they were analysed.

PCR. To identify capsule genes by PCR, bacterial lysates were diluted 10–100-fold in water and then subjected to PCR using primers and previously described reaction conditions (Pai *et al.*, 2006). The capsule-specific PCR also included primers for part of the *cpsA* gene, to serve as a positive control for the presence of pneumococcal capsule genes from most pneumococcal strains (Bentley *et al.*, 2006). The reaction products were separated by agarose electrophoresis, stained with ethidium bromide, and visualized under UV light.

Production of mAbs. mAbs were produced as previously described (Yu *et al.*, 2005). Briefly, mice were immunized with a PS conjugated to a protein, and splenocytes were harvested from immune mice for hybridization with fusion partner cells (SP2/0-Ag14). Primary wells containing antibodies to capsular PS were identified using ELISA (Yu *et al.*, 2005). Hybridoma cell lines were established by subcloning the cells in the primary wells twice by limiting dilution. The hybridoma cell lines are listed in Table 1.

Inhibition ELISA for 11A subtypes. While most isolates typed as 11A by the quellung reaction were reactive with the Hyp11AM2 mAb, some were not (Table 2). To distinguish between the two subtypes, we named the isolates typed as 11A by both methods as 11A α and those typed by the quellung reaction alone as 11A β . To investigate this serological phenomenon, we established 11A α - or 11A β -specific inhibition ELISAs, as previously described (Park *et al.*, 2007). For the 11A α inhibition ELISA, microwells of ELISA plates were coated with 11A α PS, which was purchased from the ATCC (Manassas, VA, USA) as 11A PS. To each microwell, variably diluted bacterial lysates and Hyp11AM2 mAb were added. After incubation, unbound mAb was removed, and bound mAb was determined using alkaline

Table 1. Twenty-six mAbs used for the multibead assay and their analytical specificities

Specificity is defined in the context of an inhibition-type assay.

mAb	Specificity*
Old	
Hyp6AG1	6A/6C
Hyp19AM1	19A
Hyp19FM3	19F
Hyp3M6	3
Hyp5M3	5
Hyp1G4	1
Hyp6BM1	6B
Hyp7FM1	7F/7A
Hyp9NM3	9N
Hyp9VG13	9V
Hyp14M11	14
Hyp18CM1	18C
Hyp4M3	4
Hyp23FG3	23F
Hyp6AM3	6A
New	
Hyp10AM6	10A/39
Hyp20G6	20
Hyp11AM2	11A α /11D/11F
Hyp8M2	8
Hyp2M1	2
Hyp22FM2	22F/22A
Hyp12FG3	12F/12B
Hyp15BG5	15B/(15C)
Hyp17FM6	17F/17A
Hyp33FG1	33F/33A
Hyp11AM1	11A β

*When mAbs do not cross-react fully, the serotype is listed in parentheses.

phosphatase-conjugated anti-mouse immunoglobulin antibody and nitrophenyl phosphate substrate. For the 11A β inhibition ELISA, another 11A-specific mAb, Hyp11AM1, was used and the ELISA microwells were coated with 11A β PS, which was prepared as described below.

Purification of 11A β PS. 11A β PS was purified from a 2 l culture of pneumococcal strain CDC3455-06, as previously described (Park *et al.*, 2007). Briefly, the PS in the bacterial culture supernatant was precipitated with 70 % ethanol and then purified over an ion-exchange column (DEAE-Sepharose) and a molecular mass sizing column (Sephacryl S-300). The fractions containing PS were identified by the anthrone reaction (Ashwell, 1957) and inhibition ELISA for 11A β PS.

Conjugation of PS to latex beads. Two sets of latex beads conjugated with PS were prepared using PSs purchased from the ATCC, except for the 6A and 11A β PS. 6A PS was obtained from G. Schiffman (Brooklyn, NY, USA) and 11A β PS was purified as described above. The latex bead sets contained latex beads of different sizes and with different amounts of red fluorescence, which permits one to identify each bead type by flow cytometry. The latex beads

Table 2. Validation with strains from the CDC

Quellung assay		Multibead assay	
Serotype	Number	Concordant results	Discordant results and explanations
8	12	12	0
20	12	12	0
10A	12	12	0
12F	12	12	0
15A*	3	3	0
15B	8	16	0
15C	8		
16F*	3	3	0
17F	12	12	0
22F	12	12	0
11A	12	9	Three strains (3151-06, 3455-06, 3954-06) were NT at UAB with Hyp11AM2†
33A	2	14	0
33F	12		
35B*	3	3	0
38*	3	3	0
NT at UAB‡	28	28	
4	1	1	
NT by CDC†	1	0	A strain (3069-06) showed a weak signal for serotype 8
Total	156	152	4

*Serotyped by multiplex PCR.

†NT, Non-typable. NT at UAB means that the isolate does not express one of the following 37 serotypes: 1, 2, 3, 4, 5, 6A, 6B, 7A/7F, 8, 9L/9N, 9V, 10A/39, 11A/11D/11F, 12B/12F, 14, 15A, 15B/(15C), 16F, 17F, 18C, 19A, 19F, 20, 22A/22F, 23F, 33A/33F, 35B and 38. NT by CDC indicates that the isolates do not express one of the 91 known serotypes.

‡Serotypes included in this category are: three isolates of serotype 13, one isolate of serotype 29, three isolates of serotype 31, three isolates of serotype 34, three isolates of serotype 7C, two isolates of serotype 18A, three isolates of serotype 23A, three isolates of serotype 23B, one isolate of serotype 28A, one isolate of serotype 28F, one isolate of serotype 35A, one isolate of serotype 35C and three isolates of serotype 35F.

included 10 different types of pre-labelled beads, which are available from Bangs Laboratory as the 'QuantumPlex' latex bead set. Others were unstained latex beads with carboxy or amino groups and were also purchased from Bangs Laboratory. In some cases, the unstained beads were stained with 'DiD oil' (an indodicarbocyanine dye from Molecular Probes), as we have previously described (Park *et al.*, 2000).

The PSs were conjugated to the beads using one of the three methods described below. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was used to couple PSs of serotypes 6A, 10A, 19A, 19F and 20 to the beads with amino groups, as previously described (Zielen *et al.*, 1996). Four hundred microlitres of water containing 1% bead suspension, 10 µg PS ml⁻¹ and 10 mg EDC ml⁻¹ was incubated overnight at 37 °C with shaking. To the reaction mixture was added 100 µl water containing glycine (250 mM) and glucose (250 mM). After 30 min at room temperature (RT), the beads were washed with PBS containing 0.1% Tween 20, suspended to 10⁷ beads ml⁻¹ in storage buffer (PBS, 0.01% Tween 20, 1% BSA, 0.02% azide and 0.1% glucose), and kept at 4 °C.

The PSs of serotypes 7F and 22F were conjugated using poly-L-lysine (PLL), as previously described (Pickering *et al.*, 2002). These PSs were conjugated to PLL using cyanuric chloride, and the resulting PS-PLL conjugate was purified over a Sephadex G-25 column. The PS-PLL conjugate was then conjugated to latex beads with carboxy groups using a two-step carbodiimide reaction. Carboxylated microspheres (5 × 10⁷) were mixed with 5 mg EDC ml⁻¹ and 5 mg Sulfo-NHS ml⁻¹. After 20 min, the beads were washed and mixed with 500 µl

PS-PLL. After 1 h at RT with shaking, the reaction mixture was mixed with 125 µl water containing glycine (250 mM) and glucose (250 mM). After 30 min at RT, the beads were washed with PBS containing 0.1% Tween 20, suspended to 10⁷ beads ml⁻¹ in the storage buffer, and kept at 4 °C.

The PSs of the remaining serotypes were conjugated using 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM), as previously described (Schlottmann *et al.*, 2006). Two and seven-tenths millilitres of water containing PS (1 mg ml⁻¹) and DMTMM (15 mg ml⁻¹) was kept at RT for 1 h with shaking. Activated PS was separated from free DMTMM by desalting chromatography, and the fractions containing the activated PS were identified by the anthrone reaction. Two millilitres of activated PS was mixed with 5 × 10⁷ beads with carboxy groups and was incubated overnight at RT with mixing. To the reaction mixture was added 500 µl water containing glycine (250 mM) and glucose (250 mM). After 30 min at RT, the beads were washed with PBS containing 0.1% Tween 20, suspended to 10⁷ beads ml⁻¹ in the storage buffer, and kept at 4 °C.

Multibead assay. The assay was performed as described previously using the two sets of latex bead mixture (Park *et al.*, 2000; Yu *et al.*, 2005). Set 1 contained beads coated with PSs of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9N, 9V, 11Aβ, 14, 18C, 19A, 19F and 23F. Set 2 contained beads coated with PSs of serotypes 2, 6A, 8, 10A, 11Aα, 12F, 15B, 17F, 20, 22F and 33F. Each set of bead mixture was incubated with a bacterial lysate and an appropriate mixture of mAbs. The bacterial lysate was diluted either 10-fold or 50-fold. After incubation, the

reaction mixture was washed with PBS containing 0.1% Tween 20 and mixed with fluorescein-conjugated goat anti-mouse immunoglobulin. After 30 min of incubation, the latex beads were washed and analysed for their fluorescence with a flow cytometer (FACSArray; Becton Dickinson).

RESULTS AND DISCUSSION

Development of multibead assays for 10 additional serotypes with 11 mAbs

We have developed mAbs to 10 additional serotypes to replace the polyclonal antibodies that were used for these serotypes in our previous serotyping assays (Lin *et al.*, 2006). Table 1 lists 11 new mAbs, including the two mAbs for the 11A serotype (bottom half), in addition to the old mAbs against the 14 common serotypes (top half) that have been used previously (Lin *et al.*, 2006). Two mAbs were chosen for the 11A serotype following our discovery of the two subtypes of 11A, which are described below. Hyp11AM2 is paired to bind to the 11A α -PS-coated beads included in bead set 2 (11A PS from the ATCC is labelled as 11A α PS to avoid confusion). Hyp11AM1 is paired with the 11A β -PS-coated latex beads included in bead set 1.

Cross-reaction patterns of the new mAbs suggest the existence of epitopes that have not yet been identified for pneumococcal PSs. The only epitope known to be expressed on serotypes 39 and 10A is epitope '10d' (Henrichsen, 1995). However, '10d' is known to be expressed on 10B, and Hyp10AM6 did not cross-react with 10B. Thus Hyp10AM6 may recognize a new epitope shared between only 10A and 39. In addition to this new epitope, we previously noted that Hyp18CM1 and Hyp23FG3 probably define new epitopes (Yu *et al.*, 2005) since 18C- and 23F-specific epitopes were not described in the past (Henrichsen, 1995). Furthermore, we have recently described a new serotype, 6C, the discovery of which revealed the presence of several new serogroup 6 epitopes (Park *et al.*, 2007).

In previous multibead assays, latex beads had a short shelf-life since PS was passively adsorbed to the beads. To prepare beads with a longer shelf-life, we covalently conjugated PS to the latex beads. Because one single method (the DMTMM method described above) of conjugating PS did not yield acceptable beads for all 25 different PSs, we examined two additional methods of conjugation (the EDC and PLL methods, also described above). Using these methods, we were able to produce PS-conjugated beads that were stable for more than 3 months. In many cases, the beads remained useful for more than 9 months. The sensitivity of the multibead assay was first investigated using purified PSs as inhibitors. Binding of all mAbs could be inhibited by more than 50% with 0.1 μg homologous PS ml^{-1} . This sensitivity was sufficient to allow the use of bacterial lysates diluted 10- and 50-fold for multibead assays.

While we were investigating the conjugation methods, we found that the optimal conjugation method was dependent

on the mAb itself. For instance, the PLL conjugation method yielded higher binding signals than the EDC conjugation method for mAb Hyp9VM5. However, the EDC method was better than the PLL method for mAb Hyp9VG1 (Fig. 1). Analogous differences were observed for mAbs to 22F (Fig. 1). While these findings indicate that specific conjugation methods should be selected for each mAb, they also suggest that one conjugation method could preserve a specific epitope better than another conjugation method. PS-conjugated latex beads are increasingly used for measuring concentrations of pneumococcal antibodies to many different serotypes using a flow-cytometer-like instrument (e.g. Luminex) (Pickering *et al.*, 2002). In view of our findings, before adopting a flow-cytometric bead array method for measuring pneumococcal antibody levels, one should carefully investigate the potential that the epitopes can be altered by the method used to conjugate PS to latex beads (Pickering *et al.*, 2002).

Next, the assay specificity was investigated using a panel of 147 pneumococcal isolates. The panel included 91 pneumococcal isolates representing all 91 currently known serotypes (Park *et al.*, 2007). All mAbs reacted with the homologous serotype, but several mAbs cross-reacted with other serotypes (Table 1). For instance, the mAb Hyp10AM6 was reactive with serotype 10A and was also fully cross-reactive with serotype 39. Hyp7FM1 was fully reactive with serotype 7F as well as with serotype 7A, as we previously reported (Yu *et al.*, 2005). The presence of cross-reactions was unambiguous because reactive samples always inhibited more than 67% and non-reactive samples always inhibited less than 33%. Consequently, the cross-reactions did not hinder serotype determinations.

The only exception to these unambiguous determinations was Hyp15BG5. With this mAb, all 15B isolates in the test panel inhibited more than 67% and could be identified unambiguously, but its cross-reaction with 15C isolates was highly variable. For instance, many 15C isolates inhibited about 40–60%, but some 15C isolates inhibited less than 30%. When Hyp15BG5 was examined with the purified PSs, Hyp15BG5 could be inhibited only by 15B PS and not by 15C PS (data not shown). The contradiction in cross-reactions observed with 15C PS and pneumococcal isolates can be explained since pneumococci expressing 15C can spontaneously convert to the 15B serotype with an estimated frequency of approximately 1 in 250 bacteria (Venkateswaran *et al.*, 1983) due to a genetically unstable acetylation enzyme gene in the *cps* locus (van Selm *et al.*, 2003). Thus identification of 15C isolates with Hyp15BG5 is not based on a serological cross-reaction to 15C PS but is instead based on the spontaneous but variable conversion of 15C isolates to 15B isolates.

Use of bacterial lysates for PCR to identify additional serotypes

The multibead assay is limited by the number of available mAbs. Also, the multibead assay did not reliably identify

Fig. 1. Amount of mAb bound to latex beads (y -axis, in fluorescence units) at various dilutions of the mAb (x -axis). (a) Binding of Hyp22FM1 or (b) Hyp22FM3 to latex beads conjugated to 22F PS using the EDC method (solid diamond), the PLL method (solid square) or the DMTMM method (open triangle). (c) Binding of Hyp9VM5 or (d) Hyp9VG1 to latex beads conjugated to 9V PS using the EDC method (solid diamond), the PLL method (solid square) or the DMTMM method (open triangle). 22F-conjugated beads prepared with the PLL method bind to Hyp22FM1 very well (a) but not to Hyp22FM3 (b). Also, 9V-conjugated beads prepared with the EDC method bind to Hyp9VG1 very well (c) but not to Hyp9VM5 (d). These four mAbs were not chosen for our serotyping assay.

serotype 15C due to variability in the observed cross-reactions. To supplement the multibead assay, we studied the usefulness of the bacterial lysates originally prepared for the multibead assays in a multiplexed PCR. We chose to adopt the PCR method for the five serotypes 15A, 15C, 16F, 35B and 38 because a recent CDC survey showed that these five serotypes are more common among the serotypes not covered by the multibead assay (data not shown). As shown in Fig. 2, the lysates were useful for the multiplexed PCR (Pai *et al.*, 2006) even with 100-fold dilutions. Serotype 15C could be identified with a PCR test for 15B and 15C serotypes (Fig. 2) and its weak reaction with Hyp15BG5.

Also, we could use the lysates for PCRs for different targets, including additional pneumococcal serotype identification sequences (8 and 17F/A), the pneumolysin gene and multilocus sequence typing targets (data not shown) (Enright & Spratt, 1998). In addition, we found that lysates prepared more than a year ago and kept frozen were useful for PCRs (data not shown). Thus the combination of serological and DNA testing makes the new assay system useful for epidemiological studies as well as for many

reference laboratories due to its increased capacity to distinguish many more, if not all 91, serotypes.

Validation of serotypic specificity of the new multibead assay with clinical isolates

To use clinical isolates to validate our multibead assay, we obtained a panel of 157 clinical isolates from the CDC, where they had all been typed by the quellung reaction. The panel was constructed to preferentially include the serotypes that are detected with the new mAbs, and included more than 10 isolates for each serotype in the multibead assay except for serotype 2. The isolates were analysed using the multibead assay. When the results were compared with the results of the CDC's quellung reaction tests, all the results were identical except for four discrepancies (Table 2). Three discrepancies were observed for serotype 11A, and one discrepancy was found for a strain (sample 3069-06) that was 'non-typable' at the CDC (Table 2) but was found to be weakly positive for serotype 8 at the University of Alabama at Birmingham (UAB). The non-typable strain (3069-06) was further investigated by

Fig. 2. Electrophoresis pattern of the products of a multiplexed PCR (for serotypes 15A, 15B, 15C, 16F, 35B and 38) using pneumococcal lysates expressing serotype 16F (lane 2), serotype 35B (lane 3), serotype 38 (lane 4), serotype 15A (lane 5), serotype 15B (lane 6) and serotype 15C (lane 7). These strains were obtained from the Statens Serum Institut. Lane 8 has water as a negative control, and lane 1 shows the molecular ladder. Lysates were diluted 100-fold. Serotype-specific bands were found at the expected locations (988 bp for 16F, 677 bp for 35B, 574 bp for serotype 38, 436 bp for serotype 15A and 496 bp for serotype 15B or 15C). The bands at 160 bp are the PCR product of the *cpsA* gene, which is present in the capsule gene loci of almost all pneumococcal isolates (Bentley *et al.*, 2006) and serves as a positive control for the presence of the pneumococcal capsule gene locus. This band is not detectable in some serotype 38 isolates (unpublished information from M. da G. S. Carvalho) (Bentley *et al.*, 2006).

deriving 20 isolated subcolonies from the parental stock. Fourteen of the subcolonies were non-typable, but six colonies expressed serotype 8.

Pneumococci expressing serotype 8 can spontaneously inactivate their initial transferase gene (*cap8E*) and lose capsule production by duplicating a large portion of the *cap8E* gene (Waite *et al.*, 2003). This mechanism may have been responsible for some of the subcolonies of sample 3069-06 being typed as serotype 8 and others being non-typable. Therefore, we investigated both types of colonies for the size of the *cap8E* gene using PCR. Our study found that colonies expressing serotype 8 had a normal-sized *cap8E* gene but that the non-typable colonies did not have a *cap8E* gene even though they had the pneumococcal *cpsA* gene (data not shown). Thus this sample harbours two pneumococcal strains: one expressing a non-typable serotype and the other expressing serotype 8.

When the three discrepant 11A isolates were further investigated, they were repeatedly typed as non-typable at UAB (with Hyp11AM2) but were retyped as 11A by the quellung reaction. To investigate discrepancies in 11A serotyping, we labelled the 11A isolates reacting with Hyp11AM2 as 11A α and the three non-reactive isolates as 11A β . When we examined the panel of mAbs that we had

prepared against 11A, we found that one mAb (Hyp11AM1) bound to the three 11A β isolates better than to the 11A α isolates. The three 11A β isolates inhibited the binding of Hyp11AM1 to 11A β -coated latex beads but did not inhibit the binding of any other mAbs. Also, no isolates expressing the non-11A serotype could inhibit the binding of Hyp11AM1 to 11A β -coated latex beads. Thus the multibead assay system based on 26 mAbs could correctly serotype all clinical isolates and also suggested the presence of subtypes among serotype 11A.

Discovery of subtypes among 11A isolates

To quantitatively study the 11A subtypes, we developed an 11A-subtype-specific inhibition ELISA using the two mAbs (Hyp11AM1 and Hyp11AM2) and two subtypes of 11A PS. The 11A PS from the ATCC was inhibitory in the 11A α assay but not in the 11A β assay (data not shown). Conversely, 11A β PS inhibited the 11A β assay but not the 11A α assay (data not shown). Consistent with multibead assay results, lysates of the 11D and 11F serotypes inhibited the 11A α assay, but lysates of 11B and 11C did not (data not shown). None of these lysates inhibited the 11A β assay (data not shown). When we examined all 12 CDC samples that were identified as 11A by the quellung reaction, six isolates inhibited the 11A α assay but not the 11A β assay and three isolates inhibited the 11A β assay but not the 11A α assay (Fig. 3). Interestingly, three isolates could inhibit both the 11A α assay and the 11A β assay; an example is shown in Fig. 3. These results suggest the presence of serological subtypes in serotype 11A. However, we will not propose these subtypes to be new serotypes until the biochemical and genetic bases for the subtypes of 11A are determined.

The potential discovery of serological subtypes within serotype 11A is interesting since this serotype has been extensively investigated. Identification of these new subtypes in the wake of discovering serotype 6C within the well-established serotype 6A (Park *et al.*, 2007) strongly suggests that the quellung reaction based on polyclonal antibodies may provide only limited serological specificity. We anticipate that additional subtypes will be discovered within other established serotypes and potentially among certain non-typable strains.

Operational experience with the hybrid serotyping assay using both multibead and PCR assays

We describe a hybrid serotyping system using both PCR and serological methods that shares the same sample (bacterial lysate). Since its development, our hybrid serotyping assay has already been used for typing more than 1000 samples. The multibead assay is well suited for primary screening since it has high throughput with reproducible results. Once one has appropriate instruments, the multibead assay costs less than PCR since it uses readily available latex particles, uses very small amounts of

Fig. 3. Inhibition ELISA for distinguishing the 11A subtypes. The left panel shows the binding of Hyp11AM2 to 11A α PS (y-axis in absorbance units) in the presence of various dilutions of pneumococcal lysates (x-axis in absorbance units). The right panel shows the binding of Hyp11AM1 to 11A β PS (y-axis) in the presence of various dilutions of pneumococcal lysates (x-axis). Pneumococcal isolates A (solid triangle, solid line), B (solid diamond, solid line) and 3455-06 (solid square, solid line) are 11A β strains, and isolates C (open triangle, dotted line), D (open diamond, dotted line), E (open square, dotted line) and F (solid circle, solid line) are 11A α strains. Note that isolate F inhibits both mAbs.

antibodies, and is highly automated. Indeed, the multibead assay alone is sufficient to determine the efficacy of current pneumococcal vaccines. Although we could serotype the majority of pneumococcal isolates with the multibead assay, the PCR method is a powerful complement in that it is readily adaptable to identify serotypes for which we currently have no serological reagents. This adaptability is useful when the prevalent serotypes are changing as a result of pneumococcal conjugate vaccine usage. We believe that this hybrid system, which shares one common bacterial lysate, has the capability to be highly precise, efficient and useful for laboratories with high-throughput serotyping needs.

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