JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2006, p. 383–388 Vol. 44, No. 2
0095-1137/06/$08.00+0 doi:10.1128/JCM.44.2.383–388.2006

Validation of a Multiplex Pneumococcal Serotyping Assay with Clinical Samples

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Received 12 October 2005/Returned for modification 17 November 2005/Accepted 6 December 2005

We have recently developed a rapid pneumococcal serotyping method called "multibead assay" (J. Yu et al., J. Clin. Microbiol. 43:156–162, 2005) based on a multiplexed immunoassay for capsular polysaccharides in lysates of pneumococcal cultures. The multibead assay can identify 36 serotypes (1, 2, 3, 4, 5, 6A, 6B, 7A/7F, 8, 9L/9N, 9V, 10A/10B/39/33C, 11A/11D/11F, 12A/12B/12F, 14, 15B/5C, 17F, 18C, 19A, 19F, 20, 22A/22F, 23F, and 33A/33F). More than 90% of the U.S. isolates express one of these serotypes (J. B. Robbins et al., J. Infect. Dis. 148:1136-1159, 1983). To validate the new assay, we examined 495 clinical isolates of pneumococci obtained in Brazil, Denmark, and Mexico. Pneumococci were serotyped by the Neufeld test in their countries of origin, and lysates of each strain were coded and mailed to the United States for the multibead assay at ambient temperature without any thermal protection. After breaking the code, 54 discrepancies (11% of samples) were noted, but 46 were due to nonreproducible technical problems or insufficient growth of the pneumococci. All of the isolates grew well for a second test, and therefore, the culture medium used for the multibead assay is adequate. The discrepancies persisted for eight isolates, involving the 6A, 11A, and 18C serotypes. Additional studies of the eight isolates showed that the discrepancies were due to differences in the reagents used in the multibead or Neufeld tests for these three serotypes. For instance, five isolates were typed as 6A with the Neufeld test but as nontypeable by the multibead assay. Selection of another new monoclonal antibody (Hyp6AG1) for the multibead assay resulted in all five discrepant isolates typing as 6A. This finding indicates the validity of the multibead assay and emphasizes the need to validate any new pneumococcal serotyping assay with a large number of clinical isolates from different locations. It also suggests the presence of serological subtypes among isolates expressing the 6A serotype.

Streptococcus pneumoniae is a well-known human pathogen and a major etiologic agent for pneumonia, meningitis, and otitis media as well as sepsis among primarily young children and older adults (4). *S. pneumoniae* has at least 90 serotypes, with each serotype expressing a serologically distinct polysaccharide (PS) capsule (7). Since antibodies to a capsular PS provide protection against pneumococci expressing the same capsular serotype, capsular PS is used as an immunogen in all licensed pneumococcal vaccines. One pneumococcal vaccine, called PS vaccine, contains capsular PSs from 23 commonly found serotypes and has been widely used in adults (21). A new pneumococcal vaccine, called conjugate vaccine, contains seven different capsular PSs conjugated to a protein carrier. This conjugate vaccine was introduced in 2000 to be used for young children (26). Since then, the incidence of invasive pneumococcal diseases in U.S. children has been dramatically reduced (26), and the disease incidence among adults also decreased (26). In the wake of this success, efforts are under way to develop 11 to 13 valent conjugate vaccines (27).

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The distribution of pneumococcal serotypes is useful in estimating vaccine efficacy. In an ideal situation, an effective pneumococcal vaccine would reduce the prevalence of pneumococci expressing the serotypes included in the vaccine and leave the prevalence of the pneumococci expressing nonvaccine serotypes the same. In real situations, the prevalence of the pneumococci expressing nonvaccine types increases to replace those expressing the vaccine serotypes (20). Also, the prevalence of specific serotypes can change over time for unknown reasons (6, 10). Consequently, accurate and efficient serotyping of pneumococcal isolates is important for monitoring the efficacy of pneumococcal vaccines.

Although there are various methods of typing pneumococci based on either serologic differences (3, 5, 7, 11–13, 15, 23–25) or DNA sequences (1, 2, 8, 14, 18, 22), the currently used methods are largely manual and are slow and tedious to perform. We have therefore developed a new serotyping assay method named the "multibead assay," which is based on a multiplexed inhibition-type immunoassay that can be performed semiautomated with a flow cytometer (19). Recently, we showed that the multibead assay is highly specific, using pneumococcal strains representing all 90 known serotypes (28). Although the multibead assay has been extensively characterized so far, those studies used laboratory strains that had

been collected in the United States. Thus, it was unclear how the multibead assay would perform with clinical isolates from geographic locations other than the United States. Also, it was unclear whether the culture medium used in the assay would support the growth of all clinical isolates. To validate the clinical usefulness of the multibead assay, we have conducted a blind study of the multibead assay using a large number of clinical isolates obtained in other parts of the world.

MATERIALS AND METHODS

Bacteria and bacterial lysates. The study used 495 clinical isolates. Fifty isolates were from Mexico, 100 were from Denmark, and 345 were from Brazil. Twenty-two isolates were from asymptomatic carriers of pneumococci in the nasopharynx, and 475 isolates were from patients with invasive pneumococcal infections, such as meningitis and sepsis. In addition, control pneumococcal strains expressing serotypes 11A, 11B, 11C, 11D, and 11F were purchased from Statens Serum Institut (Copenhagen, Denmark).

The lysates were prepared in their countries of origin. Three-hundred microliters of Todd-Hewitt medium with 0.5% yeast extract (THY medium) was inoculated with a single colony of pneumococci. After an overnight incubation at 37°C, 50 μl of 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. In Brazil, 400 μl of THY medium was used for bacterial growth, and 100 μl was removed to store the bacteria frozen before mixing the remaining 300 μl with 50 μl of lysing solution. In Denmark, 325 μl of THY medium and 25 μl of lysing solution were used. Bacteria were lysed by incubating the mixture at 37°C for several hours. The lysates were coded and shipped to the University of Alabama at Birmingham (UAB) laboratory for serotype testing by regular mail at room temperature (RT). Since the codes were broken only after the UAB laboratory finished the assay, the UAB laboratory performed the assay without the knowledge of the serotype determined by the reference laboratories.

Serological reagents. All the polyclonal serotyping sera were made in rabbits and were obtained from Statens Serum Institut. They include 12 serum pools for serogrouping (24) and various type- or factor-specific antisera. All the monoclonal antibodies (MAbs) were produced as described previously (28), and hybridoma culture supernatants were used.

Multibead assay. This assay was performed as described previously using two different sets of latex beads (28). One set (bead set 1) is a mixture of 14 different latex beads, each coated with one pneumococcal PS antigen. The 14 pneumococcal PS antigens were serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9N, 9V, 14, 18C, 19A, 19F, and 23F. Bead set 2 was created by coating each of 10 bead types with 1 of 10 different pneumococcal PSs (serotypes 2, 8, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F). Beads for serotypes 3, 6B, 9V, 18C, and 19F were coated with PS protein conjugates, which were made by conjugating PS obtained from ATCC (Manassas, VA) to bovine serum albumin. All other beads were coated with plain PSs obtained from ATCC. The beads were useful for 2 to 3 months when kept at 4°C.

Set 1 beads were mixed with either 5× or 20× diluted bacterial lysate and a mixture of MAbs specific for the pneumococcal capsular PSs contained on the beads. After being incubated and washed, the bead mixture was reacted with fluorescein-conjugated anti-mouse immunoglobulin antibody. Set 2 beads were used the same way as set 1 beads except that a mixture of polyclonal rabbit antisera (Statens Serum Institut) and fluorescein-conjugated anti-rabbit immunoglobulin antibody was used. After incubation, the amount of fluorescence of each bead type was determined with a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA). The fluorescence of each bead type was then used to determine its serotype. Fluorescence inhibitions greater than 67% were used as positives.

Neufeld's test. The Neufeld test was performed by the reference laboratories in Denmark, Brazil, and Mexico using the methods described in references 9, 15, and 24. They used standard serogrouping (24) and serotyping rabbit antisera from Statens Serum Institut.

Dot blot assay. To investigate discrepant results, this assay was performed as described previously (5) using pneumococcal antisera from Statens Serum Institut for the following serogroups or serotypes: 1, 4, 5, 6, 7, 8, 9, 11, 12, 14, 18, and 23. Monoclonal antibodies specific for 6A (Hyp6AM3) and 18C (Hyp18CM1) serotypes were also used in some cases. Briefly, heat-killed pneumococci grown in THY medium were spotted on strips of nitrocellulose membranes. After being dried, the strips were blocked and washed. Strips were then incubated in a diluted antiserum or MAb solution for 1 h, washed, and exposed to a diluted goat anti-rabbit or mouse immunoglobulin-peroxidase conjugate. After 1 h of incubation at RT, the strips were washed and exposed to 3-amino-9 ethylcarbazole solution. When the spots appeared, the strips were washed and evaluated.

PCRs. Pneumococci were grown in THY medium to an optical density of 0.8 at 650 nm wavelength. Chromosomal DNA was prepared using the Invitrogen Easy-DNA kit and following the given instructions, beginning with a 4-ml sample of the THY-grown pneumococci concentrated to 1 ml (Invitrogen, Carlsbad, CA). For serogroup 6 determination, PCR was performed using chromosomal DNA as the template and primers wciP-up (5′-ATG GTG AGA GAT ATT TGT CAC-3′) and wciP-down (5′-AGC ATG ATG GTA TAT AAG CC-3′). PCR thermocycling conditions were as described in Mavroidi et al. (17). A QIAGEN PCR cleanup column (QIAGEN, Valencia, CA) was used to remove excess primer from the PCRs, and the PCR was submitted as the DNA template for automated DNA sequencing using the wciP-up primer. Results were analyzed with the aid of the Sequencher (GeneCodes, Inc., Ann Arbor, MI) and the MacVector sequence analysis (Accelrys, San Diego, CA).

For serotype 11A determination, PCR for a part of the capsule gene locus was performed as described previously (17) using chromosomal DNA as the template, 1 μl of forward primer (50 pmol), and 1 μl of reverse primer (50 pmol). Primers were 11A forward (5′-GGA CAT GTT CAG GTG ATT TCC CAA TAT AGT G-3′) and 11A reverse (5′-GAT TAT GAG TGT AAT TTA TTC CAA CTT CTC CC-3′) (B. Beall [Atlanta, GA], personal communication). PCR cycling began with 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis (Tris-acetate buffer, 0.8% agarose) to determine the amplicon size.

RESULTS

Study of shipping conditions. To simplify the shipping of bacterial lysates from distant sites to the UAB laboratory for the multibead assay, we investigated whether bacterial lysates retained their activity when kept at RT or at 37°C. We found that bacterial lysates can be stored at RT for up to 1 month or at 37°C for several days without affecting the results of the multibead assay (data not shown). Thus, we used the regular postal mail system to ship all the lysates used in this study at ambient temperature without any thermal protection.

Study of 50 isolates from Mexico. The 50 isolates from Mexico were grown in THY medium, lysed, coded, and sent to UAB for typing. When the multibead assay results were compared with the Neufeld test results, results from 10 samples were discrepant. When new lysates of eight of the discrepant samples were obtained and reexamined in a blind fashion, all results matched, suggesting that the discrepancies were due largely to mislabeling. Two isolates (MX24 and MX37) that were typed to be serotypes 3 and 10A by the Neufeld test were originally typed as nontypeable (NT) by the multibead assay. Since the two serotypes should have been identified by the multibead assay, the two bacterial isolates were sent to the UAB laboratory for further study. There, they were found to grow well in THY medium, with the new lysates producing results matching the Neufeld test results. Thus, the two isolates were initially falsely identified as negatives by the multibead assay, most likely due to insufficient growth of pneumococci.

Study of 100 samples from Denmark. When the multibead assay results of 100 Denmark isolates were compared with the Neufeld test results, we found four errors in transcribing the Neufeld test results, and 1 strain (DK94) was typed as serotype 20 by the Neufeld test and as NT by the multibead assay (Table 1). In Table 1, "7F/A" means that the serotype of an isolate may be either 7A or 7F. When the DK94 isolate was regrown in THY medium and reexamined, it produced almost no inhibition (9%) at a 1:5 dilution, but it produced more inhibition at

TABLE 1. Serotyping results with both serotyping assays and final results after the investigations

Serotype[a]	No. of isolates identified by:		Final result (no. of isolates)
	Multibead assay	Neufeld's test	
1	30	30	30
2	1	1	1
3	22	22	22
4	20	20	20
5	11	11	11
6A	16[b]	21	21
6B	24	24	24
7F/A	14	14	14
8	13	13	13
9V	18	18	18
9N/L	12	12	12
10A/B/39/33C	12	12	12
11A/D/F	8[b]	10	9
12F/A/B	16	16	16
14	52	52	52
15B/C	10	10	10
17F	6	6	6
18C	28	27[b]	28
19A	18	18	18
19F	26	26	26
20	3[c]	4	4
22F/A	6	6	6
23F	19	19	19
33F/A	6	6	6
NT	104	97	97
All	495	495	495

[a] 7F/A means that the isolate may express either 7F or 7A serotype. 10A/B/39/33C indicates that the isolate may express serotype 10A, 10B, 39, or 33C.
[b] After additional studies of Brazilian isolates, it was concluded that the multibead assay failed to identify five 6A strains (with Hyp6AM3) and one 11A strain and that Neufeld's test failed to identify one 18C strain and falsely identified one strain as 11A.
[c] One Danish strain had high background signal and was not detected during the initial multibead assay.

higher dilutions (35% at a 1:20 dilution and 50% at a 1:320 dilution). This unexpected behavior suggested the presence of nonspecific binding material in the lysate of this specific isolate. When the PS in the lysate was precipitated with 70% ethanol and the ethanol precipitate was examined with the multibead assay, the precipitate produced a clear inhibition for serotype 20 (86% at a 1:5 dilution and 81% at a 1:20 dilution). Thus, the initial discrepancy was due to nonspecific binding, which was occasionally observed in the assays performed with polyclonal rabbit antisera, and there is no intrinsic problem in assay sensitivity and specificity with clinical isolates.

Study of 345 samples from Brazil. When the results of 345 Brazilian isolates obtained with the two assay methods were compared, there were 38 mismatches. When these 38 samples were reexamined by investigating test records and retesting by Neufeld's test in Brazil, 17 of the mismatches could be explained as typing mistakes or sample misidentification. One of the 17 mismatches was strain BZ652. This was initially typed as 18B but was determined to be 6A because it was typed as weakly 6A by Neufeld's test and was typed as serogroup 6 by the dot blot assay using the polyclonal rabbit antisera and MAb Hyp6AM3. When the 21 remaining mismatched samples were regrown in THY medium and retested by multibead assay, the new results of 13 isolates matched the Neufeld test results. When the original multibead assay results of the 13 isolates were reexamined, 3 isolates produced weak and incomplete inhibitions (inhibitions were less than 67%) for the appropriate serotype in the original multibead. Although 12 isolates were initially typed as NT, 1 isolate (BZ52) was initially typed as type 3. It was retyped as NT with the second sample, and the result became consistent with the Neufeld test result (Table 1).

After these reexaminations, eight discrepancies were reproducible and still unexplained (Tables 2 and 3): five isolates were typed as 6A by the Neufeld test but as NT by the multibead assay, two isolates (BZ435 and BZ705) were typed as 11A by the Neufeld test but as NT by the multibead assay, and one isolate (BZ438) was typed as NT by the Neufeld test but as 18C by the multibead assay. By the Neufeld test, BZ438 did not react with pooled sera A and Q (24), which should react with serogroup 18 pneumococci. It also did not react with several different lots of antisera specific for serogroup 18 or specific for factors 18c, 18d, 18e, and 18f. However, it produced positive dot blot results with a serogroup 18-specific polyclonal rabbit serum or with MAb Hyp18CM1 (28). Thus, the BZ438 isolate was considered to be serotype 18C.

Strains BZ435 and BZ705 were considered to be serotype 11A by the Neufeld test but not 11A, 11D, or 11F by the multibead assay. Since the standard multibead assay uses a polyclonal rabbit antiserum against serogroup 11 (28), we examined the two strains with two MAbs (Hyp11AM1 and

TABLE 2. Studies of two strains for the 11A serotype by Neufeld's test, multibead assay, PCR, and dot blot assay

Strain	Result for:[a]					
	Neufeld's test with rabbit antisera[a]	Multibead assay:			PCR	Dot blot assay with rabbit antisera[a]
		Rabbit antisera[a]	Hyp11AM1	Hyp11AM2		
BZ435	+	−	+	−	+	+
BZ705	+[b]	−	−	−	−	−
Control strain 11A	Not tested	+	+	+	+	Not tested
Control strain 11B	Not tested	−	−	−	−	Not tested
Control strain 11C	Not tested	−	−	−	−	Not tested
Control strain 11D	Not tested	+	+	+	+	Not tested
Control strain 11F	Not tested	+	+	+	+	Not tested

[a] All the rabbit sera were from Statens Serum Institut (Denmark). +, tested positive for 11A; −, tested negative for 11A.
[b] In Neufeld's test, BZ705 did not react with pooled serum T (24) and factor serum 11f, but it did react strongly with factor serum 11c and ambiguously with factor serum 11b.

TABLE 3. Studies of six strains for 6A serotype by Neufeld's test multibead assay and PCR

Strain	Serotype or allele identified by:				
	Neufeld's test with rabbit antisera	PCR for *wciP* allele[a]	Multibead assay:		
			Hyp6AM3	Hyp6AG1	Rabbit antisera
BZ17	6A	9 (1)	NT	6A	6A
BZ39	6A	9 (1)	NT	6A	6A
BZ86	6A	9 (1)	NT	6A	6A
BZ650	6A	9 (1)	NT	6A	6A
BZ652	NT (6A)[b]	2 (5)	6A	6A	6A
BZ1048	6A	Not done	NT	6A	6A

[a] The number in parentheses indicates the number of base pairs different from the proband sequence (17). BZ652 has five base pair differences that produce three amino acid differences. All these alleles express serine at amino acid residue 195. BZ1048 was not analyzed because its PCR did not work.

[b] BZ652 was initially typed as NT but was typed as weakly 6A upon reexamination.

Hyp11AM2) that are specific for serotypes 11A, 11D, and 11F and that were recently produced in the UAB laboratory (Table 2). We found that Hyp11AM1 recognizes BZ435 but not BZ705. Interestingly, Hyp11AM2 recognized neither strain, suggesting heterogeneity among the strains expressing the 11A serotype. A PCR produces 463-base-pair amplimers with strains for 11A, 11D, and 11F but not for 11B and 11C (Table 2). When both strains were tested by this PCR, BZ435 was positive, but BZ705 was not. Although the Neufeld test showed that both strains reacted with antisera specific for factor 11c, it also revealed differences between them: BZ435 but not BZ705 reacted with pooled serum T (24), with serogroup 11 antisera, and with factor serum 11f. BZ705 yielded ambiguous results for factor 11b expression, and this suggested that it could be serotype 11D. However, in a dot blot test for serogroup 11 using rabbit-typing serum, BZ435 is positive but BZ705 is negative. Considering all of these results, we conclude that BZ435 is an 11A strain and that BZ705 is not 11A, 11D, or 11F. BZ705 may belong to the 11C serotype since BZ705 expresses the 11c epitope (and reacts with 11c antisera) that is not expressed on 11B strains.

To investigate the remaining discrepant strains that may be serotype 6A, we examined the DNA sequence of the *wciP* gene based on a recent study (17). 6A or 6B capsular PS has repeating units with rhamnose linked to ribitol. The linkage is 1→3 for serotype 6A and 1→4 for 6B. The study found that the rhamnosyltransferase is likely encoded by the *wciP* gene in the capsule locus, that *wciP* for 6A encodes serine at residue 195, and that *wciP* for 6B encodes asparagine at residue 195 (17). Also, *wciP* alleles 1, 2, 7, 9, and 11 were exclusively associated with serotype 6A, while alleles 3, 4, 5, 6, 8, and 12 were associated with serotype 6B (17).

We obtained bacterial DNA from the five isolates labeled 6A as well as from BZ652, which was considered to be only weakly 6A by the Neufeld test. We then amplified a part of the *wciP* gene by PCR, sequenced the amplicon, and examined the sequence (645 base pairs). We were able to amplify only five, and their sequences were consistent with a 6A serotype because they expressed alleles associated with the 6A serotype (Table 3) and expressed serine at amino acid residue 195 (data not shown). Compared to the prototypic sequence of allele 2 of *wciP*, the *wciP* sequence of BZ652 has five base pair changes with three potential amino acid replacements. Four isolates (BZ17, BZ650, BZ39, and BZ86) express the identical *wciP* gene sequence with one identical nucleotide variation from the prototypic sequence for allele 9 and may therefore be clonally related (Table 3).

Since the DNA study suggested that these isolates may belong to the 6A serotype, we examined these isolates with the multibead assays using polyclonal rabbit antisera. All six isolates were typed as 6A (Table 3). When they were typed with 19 different 6A-specific MAbs in addition to Hyp6AM3, 1 MAb (Hyp6AG1) identified the six isolates as 6A (Table 3). When Hyp6AG1 was used to retest 45 6A isolates (21 from this study and 24 in the UAB laboratory collection), we found that this MAb identified all of them as 6A and that it did not recognize any of the 89 non-6A serotypes, including the 43 isolates expressing the 6B serotype. Thus, we conclude that all these six isolates are serotype 6A and that Hyp6AG1 recognizes all 6A isolates. Also, it is concluded that MAb Hyp6AM3 recognizes a subset of 6A isolates, although the subset is very large.

DISCUSSION

To validate the multibead serotyping method as well as the reagents used for the assay, we investigated 495 clinical isolates of pneumococci from three different geographic locations. The isolates were analyzed at the UAB laboratory without the knowledge of the serotypes determined by the three reference laboratories. The isolates represented all 23 serotypes included in the 23-valent pneumococcal PS vaccine, and each serotype was represented by a significant number of isolates (Table 1). The median number of isolates per serotype was 16. We expected about 10% to be nontypeable by the multibead assay based on the known serotype distribution of unselected isolates causing invasive diseases in the United States (21), but 20% were nontypeable. It is possible that we examined too few samples to avoid statistical fluctuations or that the serotype distribution in Brazil may be different from that in the United States. In almost all cases, the multibead assay produced unambiguous results that matched the Neufeld test results, but the results of the two assay methods differed in about 10% of the cases. Investigation of the discrepancies suggests that both assay methods are sensitive and specific. The discrepancies are due largely to human errors or inadequate bacterial growth, and only occasionally, the discrepancies are due to limitations of the reagents.

Among the 54 samples showing discrepant results, about half (32 out of 54) were due to errors in transcription, sample identification, or typing. Since, in these 32 cases, the discrepancy disappears upon repeat testing, these discrepant samples reveal no problems with either the assay itself or the reagents. These human errors are primarily associated with Neufeld test results and are reflected in other studies of the Neufeld test (9, 19). Indeed, we believe that this type of error would be universal for any intensely manual test, even if the method were based on entirely different analytical principles, such as DNA tests or other serological methods. Also, the number of this type of error would become larger as an assay leaves a research laboratory and is deployed in the field. To minimize this type

of error, we have designed our multibead assay system to minimize the handling of individual isolates and the manual analysis of data. For instance, we keep the samples in a 96-well arrangement, handle 12 samples at a time, and have automated the analysis of the assay results.

The next most significant source of discrepancies (14 samples out of 495) was caused by insufficient bacterial growth for the multibead assay. The 14 samples initially appeared as nontypeable by the multibead assay. Since these strains grew well in THY medium in the reexamination step, the inadequate growth is not a biological property. Poor bacterial growth may be due to an inadequate inoculation of the medium when a large number of bacterial specimens were processed at one time for the initial testing. Although we were initially concerned about its adequacy, we now believe that THY medium is adequate for growing clinical isolates, and our observation is consistent with a previously published report (16). Since this inadequate bacterial growth is the main problem associated with the multibead assay method, we plan to add a control for bacterial growth, such as a pH indicator. Until the effectiveness of the growth indicator is validated, we are routinely retesting the isolates identified as nontypeable. Also, if a rare isolate does not grow in THY medium, we found that pneumococci can be harvested directly from a blood agar plate and lysed for use with the multibead assay.

A small number of discrepancies were reproducible and were due to the reagents used for both of the assay systems. For instance, the multibead assay method identified only 16 strains as 6A with Hyp6AM3 but identified all 21 strains with Hyp6AG1 (Table 1). Similarly, the multibead assay identified only eight isolates as 11A with rabbit antisera but identified all nine isolates as 11A with Hyp11AM1. Also, polyclonal rabbit antisera may be inadequate for occasional isolates of 18C (1 out of 28 in this study). Since matching results could be obtained with suitable reagents, we conclude that both assay methods are sufficiently sensitive and specific and that the multibead assay is validated. Also, this experience illustrates the need to validate any new methodology as well as new reagents with a large number of clinical samples.

Since reagents must be carefully tested, we strongly believe that MAbs are superior to polyclonal antisera as reference typing reagents. In addition, MAbs generally produced fewer background signals and clearer signals than polyclonal antisera. For instance, we observed that nonspecific binding was relatively high for serotypes 10A, 20, and 33 and that nonspecific binding interfered with the serotyping of one sample (DK94). Also, MAbs may provide us with the ability to recognize new and useful epitopes. For instance, since no epitope specific for 18C or 23F has been identified with polyclonal antisera, a set of antisera must be used to determine their serotypes. But, 18C- and 23F-specific epitopes appear to exist, for the two MAbs used in this study could identify the two serotypes specifically (28). In some cases, we found evidence for subsets within an epitope. For instance, the 6b epitope (specific for the 6A serotype) may not be singular. Furthermore, MAbs may be used in other methods of typing pneumococci, such as the dot blot method, latex agglutination, or sandwich capture enzyme-linked immunosorbent assay. Monoclonal antibodies in addition to the MAbs to the 14 serotypes described previously (28) are being produced.

Our findings also suggest that there may be subtypes among 6A or 11A serotypes. Since the molecular nature of 6A and 6B serotypes was studied extensively, we may be able to identify the molecular basis for 6A subtypes. This is significantly supported by the fact that we independently identified three isolates with the same molecular basis and serological properties. Several hypotheses present themselves. Other molecules not coded by the capsule-forming genes may interfere with the formation of some epitopes of 6A PS. Alternatively, rhamnosyltransferase found in BZ652 or BZ17 may not be faithful in forming 1→3 linkage to ribose or may produce short 6A PS that does not express all epitopes associated with normal 6A PS. Some PS epitopes, such as conformational epitopes, are only expressed on long PS chains. To evaluate these possibilities, we are investigating whether 6A subtypes are associated with the *wciP* or other capsule locus genes using gene transfer methods. Also, it is interesting to consider whether pneumococcal vaccines can produce opsonizing antibodies to all subtypes of 6A.

The multibead assay is well suited for large-scale epidemiologic studies because the assay is simple, reliable, and fast. For instance, we observed only two errors during this study (one due to the background problem and the other due to a transcription error). We expect to reduce the error rates even further by replacing the polyclonal antisera with monoclonal antibodies and by automating even more with the use of instruments, such as the FACSArray or Luminex. Also, the bacterial lysates can be stored either frozen or at room temperature for a prolonged period. The presence of stored samples and the existence of original data significantly help any follow-up studies of discrepant samples. In addition, low-cost regular postal mail requiring several days for delivery is quite adequate for shipping samples, which provides a significant advantage for the multibead assay method. Also, the risk of shipping biohazardous materials is eliminated because we are not transporting viable infectious materials. Thus, the safety, simplicity, and lower cost of sample transport would enhance sample exchange among different laboratories and aid in the collection of samples from field sites. The development of well-characterized MAbs would further enhance the usefulness of the multibead assay.

ACKNOWLEDGMENTS

We acknowledge the services of the Heflin Genomics Core Facility at UAB. We are grateful to B. Beall for providing the primer sequences used for 11A serotype, to Danish clinical microbiological laboratories for submitting invasive pneumococcal isolates for national surveillance, to the Sireva-Mexico Network for providing the clinical isolates in Mexico, to A. P. S. Lemos, M. C. O. Gorla, and T. I. Yara for preparing bacterial lysates at Adolfo Lutz Institute, and to the Pan American Health Organization, Washington, D.C., for providing the pneumococcal antisera to Adolfo Lutz Institute.

M.C.C.B. was the recipient of a fellowship from the CNPq, grant no. 303348/2004-6. G.E.-A. is a Ph.D. student in Biological Sciences at the Universidad Autonoma Metropolitana, Unidad Xochimilco, and is supported by a grant from Conacyt. The work was supported by NIH funding (AI-30021) to M.H.N.

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