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Immunoblot Method To Detect *Streptococcus pneumoniae* and Identify Multiple Serotypes from Nasopharyngeal Secretions

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Conventional culture techniques are limited in the ability to detect multiple *Streptococcus pneumoniae* serotypes in nasopharyngeal (NP) secretions. We developed an immunoblot (IB) method with monoclonal antibodies (MAbs) to detect *S. pneumoniae* and to identify serotypes. NP specimens stored in skim milk-tryptone-glucose-glycerol medium were assessed by the IB method and the reference culture method (RM). In the RM, four optochin-sensitive alpha-hemolytic colonies resembling pneumococci were typed by the Quellung reaction. In the IB method, a nitrocellulose membrane blot of surface growth was reacted with a pneumococcal surface adhesion (PsaA) MAb and visualized. Of 47 NP specimens, 32 (68%) were found to be positive and 13 (28%) were found to be negative for pneumococci by both methods; each method alone yielded one positive result. The sensitivity and specificity of the IB method for the detection of pneumococci were 97 and 93%, respectively. To identify serotypes, blots were tested with serotype-specific MAbs (4, 6A, 6B, 9V, 14, 18C, 19F, and 23F). To detect the remaining serotypes, positive serotype-specific replicate blots were compared visually to an original anti-PsaA-positive blot; four unidentified colonies were subcultured and serotyped by the Quellung reaction. Fifty-eight *S. pneumoniae*-positive NP specimens containing 69 pneumococcal strains (23 serotypes) were tested; 68 (98.6%) of the strains were detected by the IB method, and 66 (95.6%) were detected by the RM. For 11 specimens found to contain two serotypes, both methods detected both serotypes in 7 (63.6%), the IB method alone detected the two serotypes in 3 (27.3%), and the RM alone detected both serotypes in 1 (9%). The IB method identified multiple clones and minor populations of pneumococci in NP secretions. This method is useful for detecting specific serotypes and carriage of multiple serotypes in epidemiologic surveillance and carriage studies.

Pneumococcal infections continue to be a leading cause of morbidity and mortality among young children and adults worldwide (4, 5, 11, 15, 18, 21, 22). Colonization by *Streptococcus pneumoniae* occurs early in childhood, often by 2 months of age, and may lead to bacteremia, pneumonia, meningitis, and repeated otitis media episodes with severe health, social, economic, and life-threatening consequences (7, 12, 13, 22, 23). A new pneumococcal heptavalent protein conjugate vaccine (Prevnar; Wyeth-Lederle Vaccines), licensed in the United States in February 2000 for use in children up to 9 years of age, has been shown to be efficacious against invasive pneumococcal disease, pneumonia, otitis media, and nasopharyngeal (NP) colonization (1, 2, 3, 8, 10)

Children are serially and simultaneously colonized in the nasopharynx by various *S. pneumoniae* serotypes. Although a single serotype usually predominates at any given time, there is ample evidence for the carriage of multiple serotypes (12). Studies of the effect of conjugate pneumococcal vaccines on NP colonization have shown a decrease in vaccine serotype (VT) colonization as well as an increase in nonvaccine serotype (NVT) colonization (17; S. K. Obaro, R. A. Adegbola, W. A. Banya, and B. Greenwood, Letter, Lancet **348:**271-272, 1996). This latter observation could reflect one of two scenarios: (i) a true increase in the risk of NVT colonization (replacement colonization) or (ii) an increased chance of identifying resident NVT pneumococci but no increase in the risk of NVT colonization (unmasking) (18). The ability to distinguish between replacement colonization and unmasking is dependent on the sensitivity of the assay for detecting the carriage of multiple serotypes.

Conventional culture techniques do not reliably detect multiple serotypes present in NP secretions if the second or third serotype is present in a much smaller proportion than the dominant type. Detection of two serotypes with 95 to 97% sensitivity would require subculturing of at least 5 CFU if the ratio of the two types were 50:50, 14 CFU would be required if the ratio were 80:20, and 59 CFU would be required if the ratio were 95:5 (14). It is clear that this level of subculturing is not practical for large field trials of pneumococcal carriage related to vaccination. Other highly sensitive methods, such as mouse inoculation, exist; however, these are even more costly and time-consuming than the serotyping of multiple colonies.

We sought to develop a method that could detect the carriage of multiple serotypes and that would be highly sensitive, less labor-intensive than the serotyping of multiple colonies, and reproducible. The method would have to identify not only serotype-specific pneumococci but also pneumococci of any

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serotype. The intent of developing such a method was specifically for use in a study evaluating the effect of a conjugate pneumococcal vaccine on NP carriage.

MATERIALS AND METHODS

MAbs. A monoclonal antibody (MAb) to the 37-kDa common pneumococcal surface adhesin protein (anti-PsaA) (hybridoma 8G12), supplied as unpurified mouse ascitic fluid, was used to detect *S. pneumoniae* on the IB (6). This procedure was needed to detect the presence of NVT pneumococci as described later. VTs 4, 6B, 9V, 14, 18C, 19F, and 23F and vaccine-associated serotype 6A were identified by using mouse MAbs (P. D. Fernsten, Hybridoma Laboratory, Wyeth-Lederle Vaccines, West Henrietta, N.Y.). Henceforth, the group including those VTs and serotype 6A is referred to as VT + 6A. The optimum working dilution for each MAb was determined by dilution to a point at which the cross-reactivity of heterologous isolates was minimized or not observed. If no cross-reactions to VT + 6A were detected, then each optimum dilution was checked against a library consisting of 22 NVT strains, other representative streptococci, nonstreptococcal oral flora, and unrelated organisms to confirm that there was no cross-reactivity.

Bacterial reference library. Isolates were obtained from the culture collections of the Streptococcal Reference Laboratory and other units at the Centers for Disease Control and Prevention, Atlanta, Ga. Representative isolates were obtained for the 7 VTs (4, 6B, 9V, 14, 18C, 19F, and 23F) and 22 NVTs (1, 3, 6A, 7F, 8, 9A, 9B, 10A, 11A, 12F, 13, 15B, 15C, 16F, 18B, 18F, 19A, 22F, 33F, 31, 35B, and 38) associated with invasive infections in the United States as well as one isolate each of *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsp. *equisimilus* (groups C and G), *Streptococcus anginosus* (group F), *Streptococcus intermedius*, *Streptococcus mitis*, *Streptococcus mitior*, *Streptococcus oralis*, *Enterococcus faecalis*, *Hemophilus influenzae* type b, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Branhamella catarrhalis*, *Corynebacterium xerosis*, *Corynebacterium minutissimum*, *Corynebacterium pseudotuberculosis*, and *Corynebacterium pseudodiphtheriticum*, two isolates each of *Dolosigranulum pigrum* and *Neisseria meningitidis*, and seven isolates of *Staphylococcus aureus*.

Specimens. Forty-seven NP specimens (i.e., NP secretions frozen in skim milk-tryptone-glucose-glycerol [STGG] transport medium) from children who were under 5 years of age, who were seen at the Beijing Childrens' Hospital, Beijing, China, and who were participating in a separate NP colonization study were used for the detection of *S. pneumoniae* colonization by the anti-PsaA MAb. Because these specimens did not adequately represent the necessary broad range of serotypes, a separate set of NP specimens stored in [STGG] transport medium (19), known to contain *S. pneumoniae* (as determined by the anti-PsaA IB method) (4), and characterized by the Quellung reaction with four colonies per sample (i.e., the reference method) were used to assess the IB method for the detection of specific *S. pneumoniae* serotypes. These specimens were collected from Navajo and White Mountain Apache children who were under 5 years of age and who were participating in an NP colonization study. We selected from these stored specimens 58 that were known to contain 31 VT pneumococcal strains (7 serotypes), 27 NVT pneumococcal strains (17 serotypes), and 3 non-*S. pneumoniae* strains (1 *D. pigrum* and 2 viridans group streptococci; these are known to cross-react with the anti-PsaA MAb). These 58 NP specimens were tested simultaneously by the RM (for a second time) and by the IB method to assess the performance of the IB method. All aspects of the laboratory work were carried out at the Centers for Disease Control and Prevention. All clinical specimens were collected after written informed consent was given by the parents or guardians of each child. These studies were approved by the institutional review boards of the Centers for Disease Control and Prevention, the Johns Hopkins Medical Institution, the Phoenix Area Indian Health Service, the Navajo Nation, the National Indian Health Service IRB, and the Beijing Children's Hospital.

NP secretions were collected with calcium alginate pediatric swabs (Fisher Scientific Co., Pittsburgh, Pa.) passed through the external nares into the nasopharynx, twisted once, and removed. The swabs were placed in 1.0-ml vials of STGG transport medium, vortexed well, and frozen at −70°C for storage. Specimens were thawed at room temperature and vortexed for at least 10 s before use. All primary cultures were grown on selective Trypticase soy agar with 5% sheep blood and 2.5 mg of gentamicin/liter (GBA; Becton Dickinson, Cockeysville, Md.). Subcultures were grown on Trypticase soy agar with 5% sheep blood but without gentamicin (BAP; Becton Dickinson). All incubations were done at 35 to 37°C in 5% CO_2 overnight in a humid incubator. Isolates were stored in sterile rabbit blood at −70°C.

Preparation of blots from cultures. An NP specimen in STGG medium (100 μl) was spread on the surface of a rotating GBA plate with a microspreader tool (Lazy-L-Spreader; Thomas Scientific, Swedesboro, N.J.). After overnight incubation, plates with 25 or more CFU visually resembling *S. pneumoniae* were selected for immunoblotting. A labeled nitrocellulose membrane (82-mm circle; Protran Unsupported BA83; pore size, 0.2 μm; Schleicher & Schuell, Keene, N.H.) was inverted on the agar surface, allowed to adhere until uniformly moist, removed with forceps, and allowed to dry impression side up at room temperature for 1 h. (For NVT detection, membranes printed with a 0.5-mm grid were used to facilitate the locations of selected colonies.) For controls, a single colony of each VT was streaked in a grid pattern on a BAP. After growth, each control plate was replica plated (Accutron replica plater; Schleicher & Schuell) to four additional plates, which were incubated overnight and blotted with membranes as described above. One blot was included in each IB run as a positive or negative control. Blots (separated with paper dividers) were stored at −20°C in airtight sealed plastic bags until used (shelf life, >1 year).

IB method. The IB method was performed with glass or inert plastic containers at room temperature (25°C) on a rocker platform changing the maximum tilt position every 5 s. All reagents were sterile and were prepared daily. Blots were removed from the freezer, thawed briefly at room temperature, and placed in blocking solution (3% Bacto Skim Milk [Difco Laboratories, Detroit, Mich.] in phosphate-buffered saline [PBS; Gibco BRL, Grand Island, N.Y.]) for 1 h to prevent nonspecific antibody binding to background antigens. Blots were washed five times for 5 min each time with PBS containing 0.05% Tween 20 (PBST; Gibco BRL). Primary antibody (either the anti-PsaA MAb or a serotype-specific MAb) diluted in PBS at the predetermined optimum working concentration was added, and the mixture was incubated for 1 h. After five washes with PBST, goat anti-mouse immunoglobulin G–horseradish peroxidase (affinity purified, heavy and light chains; Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:500 in PBS was added, and the mixture was incubated for 1 h. Blots were washed once with PBST, three times with PBS, and once with endotoxin-free water. The substrate was prepared just before use by dissolving a 3-amino-9-ethylcarbazole substrate tablet in 2.5 ml of *N*,*N*-dimethylformamide (Sigma Chemical Co., St. Louis, Mo.) and diluting the mixture in 2.5 M acetate buffer (pH 5.0) according to the manufacturer's directions. Just before use, 50 μl of 30% hydrogen peroxide per 100 ml was added. 3-Amino-9-ethylcarbazole solution was added to blots and reacted until adequate visible color development occurred (5 to 10 min). Blots were washed three times for 3 min each time with sterile water and air dried on racks at room temperature for 1 h. A background pink color will fade on drying, leaving strong rose-pink dots of insoluble precipitate representing *S. pneumoniae* colonies on a white background.

Each specimen was screened first with the anti-PsaA MAb to confirm the presence of *S. pneumoniae*. If *S. pneumoniae* was not detected, then the specimen was considered to be negative. If the specimen was found to be positive for *S. pneumoniae*, then new aliquots (diluted, if necessary, to produce distinct colonies) were plated on GBA and incubated; blots were prepared and divided in half, and each half was probed with an MAb specific for one of the seven VTs (4, 6B, 9V, 14, 18C, 19F, and 23F) or serotype 6A to identify VT + 6A present in the specimen. To identify NVTs, a new plate was prepared; specimens were incubated, replicated to fresh GBA (the number of replicas being equal to 1 plus the number of VTs determined above), and blotted as described above. The first replicate blot was probed with the anti-PsaA MAb to identify all of the *S. pneumoniae* colonies present; the second replicate blot was probed with the predominant VT-specific MAb determined above, the third replicate blot was probed with the next VT-specific MAb, and so forth. After the completion of color development, the serotype-specific blots were compared to the anti-PsaA blot and, by visual subtraction, colonies that were not represented by VT + 6A blots were presumptively identified as NVTs and were picked off the original plate. Four presumptive NVT colonies were subcultured to a BAP for growth, *S. pneumoniae* was confirmed by optochin susceptibility and bile solubility if needed, and the serotype was determined by the Quellung reaction.

The maximum number of serotypes that can be identified by this method is 12 (8 MAb plus 4 subcultured), unless more MAbs are used and/or additional CFU are subcultured and serotyped. The number of CFU that can be screened varies from 25 to 300, depending on the density of the specimen. Specimens containing fewer than 25 CFU per 100 μl do not replicate well owing to the failure of colonies to carry over to the next plate; excessive CFU density masks the definition of individual colonies and makes identification and subculturing impossible.

RM. An NP specimen in STGG medium (100 μl) was placed on a GBA plate and streaked in the traditional four-quadrant manner. After incubation, the plate was examined for alpha-hemolytic colonies resembling *S. pneumoniae*, and four colonies (especially those with different colony morphologies) were subcultured

individually to a BAP with an optochin disk (Taxo P; Becton Dickinson) and incubated. Subcultures with a positive optochin zone of inhibition (>13 mm) were serotyped by the Quellung reaction. Subcultures with an intermediate zone of inhibition (9 to 13 mm) were confirmed to be *S. pneumoniae* by a positive bile solubility test and then serotyped as described above (20).

The maximum number of CFU that can be screened by this method is four, and the maximum number of serotypes that can be identified by this method is four, unless additional CFU are subcultured and serotyped. The RM results were used to select specimens for this study of the IB method. Thus, specimens were subjected to the RM on two occasions, once as a means of selecting specimens for study and again, several months later, during the course of the comparison study.

RESULTS

Detection of *S. pneumoniae*. We tested 47 NP specimens from Beijing children for the presence of *S. pneumoniae*. Thirty-two (68%) were found to be positive for *S. pneumoniae* by both methods, 13 (28%) were found to be negative by both methods, and each method detected 1 positive specimen not detected by the other. The specimen found to be negative for *S. pneumoniae* by the IB method contained fewer than 50 CFU per ml, approaching the limits of detection of the method (25 CFU/ml). The specimen found to be negative for *S. pneumoniae* by the RM but positive by the IB method contained only *D. pigrum*, which is known to cross-react with the anti-PsaA MAb, producing a false-positive result in the IB method. The sensitivity of the IB for detecting *S. pneumoniae* was 97% (95% confidence interval, 84.2 to 99.9), and the specificity was 93% (95% confidence interval, 66.1 to 99.8).

Detection of VT and NVT. The second phase of the IB validation study probed 58 pneumococcus-positive specimens from American Indian children to identify the *S. pneumoniae* serotypes present. Based on the RM results used to select the specimens, it was known that they contained at least 58 pneumococcal strains. Using the results from both the IB method and the second run of the RM, 69 *S. pneumoniae* strains representing 23 different serotypes were identified in the 58 specimens. Of the 69 strains, 68 (98.6%) (65 found to be positive by the RM and 3 found to be negative by the RM) were identified by the IB method (and confirmed by the Quellung reaction) and 66 (95.6%) (65 found to be positive by the IB method and 1 found to be negative by the IB method) were identified by the RM. No specimens were found to be negative by both methods. Forty-seven specimens (81%) had only one serotype identified by both methods; 11 specimens (19%) had two serotypes identified. In 7 (63.6%) of the 11, the two serotypes were detected by both methods; in 3 (27.3%), the IB method alone detected the second serotype; and in 1 (9%), the RM alone detected the second serotype. Thus, the IB method identified 17.2% of specimens as containing multiple serotypes, compared to 13.8% of specimens so identified by the RM (*P* value determined by the chi-square test, 0.61). There were no specimens in this group with three or more serotypes. All isolates originally characterized for the purpose of selecting specimens were confirmed to be present by the second run of the RM. The second run of the RM identified eight specimens with two serotypes; no specimens with two serotypes were identified in the first run of the RM.

DISCUSSION

The IB method was developed as a screening tool for large numbers of NP samples in a field epidemiology study. The intent was to increase the ability to identify multiple *S. pneumoniae* serotypes present in NP secretions of children even when the second or third serotypes are present in small numbers. We have shown that the IB method is comparable to the RM for the identification of a single serotype. For the identification of a second *S. pneumoniae* serotype, the IB method appears to be somewhat more effective than the RM, but this conclusion is based on a small number of samples with more than one serotype and is not statistically significant. We have estimated the lower limit of detection by the IB method of a second serotype when two serotypes are present to be 30 CFU/ml (unpublished data). We could detect 3 CFU/100 μl when specimens with known concentrations of two serotypes were tested by the IB method; however, in practice, a loss in plate replication when the number of CFU is below 25 is variable and significant, and the situation cannot be improved due to the nature of *S. pneumoniae* colonies and the technical limitations of replication. Therefore, although the limit of the assay in theory is 30 CFU/ml, in practice we set the limit of the IB method at 250 CFU/ml.

For our field studies, we used the RM when there were 25 or fewer CFU on the plate; above that range, we used the IB method. The RM depends in part on the expertise of the technologist to recognize colony differences when subculturing two to four colonies for typing; however, different serotypes do not always have recognizable unique colony morphologies and may not be selected for subculturing. The IB method can screen 100 more colonies than the RM and thus increases the chances of identifying minor clones by 100-fold. Based on our sample, it appears that more than two serotypes are rarely present simultaneously in the populations studied at levels detectable by the IB method. By observation, one *S. pneumoniae* serotype usually predominates at a given time.

The maximum number of serotypes identified by both the RM (4) and the IB method (11) could be increased by subculturing more CFU or using additional MAbs, if available. We limited our choice of available MAbs to the VT of interest in a study of a pneumococcal conjugate vaccine. We chose to subculture 4 CFU based on methods used in clinical and public health laboratories (6–9, 20) as well as the knowledge that a substantial number of CFU must be subcultured and serotyped in order to detect minor populations of a second or third serotype (14). The use of additional MAbs would be more efficient than subculturing and typing additional isolates.

Conventional culture techniques are time-consuming and become impractical when hundreds or thousands of specimens are being processed for minor clones. Blots can be prepared ahead of time and saved in the freezer for months or even years, and the IB method can be used for multiple blots (30 to 50) simultaneously. This reduction in processing time partially offsets the multiple steps in the protocol. However, interpreting the blots is tedious and time-consuming. The method is not recommended for routine diagnostic processing when a rapid turnaround is desirable.

The use of MAbs that are highly serotype specific is critical to the success of the IB method. We attempted to develop the

method with rabbit antisera made for Quellung typing by the method of Fenoll et al. (9). Even after extensive absorption with cells of heterologous cross-reacting serotypes, capsular polysaccharide, and unencapsulated *S. pneumoniae* strain R36A, we were unable to remove false-positive cross-reactions for some sera. Most remarkable were serogroups containing factors, such as serogroup 18, that were difficult to separate, as also noted by Fenoll et al. (9).

The anti-PsaA MAb was necessary to identify all pneumococcal colonies so that those not reacting with the VT-specific MAbs could be readily identified. The anti-PsaA MAb was previously characterized as reacting with 89 of 90 pneumococcal serotypes, including the 7 types in the licensed conjugate vaccine (6). However, in the IB method, the MAb did react with serotype 16F, the type missed by anti-PsaA MAb 8G12 in the previous report. The anti-PsaA MAb was believed to be exquisitely specific for *S. pneumoniae*. However, when applied to the identification of *S. pneumoniae* in NP flora of hundreds of individuals, we found occasional false positives (<2%) identified as *D. pigrum* and viridans group streptococci. These organisms were anti-PsaA positive but were nonreactive with serotype-specific MAbs and were nontypeable by the Quellung reaction. Conventional optochin susceptibility, bile solubility, and other phenotypic tests were needed to confirm that these isolates were not *S. pneumoniae*. With experience, it was possible to visually determine that these colonies were not *S. pneumoniae* owing to their pinpoint size. It was shown previously that some viridans group streptococci contain proteins with homology to PsaA (6, 16). Initially, we also demonstrated cross-reactivity between the anti-PsaA MAb and some clinical isolates of *S. aureus*. This reaction is thought to be a result of nonspecific binding of protein A in the cell wall and was reported previously (6). However, most *S. aureus* isolates do not grow on GBA, and the false-positive reaction did not occur in the IB method.

The licensed pneumococcal conjugate vaccine (Prevnar) has been shown to confer protection against invasive pneumococcal disease and otitis media caused by the seven serotypes in the vaccine. We tested the IB method with these serotypes and serotype 6A, which is the most common type carried in the nasopharynx of the American Indian population studied and a type for which the heptavalent pneumococcal conjugate vaccine offers cross protection. In other countries, pneumococcal conjugate vaccines may have different serotype compositions; the target serotypes in the IB method can be changed to reflect the serotypes of interest if MAbs against those serotypes are available.

We conclude that the IB method can be used in field epidemiology trials to detect *S. pneumoniae* and to identify specific serotypes, including minor populations. It offers simultaneous screening of multiple colonies, easy volume processing, evaluation of multiple specimens, and a permanent record for reference and review. In its current format, the method is not suitable for diagnostic testing with a rapid turnaround. With additional development, the IB method could be adapted for epidemiologic surveillance to detect a specific serotype and could be used for the direct identification of NVTs as MAbs against a broader range of serotypes become available. Extensive data on the diagnostic accuracy of each new MAb will be required. However, the method is operator dependent and time-consuming and still requires a culture step, which in itself may alter the proportions of multiple serotypes identified compared with those originally present in the nasopharynx. Further work is needed to develop an optimum method to identify carriers of multiple strains and to assess the true frequencies of carriers of multiple serotypes directly from NP secretions without the need for a culture step.

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