A 22-plex Chemiluminescent Microarray for Pneumococcal Antibodies

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Abstract

We developed a chemiluminescent multiplexed microarray that simultaneously determines IgG antibody concentrations to 22 pneumococcal polysaccharide (PnPs) serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 23F, and 33F). We compared the microarray with an enzyme-linked immunosorbent assay (ELISA) for 9 of the 22 serotypes (1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F). Correlation coefficients (r²) for the comparison of the microarray with ELISA ranged from 0.91 to 0.97 for the 9 serotypes. The microarray detected more than 4-fold increases in antibody concentrations in serum samples from before and 1 month after administration of pneumococcal vaccine for all 22 serotypes tested. The mean interassay and intra-assay coefficients of variation for 12 serum samples for the 22 serotypes were 7.6% and 6.0%, respectively. Inhibition-of-binding studies showed more than 90% inhibition by homologous serotypes and, with few exceptions, less than 25% inhibition by heterologous serotypes. The microarray multiplexing technology is an attractive alternative to ELISA for antibody responses to 23-valent PnPs vaccines.

Type-specific IgG antibodies to the capsular polysaccharides of Streptococcus pneumoniae protect against invasive diseases by opsonizing the organism.1,2 Type-specific anticapsular polysaccharide antibodies also protect against infection by preventing the acquisition and carriage of the pneumococci.2-4 People with one of a variety of immunodeficiency disorders do not produce antibodies to polysaccharide antigens and, therefore, experience chronic or recurring respiratory infections caused by S pneumoniae and other encapsulated bacteria.5-7 Among the immunodeficiency disorders known to cause a diminished antipolysaccharide response are ataxia telangiectasia, Wiskott-Aldrich syndrome, common variable hypogammaglobulinemia, and DiGeorge syndrome.6,8 In addition, some people may not produce antibodies to polysaccharides even though their serum immunoglobulin levels are normal.8 For example, about 6.5% of children with recurrent respiratory infections and normal immunoglobulin levels do not respond to pneumococcal polysaccharide (PnPs) antigens9,10 and may have what is commonly referred to as specific polysaccharide antibody deficiency syndrome.11

The antibody response to vaccination with 23-valent PnPs vaccine is often used to assess a patient’s ability to produce antipolysaccharide antibodies.11-13 The 23-valent PnPs vaccines contain serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F.14 A 7-valent conjugated pneumococcal vaccine, Prevnar (Wyeth, Philadelphia, PA), was introduced in 2000 for infants and toddlers younger than 2 years and contains serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F.15

Pickering et al16 first described a multiplexed microsphere-based flow cytometric assay that allowed for simultaneous quantitation of antibodies to 14 pneumococcal
serotypes. Since that report, other laboratories have used similar protocols for simultaneous determination of antibodies to 9, 12, and 23 serotypes. Herein, we describe a new chemiluminescent microarray for simultaneous quantitation of antibodies to 22 PnPss in a single assay. This assay is based on microplate-type technology but still allows serum antibody concentrations for 22 serotypes in the polysaccharide vaccine to be determined simultaneously with a single dilution of patient serum in 1 well of a microtiter plate.

**Materials and Methods**

**Pneumococcal Polysaccharides**

The PnPss were purchased from American Type Culture Collection, Manassas, VA. Pneumococcal C-polysaccharide (C-Ps) was purchased from Statens Serum Institut, Copenhagen, Denmark. Pneumococcal ELISA absorbent (PNA), a crude cell wall polysaccharide preparation, was provided by Wyeth Lederle Vaccines, West Henrietta, NY.

**Pneumococcal Antisera**

US human antipneumococcal standard reference serum, lot 89SF-2, was obtained from the Center for Biological Evaluation and Review, US Food and Drug Administration, Bethesda, MD. The 89-S reference standard consists of a pool of serum samples from 17 adults vaccinated with the 23-valent PnPss vaccine. Antibody concentrations assigned to 89-S by Quataert et al. were used to determine the IgG concentrations of unknown samples for pneumococcal serotypes.

A panel of 50 calibration serum samples was obtained from the National Institute for Biological Standards and Control, Potters Bar, England. The 50 serum samples were collected from adults vaccinated with the 23-valent PnPss vaccine and were kindly provided by David Goldblatt, Institute of Child Health, London, England. The panel was originally used for validation of the ELISA for pneumococcal vaccine testing, the purpose for which it was intended. After establishing comparability of our ELISA method with the established assay, we subsequently used these serum samples to compare the microarray with the consensus ELISA. Serum samples obtained from patients older than 2 years from before and 1 month after administration of pneumococcal vaccine were submitted to ARUP Laboratories, Salt Lake City, UT, for pneumococcal antibody testing. All samples were deidentified according to protocols approved by the University of Utah Institutional Review Board (No. 7275).

**ELISA for Anti-PnP**

The ELISA was performed as previously described. PnPss antigens were diluted in phosphate-buffered saline, pH 7.2 (PBS), and absorbed to medium binding 96-well microtiter plates (Corning Costar 9017, Corning, Acton, MA) by incubation at 37°C for 5 hours. Test serum samples and 89-S reference serum were diluted in PBS with 0.05% Tween 20 (Sigma Chemical, St Louis, MO), 1 µg/mL of PNA, and 2 µg/mL of PnPss 22F. Two-fold dilutions of the 89-S standard serum and dilutions of test serum samples were added to pre-washed, PnPss-coated microtiter plates. Following a 2-hour incubation at room temperature, plates were washed with tris(hydroxymethyl)aminomethane (Tris)-buffered saline, pH 7.2, with 0.1% Brij 35 (Sigma Chemical), and alkaline phosphatase–conjugated goat antihuman IgG (γ chain-specific; Jackson Immunoresearch, West Grove, PA) was added. After a second 2-hour incubation at room temperature, plates were washed again, and 100 µL of a 5-mg/mL solution of p-nitrophenyl phosphate in 1 mol/L of diethanolamine, pH 9.8, with 5 mmol/L of magnesium chloride was added to each well. The substrate reaction was stopped after a 2-hour incubation at room temperature by addition of 100 µL of 3N sodium hydroxide.

**Microarray for Pneumococcal Antibodies**

Purified PnPss were printed in a 5 × 5 matrix in each well of 96-well black polystyrene microtiter plates. All serotypes that compose the 23-valent PnPss vaccines were printed in the 5 × 5 matrix. The 2 other spots in the 5 × 5 matrix contained C-Ps and a buffer blank. Serum samples and 89-S standard were diluted in sample diluent containing 10 µg/mL of C-Ps and 10 µg/mL of PnPss 22F. Serial dilutions of the 89-S standard were added in duplicate to each run. Plates were incubated for 30 minutes at 37°C and washed 3 times. After addition of peroxidase-conjugated antihuman IgG to each well, plates were incubated again for 30 minutes at 37°C and washed 5 times with Tris-buffered saline containing 0.05% Tween 20. Chemiluminescent substrate consisting of luminol (3-aminophthalhydrazide), hydrogen peroxide, and enhancers (Quansys Biosciences, Logan, UT) was added to each well containing standard or sample. Immediately after addition of the substrate, the plate was placed in a dark chamber designed and constructed at Quansys Biosciences. A high-resolution (8.1 million pixels) image was taken and captured using a Canon EOS 20D camera and Canon EOS viewer utility software (Canon USA, Lake Success, NY). The plate image was processed using software provided by Quansys Biosciences. Antipneumococcal antibody concentrations of unknown samples were determined by comparing their pixel intensities with those of the 89-S reference standard run simultaneously.

**Competitive Inhibition-of-Binding Studies**

Serum samples from 12 subjects who had received pneumococcal vaccine were pooled, and the pooled serum was diluted 1:100 in sample diluent with 10 µg/mL of C-Ps and PnPss 22F. The diluted serum was preincubated separately with
each of the 22 purified PnPs serotypes in the microarray at a final concentration of 100 µg/mL. Following a 1-hour incubation at room temperature, 50 µL of each dilution containing a different polysaccharide inhibitor was assayed by the microarray protocol described in the preceding section. The pixel intensity was measured for each well containing a PnPs inhibitor and a control well containing no PnPs inhibitor. The percentage of inhibition for each of the 22 PnPs serotypes tested for each inhibitor was calculated by using the following formula:

\[
1 - \frac{\text{Pixel Intensity of PnPs Inhibitor}}{\text{Pixel Intensity of Control (No Inhibitor)}} \times 100
\]

**Results**

**Microarray Development**

All 23 of the serotypes that constitute the pneumococcal PnPs vaccine were printed in a 5 × 5 matrix in each well of black polystyrene microtiter plates. [Figure 1A](#) shows the location of each of the 23 PnPs serotypes within the matrix. Position 24 was printed with C-Ps as a control, and position 25 was printed with PBS as a blank. [Figure 1B](#) and [Figure 1C](#) show the photographic image after completion of the assay procedure with the 89-S standard. The image in Figure 1B was without C-Ps and PnPs 22F in the sample diluent, and the image in Figure 1C shows results with C-Ps and 22F in the sample diluent. As can be seen, antibody binding to 22F and C-Ps on the plate was inhibited by C-Ps and 22F in the sample diluent. [Figure 2](#) shows typical standard curves generated from 2-fold dilutions of the 89-S standard. Shown are the dilutions of the standard plotted against the pixel intensity for 9 of 22 serotypes.

**“Pre” and “Post” Serum Samples**

[Figure 3](#) shows the microarray results for 4 representative pairs of serum samples before (pre) and after (post) administration of 23-valent PnPs vaccine. The microarray detected greater than 4-fold antibody responses to all 22 serotypes tested. The IgG antibody response to individual serotypes, however, varied among the 4 patients. For example, patients 2 and 3 had poor responses to serotype 10A with post/pre ratios of 3 and 2, respectively, whereas patients 1 and 4 had post/pre ratios to serotype 10A of 6 and 17, respectively. Likewise, patient 3 had a poor response to serotype 6B with a post/pre ratio of 2, whereas patient 2 had a post/pre ratio of 30 for serotype 6B. Several responses were less than 4-fold between pre and post vaccine serum samples owing to high levels of preexisting antibody, such as with serotypes 19F and 23F in patient 1 and serotypes 6B and 14 in patient 4.13

**Competitive Inhibition of Binding**

To access the type specificity of the microarray, we combined 12 post PnPs vaccine serum samples to produce a pooled serum sample with high-titer antibody to all 22 serotypes in the microarray. The serum was diluted in sample diluent containing PnPs 22F and C-Ps and preincubated individually with purified PnPs of each of the 22 serotypes before testing by the microarray. As shown in [Figure 4](#), the pooled serum was inhibited from binding to each of the 22 serotypes by the homologous serotype by 91.3% or more. With few exceptions, inhibition of binding by heterologous serotypes was less than 25%. Greater than 50% inhibition by
heterologous serotypes was observed for only 6 of the data points, and 4 of these were less than 53.5%. High heterologous inhibition rates of 65.1% and 86.0% were seen for inhibition of serotype 9V by serotype 4 and serotype 33F by serotype 23F, respectively.

Microarray Comparison With ELISA

We compared the microarray system with the consensus ELISA method currently recommended for pneumococcal vaccine immunogenicity testing. A panel of 50 Goldblatt reference serum samples was tested against 22 pneumococcal serotypes simultaneously by the microarray and against 9 serotypes (1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F) individually by the conventional ELISA. Correlations between the microarray and ELISA for 9 serotypes are shown in Figure 5. Correlation coefficients ($r^2$) were 0.94 or more for all serotypes tested except PnPs 1 and PnPs 9V, which were 0.91 and 0.92, respectively.

Microarray Precision

The interassay and intra-assay variability of the microarray system was assessed using 12 serum samples from the 50 serum sample calibration panel with a range of anti-PnPs titers. The intra-assay variability was assessed by testing the 12 serum samples in triplicate in a single assay. The interassay coefficient of variation (CV) for the 3 assays ranged from
Figure 3 | Microarray results for IgG concentrations in serum samples before (pre) and 1 month after (post) administration of pneumococcal vaccine. IgG concentrations determined by the microarray system are shown for 22 pneumococcal serotypes in serum samples from 4 people for paired pre and post pneumococcal vaccine administration.

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Figure 4 | Diagram of inhibition of binding of a pooled serum by individual pneumococcal polysaccharide (PnPs) antigens. The percentage of inhibition shown is the percentage of control wells without a PnPs inhibitor. Homologous inhibition (>91.3%) is shown diagonally in black grids from top left to bottom right. Heterologous inhibition (<25%) is shown in white grids. Light and medium-shaded grids show heterologous inhibition of 25%-49% and 50%-80%, respectively.
0.2% to 40.1% for all 22 serotypes. The mean CV for all 264 triplicates was 7.6%. Of the 12 serum samples tested in triplicate within the same run, CVs ranged from 0.2% to 20.6% for the 22 serotypes. The mean CV for all 264 triplicates was 6.0%. Only 4 of 264 intra-assay triplicates and 7 of 264 interassay triplicates were more than 20%.

**Discussion**

Our microarray for simultaneous quantification of IgG concentrations to 22 PnPs serotypes compared well with a consensus ELISA widely used for pneumococcal vaccine testing. Correlation coefficients \( (r^2) \) for the 9 serotypes compared ranged from 0.91 to 0.97, with an average of 0.94 for these 9 serotypes (Figure 5). The ELISA used in this study was similar to the protocol adopted by a World Health Organization Expert Committee on Biological Standardization in 2003 as a reference for laboratories testing antibody responses to pneumococcal antibodies.\(^{22-24}\)

Since originally described, the ELISA protocol has undergone several revisions. Original ELISA procedures for pneumococcal antibody testing overestimated the type-specific
antibody concentration and showed poor correlation with vaccine efficacy.22 C-Ps was added to the sample diluent as an absorbent to remove antibodies to C-Ps.25,26 Subsequently, the addition of PnPs 22F as an absorbent was found to remove additional non-serotype-specific antibodies and improve the correlation between ELISA and opsonophagocytosis assays.27-29 Investigators at Merck Research Laboratories (Wayne, PA) use a different approach. They add PnPs 25 and PnPs 72 as absorbents instead of PnPs 22F.19,30 This approach allows all 23 serotypes in the PnPs vaccine, including PnPs 22F, to be tested. Because PnPs 25 and PnPs 72 were not commercially available, we used C-Ps and PnPs 22F as absorbents in the sample diluents for the microarray assay. The ELISA sample diluent contained PnPs 22F and PNA, a C-Ps preparation provided by Wyeth Lederle Vaccines.

The microarray described in this report and the conventional ELISA are solid-phase immunoassays in which PnPs antigens are absorbed to 96-well polystyrene plates. Antibodies to PnPs antigens in patient serum samples are detected by using enzyme-labeled antihuman IgG second antibody. Therefore, the kinetics and dynamic ranges of the 2 methods are similar. The methods differ in 2 respects. First, with the microarray, each PnPs antigen is spotted in a precise location within each well. All 23 PnPs serotypes, along with a C-Ps control, are spotted in each well in a 5 × 5 matrix. With ELISA, only 1 antigen is absorbed per well. The second difference is that ELISA uses a colorimetric substrate that diffuses throughout the well, whereas the microarray uses a light-emitting chemiluminescent substrate. Because the photons of light are instantaneous and transient, the signal is localized to a specific spot and the signal from each PnPs antigen can be differentiated and quantitated. The amount of light emitted from each spot is quantitated by first capturing a digital image and converting it into pixel intensities. The pixel intensity of each spot is directly proportional to the amount of antibody bound.

The antibody response to vaccination with the 23-valent PnPs vaccine is routinely used to diagnose a deficiency in the ability to produce antibodies to polysaccharide antigens. Although not clearly established, a 4-fold or greater rise in antibody concentration between pre and post vaccine serum samples is seen by many investigators as a good response to PnPs antigens.31,32 As can be seen in Figure 3, the microarray detected 4-fold or greater antibody responses to all 22 of the PnPs serotypes tested. None of the 4 patients represented, however, responded with 4-fold increases to all 22 serotypes, even those considered to be more immunogenic such as 3 and 14.31 As noted, there were also significant differences in the response to individual serotypes among the 4 patients. These results are consistent with those of previous studies. Go and Ballas31 summarized results of 23 studies of the antibody response to PnPs serotypes in healthy adult control subjects.

None of 12 serotypes in 15 studies tested by radioimmunoassay had a 4-fold antibody response in all patients. In 7 studies in which antibody responses were tested by ELISA, only serotype 2 of 13 serotypes tested had a 4-fold antibody response in all patients. In another study, 50% of healthy adults failed to respond to 1 of 10 PnPs serotypes and 9% failed to respond to 5 or more of 10 serotypes.32 Considering the variability in antibody responses to individual PnPs serotypes, multiple serotypes should be tested when evaluating antibody responses to the 23-valent PnPs vaccine.11,13 Healthy children 2 through 7 years old should respond to 50% of the serotypes tested, and older persons should respond to 70% of the serotypes tested.33 The microarray described herein tests for 22 of the 23 serotypes in the 23-valent PnPs vaccine. This gives a more comprehensive view of the patient’s anti-PnPs response. Testing multiple serotypes by the conventional ELISA, on the other hand, requires a separate assay for each serotype, which is very consuming of time, labor, and costs.

Although we did not test our microarray against pediatric samples in this study, the microarray format might also be adapted for testing antibody responses of infants to pneumococcal conjugate vaccines. Pediatric serum samples may behave differently, however, from the adult serum samples that we used in this study, and additional studies would be needed to evaluate the suitability of the microarray for this application.

Pickering et al16 first reported a multiplexed microsphere assay for simultaneous determination of antibodies to 14 pneumococcal serotypes based on technology from Luminex Corporation, Austin, TX. Other laboratories have subsequently reported Luminex-based multiplexed assays for 9, 12, and 23 pneumococcal serotypes.17-19

As previously observed by Pickering et al,16 the Luminex technology has a much greater dynamic range than ELISA-based assays, thus reducing the need for multiple dilutions of each sample. Because the microarray system is an ELISA-based system, the dynamic range of the system is similar to that of ELISA, which is a drawback of this new technology. A major disadvantage of the Luminex technology, however, is the requirement that PnPss be covalently attached to the microspheres. This is accomplished by introduction of primary amino groups into the polysaccharide structure and subsequently coupling the amino-modified polysaccharides to the carboxyl groups attached to the microspheres. Chemical alteration of the polysaccharide structure raises the possibility that critical type-specific epitopes may be altered or destroyed or that new epitopes might be introduced. For example, Biagini and coworkers17 could not demonstrate homologous inhibition for 9 of 24 serotypes coupled to Luminex microspheres by the peridote method. This suggests that the type-specific epitopes were destroyed.
during the coupling procedure. They suggested that the periodate levels might need to be titrated for each serotype but did not provide procedures for doing this. Coupling procedures may be difficult to standardize for all 23 serotypes and to control from lot to lot.

The chemiluminescent microarray system correlated well with a standardized ELISA used for pneumococcal vaccine testing and with a calibration serum panel. The assay had good interassay and intra-assay reproducibility. The ability to quantitate IgG antibody concentrations for up to 22 serotypes simultaneously would reduce the time, resources, and cost of the current method of testing each serotype individually by ELISA. The microarray system might also be a more desirable multiplexing technology for pneumococcal antibody testing than the previously described Luminex system because PnPs antigens are not modified, but this may be offset by the fact that the dynamic range of the assay is less than with the Luminex system, thus requiring more frequent diluting and retesting of samples.

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