Development and Characterization of a Multiplex Bead-Based Immunoassay To Quantify Pneumococcal Capsular Polysaccharide-Specific Antibodies

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Enzyme-linked immunosorbent assay (ELISA), the traditional antibody quantification technique, has several limitations, especially when used to evaluate multivalent and/or infant vaccines. We have developed a multiplex bead-based antibody quantification assay (MBIA) to measure antibody response to multiple pneumococcal (Pn) serotypes (St) in a single assay. MBIA was compared with the WHO ELISA using a WHO panel of 12 international calibration sera for 7 Pn Sts. An agreement of 75 to 92% was obtained for all 7 Sts. MBIA exhibited good robustness, with the assay variability at ≤16%. A major contributor to MBIA variability was the cell wall polysaccharide (CWPs) content in Pn St-specific capsular Ps. This necessitated careful CWPs (20 μg/ml) preadsorption of sera. MBIA is specific, robust, and reproducible and offers high throughput. The use of MBIA will greatly reduce the cost and time required to evaluate the immune response to multiple Pn Sts and could help promote the licensure of future Pn and other multivalent vaccines.

A number of immunoassays have been developed to measure the concentration of serotype (St)-specific human IgG anti-pneumococcal (anti-Pn) capsular polysaccharide (Ps) antibodies following induced and natural immunization. The standard measurement of Pn antibody in most laboratories today relies on the World Health Organization (WHO) enzyme-linked immunosorbent assay (ELISA) (3, 17). This standardized assay has several limitations that result in substantial laboratory effort at a relatively high cost. It is labor-intensive and can measure antibodies to only one antigen at a time. The singleplex aspect of ELISA testing also results in increased use of patient samples (4, 10, 11). Although an increased volume of serum (10 μl/St) is not troublesome to achieve with adults, it is with infants. In such situations, a multiplex immunoassay that measures specific antibody to several antigens simultaneously from a single small sample (i.e., 10 μl) is highly advantageous (15). The use of the multiplex assay to measure serum IgG reduces the time required per assay analyte by 80%, thereby markedly increasing throughput and greatly reducing the assay cost. In addition to increased automation and efficiency, numerous studies have shown serological multiplexing procedures to be sensitive, precise, and accurate (1, 5, 7). Furthermore, multiplex assays offer an efficient laboratory approach that can serve as a primary antibody concentration assay for analyzing imposing numbers of patient sera associated with large-scale vaccine efficacy trials and epidemiological studies.

In this study, we evaluated an in-house multiplex bead-based immunoassay (MBIA) to quantify anti-Pn Ps IgG to multiple serotypes. The multiplex assay is based on Luminex’s xMAP technology (Luminex, Austin, TX). The primary objective of this study was to characterize MBIA and elucidate the correlation between MBIA and an in-house ELISA with the WHO reference panel of 12 pneumococcal calibration sera (12). Additional MBIA and in-house ELISA validations were performed with paired maternal sera (n = 50) from women vaccinated once with a 23-valent Pn Ps vaccine during their third trimester of pregnancy. Assay modification in terms of changes in cell wall Ps (CWPs) concentration in the serum preadsorption buffer was necessary for a few Pn Sts. MBIA exhibited excellent correlation with in-house ELISA for Pn St-specific Ps IgG in the 12 WHO calibration sera and clinical specimens.

MATERIALS AND METHODS

Standard and serum samples. The Food and Drug Administration’s (FDA) Pn human serological reference standard, 89SF, was used as the standard for all in-house ELISA and MBIA; it was never used as an unknown source. The WHO Pn calibration human serum panel (n = 12) was used for primary assay correlation and validation studies. Secondary assay validations were carried out with paired sera (n = 50) from pregnant women who received one dose of the 23-valent Pn Ps vaccine during their third trimester of pregnancy. Five in-house quality control (QC) sera generated from 23-valent Pn Ps-vaccinated laboratory technicians were also used in selected assay development experiments to conserve clinical sera.

Pn Ps IgG ELISA. The IgG ELISA procedure was performed according to the WHO consensus protocol, with modifications (9, 17). Briefly, 89SF was prediluted (1:20) with phosphate-buffered Tween solution containing 20 μg/ml CWPs (PBST + CWPs), subsequently adsorbed once with CWPs, and run in duplicate on each plate. Similarly, QC, calibration, and maternal sera were diluted (1:300) with PBST + CWPs that included 20 μg/ml 22F Ps (PBST + CWPs + 22F Ps). All samples were serially diluted 2-fold seven times in respective buffers in a non-ELISA 96-well plate. The diluted sera (100 μl) from each well were transferred to the corresponding well on an ELISA plate coated with 5 μg/ml Pn St-specific capsular Ps. The plates were incubated for 2 h at room temperature and washed four times with 100 μl PBST. Horseradish peroxidase-conjugated goat anti-human...
IgG (1:1,500 dilution in PBS with Tween 20) was added to each well (100 μl) as a secondary antibody, followed by incubation and a washing procedure. After 1 h of incubation at room temperature and four washes, 100 μl/well TrueBlue peroxidase substrate (TMB Sureblue; KPL) was added to initiate a peroxidase-catalyzed color reaction. After 15 to 20 min, the reaction was stopped with hydrochloric acid (1 N HCl) and color intensity was measured in an ELISA reader (ELx 800; BioTek). KC Junior software (BioTek) was used for data acquisition and ELISA3.2 (CDC, Atlanta, GA) for data analysis.

**MBIA for Pn Ps.** Purified capsular Pn Ps (ATCC, Manassas, VA) and CWPss (SSI, Denmark) were activated and conjugated to fluorescent beads with minor modifications of previously published techniques (5, 11). Primary modifications pertained to the amount of Ps conjugated to the microsphere (25 μg/ml to 50 μg/ml, St dependent) and conjugation time (overnight at room temperature for all Sts). Dilutions and preadsorption of standard, 89SF, QC, calibration, and maternal sera were carried out similarly to ELISA. After optimization studies with concentrations of CWPss that ranged from 20 μg/ml to 100 μg/ml, preadsorption buffer was supplemented with 20 μg/ml CWPss. Fifty microliters/well of the bead mix (5,000 beads/St/well) was transferred to appropriately labeled prewet multiscreen plates, and the plates were aspirated gently with a vacuum aspirator. Serum dilutions (50 μl/well) were transferred to a filter plate and incubated for 1 h at room temperature with agitation at 150 rpm. After incubation, the plates were washed 3 times with 100 μl/well assay buffer. Fifty microliters/well of phycoerythrin-labeled goat anti-human IgG reporter antibody (Jackson ImmunoResearch, West Grove, PA) was added and incubated for 1 h at room temperature with agitation at 150 rpm. After three washes with assay buffer, beads in the filter plate were resuspended in 75 μl/well assay buffer and read in the Luminex 100 reader. IgG concentration was calculated with the 89SF standard using the Masterplex QT 3.2 program (MiraBio, San Francisco, CA).

**Statistical methods.** Statistical analyses were performed using SigmaStat version 3.5, software (Systat Software, Inc., Chicago, IL). Additional descriptive statistics were determined using Excel 2010 (Microsoft Inc., Seattle, WA). Each sample was run in duplicate on each plate. Coefficient of variance (CV) between duplicate wells and between sample dilutions was monitored to accept or reject a sample or plate for both ELISA and MBIA. Once the plate was run, we looked at the CV for concentrations determined between dilution wells, with a cutoff of 20%. Nonparallel dilutions, where the CV between the duplicates was >20% for unknowns, were edited out. If a sample had >3 nonparallel dilutions, it was rejected. In a test run, if the QC failed, the plate was summarily rejected and the test repeated. A nonparallel reference standard dilution was the major reason for plate rejection in the ELISA, and this was ≤5% for 6 Sts except St 6B, for which it was 20%. The reason for the higher plate rejection rate for St 6B was improper plate coating. Addressing this with a new Ps batch brought down the plate rejection rate on par with the other 6 Sts. Similarly, for MBIA, Sts 18C and 23F had higher plate failure rates (10% and 33%, respectively) due to Ps-bead conjugation errors. The rate of sample rejection, based on parallelism acceptance criteria, was 11% to 20% for both ELISA and MBIA. Among the Sts tested, the highest variation was observed for Sts 14 and 23F (20%) with ELISA.

The in case of MBIA, Sts 19F and 23F (20%) exhibited higher variation, followed by Sts 4 and 14 (19%). We used the Westgard 1s rule as a guideline for run acceptance and rejection decisions. In a Levey-Jennings chart, control limits were set at ±2 standard deviations (SD) from the mean IgG concentration for the respective St. A run was rejected when a single control measurement exceeded the ±2-SD control limit. The limit of detection (LOD) and limit of quantitation (LOQ) for MBIA were calculated using the method published by Lal et al. (5). Blank median fluorescence intensities (MFIs) for respective Sts were averaged from 30 test runs, and a mean + 2 SD was calculated for each St. The St-specific IgG concentration (pg/ml), corresponding to the MFI (mean + 2 SD), was set as the LOD for the respective St. The LOQ for individual Sts was set as 2× the LOD.

**Compliance with WHO and FDA guidelines.** To ensure compliance with the FDA and WHO guidelines, and to demonstrate the accuracy of the MBIA, we tested it against a panel of 12 WHO reference or calibration sera with the 89SF standard and compared the results to a set of consensus IgG concentrations published previously. The WHO serum panel was tested for Pn St 4-, 6B-, 9V-, 14-, 18C-, 19F-, and 23F-specific IgG simultaneously with MBIA (7-plex) and individually with ELISA. New assay methodology is considered acceptable by the FDA and WHO guidelines if ≥75% of the serum samples tested provide values that are within ±40% of published target values (i.e., assigned IgG concentrations) for a specific St (9, 12).

**Correlation of MBIA with ELISA.** Adult serum from immunized mothers was evaluated for anti-Pn Ps-specific IgG to Sts 4, 6B, 9V, 14, and 19F using both ELISA and MBIA. To help minimize the cost and laboratory effort for providing a valid comparison between the ELISA and MBIA platforms, 5 rather than 7 Sts were selected for this analysis. Since the purpose of the study was to evaluate the level of agreement between the two assays, we randomly selected these five Pn Ps sera for testing. The relationship between MBIA and ELISA was deduced and r² discussed.

**Test for MBIA robustness.** QC sera were prepared from plasma units obtained from adult donors 20 to 45 years of age. The donors were vaccinated with a 23-valent Pn Ps vaccine, and serum was collected 30 days postvaccination. Five different QC sera were used in a run consisting of high, medium, and low antibody responders with a single QC sample on each plate. QC sera were used to monitor the day-to-day performance of the MBIA and assay acceptance. A Levey-Jennings chart was generated with Pn St-specific IgG concentrations on different days over a period of 25 days for QC1 and QC3.

**Test for MBIA reproducibility.** The reproducibility of the MBIA was determined by calculating the intraplate percent CV obtained for 89SF during a single week. A total of 200 samples were run as duplicates, with 8 dilutions in each plate assay. The mean percent CV was calculated for each of 9 Pn Sts during this period.

**RESULTS**

**Ps beads conjugation efficiency.** Serotype-specific Pn Ps beads were effectively conjugated and revealed consistently high MFIs (>10,000) for 89SF (1:20 dilution). The conjugation efficiency was monitored based on the consistency and nondecay of signal in terms of MFI and between-well CVs for 89SF over a 3-year period (2008 to 2010). Data pertaining to five Pn Sts are presented in Table 1. The CVs were consistently low for all 5 Sts, with the most recent batch of beads (year 2010) providing the lowest CVs for Sts 4, 6B, and 14.

**Compliance with WHO and FDA guidelines.** A panel of 12 WHO reference or calibration sera (i.e., Goldblatt sera) were tested for 7 Pn St-specific IgG using the MBIA (7-plex) and com-
FIG 1 Scatter plot to deduce the correlation between MBIA and WHO ELISA anti-pneumococcal polysaccharide IgG assignments for the WHO International pneumococcal calibration serum panel (12 sera). x axis, log_{10} of MBIA anti-pneumococcal serotype-specific IgG concentration (μg/ml); y axis, log_{10} of WHO ELISA anti-pneumococcal serotype-specific IgG concentration (μg/ml) assignments. The solid line represents the linear regression trend line and the dashed line the line of identity. Values for serotype 14 are not shown, as the $r^2$ was ≥0.99. Serotype 14 exhibited the highest correlation ($r^2 > 0.99$), followed by serotypes 19F and 23F ($r^2 = 0.94$ and $0.91$, respectively). Serotype 18C exhibited poor correlation, with the $r^2$ at 0.488.
TABLE 2 MBIA compliance with WHO assignments for pneumococcal serotypes 4 and 6B$^a$

| WHO panel | Concn (µg/ml) of IgG to: | Serotype 4 | Serotype 6B |
|-----------|------------------------|-----------|-----------|
|           | WHO assigned value      | A   | B   | C   | MBIA assigned value | A   | B   | C   |
| 730       | 7.2                    | 10.71| 13.66| 12.08| 4.9                | 5.44| 5.1  | 4.14 |
| 734       | 9.7                    | 7.72 | 14.99| 8.09 | 2.2                | 1.89| 1.2  | 1.41 |
| 738       | 2.3                    | 2.5  | 2    | 2.6  | 12.9               | 10.5| 11.9 | 11.1 |
| 742       | 6.2                    | 4.69 | 4.58 | 3.74 | 9.9                | 11.9| 11.7 | 11.98|
| 744       | 1.8                    | 0.74 | 1.2  | 1.54 | 23.3               | 17.2| 16.7 | 8.04 |
| 732       | 9.7                    | 12.54| 13.29| 11.45| 10.2               | 10.7| 12.7 | 22.45|
| 734       | 14.6                   | 11.42| 15.6 | 16.76| 3.3                | 2.29| 8.1  | 4.49 |
| 760       | 2.3                    | 2.56 | 2.6  | 2.73 | 2.1                | 0.97| 1.26 | 1.41 |
| 764       | 4                      | 5.22 | 5.3  | 4.41 | 23.2               | 45.23|73.8 |63.81 |
| 768       | 0.7                    | 0.43 | 0.29 | 0.52 | 2.5                | 2.54| 2.5  | 2.24 |
| 770       | 2.5                    | 2.53 | 2.2  | 1.31 | 8.3                | 7.93| 11.6 |10.98 |

$^a$ MBIA values are from 3 individual runs (A, B, and C). For serotype 4, 83, 75, and 92% of the values for runs A, B, and C, respectively, fell within ±40% of the assigned value; for serotype 6B, 75, 75, and 75% of the values for runs A, B, and C, respectively, fell within ±40% of the assigned value.

TABLE 3 MBIA compliance with WHO assignments for pneumococcal serotypes 9V and 14$^a$

| WHO panel | Concn (µg/ml) of IgG to: | Serotype 9V | Serotype 14 |
|-----------|------------------------|-----------|-----------|
|           | WHO assigned value      | A   | B   | C   | MBIA assigned value | A   | B   | C   |
| 730       | 1.5                    | 0.76 | 0.65 | 0.68 | 66.10 | 14.59| 9.7  | 11.39|
| 734       | 7.7                    | 6.8  | 6.3  | 7   | 322.10| 392.67|450  |371.34|
| 738       | 3.2                    | 2.81 | 4.3  | 2.05| 18.40 |65.4  |16.5  | 18  |
| 742       | 2.1                    | 2.36 | 2.3  | 2.24| 7.40  |6.88  |4.4   | 4.41 |
| 744       | 10.9                   | 3.29 | 8.7  | 6.95| 3.70  |2.54  |3.31  | 3.66 |
| 748       | 4.2                    | 5.28 | 3.7  | 2.65| 10.60 |9.9   |12.3  | 9.95 |
| 752       | 17.8                   | 18.8 | 60.3 | 65.16| 27.80 |39.4  |98    |85.06 |
| 754       | 15.8                   | 10.84| 21.7 | 39.37| 160.80|102.4 |156.8 |214.99|
| 760       | 1.0                    | 1.02 | 0.89 | 1.35| 19.20 |21.21 |30.8  |19.75 |
| 764       | 8.3                    | 4.99 | 7.2  | 5.11| 17.2  |10.91 |12.2  | 11.99|
| 768       | 4.6                    | 2.77 | 3.9  | 3.61| 14.00 |7.77  |10.2  | 8.95 |
| 770       | 4.2                    | 4.1  | 2    | 3.19| 115.60|134.86|146.46|

$^a$ MBIA values are from 3 individual runs (A, B, and C). For serotype 9V, 75, 75, and 75% of the values for runs A, B, and C, respectively, fell within ±40% of the assigned value; for serotype 14, 75, 75, and 83% of the values for runs A, B, and C, respectively, fell within ±40% of the assigned value.

TABLE 4 MBIA compliance with WHO assignments for pneumococcal serotypes 18C and 19F$^a$

| WHO panel | Concn (µg/ml) of IgG to: | Serotype 18C | Serotype 19F |
|-----------|------------------------|-----------|-----------|
|           | WHO assigned value      | A   | B   | C   | MBIA assigned value | A   | B   | C   |
| 730       | 3.2                    | 2.54 | 2.23 | 1.99 | 9.7                | 9.1 | 11.5 | 6.47 |
| 734       | 6.8                    | 7.68 | 34.7 | 19.39| 11.3               | 7.4 | 15.49| 8.98 |
| 738       | 6.1                    | 5.4  | 7.2  | 5.9  | 2.5                | 2.1 | 2    | 2.35 |
| 742       | 11.5                   | 7.22 | 8.1  | 7.35 | 11.5               | 6.2 | 6.9  | 8.06 |
| 744       | 9.5                    | 7.16 | 9.1  | 9   | 9.5                | 2.97| 3.2  | 8.5  |
| 748       | 10.6                   | 10.3  |10.89 |12.87| 16.5               | 17.8|34.6  |23.96|
| 752       | 9.9                    | 9.6  | 37.6 | 32.5 | 64.1               | 131.1|215  |190.52|
| 754       | 4.9                    | 4.37  |9.9  | 6.38 | 14.1               | 8.55|19.2 | 19.1 |
| 760       | 3.3                    | 2.82  |4.4  | 2.84 | 6.8                | 7.7 | 7.6  | 9.28 |
| 764       | 6.4                    | 23.34 |5.45 | 18.41| 21.7               | 30.19|30.1 |35.97|
| 768       | 1.5                    | 1.78  |1.15 | 1.62 | 3.5                | 3.2 | 2.6  | 3.43 |
| 770       | 2.8                    | 3.41  |1.69 | 1.83 | 7.3                | 10.12|9.38 |10.46|

$^a$ MBIA values are from 3 individual runs (A, B, and C). For serotype 18C, 92, 75, and 75% of the values for runs A, B, and C, respectively, fell within ±40% of the assigned value; for serotype 19F, 75, 75, and 75% of the values for runs A, B, and C, respectively, fell within ±40% of the assigned value.

nized mothers was evaluated for anti-Pn Ps-specific IgG to Sts 4, 6B, 9V, 14, and 19F to evaluate the correlation between our in-house WHO ELISA and the bead-based multiplex assay. Table 6 indicates a linear relationship in the Pn Ps St-specific IgG concentrations when measured by ELISA and MBIA, as well as demonstrating a statistically significant positive relationship. The $r^2$ values were consistently high (≥0.84), with no significant difference ($P ≥ 0.528$) among the 5 Pn Sts. In addition, this positive relationship provides the necessary statistical strength for MBIA to predict the protective antibody levels similarly to ELISA as defined by the WHO in test subjects (Table 7).

Test for MBIA robustness. Robustness was tested on two counts. Quality control 1 and QC 3 were tested every day for a period of 25 consecutive days and the anti-Pn St 4, 6B, 9V, 14, and 19F IgG concentrations plotted in a Levey-Jennings chart (data...
not shown). The assay is robust for IgG values among all tested Sts in QC 1 and QC 3 and, with one exception, fell within ±2 SD of the expected values. Additional robustness testing with different operators and reagent lots is in progress as a part of an extensive validation process.

Test for assay reproducibility. The reproducibility of the MBIA was determined by calculating the intraplate CV obtained for the 89SF MFI during a single week. The results are shown in Fig. 2. All CVs were less than 16% for any given day, with the mean intraplate CV per St ranging from 6.2% for Sts 18C and 23F to 9.1% for St 5. Additional 89SF CV data were analyzed over a period of 3 years for MFI. During this time, the CVs for each of the nine serotypes measured were <9%.

**DISCUSSION**

This study describes the development and characterization of a multiplex bead-based immunoassay (MBIA) to quantify Pn St-specific IgG. The MBIA used in this clinical study was able to provide a sensitive, efficient, and high-throughput analysis of a large number of serum samples from both infants and adults. The reproducibility of the multiplex assay values was evaluated daily and over a 3-year period. The intra-assay variability for 89SF was consistently low, with CVs of less than 8.93% for any given St on any given day. These results suggest that the multiplex assay shows good precision in measuring St-specific anti-Pn IgG. To further assist in monitoring the day-to-day consistency of the multiplex assay and to help normalize the results, a QC panel of 5 sera containing low-, medium-, and high-titer anti-Pn antibody was run daily on each plate. The fact that 3 different preparations of conjugate beads were used during the analysis period also provided evidence that batch-to-batch variation during bead conjugation was minimal, with no significant impact on assay results.

The MBIA platform introduces a number of improvements compared to ELISA. The multiplex assay is valuable in situations where small volumes of serum (5 to 10 μl) are available for analyzing large multiples of Sts, as was the case in this study. These volumes of serum are much less than what is needed to obtain similar results using the ELISA (25 to 50 μl) (10, 11, 13). In our hands, the MBIA provided a 5-fold reduction in the required amount of serum and a 29-fold reduction in the required amount of Ps. Other studies have reported reductions as high as 25- and 200-fold for serum and Ps, respectively (8). Multiplexing of serum samples for IgG antibody also reduces the assay time required for each analyte by 80% and markedly increases sample throughput (4, 5, 7). In contrast to ELISA, the multiplex technology allows for the simultaneous analysis of as many as 100 distinct antigens in a single microtiter well and the potential for high-throughput screening of up to 1,000 sera per day (16). Finally, in terms of cost, the multiplex assay is approximately seven times less expensive than the ELISA for analyzing five Sts from each serum sample.

In our study, assessment of the maternal antibody response in the multiplex assay indicated that serum samples with high concentrations of nonspecific, cross-reactive antibodies produced additional fluorescent signals that were confounding and often masked the true type-specific antibody response to capsular Ps. It is possible that much of this background noise may be attributable to the sensitivity of the multiplex assay. One approach for eliminating this problem is to develop chemically assayed true standards. Another approach involves improving the quality of commercially available Pn Ps antigen as close to reagent grade as possible and eliminating the use of clinical-grade or vaccine grade material as a target for *in vitro* immunoassays.

Due to the presence of variable amounts of CWPs and protein contaminants associated with each Pn St as well as variations in the production of anti-CWPs antibody by individual donors (5, 14), it has been nearly impossible to create assay conditions in...
which all of the confounding antibody effects have been removed across all possible antibody levels and from all donors. The effect of CWPs antibodies on a given serum IgG measure is dependent on what St is being tested, the time allowed for CWPs adsorption, and a patient’s CWPs antibody level. Because the concentration of CWPs can be extremely high in some situations, the ability to adsorb out all of the contaminants is often limited regardless of the amount of time and adsorbent used in the procedure. Samples with low levels of IgG antibody to Ps can be drastically reduced by overly long CWPs adsorption, and samples with high CWPs antibody levels can demonstrate very high Ps IgG levels where the CWPs are not completely removed by adsorption. In situations where CWPs has been used to adsorb out nonspecific activity, it is still possible to have up to 25% anti-CWPs remaining in the sera (11, 17). It is also possible that adsorption with high concentrations of CWPs may have little effect on reducing nonspecific antibody activity due to the presence of other contaminating cell wall factors.

Validation of a new serological assay is often challenged by the requirements to meet the assigned antibody titer obtained with an alternate and highly regarded technique. While this is highly plausible when comparing the reliability and usefulness of a technique with similar chemistry and reaction dynamics, it becomes a real challenge for techniques with clear differences in their operating principles. This is further compounded by the need to meet historical IgG assignments that were generated 15 to 20 years ago. Realizing this challenge, previous studies attempted to demonstrate a correlation between an in-house ELISA and an in-house multiplex bead-based assay with a set of fresh in-house sera as a surrogate to “true” validation. Despite these well-known differences, regulatory authorities insist on meeting the historical ELISA assignments with the newly developed serological technique as the primary validation criteria for Pn Ps IgG quantification (3, 12). In this background, we have made an attempt to meet the historical WHO Pn St-specific IgG assignments for 12 international calibration sera and have been successful in our efforts. MBIA has returned IgG concentrations for all seven Pn Sts within the compliance criteria (Tables 2 to 5). In short, the MBIA data presented here met the WHO qualification guidelines, with 75 to 92% of the serum panel showing strong agreement to preestablished WHO values and falling within the acceptable 40% error range. Many other assay protocols published in the literature have failed to meet the qualification criteria for the same Pn Sts evaluated in this study (1, 2, 6, 19).

Recently, Whaley et al. (18) compared an MBIA-like assay with two other multiplex assays. They examined several different laboratory parameters, including assay accuracy, where the results from each platform were compared against the assigned values of the 12 WHO reference sera. All three assays performed in a similar fashion as determined by comparing their individual mean IgG concentrations to the WHO assigned reference serum values. Furthermore, all three assays had significantly wide spreads for CVs, and none of them reached total agreement with the WHO-recommended values. In fact, only the MBIA-like assay had any Sts that met the WHO requirement of at least 75% of the samples tested returning values within the 40% error range. On the other hand, Flow Applications, Inc., has developed a multiplex assay platform that successfully demonstrated concurrence with the WHO criteria for 7 Pn Sts associated with the first licensed Prevnar vaccine (Wyeth Lederle, NY).

Other comparative results in our study showed a highly significant linear relationship and equivalence of the anti-Pn IgG concentrations when measured by the WHO in-house ELISA and MBIA using 89SF as a standard. Additional correlation values were observed between the two assays based on sera obtained from pregnant women vaccinated with a 23-valent Pn Ps vaccine as part of a randomized, double-blind, placebo-controlled clinical trial (20). The results showed excellent agreement between the two assays, with r² values ranging from 0.84 to 0.97 for the 5 Sts tested. This relationship is critical when attempting to bridge an unknown multiplex assay with a standardized and validated ELISA, the latter being pivotal in providing serological data for the licensure of the 7-valent Pn conjugate vaccine (3). The high level of agreement between the ELISA and MBIA further suggests that following additional characterization and complete validation of the MBIA, it might be possible for the two assays to be used interchangeably when measuring the immune response to various Pn vaccines in a clinical setting.

To conclude, the MBIA represents a comprehensive multiplex assay system that can be applied to virtually any application that requires analysis of anti-Ps antibody at both the basic and clinical levels. Assay parameters such as the FDA 89SF, WHO International calibration serum panel, in-house QC sera, and pre- and postvaccination immune sera indicate that the MBIA has good precision, accuracy, and reproducibility, the three attributes critical for any assay system. The intra-assay variation for 89SF, when measured over a 3-year period, was very low for all Sts (<9%) and in some cases 1% or less, which again is indicative of a highly reproducible assay. Most importantly, the MBIA met the requirements for assay qualification based on the FDA- and WHO-preestablished criteria for concordance.

Many factors influence the success of a multiplex anti-Pn IgG assay. These include (i) CWPs and protein contaminants and their concentration in the target antigen; (ii) assay conditions; (iii) serum antibody composition (e.g., specific versus nonspecific, IgG versus IgM, and high versus low affinity) in each donor sample; (iv) the Sts being tested; (v) the Ps conjugation procedure; (vi) the extent to which confounding variables, such as CWPs antibodies and antibodies to cell wall proteins, are removed from sera; and (vii) the assay protocol and the data analysis process used in the study. While the multiplex assay offers clear-cut advantages over ELISA, careful optimization of the various above-cited factors is required for its clinical use. Careful optimization, stringent validation, and continuous monitoring are the most critical factors for the clinical exploitation of this unique multiplex, high-throughput serological technique, MBIA.

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