Comparison of a New Multiplex Binding Assay versus the Enzyme-Linked Immunosorbent Assay for Measurement of Serotype-Specific Pneumococcal Capsular Polysaccharide IgG

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The measurement of serotype-specific anti-capsular polysaccharide antibodies remains the mainstay of pneumococcal (Pn) vaccine evaluation. New methods that allow the simultaneous measurement of antibodies to several antigens in small volumes of serum, and that agree well with existing techniques, are urgently required to support the increasing number of concomitant vaccines delivered in the infant immunization schedules and the use of extended-valency Pn vaccines. We therefore compared a relatively new multiplexed platform for measuring anti-Pn antibodies with the existing WHO consensus enzyme-linked immunosorbent assay (ELISA). A panel of 50 pediatric samples (34 collected after receipt of a heptavalent pneumococcal conjugate vaccine [PCV7] and 16 without PCV7) was analyzed across two different laboratories using a new multiplex electrochemiluminescence (ECL)-based detection assay developed for the quantitation of IgG serotype-specific antipneumococcal antibodies, and the results were compared to those obtained using the WHO consensus ELISA. For the seven serotypes measured, there was good agreement between the techniques and laboratories. The most notable difference was found between the ECL assay and the ELISA: concentrations surrounding 0.35 g/ml were in excess of 92%, and agreement on average antibody concentrations was within 31%.

We conclude that the Meso Scale Discovery (MSD) platform provides a promising new technique for the simultaneous measurement of antipneumococcal antibodies.

Antibodies specific for the capsular polysaccharide of Streptococcus pneumoniae are critical for protection against infection with the pneumococcus and are thus the key parameter measured for assessment of the performance of vaccines designed to prevent pneumococcal infections. During the development phase of pneumococcal conjugate vaccines (PCV), discussions on standardizing the measurement of IgG specific for the capsule, by following the same process used for the standardization of Haemophilus influenzae type b (Hib) assays, had already been initiated (6). Initial efforts to define a standard assay were superseded by the licensing of the first PCV on the basis of efficacy and the accompanying serology performed in the laboratories of Wyeth (2). Subsequent international efforts were therefore focused on establishing guidelines for the performance of a pneumococcal enzyme-linked immunosorbent assay (ELISA) that could match the data obtained by the Wyeth ELISA (11, 15). The importance of matching the data obtained with the Wyeth assay was underlined with the publication of correlates of protection derived from three efficacy studies, each of which had antibodies measured by the Wyeth assay (4, 13). These correlates were incorporated into guidelines for licensing new vaccines that rely on assessment of the proportions of samples achieving antibody concentrations above the protective threshold of 0.35 μg/ml by the World Health Organization (WHO) reference ELISA (16a). The guidelines also state that it may be acceptable for manufacturers to employ an alternative threshold value when using a specific in-house assay, provided it can be demonstrated by a well-conducted bridging study to correspond to an IgG concentration of 0.35 μg/ml in the WHO reference ELISA (17, 18). Therefore, it is critical to use assays that are bridged and are comparable to the original Wyeth assays.

More recently, as evaluation of extended-valency vaccines containing 10 or 13 serotypes has been required, the labor-intensive ELISA has come under scrutiny. The need for serological analysis of vaccines administered concomitantly has meant that currently, from a single infant blood sample, as many as 25 separate assays may be required during a vaccine
trial. Clearly, less labor-intensive, assays with faster throughput, that require less sample volume are required. To this end, multiplexing of pneumococcal assays has been explored.

The first assay to be described involved the simultaneous analysis of 14 serotypes utilizing fluorescent beads with different combinations of fluorochromes bound individually to the pneumococcal serotypes (10). Subsequent descriptions have extended the application to 22 (9) and 23 (1) serotypes, and while the fluorescent-bead method has numerous benefits over the ELISA used to measure the levels of IgG specific for pneumococcal capsular polysaccharides (PnPs), including increased speed, smaller sample volumes, equivalent or better sensitivity, and increased dynamic range, concern remains about the agreement between the two assays at the critical low end of the determination range (5).

More recently, a solid-phase assay based on electrochemiluminescence (ECL), which permits the simultaneous detection of IgG specific for as many as 10 pneumococcal serotypes, has been described (7). ECL-based techniques provide an alternative to conventional colorimetric methods, allowing high sensitivity, good reproducibility, and generally low levels of interference from components in complex matrices, such as serum or plasma. This Pn ECL assay is based on the Meso Scale Discovery (MSD) technology, which employs disposable multiplex microtiter plates (multiarray plates; MSD, Gaithersburg, MD) that include integrated screen-printed carbon ink electrodes on the bottoms of the wells. In contrast to other multiplex platforms, such as the Luminex platform, the ECL technology is advantageous in that it does not require conjugation of the PnPs to the solid phase, since the Ps bind directly to the carbon surface, minimizing the potential impact on Pn antigenicity. In this sense, the ECL platform resembles the WHO reference ELISA more closely than it resembles other multiplex platforms (15).

The purpose of this study was to compare three assays for the detection and quantitation of anti-Pn IgG antibodies (Ab) to types 4, 6B, 9V, 14, 18C, 19F, and 23F. The three assays were (i) the Merck Pn-8 ECL assay, performed by PPD Vaccines and Biologics (Wayne, PA) on behalf of Merck Sharp & Dohme Corp.; (ii) the WHO ELISA, performed in the laboratory of David Goldblatt at the Institute of Child Health (ICH), London, United Kingdom; and (iii) the ICH Pn-8 ECL assay, also performed at the ICH under the direction of David Goldblatt.

The primary objectives of this study for each of the serotypes evaluated were (i) to assess the concordance between the three antipneumococcal antibody assays, (ii) to assess the serostatus agreement between these assays based on a serostatus cutoff of 0.35 pg/ml, and (iii) to estimate the assay variability and precision (relative standard deviation [%RSD]) for each assay method. This is the first study to compare the ECL assay with the WHO reference ELISA. The Merck and ICH Pn-8 ECL assays followed the same protocol.

MATERIALS AND METHODS

CPS. C polysaccharide (CPS) is a pneumococcal cell wall polysaccharide obtained from the Statens Serum Institut, Copenhagen, Denmark.

PnPs. All PnPs powders for serotypes 3, 4, 6B, 9V, 14, 18C, 19F, 23F, 25, and 72 were manufactured and received from Merck Manufacturing Division, West Point, PA. The PnPs powder for serotype 22F was obtained from the American Type Culture Collection (ATCC). Each PnPs was reconstituted in sterilized pyrogen-free water. The final concentration for each PnPs following reconstitution was 1 mg/ml. PnPs serotype 25 (PnP25) and PnP45 (PnP72) are utilized in the Pn ECL assay for serum preadsorption in order to improve assay specificity.

Sera for standard. The U.S. FDA Pn reference standard, lot 89SF-2, was prepared by Lederle-Praxis Biologicals from 17 individual high-titer sera from adults following vaccination with Pnu-Imune (a 23-valent Pn vaccine from Lederle), Menomune (a meningococcal polysaccharide vaccine; Connaught), and ProfHib (a Haemophilus influenzae conjugate vaccine; Connaught).

Pediatric serum samples. The concordance between the 3 assays was assessed using 50 pediatric samples (from 34 infants immunized with at least one dose of Prevnar [the pneumococcal 7-valent formulation] and 16 naive infants). The immunized infants were 7-month-olds from the United States, and the naive subjects were from Finland and were 7 months old or younger. Each of the 50 samples was tested across 3 runs in the Merck Pn ECL assay, 3 runs in the ICH Pn ECL assay at the ICH, and 2 runs in the WHO reference ELISA.

Overview of the Meso Scale Discovery assay method. The Meso Scale Discovery (MSD) technology is based on ECL detection utilizing a Sulfo-tag label that emits light upon electrochemical stimulation. The mechanism for the generation of ECL from ruthenium tris(bipyridine) complexes at an oxidizing electrode in the presence of tripropylamine (TMA) read buffer is described previously (3). Using a dedicated ECL plate reader, an electrical current is placed across the plate-associated electrodes, resulting in a series of electrically induced reactions leading to a luminescent signal. The multiplex configuration used in development and validation was 10 spots/well in a 96-well plate format. Each well was coated with 5 ng per spot (unless specified otherwise for optimization studies). Each PnPs (s, 4, 6B, 9V, 14, 18C, 19F, 23F) was bound to separate wells and contained two bovine serum albumin (BSA) spots, which were used to assess the background reactivity of the assay (i.e., the response associated with serum and a labeled secondary antibody in the absence of PnPs). The assay standard (89SF-2), controls, and test sera were diluted at appropriate dilutions in phosphate-buffered saline (PBS) containing 0.05% Tween 20, 1% BSA, 5 µg/ml CPS, 10 µg/ml Pn25, and 10 µg/ml PnP45 (72) and were incubated overnight at 4°C (2 to 8°C) or at ambient temperature for 45 min. Each antigen-coated plate was incubated at ambient temperature for 1 h on a shaker platform with a blocking agent. Plates were washed with 0.05% PBS-Tween (PBS-T), and 25 µl per well of theeadsorbed and diluted test sera was added and incubated for 45 min at ambient temperature on a shaker platform. Plates were washed with 0.05% PBS-T; an MSD Sulfo-tag-labeled-antihuman IgG secondary antibody was added to each well; and the mixture was incubated for 1 h at ambient temperature on a shaker platform. Plates were washed with 0.05% PBS-T, and 50 µl of MSD read buffer T (4°C) (with surfactant) diluted 1:4 in water was added to each well. The plates were read using an MSD sector imager, model 2400 or 6000. The concentrations of antibodies in test samples were determined by referencing their ECL responses against a standard curve generated from the sera diluted 89SF-2 reference serum.

Overview of the WHO ELISA method. The WHO ELISA method employed by the WHO has been described previously and can be accessed online at http://www.vaccine.ub.edu/ELISAProtocol/89SF-Pdf.pdf. Briefly, medium-binding 96-well microtiter plates (Greiner) were precoated with various concentrations of purified ATCC pneumococcal polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Human sera were absorbed with 10 µg/ml CPS (Statens Serum Institut) and 5 µg/ml serotype 22F capsular polysaccharide (American Type Culture Collection) for 30 min at room temperature to neutralize antibody binding to CPS and other contaminants found in the pneumococcal polysaccharide coating antigens. International reference standard 89SF-2 (distributed by the U.S. Food and Drug Administration, Bethesda, MD) was absorbed with 10 µg/ml CPS alone (12). Serial dilutions of absorbed sera were added to Ps-coated plates. Serotype-specific antibody was detected using an alkaline phosphatase-labeled goat anti-human secondary antibody (Biosource), followed by addition of the substrate, p-nitrophenyl phosphate (Sigma). The optical density of each well was measured at 405 nm and 620 nm (reference) using an ELISA plate reader. The optical density of each sample well was compared to that of the standard in order to determine the concentration of antibody in the human serum by using a 4-parameter logistic (4PL) model.

Assay concordance experiments. The objective of the concordance experiment was to assess the concordance of the Pn ECL assay concentrations with those generated by the international WHO ELISA for a set of 50 samples from pediatric subjects. Each of the 50 samples was tested in duplicate across three independent runs by each Pn ECL assay (the Merck and ICH assays) at the 1:1,000 dilution. Each of the 50 samples was also tested in duplicate within each of two independent runs by the WHO Pn ELISA. The experiments were per-
formed at two separate laboratories: the ICH and PPD. Concordance among the three assays was assessed for serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Because serotype 3 was measured by the ECL assays and not by the WHO ELISA, concordance for serotype 3 was assessed between the Merck and ICH ECL assays but not between the ECL assays and the ELISA.

Statistical methods. (i) Standard-curve modeling. For the WHO ELISA and the Pn ECL assays, concentrations of antibodies in test samples were determined by referencing the response of the samples against a standard curve generated from the serially diluted 89SF-2 reference serum. For the Pn WHO ELISA, the standard curve was fit using the 4PL regression function. For the Pn ECL assay, the mean of the reference serum dilation–response curve was modeled using the 4PL regression function, and the corresponding variance was modeled using the power of the mean variance function (8). The value of the variance function parameter was fixed at 0.9. The Pn ECL assay standard-curve fit was carried out using the NLIN procedure in SAS.

(ii) Quantitative concordance between assay methods. Quantitative concordance between assay methods was assessed separately for each serotype. For each sample, an “average” concentration (either a geometric mean concentration [GMC] or a median concentration) was determined across the runs within each assay method, and all interassay comparisons were performed on the averaged antibody concentrations. The GMC was used in the interassay comparisons when all of the results for a sample were within an assay’s quantifiable range; otherwise, the median concentration for that sample was used.

Differences between assay methods were assessed using the linear statistical relationship method (14). The fitted concordance line was used to estimate the fold difference between the Pn ECL assay and WHO ECL methods at a concentration of 0.35 μg/ml in the WHO ELISA. Additionally, an average percentage of difference between assay methods was calculated for the subset of samples that had quantifiable concentrations within each of the methods being compared.

(iii) Qualitative concordance between assay methods. Qualitative concordance between the Pn ECL assay and WHO ELISA methods was evaluated through the construction of cross-classification tables. Agreement in serostatus assignment between assay methods was assessed using a serostatus cutoff point of 0.35 μg/ml for each assay. The agreement rate between assay methods was estimated by the number of concordant samples (either positive in both assays or negative in both assays) divided by the total number of samples tested. Imbalance in the distribution of discordant samples was assessed using McNemar’s exact test. Cohen’s kappa coefficient, a measure of agreement in serostatus assignment beyond that which might occur due to chance alone, was also determined for each cross-classification table. Agreement in serostatus assignment between the Pn ECL assay and WHO Pn ELISA methods was also assessed by using the threshold value in the Pn ECL assay that corresponded to 0.35 μg/ml in the WHO Pn ELISA as determined by the fitted concordance line.

(iv) Assay variability and precision (%RSD). The precision of each assay method was assessed separately for each serotype. Test samples with quantifiable individual antibody concentrations were used to assess the assay variability and precision. Since each sample was tested once per run, across three independent runs in the Merck and ICH ECL assays and two independent runs in the WHO ELISA, the total variability within each assay (σ²) was composed of the run-to-run variability (σ²r) and the run-by-sample variability (σ²rs). Variance component estimates were obtained by applying the MIXED procedure in SAS on the individual log concentrations. The total variability within each assay was estimated by the equation σ² = σ²r + σ²rs, and the assay precision (expressed as the relative standard deviation [%RSD]) was calculated as σ²/(σ² − 1) × 100%.

RESULTS

Quantitative concordance. The averaged results (GMCs or medians) for each test sample were used to assess quantitative concordance between assay methods. Figure 1 graphically portrays the comparisons between the Merck ECL assay and the WHO ELISA by serotype. The concentrations obtained by the Merck ECL assay were about 1.15- to 2.0-fold higher than those obtained by the WHO ELISA, and the magnitude of the difference was serotype dependent. The estimates of concordance slope, average percentage of difference, and fold difference between assays at 0.35 μg/ml for the combined vaccinated and unvaccinated sample sets are summarized in Table 1. For each serotype evaluated, the difference between the Merck ECL assay and the WHO reference ELISA was fairly consistent throughout, with the concordance slopes across the seven serotypes ranging from 0.94 to 1.19. In the comparison of the ICH ECL assay with the WHO ELISA, the concordance slopes across the seven serotypes ranged from 0.92 to 1.38, and in the comparison of the ICH ECL assay with the Merck ECL assay, the concordance slopes across the eight serotypes ranged from 1.00 to 1.15. Thus, across the set of serotypes evaluated and the interassay comparisons performed, the concordance slope estimates were predominantly close to 1. As shown in Table 1, the Merck and ICH ECL assays tended to yield higher concentrations than the ICH WHO ELISA; the most appreciable increases were those for serotypes 6B, 9V, 18C, and 23F. Relative to the ICH WHO ELISA, the average increases in antibody concentration across these four serotypes ranged from 74 to 102% for the Merck ECL assay and from 48% to 94% for the ICH ECL assay. For the other three serotypes, the average difference in antibody concentration among the three assays did not exceed 38%. Table 1 also contains estimates of the fold difference in antibody concentration in the region of 0.35 μg/ml that were determined on the basis of the fitted concordance line. The fold difference estimates in the region of 0.35 μg/ml typically mirror the average fold difference estimates, which is not unexpected given the proximity of the concordance slopes to 1. Allowing for the reductions in both sample size and range of antibody concentration responses for the unvaccinated sample set, the differences between assays were fairly comparable for the vaccinated and unvaccinated sample sets.

Qualitative concordance. Serostatus agreement rates between the Merck ECL assay and the WHO ELISA, obtained by using a 0.35-μg/ml cutoff in both assays (Table 2), were ≥90% for each serotype except 6B (82%). Notably, all of the 9 discordant results for type 6B were from vaccinated subjects and were positive by the Merck ECL assay and negative by the WHO ELISA. The agreement rate over the combined set was 91.7%. For the combined set, there was statistically significant evidence of imbalance in the distribution of discordant samples: 26 samples tested negative by the WHO ELISA and positive by the Merck ECL assay, compared to only 3 samples testing positive by the WHO ELISA and negative by the Merck ECL assay. This difference is consistent with the fact that the concentrations were 1.15- to 2.0-fold higher in the Merck ECL assay than in the WHO ELISA. In the comparison of the Merck ECL assay with the WHO ELISA, Cohen’s kappa coefficient was 0.64 for serotype 6B and >0.75 for each of the other 6 serotypes, indicating that the agreement in serostatus assignment between the Merck ECL assay and the WHO ELISA far exceeds that which might be obtained by chance alone. Serostatus agreement rates were also determined between the ICH ECL assay and the other two assays by using a cutoff of 0.35 μg/ml for each assay. The overall agreement rates were 94.2% between the ICH ECL assay and the WHO ELISA and 94.3% between the ICH and Merck ECL assays (data not shown for comparisons of the ICH ECL assay with the other two assays).

Serostatus agreement rates between the Merck ECL assay and the WHO ELISA were also assessed using the threshold value corresponding to 0.35 μg/ml in the WHO reference ELISA as the Merck ECL assay cutoff and 0.35 μg/ml as the
FIG. 1. Comparisons of the concentrations of antibodies to the 7 serotypes contained in Prevnar that were found in test samples from either naïve or Prevnar-vaccinated individuals by the Merck ECL assay versus the ICH WHO reference ELISA.
WHO ELISA cutoff (Table 3). The agreement rate obtained by using the calculated Merck ECL assay threshold value was >90% for each serotype and 94.6% over the combined set. Thus, by applying threshold values in the Merck ECL assay that correspond to 0.35 \( \mu \text{g/ml} \) in method 2. The threshold value was determined by using the estimated linear statistical relationship between method 1 and method 2.

\[ \text{Threshold value} = 0.35 \times \left( \frac{\text{g/ml in method 2}}{\text{g/ml in method 1}} \right) \]

Assay variability and precision (%RSD). For each sample with quantifiable concentrations, the GMCs and %RSD across the repeated tests within an assay method were calculated. Figure 2 shows the %RSD plotted against the corresponding GMC for individual samples, identified by vaccination status and assay method. When evaluated on the combined set of samples, the variability among the repeat test results for each serotype was lower in the Pn ECL assay than in the WHO reference ELISA. For the combined set of samples, the %RSD for both the Merck and ICH ECL assays was <20% for each serotype evaluated (detailed results not shown).

### Table 1. Summary of quantitative concordance results

| Serotype | Method 1 | Method 2 | Concordance slope (95% CI) | Avg difference (%) | Threshold value (\( \mu \text{g/ml} \)) | Fold difference at 0.35 \( \mu \text{g/ml} \) |
|----------|----------|----------|-----------------------------|-------------------|-------------------------------------|----------------------------------|
| Merck ECL assay | WHO ELISA | 4 | 33 | 1.05 (0.94, 1.18) | 15.3 (−3.7, 37.3) | 0.40 | 1.14 |
| 6B | 40 | 0.94 (0.84, 1.04) | 101.7 (79, 128) | 0.69 | 1.98 |
| 9V | 38 | 1.00 (0.94, 1.07) | 89.7 (72, 109) | 0.66 | 1.89 |
| 14 | 45 | 0.96 (0.89, 1.03) | 31.9 (19, 46) | 0.50 | 1.42 |
| 18C | 38 | 1.14 (1.03, 1.25) | 98.1 (71, 130) | 0.71 | 2.02 |
| 19F | 43 | 1.19 (1.07, 1.32) | 38.0 (18, 61) | 0.40 | 1.16 |
| 23F | 45 | 1.03 (0.93, 1.15) | 74.3 (50, 103) | 0.61 | 1.74 |

| Serotype | ICH ECL assay | WHO ELISA | Merck ECL assay | ICH ECL assay | 4 | 33 | 1.12 (1.05, 1.19) | 13.4 (1, 27) | 0.39 | 1.11 |
|----------|--------------|----------|----------------|--------------| 6B | 36 | 0.92 (0.8, 1.05) | 47.5 (28, 70) | 0.51 | 1.46 |
| 9V | 35 | 1.12 (1.05, 1.19) | 93.8 (73, 117) | 0.65 | 1.85 |
| 14 | 44 | 1.05 (0.99, 1.10) | 8.8 (0, 18) | 0.35 | 1.00 |
| 18C | 32 | 1.24 (1.09, 1.40) | 73.3 (42, 112) | 0.63 | 1.79 |
| 19F | 34 | 1.38 (1.23, 1.54) | 20.1 (0, 44) | 0.28 | 0.80 |
| 23F | 42 | 1.12 (1.04, 1.21) | 53.5 (36, 73) | 0.54 | 1.53 |

| Serotype | ICH ECL assay | Merck ECL assay | 3 | 36 | 1.04 (0.86, 1.27) | −30.6 (−37, −23) | 0.25 | 0.71 |
|----------|--------------|----------------| 4 | 32 | 1.11 (1.05, 1.17) | −13.1 (−22, −4) | 0.29 | 0.84 |
| 6B | 38 | 1.00 (0.91, 1.10) | −27.1 (−34, −20) | 0.26 | 0.73 |
| 9V | 37 | 1.13 (1.07, 1.19) | 0.1 (−10, 12) | 0.31 | 0.89 |
| 14 | 45 | 1.09 (1.04, 1.14) | −17.7 (−24, −11) | 0.24 | 0.69 |
| 18C | 32 | 1.13 (1.06, 1.21) | −15.9 (−25, −5) | 0.27 | 0.78 |
| 19F | 34 | 1.15 (1.08, 1.25) | −17.1 (−25, −8) | 0.23 | 0.66 |
| 23F | 41 | 1.10 (1.04, 1.16) | −14.1 (−22, −6) | 0.28 | 0.81 |

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\[ a \] Number of test samples with concentrations (within the quantifiable range) used to estimate the concordance slope and the average percentage of difference.  
\[ b \] CI, confidence interval.  
\[ c \] The percentage of difference for an individual sample was calculated as 100% \( \times \) [(concentration by method 1 − concentration by method 2)/concentration by method 1].  
\[ d \] The concentration obtained by method 1 that corresponds to 0.35 \( \mu \text{g/ml} \) in method 2. The threshold value was determined by using the estimated linear statistical relationship between method 1 and method 2.  
\[ e \] Ratio of the threshold value in method 1 to 0.35 \( \mu \text{g/ml} \) in method 2.
The purpose of this study was to compare three assays for the detection and quantitation of antipneumococcal IgG antibodies (anti-Pn Ab) and in particular to assess the level of agreement between the accepted international reference assay for measuring pneumococcal serotype-specific IgG, the so-called WHO reference ELISA, and a new technique, ECL. A considerable amount of effort has gone into standardizing and controlling the performance of the ELISA used to measure antipneumococcal antibodies. An ELISA was used in the evaluation of the first pneumococcal conjugate vaccine to be licensed in 2000 (Prevnar; Pfizer). This vaccine was licensed on the basis of efficacy (2), but serological correlates of protection were derived from this and other pivotal efficacy studies (4).

The relationship of the Merck Pn ECL method to the WHO ELISA was first assessed on the WHO pneumococcal quality control (QC) calibration panel both at Merck and independently at the ICH (7). The WHO QC panel has been used by several laboratories to compare the abilities of different assays to produce the assigned antibody concentrations within an acceptable tolerance. It consists of 12 serum samples from adult subjects with antibody concentrations assigned as determined by the WHO reference ELISA. The WHO QC panel was used to test the Pn ECL assay for the seven Prevnar serotypes, and the resulting concentrations were compared to the assigned ELISA concentrations. The results of this comparison demonstrated excellent concordance between the two assay formats. For each of the seven Prevnar serotypes, the GMCs for at least 9 of the 12 QC samples fell within ±40% of the published concentrations, thus meeting the preestablished WHO criteria for concordance (7). A study that compared three different multiplexed bead-based PnP immunoaessays, including a commercial Luminex PnP assay, to the WHO ELISA, also using the WHO QC reference panel, reported that these assays did not meet the WHO-established criteria for concordance (16).

The benefits of multiplex assays, such as chemiluminescence- or Luminex-based assays, over the ELISA methodology have been described previously for PnP assays; they include smaller sample volumes, speed, equivalent or better sensitivity, increased dynamic range, and the ability to multiplex (9, 10). Moreover, compared with the ELISA, the Pn ECL assay allows a 25-fold reduction in the volume of serum required for the reference standard and test samples (an important factor in pediatric clinical trials, of multivalent vaccines, and with concomitant administration of other vaccines) and a 200-fold reduction in the amount of polysaccharide required. Furthermore, the broader dynamic range of the Pn ECL assay has the potential to minimize sample retesting for high-concentration samples.

The study described in this report evaluated the relationship between the two assay formats on a panel of pediatric sera. Specifically, sera obtained from infants who had received at least one dose of the Prevnar vaccine and sera from naïve subjects were selected in an attempt to capture a broad range of antibody concentrations. The most notable difference among the assays was that the Merck and ICH ECL assays tended to yield higher concentrations than the ICH WHO ELISA; the most appreciable increases were those for serotypes 6B, 9V, 18C, and 23F. Despite these higher concentrations, when a serostatus analysis was done, there was good agreement between the ELISA and the ECL assays. Furthermore, the bridging study described here allows for the determination of the threshold value of the Pn ECL assay corresponding to 0.35 μg/ml in the WHO reference ELISA, thereby maintaining the link between immune responses to vaccination.

### DISCUSSION

The purpose of this study was to compare three assays for the detection and quantitation of antipneumococcal IgG antibodies (anti-Pn Ab) and in particular to assess the level of agreement between the accepted international reference assay for measuring pneumococcal serotype-specific IgG, the so-called WHO reference ELISA, and a new technique, ECL. A considerable amount of effort has gone into standardizing and controlling the performance of the ELISA used to measure antipneumococcal antibodies. An ELISA was used in the evaluation of the first pneumococcal conjugate vaccine to be licensed in 2000 (Prevnar; Pfizer). This vaccine was licensed on the basis of efficacy (2), but serological correlates of protection were derived from this and other pivotal efficacy studies (4).

ELISA measurements thus provide the critical link between the level of IgG measured in serum and the clinical efficacy of the PCV. The antibody levels are even more important now that pneumococcal conjugate vaccines are licensed on the basis of immunogenicity alone, as opposed to both immunogenicity and efficacy; thus, the technique used to measure such antibodies is critical, and the values obtained must be comparable to those from the original efficacy study. In light of this, the study described in this report assumes great importance.

It was encouraging to see that the agreement between two independent laboratories running the same ECL assay was excellent, demonstrating the reproducibility of the technology and the relative ease with which agreement can be reached between laboratories in a controlled setting.

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The benefits of multiplex assays, such as chemiluminescence- or Luminex-based assays, over the ELISA methodology have been described previously for PnP assays; they include smaller sample volumes, speed, equivalent or better sensitivity, increased dynamic range, and the ability to multiplex (9, 10). Moreover, compared with the ELISA, the Pn ECL assay allows a 25-fold reduction in the volume of serum required for the reference standard and test samples (an important factor in pediatric clinical trials, of multivalent vaccines, and with concomitant administration of other vaccines) and a 200-fold reduction in the amount of polysaccharide required. Furthermore, the broader dynamic range of the Pn ECL assay has the potential to minimize sample retesting for high-concentration samples.

The study described in this report evaluated the relationship between the two assay formats on a panel of pediatric sera. Specifically, sera obtained from infants who had received at least one dose of the Prevnar vaccine and sera from naïve subjects were selected in an attempt to capture a broad range of antibody concentrations. The most notable difference among the assays was that the Merck and ICH ECL assays tended to yield higher concentrations than the ICH WHO ELISA; the most appreciable increases were those for serotypes 6B, 9V, 18C, and 23F. Despite these higher concentrations, when a serostatus analysis was done, there was good agreement between the ELISA and the ECL assays. Furthermore, the bridging study described here allows for the determination of the threshold value of the Pn ECL assay corresponding to 0.35 μg/ml in the WHO reference ELISA, thereby maintaining the link between immune responses to vaccination.

### TABLE 3. Merck ECL assay versus WHO ELISA serostatus cross-classification

| Serotype | No. of samples tested | No. of samples with Merck ECL assay/WHO ELISA results of: | Agreement (%) | Kappa (95% CI) | P value by McNemar’s exact test |
|----------|----------------------|--------------------------------------------------------|---------------|----------------|--------------------------|
| 4        | 50                   | +/+                                                   | 96.0          | 0.91 (0.80, 1.00) | 0.500                    |
| 6B       | 50                   | +/-                                                   | 90.0          | 0.77 (0.58, 0.96) | 0.375                    |
| 9V       | 50                   | ++                                                    | 98.0          | 0.95 (0.87, 1.00) | 1.000                    |
| 18C      | 50                   | +/+                                                   | 98.0          | 0.95 (0.85, 1.00) | 1.000                    |
| 14       | 50                   | +/+                                                   | 90.0          | 0.79 (0.62, 0.96) | 1.000                    |
| 19F      | 50                   | +/+                                                   | 90.0          | 0.77 (0.57, 0.96) | 1.000                    |
| 23F      | 50                   | +/+                                                   | 100.0         | 1.00 (1.00, 1.00) | 1.000                    |

Combined

| Naïve | 112 | 12 | 95 | 0 | 5 | 95.5 | 0.80 (0.64, 0.97) | 0.063 |
| Prevnar | 238 | 184 | 40 | 9 | 5 | 94.1 | 0.81 (0.72, 0.91) | 0.424 |
| All | 350 | 196 | 135 | 9 | 10 | 94.6 | 0.89 (0.84, 0.94) | 1.000 |

* a The Merck ECL cutoff is the threshold value corresponding to 0.35 μg/ml in the WHO reference ELISA, and the WHO ELISA cutoff is 0.35 μg/ml.

b Naïve, sera from unvaccinated individuals; Prevnar, sera from individuals vaccinated with Prevnar.
FIG. 2. Precision profiles (relative standard deviation [%RSD]) for test samples with quantifiable concentrations. The samples are identified by vaccination status and assay method. The variabilities in the Merck and ICH ECL assays did not exceed that in the WHO reference ELISA. Averaged over the set of pediatric samples, the %RSD among the repeat test results within each Pn ECL assay was <20% for each serotype.
and the demonstration of protective efficacy against invasive Pn disease conferred by the 7-valent conjugate pneumococcal (7vPNc) vaccine. Such an approach follows WHO guidelines stating that it may be acceptable for manufacturers to employ an alternative threshold value when using a specific in-house assay, provided that this value can be demonstrated by a well-conducted bridging study to correspond to an IgG concentration of 0.35 µg/ml in the WHO reference ELISA. In the present study, the agreement rates between the Pn ECL assay and the WHO ELISA were improved by applying the threshold values determined. Moreover, Merck is pursuing a formal study, involving more than 200 pediatric samples selected from a variety of geographic regions, to bridge the Pn ECL assay to the WHO reference assay (17, 18). These data will be used to formally define the threshold values in the ECL assay that correspond to 0.35 µg/ml in the WHO ELISA for each of the serotypes in Prevnar 13. By the application of threshold values in the Pn ECL assay that correspond to 0.35 µg/ml in the WHO ELISA, the Pn ECL assay is expected to yield seroconversion rates similar to those that would be obtained in the WHO ELISA.

The multiplexed pneumococcal capsular polysaccharide ECL assay provides a highly sensitive and specific technique for measuring antibodies, with the advantage of providing rapid results using small amounts of serum.

While we acknowledge and appropriately account for the small yet consistent elevations in anti-Pn IgG concentrations determined by the Pn ECL assay relative to the WHO reference ELISA, the Pn ECL assay appears to be an acceptable alternative to the WHO reference ELISA for quantitating IgG responses to Pn serotypes in pediatric sera.

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