Abstract

Aim: To re-optimize the pneumococcal (Pn) electrochemiluminescence (ECL) assay and to validate and bridge the enhanced assay to the World Health Organization (WHO) enzyme-linked immunosorbent assay (ELISA), to support the Phase III clinical trial program for V114, a 15-valent Pn conjugate vaccine. **Methods**: The Pn ECL assay was re-optimized, validated, and formally bridged to the WHO ELISA. **Results**: The enhanced Pn ECL assay met all pre-specified validation acceptance criteria and demonstrated concordance with the WHO ELISA. The corresponding threshold value remains at 0.35 µg/ml for all 15 serotypes. **Conclusion**: The enhanced Pn ECL assay has been validated for the measurement of antibodies to 15 Pn capsular polysaccharides and is concordant with the WHO ELISA, supporting its use in clinical trials.

Word count: 120

Key words: electrochemiluminescence; ELISA; Meso Scale Discovery; multiplex; optimization; pneumococcal; vaccine; validation

Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a Gram-positive, facultative anaerobic organism that causes acute bacterial infections, including pneumonia, meningitis, septicemia and otitis media [1,2]. *S. pneumoniae* has a highly variable polysaccharide (Ps) capsule, which is the primary basis for its pathogenicity, with more than 90 distinct serotypes documented [1].

V114 is a 15-valent adjuvanted pneumococcal (Pn) conjugate vaccine (PCV) currently in Phase III clinical trials for the prevention of *S. pneumoniae* disease. V114 includes Ps from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F, extending the serotype coverage beyond existing PCVs [3]. In order to efficiently measure the immunogenicity of V114 across a broad range of serotypes in large numbers of samples from clinical trials, high-throughput assays are desirable. The electrochemiluminescence (ECL) detection assay for the multiplex quantitation of anti-PnPs antibodies, based on Meso Scale Discovery (MSD) technology, was first developed and validated by Merck & Co., Inc., Kenilworth, NJ, USA, in 2009 for the quantitation of immunoglobulin G (IgG) serotype-specific antibodies induced by Pn vaccines [4], replacing the previously used single-plex ELISA. The assay uses disposable multi-spot microtiter plates that include integrated screen-printed carbon ink electrodes on the base of the wells, enabling direct binding of PnPs. Sample antibody concentration is determined by referencing the ECL response of the sample against the ECL response of a fitted standard curve generated from the serially diluted World Health Organization (WHO) Pn international reference standard, lot 007sp. The assay allows for the simultaneous quantitation of IgG responses to multiple Pn serotypes in small sample volumes [4].

The ECL assay was re-optimized with a view to improving MSD plate lot-to-lot consistency and assay processes, including automation. As this represents a modification of a previously published technique [4], with the addition of seven more serotypes, Merck & Co., Inc., Kenilworth, NJ, USA, in collaboration with PPD® Laboratories Vaccine Sciences Lab, Richmond, VA, USA, undertook a series of validation experiments to confirm its suitability for the detection and quantitation of anti-Pn IgG antibodies in human serum in Phase III clinical trials. The WHO Expert Committee on Biological Standardization (ECBS) recommends that in-house assays used in immunogenicity studies designed to evaluate protection against invasive Pn disease be bridged to the WHO reference ELISA [5,6]. In accordance with this recommendation, the assay threshold values for the enhanced Pn ECL assay that correspond to the 0.35 µg/ml threshold measured using the WHO reference ELISA for each of the serotypes in V114 were determined.

Experimental

Reagents

All reagents used were as described previously [4], with some additional polysaccharides as follows:

C-polysaccharide (CPs), a Pn cell wall Ps, was obtained from the Statens Serum Institut, Copenhagen, Denmark. All PnPs powders for serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, 25, 33F, and 72 were manufactured and received from the manufacturing division of Merck & Co., Inc., West Point, PA, USA. The coating antigens for the fifteen V114 serotypes are process performance qualification lots that met all release-testing requirements for commercial manufacturing. Each PnPs was reconstituted in sterilized pyrogen-free water. The final concentration for each PnPs following reconstitution was 1 mg/ml for vaccine serotypes and 2 mg/ml for serotypes 25 and 72.

Reference standard

The WHO Pn international reference standard serum, lot 007sp (National Institute of Biological Standards and Control, Potters Bar, Hertfordshire, UK) was used for preparing standard curves. The 007sp reference standard is a pooled serum from 278 healthy volunteers vaccinated with the 23-valent PnPs vaccine and has replaced the previously established standard 89SF for quantitation of human IgG antibodies specific for *S. pneumoniae* capsular Ps [7].

Study samples

Anonymized serum samples used for the validation of the Pn ECL assay were acquired as part of the V114 Phase I and II clinical trial programs. The samples were selected to be representative of the intended Phase III population. Assay precision was assessed using 60 adult and pediatric serum samples selected to span the range of anti-Pn antibody levels. To assess assay selectivity, antibody-depleted human serum (ADHS) and nine serum samples identified to have low or negative anti-Pn concentrations were spiked with varying concentrations of the WHO 007sp reference standard. Assay specificity was assessed using nine adult serum samples identified to span the range of anti-Pn antibody levels, and dilutional linearity was assessed using 20 adult serum samples identified to have high anti-Pn antibody levels.

The WHO bridging study included 116 anonymized pediatric serum samples from infants immunized with three doses of V114 (adjuvanted or non-adjuvanted), collected from within the USA during Phase I and II clinical trials. The samples were representative of similar samples in the V114 clinical Phase III program. The samples were selected with antibody concentrations that spanned the entire range of response, with a concerted effort to secure samples with serotype-specific IgG concentrations close to the WHO reference ELISA threshold value of 0.35 µg/ml in order to best assess the concordance between the two assays in the region of the threshold value. In addition, serum samples with known antibody concentrations from 12 adults vaccinated with the 23-valent PnPs vaccine (Goldblatt panel) [7] were used as a calibration panel to determine the concordance between the Pn ECL assay and the WHO reference ELISA.

Assay re-optimization

The original Pn ECL assay has been described previously [4] and the enhanced assay is schematically represented in Figure 1. For the Pn ECL, reference standard, quality control samples, and test sera were diluted at appropriate dilutions in phosphate-buffered saline containing 0.05% Tween 20, 1% bovine serum albumin, 5 µg/mL CPs, 10 µg/mL Pn 25, and 10 µg/mL Pn 72. In the WHO ELISA, PnPs 22F is used for pre-adsorption of samples to improve the specificity to the Pn serotypes assessed in the assay; however, because V114 includes PnPs 22F, the Pn ECL assay was developed using PnPs 25, 72, and CPs as an alternative to PnPs 22F for pre-adsorption of samples.

Since cross-reactivity within a serogroup can be observed on MSD plates, serotypes within a serogroup were separated across the two panels used in the assay. The 15 serotypes in V114 were assayed in groups of seven (types 1, 5, 6A, 7F, 19A, 22F, and 33F) and eight (types 3, 4, 6B, 9V, 14, 18C, 19F, and 23F), and the assays are termed Pn7 ECL and Pn8 ECL, respectively.

Two changes to the original Pn ECL assay were made in this study. First, the concentration of PnPs coated onto the MSD 10-spot High Bind plates was increased from 5 ng/spot to 20 ng/spot for each serotype to improve consistency of response across plate lots. The PnPs were coated at 2.5, 5, 10, and 20 ng/spot, and each PnPs was assessed independently. Since all fifteen PnPs reached plateaus at 10–20 ng/spot and lower coating concentrations yielded variable signals, 20 ng/spot was recommended for each of the 15 types. Second, the TECAN EVO Workstation was implemented, including an optimized script that standardized liquid classes and

mixing techniques, updated standard curve preparation, and updated the sample preparation dilution scheme to serial 1:10 dilution steps.

Validation of the ECL assay

For each of the 15 serotypes in V114, the objectives of the validation were to confirm the limit of detection (LOD) and quantifiable range (limits of quantitation; LOQ), and to assess the precision, accuracy, specificity, selectivity, and dilutional linearity of the assay. Each serotype was evaluated separately.

Precision

Intra-assay precision (the within-run variation representing the repeatability of the assay under the same conditions) and inter-assay precision (representing the between-run variation attributable to differences in assay conditions, such as different analyst, day, and reagent), were estimated over 16 runs by two analysts. The intermediate assay precision was calculated as the sum of the intra- and inter-assay variance components.

Each analyst performed their runs across separate days over at least a 3-week period. The set of 60 adult and pediatric serum samples were tested at dilutions of 1:1,000 and 1:10,000 in each of the 16 assay runs. Four samples were tested on each plate within each assay run to assess intra-assay precision. The remaining 56 samples were tested once within each assay run. To account for potential well location effects, the placement of the 60 samples within the plate was randomized within each run. Variability estimates were obtained on the natural log (ln)-transformed antibody concentrations using the MIXED procedure in SAS/STAT® software, Version 9.4 for Windows. Copyright© 2002–2012 SAS Institute Inc. SAS

and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA. The pre-specified acceptance criterion for intermediate precision was that the percent geometric coefficient of variation (GCV), where %GCV = $100 \times \left(e^{\sqrt{\hat{\sigma}^2}} - 1\right)$ and $\hat{\sigma}^2$ denotes the sum of the intra- and inter-assay variance components) be <25% for each serotype.

Accuracy

Accuracy, the closeness of agreement between a test result using the Pn ECL method and its theoretical true value or the accepted reference value, was assessed across a 12-point 2.5-fold dilution series (1:1 to 1:23,842) of the WHO Pn reference standard, lot 007sp. Samples were tested twice on each of two plates across four assay runs, and antibody concentrations were determined by interpolating each of the 12 points off the 11-point reference standard that was also tested on each plate.

For each point, accuracy was assessed in terms of percent recovery, calculated as (*observed concentration/expected concentration*) x 100%, where the *observed concentration* is the interpolated antibody concentration and the *expected concentration* is the concentration of the WHO Pn reference standard corresponding to the dilution at which the sample was tested. At each of the 12 expected concentrations, the geometric mean percent recovery across the 16 estimates and its associated 90% confidence interval were determined. For each serotype, the pre-specified acceptance criterion was a recovery range of 80% to 125% for samples with expected concentrations within the LOQ.

Dilutional Linearity

Dilutional linearity (dilutability) is an attribute of a biological assay that demonstrates that a test sample can be diluted through a series, yielding equivalent dilution-corrected antibody concentrations across that series. To evaluate the dilutional linearity of the Pn ECL assay, the 20 serum samples identified to have high anti-Pn antibody concentrations were tested across a 9-point dilution series between 1:1,000 and 1:400,000 (1:1,000, 1:2,000, 1:4,000, 1:10,000, 1:20,000, 1:40,000, 1:100,000, 1:200,000, and 1:400,000). The dilution bias was assessed across the range of dilutions evaluated based on regressing the log_{10}-transformed dilution-corrected antibody concentrations against the log_{10}-transformed dilutions. For each serotype, the dilution fold-bias per 10-fold dilution was calculated as 10^b, where *b* represents the estimate of the average dilution effect (slope) from the model. A dilution bias of less than ±2-fold per 10-fold increase in dilution was the pre-specified acceptance criterion for dilutional linearity of the assay.

Selectivity

The selectivity of the assay, defined as the ability of the assay to measure and differentiate the analyte in the presence of components that may be expected to be present in the sample, was assessed by spiking 2-fold serial dilutions (50%, 25%, 12.5%, 6.25%, 3.125%, 1.5625%, and 0.78125%) of the WHO Pn reference standard, lot 007sp, into each of the nine serum samples that were pre-screened to have low or negative antibody concentrations to the serotypes in the assay. All samples were tested at a dilution of 1:1,000. The recovered concentration directly associated with the 007sp spike was determined for each sample. The percent recovery was calculated as (*measured concentration/expected concentration*) x

100%, where the *measured concentration* was the interpolated antibody concentration after correcting for the incurred level in the sample, and the *expected concentration* was the concentration spiked into the sample. An overall estimate of percent recovery across the nine samples was also derived for each combination of serotype and spike concentration. For each serotype, the pre-defined acceptance criterion for selectivity was that the overall percent recovery for spike concentrations within the LOQs be between 80% and 125%.

Limit of detection

The LOD of an assay is defined as the lowest concentration that has a high probability of producing a response that can be distinguished from the background response (i.e., the response at zero concentration). The LOD was set at the lowest antibody concentration at which the assay response was significantly higher (with >95% probability) than the background response. The LOD was determined by spiking 2-fold serial dilutions (3.125–0.048828125%) of WHO Pn reference standard, lot 007sp, into ADHS and comparing the associated ECL responses to the ECL response of the ADHS without spike. One master spike series was created, and five aliquots of the series were independently tested across one assay run.

The LOD was determined based on the individual differences in ECL responses between the spiked samples and the sample without spike. The LOD was the lowest spike level that provided a statistically significant increase in ECL response above the unspiked sample, where significance was based on a *t* distribution at the 5% significance level and was determined using the mean and standard deviation of the individual differences. The pre-specified acceptance criterion for LOD was ≤0.05 µg/ml for each serotype.

Analytical specificity

Analytical specificity is the ability of an analytical method to determine only the component it purports to measure in the presence of other components expected to be in the sample. The analytical specificity of the Pn ECL assay was determined by competitive inhibition, using homologous PnPs for homologous competition, and plate-specific heterologous PnPs and *Neisseria meningitidis* W135 Ps (MnPs) for heterologous competition. Nine serum samples selected to span the quantifiable range of the assay were tested at 1:1,000 and 1:10,000 dilution across two runs performed by one analyst. Samples were tested uncompeted, mock competed with sterile water, and competed with 10 µg/ml of each individual PnPs per serotype or 10 µg/ml MnPs.

The specificity of the assay was determined by comparing the antibody concentration of serum pre-adsorbed with PnPs and MnPs to the antibody concentration of the serum samples that were pre-adsorbed in water. Percent inhibition was calculated as $100\% \times \left(1 - \frac{PnPs\ or\ MnP\ Adsorbed\ Ab[C]}{Water\ Adsorbed\ Ab[C]}\right)$. The pre-specified acceptance criteria were ≥75% evaluable samples with ≥75% inhibition when adsorbed with the homologous Ps, and ≥75% evaluable samples with ≤25% inhibition when adsorbed with MnPs.

Limits of quantitation

The lower LOQ (LLOQ) and upper LOQ (ULOQ) define the antibody concentration range over which the assay is acceptably accurate and precise (i.e., the quantifiable range). The assay LOQs were determined by evaluating the precision profile of the calibrated antibody concentrations of the test samples and the accuracy of the

assay. The final LOQs were determined according to the following criteria: (1) the LLOQ must be greater than or equal to the LOD; (2) the LLOQ and the ULOQ must lie between the concentrations associated with the second and tenth standard curve points; (3) the percent recovery estimates from the accuracy experiment must be between 80% and 125% throughout the determined quantifiable range; and (4) ≥80% of samples with geometric mean antibody concentrations within the LOQs must have intermediate precision <25% GCV.

The pre-specified acceptance criterion was that the LLOQ must be ≤0.1 µg/ml for each serotype.

Bridging to the WHO reference ELISA

The validated, enhanced Pn ECL assay was bridged to the WHO reference ELISA according to the WHO ECBS recommendation [5]. Feedback by both the European Medicines Agency and the US Food and Drug Administration was also incorporated into the study design. To this end, samples were selected with antibody concentrations that spanned the entire range of response.

All testing for the WHO ELISA was performed in the laboratory of Professor David Goldblatt at the Institute of Child Health (ICH) in the UK, according to ICH procedures for the WHO Reference ELISA, the method for which has been described in detail elsewhere [6]. Briefly, serum samples were mixed before analysis with an adsorbent-containing CPs and 22F capsular Ps (except in the case of the 22F assay, which used CPs-multi, a product that retains the ability to measure antibodies directed against 22F, as the adsorbent) to reduce the level of non-specific binding in the assay. ELISA plates were coated with PnPs by adsorbing individual

PnPs serotype antigens to microtiter plates. Dilutions of adsorbed human sera were subsequently added to the ELISA plates. The serotype-specific antibody bound to the ELISA plate was detected with anti-human IgG antibody conjugated with alkaline phosphatase, followed by addition of the substrate, *p*-nitrophenyl phosphate. The optical density of each well was measured at 405 nm, and at 620 nm for reference using an ELISA plate reader. The level of antibody in the human serum was calculated by comparing the optical density of the sample wells to that of the WHO reference standard, lot 007sp.

Each of the 128 serum samples (116 pediatric samples and 12 adult samples) were tested across three independent runs in the Pn ECL assay and across three independent runs in the WHO reference ELISA. All testing for the Pn ECL was performed at PPD® Laboratories Vaccine Sciences Lab, Richmond, VA, USA, using the validated assay described in this manuscript. Within each of the Pn ECL runs, samples were tested in duplicate at the 1:1,000 dilution (or further dilution if necessary). Within each of the WHO reference ELISA runs, samples were tested in a series of eight 2.5-fold dilutions starting at the 1:50 dilution.

Comparisons between the Pn ECL and WHO ELISA results were performed on the *ln*-transformed median concentrations across the three runs within each laboratory. For each serotype, the set of paired test sample results were used to estimate the concordance slope, the average fold difference, and the coefficients of accuracy, correlation, and concordance. The pediatric samples were used to estimate the aggregate and the serotype-specific Pn ECL thresholds corresponding to the WHO reference ELISA value of 0.35 µg/ml. Using the serotype-specific median concentrations, serostatus agreement between assays was separately

assessed for the pediatric samples, for the adult samples, and for the subset of pediatric samples with concentrations close to 0.35 µg/ml. Details of the methods used to estimate the concordance line and serostatus agreement between assays have been reported previously [8,9]. In addition, the reverse cumulative distribution function (RCDF) method [10,11] was used to identify the Pn ECL threshold value corresponding to 0.35 µg/ml in the WHO reference ELISA. The RCDF threshold values were determined for each serotype and for the combined set of 15 serotypes in the aggregate value.

Cross-classification tables were constructed to compare assays in terms of their agreement rate at 0.35 µg/ml. The number of discordances in the serostatus assignment between the two assays was indicated by the off-diagonal entries within the cross-classification tables. The level of imbalance in the discordance between the two assays was statistically assessed using a McNemar's exact test. Cohen's kappa coefficient was also calculated, providing an estimate of the agreement between assays beyond which might exist due to chance alone. The value of the kappa coefficient ranges from –1.0 to 1.0. Agreement consistent with chance alone yields a kappa coefficient near 0, whereas agreement far exceeding chance alone yields a kappa coefficient approaching 1.0.

The Pn ECL and WHO ELISA results for the 12 adult bridging panel samples were also assessed against their published concentrations for 13 of the 15 serotypes (excludes 22F and 33F).

Results & discussion

The enhanced Pn ECL assay for quantitation of serotype-specific Pn antibodies directed against serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F met all of the pre-specified acceptance criteria for each of the parameters assessed (Table 1) and was therefore considered validated. The assay was reviewed and approved by the Center for Biologics Evaluation and Research (CBER), within the US Food and Drug Administration.

Precision

The intermediate assay precision ranged from 16.8% to 24.1% GCV across the 15 serotypes and met the pre-specified acceptance criterion of <25% GCV for each serotype (Table 1); therefore, the assay was deemed to be acceptably precise. Individual precision plots are shown in Supplementary Figure 1.

Accuracy

Percent recovery estimates of the WHO Pn reference standard were between 92% and 122% for samples with expected concentration levels within the quantifiable range of the assay for each serotype (Table 1). As such, the assay met the pre-specified acceptance criterion that recovery be between 80% and 125% for each serotype. Overall recovery estimates are represented graphically in Supplementary Figure 2.

Dilutional linearity

Across the 15 serotypes, the overall estimates of dilution bias ranged from 1.06-fold per 10-fold dilution to 1.36-fold per 10-fold dilution (Table 1). Individual dilution plots

for all serotypes are presented in Supplementary Figure 3. The assay met the pre-specified acceptance criterion that the dilution bias per 10-fold increase in dilution be less than 2-fold and was therefore deemed to be acceptably linear.

Selectivity

The overall recovery estimates ranged from 93% to 116% across the 15 serotypes, falling within the pre-specified acceptance range of 80% to 125% (Table 1), rendering the assay acceptably selective. The individual and overall percent recovery estimates across the range of spike concentrations are graphically presented by serotype in Supplementary Figure 4.

Limit of detection

The LOD for each serotype was ≤0.02 µg/ml (Table 1), which met the pre-specified acceptance criterion that the LOD must be ≤0.05 µg/ml.

Analytical specificity

As shown in Table 1, for each serotype, all of the samples (100%) had ≥75% inhibition when adsorbed with the homologous PnPs, ≥94.4% of the samples had ≤25% inhibition when adsorbed with the heterologous MnPs, and ≥88.2% of the samples had ≤25% inhibition when adsorbed with any of the other plate-specific heterologous PnPs. As the pre-specified acceptance criteria were met for both homologous and heterologous inhibition, the assay was considered adequately specific for each of the 15 serotypes.

Limits of quantitation

Each serotype met the pre-specified criterion that the LLOQ be ≤0.1 µg/ml (Table 1); thereby showing that the assay can quantitate with adequate precision and accuracy well below the clinical threshold of 0.35 µg/ml.

Bridging to the WHO reference ELISA

A formal bridging study was conducted to establish the Pn ECL threshold values corresponding to 0.35 µg/mL in the WHO ELISA for each of the 15 serotypes in V114. The LLOQ in the Pn ECL assay was 0.10 µg/ml for serotype 5 and 0.05 µg/ml for all other serotypes (Table 1), while for the WHO reference ELISA, the LLOQ was 0.15 µg/ml for all 15 serotypes.

In general, for the 15 serotypes in V114, the Pn ECL assay and the WHO reference ELISA resulted in similar concentrations throughout the range of response. The concordance slope estimates ranged from 0.97 to 1.33 for the set of pediatric samples, where a slope of 1 indicates that the response ratio between the assays is consistent throughout the range of response (Table 2). The most notable difference between assays was for serotype 5, in that the Pn ECL assay tended to provide higher measured concentrations than the WHO reference ELISA for the pediatric set of samples (serotype 5 antibody concentrations were 52% higher in the Pn ECL assay compared with the value attained with the WHO reference ELISA). For the other 14 serotypes, the difference in antibody concentration between the Pn ECL and the WHO reference ELISA was between –22% and 29%. The fold difference between the assays at 0.35 µg/ml ranged from 0.63 (serotype 19F) to 1.49 (serotype 5), with an overall fold difference of 1.01 when taken over all 15 serotypes.

For the set of pediatric samples, the serotype-specific Pn ECL threshold values equivalent to the WHO reference ELISA value of 0.35 µg/ml obtained using the concordance method ranged from 0.22 µg/ml to 0.52 µg/ml, with an aggregate threshold of 0.35 µg/ml across all 15 serotypes, while the RCDF method produced threshold values ranging from 0.24 µg/ml to 0.56 µg/ml (Supplementary Figure 5), with an aggregate value of 0.38 µg/ml (Figure 2). The RCDF curves display the proportion of subjects tested whose serotype concentration exceeded a specified concentration.

The rate of agreement in serostatus assignment was assessed on test samples using the assay-specific median concentrations for each test sample. Using the serostatus threshold of 0.35 µg/ml for the Pn ECL and the WHO reference ELISA assays, the agreement rates in serostatus assignment were greater than 80% for all serotypes in the pediatric sample panel. Using either the concordance threshold value or the RCDF threshold value for individual serotypes resulted in only a slight improvement in serostatus agreement rates as compared with the 0.35 µg/ml threshold. Using a serostatus threshold of 0.35 µg/ml for both assays, the 2×2 cross-classification tables for the set of pediatric samples are provided by serotype in Table 3. Across the 15 serotypes, the agreement rate in serostatus between the two assays ranged from 80.2% to 96.6% and Cohen's kappa coefficient ranged from 0.474 to 0.908, with aggregate values of 89.4% and 0.782, respectively (Table 3). The overall McNemar's exact *P* value was 0.046.

For the subset of pediatric samples having WHO reference ELISA concentrations close to 0.35 µg/ml, the serostatus agreement rate using the 0.35 µg/ml threshold for the Pn ECL ranged from 59.5% to 87.9% across the

15 serotypes. For this subset of samples, using either the concordance threshold value or the RCDF threshold value for individual serotypes as compared with the 0.35 µg/ml threshold resulted in improvement in serostatus agreement rates of ≥8 percentage points in six of the 15 serotypes (data not shown).

For the panel of 12 adult samples (Goldblatt panel), ≥75% of samples had concentrations within ±40% of the published values [7] for eight of the 13 serotypes with the WHO reference ELISA; there are no published results for serotypes 22F and 33F in the panel of adult sera. For the other five serotypes, 42% to 58% of samples had concentrations within ±40% of the published values. For the Pn ECL assay, ≥75% of samples had concentrations within ±40% of the published values for six of the 13 serotypes; while 42% to 67% of samples were within ±40% of the published values for the other seven serotypes. The pairwise comparisons between the published concentrations and those derived by the ECL and WHO reference ELISA assays for this panel of adult sera are presented in Figure 3.

All 128 pediatric and adult samples with quantifiable individual concentrations were used to assess the assay variability and precision. The relative standard deviation for precision in the Pn ECL assay was <12% across the 15 serotypes, while for the WHO reference ELISA the relative standard deviation was <24%. Although limited in the number of independent runs per laboratory, for each of the serotypes, the variability of the Pn ECL assay was either comparable to, or notably less than, the WHO reference ELISA.

Given the proximity of the aggregate Pn ECL threshold values to the WHO reference ELISA threshold of 0.35 µg/ml, and that the serotype-specific Pn ECL threshold values were within 1.60-fold of 0.35 µg/ml for each of the 15 serotypes with

agreement rates between assays only slightly improved when using the serotype-specific threshold values for the Pn ECL as compared to using the 0.35 µg/ml threshold, the single Pn ECL threshold value of 0.35 µg/ml can reasonably be applied to each of the 15 evaluated serotypes in V114.

Overall discussion

In order to facilitate high-throughput sample testing in Phase III trials, we made modifications to the Pn ECL assay to improve MSD plate lot-to-lot consistency and to add automation using the TECAN EVO workstation, including an optimized script to allow for standardization of liquid classes and mixing techniques, and enhanced standard curve and sample preparation.

In a series of validation experiments, all of the pre-specified acceptance criteria were met, and the assay is considered validated. The assay was securely within the pre-specified acceptance threshold of <25% GCV for intermediate precision for each serotype, with values ranging from 16.8% to 24.1% GCV across the 15 serotypes. In addition, the accuracy, selectivity, and dilutional linearity were also generally well within their pre-specified validation criteria. Moreover, the assay showed specificity for the individual serotypes in almost 100% of samples, with a validation requirement for ≥75% of samples to exhibit this characteristic.

The WHO reference ELISA has been adopted as the benchmark for measuring the efficacy of Pn vaccines for the past two decades, having been used in the evaluation of the first licensed PCV [12,13]. In order to preserve the link between the level of Pn serotype-specific IgG in serum samples and the clinical efficacy of Pn vaccines in the context of the original study, the WHO ECBS recommends that all

in-house immunogenicity assays be bridged to the WHO reference ELISA [5,6]. We therefore compared the performance of our enhanced Pn ECL assay with that of the WHO reference ELISA against a panel of 116 pediatric serum samples and a further panel of 12 adult serum samples (Goldblatt panel) with known, published concentrations [7]. Overall, the concordance resulted in fairly similar antibody concentrations throughout the range of response. We also determined the individual threshold values for each of the 15 serotypes measured with the Pn ECL assay that correspond to the internationally accepted threshold value of 0.35 µg/ml in the WHO reference ELISA. We found that agreement between the two assays was only marginally improved by using the individual serotype-specific Pn ECL threshold values; therefore, we recommend using the aggregate threshold value of 0.35 µg/ml for all 15 serotypes. Although the concordance is not exact, this study allows us to better understand the relationship between the two assays.

The Pn ECL gives comparable results to the WHO reference ELISA with the benefit of speed, increased dynamic range, higher throughput, smaller sample volumes, and the ability to multiplex [4]. Automation provides the additional benefit of decreased variability and standardized workflow, making the automated Pn ECL assay particularly suitable for large-scale clinical trials. The results of this study support use of the Pn ECL assay for immunogenicity endpoints in Phase III studies.

Conclusion

The enhanced Pn ECL assay is a validated immunogenicity assay for the measurement of serotype-specific antibodies to 15 different capsular Ps, with proven specificity, selectivity, accuracy, dilutional linearity, and precision. The assay has been formally bridged and is concordant to the WHO reference ELISA, and has the benefits of speed, small sample volumes, large dynamic range, and the ability to multiplex, making it suitable and advantageous for use in large clinical trials of Pn vaccines.

Future perspective

This enhanced, validated, and WHO reference ELISA-bridged Pn ECL assay will be a valuable and efficient tool in assessing outcomes in Phase III clinical trials of V114. Owing to the simplicity of optimization, the technology can easily be expanded to the serological analysis of other multivalent vaccine formulations. Together with the microcolony-based high-throughput multiplexed opsonophagocytic killing assay also reported in this special focus issue, we have at our hands a robust armamentarium for assessing anti-Pn serostatus and vaccine-conferred immunogenicity with high-throughput speed and accuracy.

Executive summary

- The Pn ECL assay was re-optimized, validated, and bridged to the WHO reference ELISA, to assess its suitability as an immunogenicity assay in Phase III clinical trials of V114, a 15-valent PCV.

- The enhanced Pn ECL assay met all pre-specified acceptance criteria confirming that the assay is acceptably precise, accurate, selective, linear, and specific for the 15 serotypes present in V114.

- The assay was successfully bridged to the WHO reference ELISA using both pediatric and adult samples, and the variability of the Pn ECL assay was observed to be comparable to, or notably less than, that of the WHO reference ELISA. The threshold value corresponding to that of the WHO reference ELISA remains at 0.35 µg/ml, simplifying transition to the enhanced assay.

- The enhanced Pn ECL assay is suitable for assessing seropositivity and seroconversion in large numbers of samples in clinical trials, with the benefits of speed, small sample volumes, large dynamic range, and the ability to multiplex.

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Author contributions

KMN, JMA, AHH, CJB, RG, TG, DG and RDM contributed to the conception and design of the study. AHH, CJB, PGA, RG, TG and DG contributed to the acquisition of data. KMN, YZ, JMA, RG, TG, DG and RDM contributed to the interpretation of the results. All authors contributed to drafting and/or revision of the manuscript.

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Table 1. Validation assay parameters and acceptance criteria.

	Serotype	LOD (µg/ml)	LOQ[†] (µg/ml)	Intermediate precision (% GCV)	Accuracy (% recovery range)[‡]	Dilutional linearity (fold bias)[§]	Selectivity (% recovery)	Homologous specificity (%)[‖]	Heterologous MnPs specificity (%)[¶]	Heterologous PnPs specificity (%)[#]
Pn7 ECL	1	0.0042	0.05, 8.50	19.0	106–122	1.24	104	100	100	≥93.8%
	5	0.0037	0.10, 3.68	20.5	101–112	1.20	116	100	100	≥93.3%
	6A	0.0019	0.05, 3.93	23.0	103–112	1.19	93	100	100	≥94.4%
	7F	0.0041	0.05, 8.30	24.1	111–121	1.17	106	100	100	≥94.4%
	19A	0.0068	0.05, 13.87	20.7	110–118	1.06	102	100	100	≥100%
	22F	0.0051	0.05, 10.54	20.6	108–121	1.36	99	100	94	≥93.8%
	33F	0.0043	0.05, 8.83	17.8	110–119	1.21	94	100	100	≥100%
Pn8 ECL	3	0.0007	0.05, 1.45	20.1	92–112	1.20	108	100	100	≥100%
	4	0.0016	0.05, 1.73	19.9	97–105	1.23	105	100	100	≥92.9%
	6B	0.0044	0.05, 3.41	18.9	97–116	1.12	100	100	100	≥100%
	9V	0.0031	0.05, 6.44	19.2	100–119	1.22	103	100	100	≥100%
	14	0.0185	0.05, 15.20	17.9	113–119	1.29	108	100	100	≥100%
	18C	0.0036	0.05, 7.30	16.8	109–121	1.08	104	100	100	≥88.2%
	19F	0.0071	0.05, 14.61	20.9	104–119	1.14	95	100	100	≥100%

	23F	0.0029	0.05, 5.95	18.4	102–114	1.19	101	100	100	≥87.5%
Acceptance criteria		≤0.05	LLOQ ≤0.10	<25	80–125	<2-fold	80–125	≥75	≥75	NA

†For samples diluted 1:1000. The LOQs corresponding to all subsequent dilutions should be adjusted by the appropriate dilution factor.

‡Percent recovery range for samples with expected concentrations within the LOQs.

§Overall fold bias per 10-fold dilution.

‖Percentage of evaluable samples with ≥75% inhibition when adsorbed with homologous polysaccharide.

¶Percentage of evaluable samples with ≤25% inhibition when adsorbed with heterologous MnPs.

#Percentage of evaluable samples with ≤25% inhibition when adsorbed with plate-specific heterologous PnPs. The percentage shown is the minimum percentage across the six (Pn7 ECL) or seven (Pn8 ECL) heterologous PnPs on the plate.

GCV: Geometric coefficient of variation; LLOQ: Lower limit of quantitation; LOD: Limit of detection; LOQ: Limit of quantitation; MnPs: *Neisseria meningitidis* W135 polysaccharide; NA: not applicable (as there were no pre-specified acceptance criteria on the specificity of the assay to the plate-specific heterologous PnPs).

Table 2. Concordance between the ECL assay and the WHO reference ELISA.

			Concordance slope		Average % difference		Fold difference	Agreement coefficient		
	Serotype	N1[†]	Slope	95% CI	% difference	95% CI	at 0.35 µg/ml[‡]	Correlation	Accuracy	Concordance
ECL vs. ELISA	1	91	1.05	1.00, 1.11	10.3	4.8, 16.0	1.09	0.97	0.99	0.96
	3	73	1.17	0.95, 1.43	3.5	–11.5, 21.0	1.00	0.75	0.99	0.75
	4	75	1.11	1.04, 1.18	15.4	9.4, 21.8	1.11	0.97	0.98	0.95
	5	102	1.07	0.93, 1.22	52.1	40.6, 64.6	1.49	0.83	0.84	0.70
	6A	89	1.29	1.13, 1.46	–10.3	–20.2, 0.8	0.74	0.86	0.97	0.83
	6B	93	1.05	1.00, 1.10	–4.3	–8.6, 0.2	0.91	0.97	1.00	0.97
	7F	107	0.99	0.95, 1.03	22.4	18.4, 26.6	1.23	0.98	0.98	0.96
	9V	88	1.33	1.22, 1.46	27.8	16.8, 39.7	1.20	0.92	0.93	0.86
	14	109	0.97	0.94, 1.01	20.9	16.0, 26.0	1.26	0.98	0.99	0.97
	18C	77	1.11	1.04, 1.18	28.6	22.5, 35.0	1.26	0.96	0.94	0.90
	19A	94	1.28	1.14, 1.43	–12.7	–21.8, –2.5	0.77	0.87	0.97	0.85
	19F	109	1.22	1.15, 1.28	–21.4	–27.2, –15.1	0.63	0.96	0.96	0.93
	22F	116	1.05	0.98, 1.13	1.5	–5.2, 8.7	0.94	0.94	1.00	0.93
	23F	72	1.08	1.00, 1.17	–7.8	–13.9, –1.2	0.89	0.95	0.99	0.95
	33F	116	1.01	0.97, 1.06	–8.0	–11.4, –4.4	0.91	0.97	1.00	0.97
	Overall	1411	1.08	1.05, 1.10	6.0	3.7, 8.2	1.01	0.93	1.00	0.93

†N1 is the number of test samples with quantifiable concentrations in both assays, used to estimate the concordance slope and the average % difference.

‡To calculate the fold difference at the serostatus threshold of 0.35 µg/ml, 0.35 µg/ml was used as the concentration of the WHO ELISA.

CI: Confidence interval; ECL: Electrochemiluminescence; ELISA: Enzyme-linked immunosorbent assay; WHO: World Health Organization.

Table 3. Serostatus cross-classification 2x2 tables between the Pn ECL assay and the WHO reference ELISA assay for pediatric samples using a serostatus threshold of 0.35 µg/ml for each assay.

		ELISA								
		Serotype 1			**Serotype 3**			**Serotype 4**		
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
ECL	Neg	59	**4**	63	63	**5**	68	67	**3**	70
	Pos	**8**	45	53	**14**	31	45	**5**	41	46
	Total	67	49	116	77	36	113	72	44	116
	Agreement rate			89.7%			83.2%			93.1%
	Kappa			0.790			0.637			0.855
	McNemar's Exact P value			0.388			0.064			0.727
		Serotype 5			**Serotype 6A**			**Serotype 6B**		
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
	Neg	28	**1**	29	46	**11**	57	41	**3**	44
	Pos	**22**	65	87	**5**	54	59	**2**	70	72
	Total	50	66	116	51	65	116	43	73	116
	Agreement rate			80.2%			86.2%			95.7%
	Kappa			0.574			0.724			0.908
	McNemar's Exact P value			<0.001			0.210			1.000
		Serotype 7F			**Serotype 9V**			**Serotype 14**		
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
	Neg	22	**0**	22	59	**4**	63	18	**0**	18
	Pos	**12**	82	94	**10**	42	52	**8**	90	98
	Total	34	82	116	69	46	115	26	90	116
	Agreement rate			89.7%			87.8%			93.1%
	Kappa			0.722			0.752			0.777
	McNemar's Exact P value			<0.001			0.180			0.008
		Serotype 18C			**Serotype 19A**			**Serotype 19F**		
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
	Neg	69	**1**	70	47	**18**	65	29	**21**	50
	Pos	**6**	40	46	**5**	46	51	**1**	65	66
	Total	75	41	116	52	64	116	30	86	116
	Agreement rate			94.0%			80.2%			81.0%
	Kappa			0.872			0.608			0.594
	McNemar's Exact P value			0.125			0.011			<0.001
		Serotype 22F			**Serotype 23F**			**Serotype 33F**		
		Neg	Pos	Total	Neg	Pos	Total	Neg	Pos	Total
	Neg	3	**2**	5	70	**3**	73	8	**2**	10
	Pos	**4**	107	111	**2**	41	43	**2**	104	106
	Total	7	109	116	72	44	116	10	106	116
	Agreement rate			94.8%			95.7%			96.6%
	Kappa			0.474			0.908			0.781
	McNemar's Exact P value			0.688			1.000			1.000
		Overall								
		Neg	Pos	Total						
	Neg	629	**78**	707						
	Pos	**106**	923	1029						
	Total	735	1001	1736						
	Agreement rate			89.4%						
	Kappa			0.782						
	McNemar's Exact P value			0.046						

The specified threshold for both assays was 0.35 µg/ml.

ECL: Electrochemiluminescence; ELISA: Enzyme-linked immunosorbent assay; Neg: Negative; Pn: Pneumococcal; Pos: Positive; WHO: World Health Organization.

Figure 1. Schematic overview of the enhanced ECL assay as previously described [4].

ECL: Electrochemiluminescence; IgG: Immunoglobulin G; MSD: Meso Scale Discovery; PnPs: Pneumococcal polysaccharide.

Figure 2. Sample median concentration reverse cumulative distribution functions for aggregate threshold value across the 15 serotypes in V114.

Results were obtained from a panel of 116 sera from infants immunized with three doses of V114.

ECL: Electrochemiluminescence; ELISA: Enzyme-linked immunosorbent assay.

Figure 3. Pairwise comparison between published concentrations [7] and those derived by the Pn ECL and the WHO reference ELISA for a panel of 12 adult sera.

The black 45-degree solid line is the perfect match line Y=X. The black 45-degree dashed lines are 0.6-fold and 1.4-fold reference lines. The red vertical and horizontal reference lines represent 0.35 µg/ml. The R^2 values from the simple linear regression fit applied to the log transformed data in these figures are 0.91 (ECL vs. published), 0.93 (ELISA vs. published), and 0.91 (ECL vs. ELISA). The corresponding correlation coefficients are 0.95, 0.97 and 0.95, respectively.

ECL: Electrochemiluminescence; ELISA: Enzyme-linked immunosorbent assay; Pn: Pneumococcal; WHO: World Health Organization.

Supplementary Figure 1. Precision profiles for samples with final antibody concentrations within the determined LOQs.

GCV: Geometric coefficient of variation; LOQ: Limit of quantitation.

Supplementary Figure 2. Accuracy estimates across the evaluated concentrations of the WHO reference standard.

Different symbols represent different runs, plates and preparations.

CI: Confidence interval; LOQ: Limit of quantitation; WHO: World Health Organization.

Supplementary Figure 3. Dilution bias across all dilutions by sample.

Numbers indicate sample number in each experiment, indicated by the different colors.

Supplementary Figure 4. Selectivity results for nine individual samples spiked with a series of concentrations of the WHO reference standard.

Different symbols represent different samples.

CI: Confidence interval; LOQ: Limit of quantitation; WHO: World Health Organization.

Supplementary Figure 5. Median sample concentration reverse cumulative distributions functions by serotype.

The pink line represents the sample response with the WHO reference ELISA, while the blue line represents the response with the Pn ECL assay. Corresponding threshold values for the two assays are shown on the x-axis in pink and blue, respectively.

ECL: Electrochemiluminescence; ELISA: Enzyme-linked immunosorbent assay; Pn: Pneumococcal; WHO: World Health Organization.