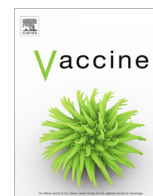


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Comparison between a new multiplex electrochemiluminescence assay and the WHO reference enzyme-linked immunosorbent assay to measure serum antibodies against pneumococcal serotype-specific polysaccharides



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ABSTRACT

Background: Two electrochemiluminescence (ECL) assays were developed which, together, can simultaneously measure serum antibodies against pneumococcal capsular polysaccharides (PnPS) for 17 serotypes. The assays were validated for the 13 PnPS included in the 13-valent pneumococcal conjugate vaccine (PCV13). As recommended by the World Health Organization (WHO), we compared the ECL assays with the WHO reference enzyme-linked immunosorbent assay (ELISA) and derived a threshold corresponding to the 0.35 µg/mL threshold established for the WHO reference ELISA for the non-inferiority comparison and licensure of new PCVs against invasive pneumococcal disease.

Methods: A panel of 452 serum samples from children vaccinated with one of the three licensed PCVs was assessed with the ECL assays and the WHO reference ELISA. The ECL assay threshold for the aggregated seven PnPS included in the 7-valent PCV (PCV7) and serotype-specific thresholds were determined using a receiver operating characteristics (ROC) curve-based approach and Deming regression. To evaluate concordance between the ECL assays and the WHO reference ELISA, serostatus agreement rates between both assays and geometric means of the ratios (GMRs) of concentrations obtained with both assays were calculated.

Results: The thresholds for the seven aggregated PCV7 serotypes obtained with the ROC curve-based approach and Deming regression approximated 0.35 µg/mL (0.38 and 0.34 µg/mL, respectively). Individual thresholds for the PCV13 serotypes ranged between 0.24 and 0.51 µg/mL across both approaches. Serostatus agreement rates using a 0.35 µg/mL threshold for both assays were ≥86.9% for all PCV13 serotypes. GMRs ranged between 0.85 and 1.25 for 11/13 serotypes and were <1.29 for the two remaining serotypes.

Conclusion: The ECL assays were comparable to the WHO reference ELISA and offer a sensitive, time- and serum volume-saving method to quantify serotype-specific anti-PnPS antibodies in pediatric sera. A 0.35 µg/mL threshold will be used for each PCV13 serotype to assess PCV immunogenicity in clinical trials.

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Abbreviations: BSA, bovine serum albumin; CI, confidence interval; CWPS multi, pneumococcal cell wall polysaccharide multi; DPBS, Dulbecco's phosphate buffered saline; DPBST, Dulbecco's phosphate buffered saline containing 0.05% Tween-20; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; GMR, geometric mean of the ratios; ICH, Institute of Child Health; IgG, immunoglobulin G; IPD, invasive pneumococcal disease; LLOQ, lower limit of quantitation; MSD, Meso Scale Discovery; PCV, pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; PCV7, 7-valent pneumococcal conjugate vaccine; PCV9, 9-valent pneumococcal conjugate vaccine; PHiD-CV, pneumococcal non-typeable *Haemophilus influenzae* protein D conjugate vaccine; PnPS, pneumococcal capsular polysaccharide; RCDC, reverse cumulative distribution curve; ROC, receiver operating characteristics; ULOQ, upper limit of quantitation; WHO, World Health Organization.

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1. Introduction

Streptococcus pneumoniae causes invasive infections, such as meningitis and sepsis, and is also a major bacterial cause of mucosal infections, including otitis media and pneumonia. At least 97 different pneumococcal serotypes are known to circulate, but only a small proportion is responsible for the majority of invasive infections [1]. These serotypes differ in the chemical composition and antigenicity of their polysaccharide capsule, the bacteria's most important virulence factor [1].

Pneumococcal conjugate vaccines (PCVs), in which pneumococcal capsular polysaccharides (PnPS) are covalently linked to carrier proteins, have had a major impact on the burden of pneumococcal disease [2,3]. A 7-valent PCV (PCV7, Pfizer Inc.) including PnPS of seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) was licensed based on efficacy results from several clinical trials [4–6]. PCV7 has meanwhile been replaced by two higher-valent PCVs: the pneumococcal non-typeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV, GSK), containing PnPS of serotypes 1, 5, and 7F in addition to the PCV7 PnPS, and the 13-valent PCV (PCV13, Pfizer Inc.), containing PnPS of three more serotypes (3, 6A, and 19A) [2].

To accelerate access to the new PCVs, PHiD-CV and PCV13 were licensed based on randomized trials comparing their immunogenicity with that of PCV7 [7–9]. The primary endpoint in such studies was based on serotype-specific anti-PnPS immunoglobulin G (IgG) antibody concentrations measured by enzyme-linked immunosorbent assay (ELISA) approximately 1 month after the primary vaccination series. Indeed, to license new PCVs for immunization against invasive pneumococcal disease (IPD), the World Health Organization (WHO) recommended that the immunogenicity of the new vaccine is directly compared for non-inferiority with that of PCV7 to bridge the efficacy of PCV7 against IPD to the new vaccine [10,11]. Based on pooled efficacy and serology results aggregated for the seven PCV7 serotypes obtained from three IPD efficacy studies with PCV7 and a 9-valent PCV (PCV9, not licensed), a threshold IgG antibody concentration of 0.35 µg/mL measured by ELISA was recommended as a benchmark when comparing antibody responses between PCVs [10–12]. To standardize the measurement of serum anti-PnPS IgG antibody concentrations, the WHO established a reference ELISA, which included a 22F PnPS pre-absorption step to increase specificity and for which the 0.35 µg/mL threshold was retained [11,12]. The WHO recommends that any laboratory-specific modifications to the WHO reference ELISA or any alternative assays developed to measure serotype-specific anti-PnPS IgG antibody concentrations are adequately bridged to the WHO reference assay to maintain the link between immune responses to vaccination and the demonstration of protective efficacy of PCV7 against IPD. If alternative assay-specific thresholds are proposed, robust justification of threshold equivalence is needed [11], as was done for instance for GSK's 22F-inhibition ELISA [13,14].

Infant immunization studies often involve co-administration of PCVs with combination vaccines, thus more than 30 different assays per infant may need to be performed to determine the immune response to all antigens in the administered vaccines. In addition, new PCVs including more than 13 PnPS are being developed, further increasing the number of antigens to be assessed in the limited amount of serum that can be collected from an infant. Therefore, assays that are less time consuming, less labor-intensive, and less serum volume demanding than the currently used ELISAs are needed. A variety of alternative assays have been developed to overcome the limitations of ELISA, including multiplexed bead assays and chemiluminescence-based solid phase assays [15–20]. GSK has developed two complementary multiplex electrochemiluminescence (ECL) assays based on the Meso Scale

Discovery (MSD) technology, allowing simultaneous measurement of antibodies to multiple PnPS using small volumes of serum (<10 µL for all serologies). In these assays, serotype-specific serum IgG antibodies bound to PnPS on the surface of multispot microplates are detected using anti-human IgG secondary antibodies with an electrochemiluminescent tag. In the presence of electrical stimulation this tag emits light at an intensity that increases with the amount of bound anti-PnPS IgG antibodies and hence with the concentration present in the serum sample [21,22]. The newly developed ECL assays combine 17 PnPS (9 in the first assay and 8 in the second) and were qualified and validated for the 13 PnPS contained in PCV13, in line with guidelines developed by the International Conference on Harmonization and the Center for Drug Evaluation and Research [23,24]. The aim of the current study was to compare the ECL assays performed at GSK (Rixensart, Belgium) with the WHO reference ELISA performed at the Institute of Child Health (ICH; London, United Kingdom), one of the two WHO reference laboratories for pneumococcal serology. We derived a threshold based on the seven PnPS included in PCV7 corresponding to the 0.35 µg/mL WHO reference ELISA threshold and performed a bridging between the assays on the 13 PnPS included in PCV13.

2. Materials and methods

2.1. PnPS

Lyophilized PnPS for each PCV13 serotype to coat the microplates for the ECL assays and for the WHO reference ELISA were obtained from the American Type Culture Collection (Manassas, VA, USA). Each PnPS was reconstituted at GSK (for the ECL assays) or ICH (for the WHO reference ELISA) according to suppliers' instructions at a final concentration of 1 mg/mL and kept frozen at –20 °C or –80 °C until used.

2.2. Standard serum and controls

The human pneumococcal standard reference serum, 007sp (National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK), which has assigned anti-PnPS antibody concentrations for the PCV13 serotypes [25,26], was used to generate a standard curve to determine antibody concentrations in the ECL assays and WHO reference ELISA. Lyophilized serum was reconstituted at GSK or ICH.

For the ECL assays, an IgG-depleted negative control serum (Valley Biomedical, Winchester, VA, USA) containing no detectable anti-PnPS antibodies as measured by the multiplex ECL assays and two positive controls containing known levels of anti-PnPS (*Gammagard S/D*, Baxter, Lessines, Belgium; *Biomnis*, France Dom-Tom) were also used.

2.3. Panel of sera used for assay comparisons

The analyses to determine the ECL assay threshold and the bridging analyses were performed on data generated from a panel of sera collected from infants and children vaccinated with PCV7, PHiD-CV, or PCV13 in 11 clinical trials previously performed by GSK, predominantly in Europe. All trials had been conducted in accordance with Good Clinical Practice guidelines and included informed consent. Samples were selected based on volume availability and on anti-PnPS IgG concentrations that allowed coverage of a broad analytical range. Within this panel, 452 serum samples were analyzed for antibodies against PCV7 PnPS, of which 191 samples were also tested for antibodies against serotypes 1, 5,

and 7F PnPS, of which 53 samples were also tested for antibodies against serotypes 3, 6A, and 19A PnPS.

2.4. ECL assay method

The two multiplex ECL assays developed by GSK are based on the MSD technology, which uses an electrochemiluminescent ruthenium tris(bipyridine)-labeled anti-human IgG antibody to detect serum IgG antibodies bound to specific PnPS immobilized on multispot microplates [21,22]. PnPS of serotypes 3, 4, 6B, 9V, 14, 18C, 19F, and 23F (ECL1) and 1, 5, 6A, 7F, and 19A (ECL2) were immobilized at MSD (Rockville, MD, USA) by passive adsorption in spots on the high-binding carbon electrode surface on the bottom of each well of 96-well multispot microplates (10 spots per well). Coated microplates were stored at 2–8 °C until use and were shown to be stable for more than 1 year at this temperature.

Samples were diluted at appropriate dilutions (1:1000 for test serum, positive and negative control samples or 1:10,000 in case of high concentrations; serial dilutions to obtain 11 standard points for the 007sp reference serum) in Dulbecco's phosphate buffered saline (DPBS; pH 7.4), containing 0.05% Tween-20, 1% bovine serum albumin (BSA), and 20 µg/mL pneumococcal cell wall polysaccharide multi (CWPS multi; Statens Serum Institute, Copenhagen, Denmark) and incubated overnight (16–24 h) at 2–8 °C to allow pre-absorption of the samples with CWPS multi. No pre-absorption with 22F PnPS was done. Microplate wells were blocked with 150 µL DPBS containing 5% BSA for 1 h at room temperature on an orbital shaker. Wells were washed three times with 300 µL DPBS (pH 7.4) containing 0.05% Tween-20 (DPBST). 30 µL of the diluted and pre-absorbed samples were added to the blocked pre-coated wells and plates were incubated for 1 h at room temperature on an orbital shaker. Wells were washed three times with 300 µL DPBST and incubated with 30 µL ruthenium tris(bipyridine)-labeled goat anti-human IgG antibody working solution (antibody: Southern Biotech, Birmingham, AL, USA; label: MSD, Rockville, MD, USA; conjugated at GSK) for 1 h at room temperature on an orbital shaker. After another three 300 µL washing steps with DPBST, 150 µL ECL read buffer containing tripropylamine (MSD, Rockville, MD, USA) was added in each well and plates were read as soon as possible (within 1 h) after adding the read buffer. Reading was done with an ECL plate reader, which applies an electrical current across the carbon electrodes at the bottom of the wells. In the presence of tripropylamine, this triggers a redox reaction of the ruthenium tris(bipyridine) tag and the generation of a luminescent signal [21]. The signal was read at 620 nm with a high-resolution cooled camera detector with no cross-talk between spots (MSD SECTOR Imager 6000 or Imager S600).

Samples were tested at a single dilution in duplicate wells on each plate, except for the 11 reference serum standard points, which were tested in single wells on each plate. The mean ECL values of the duplicate samples were converted to concentrations in µg/mL by interpolation on the reference standard curve which was fit using a weighted four-parameter logistic regression model.

2.5. WHO reference ELISA method

The WHO reference ELISA was performed at ICH as previously described [27]. In brief, 96-well microtiter plates were coated with PnPS for the 13 PCV13 serotypes. Sera were pre-absorbed for 30 min at room temperature with 10 µg/mL CWPS and 5 µg/mL serotype 22F PnPS. PnPS-coated plates were incubated with the diluted pre-absorbed samples, washed, and incubated with alkaline phosphatase-labeled goat anti-human secondary antibody. Substrate (*p*-nitrophenyl phosphate) was added to induce a colorimetric reaction. The optical density of each well was measured at 405 nm and 690 nm using an ELISA plate reader and compared

with the reference standard to determine the concentration in µg/mL using a weighted standardized curve-fitting four-parameter logistic regression model.

The lower limits of quantitation (LLOQs) for the WHO reference ELISA were <0.150 µg/mL for all serotypes and the upper limits of quantitation (ULOQs) have not been defined. Inter-assay precision for the WHO reference ELISA is summarized in [Supplementary table 1](#).

2.6. Statistical analyses

All statistical analyses were performed using SAS version 9.2, 9.3 or 9.4, depending on the analysis (SAS Institute Inc., Cary, NC, USA). The ROC curves were drawn in R version 3.3.1 with the pROC package.

Methods used to determine the performance characteristics of the ECL assays are described in the [Supplementary methods](#).

2.7. Determination of a threshold for the ECL assays

Two statistical methods were used to determine the ECL assays' threshold corresponding to the previously established ELISA threshold of 0.35 µg/mL: a receiver operating characteristics (ROC) curve-based approach and Deming regressions.

As a primary analysis, the ROC curve-based approach was used to derive the threshold for the aggregated seven PCV7 serotypes based on data generated from the panel of samples from PCV7/PHiD-CV/PCV13-vaccinated infants and children. The ROC analysis was also used to derive thresholds for the aggregated 10 PHiD-CV serotypes, the aggregated 13 PCV13 serotypes, and the thresholds for each individual PCV13 serotype. A ROC curve plots the proportion of true positives (sensitivity) versus the proportion of false positives (1 – specificity) at various threshold levels ([Fig. 1](#)). Logistic regressions were used to model the probability of the samples to have a concentration <0.35 µg/mL or ≥0.35 µg/mL in the WHO reference ELISA based on their log₁₀ concentrations in the ECL assays. The predicted probabilities were then used to calculate sensitivities and specificities according to different cut-off values in the ECL assays, which allowed drawing the ROC curves. The ROC curves were used to choose the optimal thresholds based on our tolerance for false positives (<0.35 µg/mL predicted to be ≥0.35 µg/mL in the WHO ELISA) and on our target for true positives (≥0.35 µg/mL predicted to be ≥0.35 µg/mL in the WHO ELISA), i.e., looking for equal sensitivities and specificities.

Deming regressions [28] were performed as supportive analyses. These account for the continuous characteristic and the measurement error of concentrations from both assays. An error variance ratio of 1 was assumed along the range of concentrations. The analysis was performed on paired values between the assays' LLOQs and ULOQs (the latter applicable to the ECL assays only).

Confidence intervals (CIs) for the ROC curve-based approach and for Deming regressions were determined by bootstrapping.

To compare the different aggregate thresholds (0.35 µg/mL and those obtained with the ROC curve-based approach and Deming regression for the aggregated PCV7 serotypes), we looked at how the different thresholds affected the contingency tables between the ECL assays and the WHO reference ELISA in terms of the serostatus of the samples for the aggregated PCV7 serotypes. We calculated agreement rates between the ECL assays and the WHO reference ELISA in terms of serostatus of the samples by dividing the number of concordant paired samples (i.e., samples with concentrations either above the thresholds with both assays or below the threshold with both assays) by the total number of tested valid paired samples. For each threshold, we also calculated the difference between the proportion of samples that were positive in the WHO ELISA and the proportion of samples that were positive in

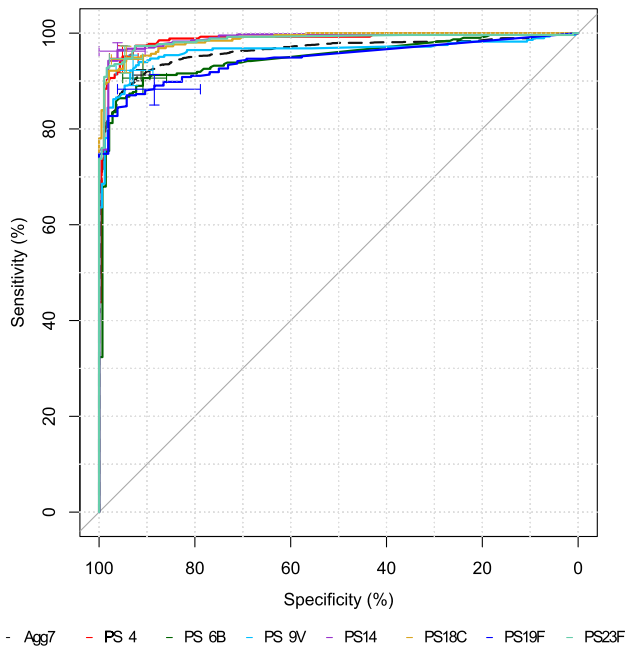


Fig. 1. ROC curves from logistic model predictions of WHO ELISA status based on ECL assay concentrations for aggregated and individual PCV7 serotypes. ROC, receiver operating characteristics; WHO, World Health Organization; ELISA, enzyme-linked immunosorbent assay; ECL, electrochemiluminescence; PCV7, 7-valent pneumococcal conjugate vaccine; Agg7, aggregated seven PCV7 serotypes; PS, polysaccharide. The X axis displays the specificity from the highest value (on the left) to the smallest value (on the right). Threshold values corresponding to observations of the curves closest to the upper left corner are the most performant. 95% bootstrapped confidence intervals of sensitivities and specificities allow spotting the performances of the optimal cut-points identified by minimizing the absolute values of the differences between sensitivities and specificities.

the ECL assays. The differences between these proportions allows assessing the symmetry of the discordants since the proportion of samples that are positive in both assays is used in the calculation of both proportions.

2.8. Technical bridging: qualitative and quantitative concordance

We assessed both qualitative and quantitative concordance between the ECL assays and the WHO reference ELISA. The analysis was based on the final ECL assay threshold aggregated for the seven PCV7 serotypes and the 0.35 $\mu\text{g}/\text{mL}$ threshold for the WHO reference ELISA.

For the qualitative concordance assessment, agreement rates (overall, positive, and negative) between the assays were estimated for each PCV13 serotype in terms of serostatus of the samples by dividing the number of concordant paired samples by the total number of tested valid paired samples (overall agreement) or by dividing the number of concordant paired samples above/below both thresholds by the total number of tested valid paired samples above/below the ELISA threshold (positive/negative agreement). McNemar *p*-values were calculated to assess the imbalance in the distribution of discordant samples. The acceptance criteria for the qualitative part of the technical bridge were an overall agreement at the final aggregate threshold for the ECL assays of $\geq 80\%$ for all 13 PCV13 serotypes and of $\geq 90\%$ for at least 66% of the 13 serotypes.

For the quantitative concordance assessment, we calculated the geometric means of individual ratios (GMR; ECL assay divided by WHO reference ELISA) of pairs of concentration values falling above the respective LLOQs in both assays and below the ULOQs in the ECL assays. The GMR analysis was supported by the slope

of the Deming regression line and the Deming plot of the bias presenting the individual ratios (ECL assay concentration over WHO reference ELISA concentration) over the analytical range. The acceptance criteria for the quantitative part of the technical bridge were GMRs between 0.67 and 1.50 for all 13 serotypes and between 0.80 and 1.25 for at least 66% of the 13 serotypes.

3. Results

3.1. Performance characteristics of the ECL assays

The main performance characteristics of the ECL assays, as established during the qualification and validation processes, are summarized in [Supplementary tables 2–4](#). The LLOQs by PnPS ranged between 0.061 and 0.199 $\mu\text{g}/\text{mL}$; the ULOQs between 7.344 and 184.000 $\mu\text{g}/\text{mL}$ ([Supplementary table 2](#)). Precision and linearity were demonstrated between the LLOQs and ULOQs ([Supplementary table 3](#)). Acceptance criteria for inter-assay precision (i.e., coefficient of variation $\leq 30\%$) were met for all 13 PCV13 PnPS ([Supplementary table 3](#)). Linearity was demonstrated for all 13 PnPS, i.e., $\geq 75\%$ of the deviations from linearity fell between the acceptance limits of 0.80–1.25 ([Supplementary table 3](#)). Accuracy, assessed on the WHO quality control panel of 12 serum samples from vaccinated adults [25,29], was acceptable: for nine serotype-specific PnPS, antibody concentrations measured with the ECL assays fell within $\pm 40\%$ of their assigned concentrations using the WHO reference ELISA for at least 9 out of 12 tested samples ([Supplementary table 4](#)).

3.2. Determination of a threshold for the ECL assays

Using the ROC curve-based approach ([Fig. 1](#)), the aggregate threshold for the seven PCV7 serotypes was 0.38 $\mu\text{g}/\text{mL}$ (95% CI: 0.366–0.402) ([Table 1](#)). The aggregate thresholds for the PHiD-CV and PCV13 serotypes were 0.38 $\mu\text{g}/\text{mL}$ (95% CIs: 0.366–0.399 and 0.367–0.401, respectively). No individual thresholds could be derived for serotypes 3, 6A, and 19A because of too low numbers of samples with antibody concentrations $< 0.35 \mu\text{g}/\text{mL}$ in the WHO reference ELISA. Individual thresholds for the other serotypes ranged between 0.24 $\mu\text{g}/\text{mL}$ (for serotype 6B) and 0.51 $\mu\text{g}/\text{mL}$ (for serotype 4) ([Table 1](#)).

Using Deming regression, the estimated aggregate threshold for the seven PCV7 serotypes was 0.34 $\mu\text{g}/\text{mL}$ (95% CI: 0.330–0.348) ([Table 1](#), [Fig. 2](#)). Aggregate thresholds for the PHiD-CV and PCV13 serotypes were 0.34 $\mu\text{g}/\text{mL}$ (95% CIs: 0.332–0.348 and 0.333–0.348, respectively). Estimated thresholds for the individual PCV13 serotypes ranged between 0.25 $\mu\text{g}/\text{mL}$ (for serotypes 6B and 19F) and 0.45 $\mu\text{g}/\text{mL}$ (for serotype 5) ([Table 1](#)). No individual threshold was derived for serotype 19A due to a lack of observations in the threshold area.

Given the wide analytical range of the ECL assays ([Supplementary table 2](#)), the aggregate threshold values for the ECL assays obtained with the ROC curve-based method and Deming regression were very close to and on either side of the 0.35 $\mu\text{g}/\text{mL}$ IPD licensure threshold established for the WHO reference ELISA. To evaluate whether an anti-PnPS concentration of 0.35 $\mu\text{g}/\text{mL}$ could be used as the aggregate threshold for the ECL assays, we evaluated how the three different thresholds (0.38 $\mu\text{g}/\text{mL}$, 0.34 $\mu\text{g}/\text{mL}$, and 0.35 $\mu\text{g}/\text{mL}$) impacted the contingency tables between the ECL assays and the WHO reference ELISA in terms of the serostatus of the samples for the aggregated PCV7 serotypes ([Supplementary table 5](#)). Agreement rates between the assays were 91.2% when using 0.38 $\mu\text{g}/\text{mL}$ as ECL threshold and 91.4% when using 0.34 or 0.35 $\mu\text{g}/\text{mL}$ as ECL thresholds. The difference between the proportion of samples that were positive in the WHO ELISA and the pro-

Table 1
Estimated ECL assay thresholds for aggregated and individual serotypes obtained through the ROC curve-based method and through Deming regression.

Serotype	ROC curve-based method				Deming regression	
	N	ECL assay threshold, $\mu\text{g/mL}$ (95% CI)	Specificity, % (95% CI)	Sensitivity, % (95% CI)	N	ECL assay threshold, $\mu\text{g/mL}$ (95% CI)
Aggregated PCV7 serotypes	3149	0.38 (0.366; 0.402)	91.22 (89.49; 92.96)	91.24 (90.04; 92.44)	2629	0.34 (0.330; 0.348)
Aggregated PHiD-CV serotypes	3718	0.38 (0.366; 0.399)	91.20 (89.59; 92.74)	91.17 (90.04; 92.30)	3078	0.34 (0.332; 0.348)
Aggregated PCV13 serotypes	3877	0.38 (0.367; 0.401)	91.33 (89.73; 92.86)	91.37 (90.27; 92.44)	3235	0.34 (0.333; 0.348)
1	190	0.34 (0.292; 0.401)	95.15 (92.23; 99.03)	95.40 (90.80; 98.85)	139	0.31 (0.292; 0.327)
3	53	NA	NA	NA	52	0.27 (0.219; 0.320)
4	451	0.51 (0.459; 0.557)	95.11 (91.85; 97.83)	95.13 (91.76; 97.38)	379	0.40 (0.378; 0.418)
5	188	0.45 (0.407; 0.506)	87.91 (80.22; 94.51)	87.63 (80.41; 93.81)	152	0.45 (0.428; 0.472)
6A	53	NA	NA	NA	52	0.34 (0.231; 0.483)
6B	451	0.24 (0.226; 0.273)	90.85 (85.92; 95.07)	90.61 (87.38; 93.85)	371	0.25 (0.237; 0.266)
7F	191	0.29 (0.243; 0.343)	90.77 (83.08; 96.92)	91.27 (85.71; 96.03)	158	0.28 (0.259; 0.301)
9V	452	0.50 (0.431; 0.566)	92.90 (88.76; 96.45)	92.58 (89.05; 95.05)	381	0.42 (0.387; 0.455)
14	451	0.36 (0.338; 0.450)	96.15 (90.38; 100.00)	96.24 (94.24; 97.99)	407	0.30 (0.276; 0.321)
18C	449	0.50 (0.462; 0.593)	94.33 (90.72; 97.42)	94.51 (91.76; 97.25)	351	0.42 (0.402; 0.447)
19A	53	NA	NA	NA	53	NA
19F	445	0.35 (0.260; 0.409)	88.46 (78.85; 96.15)	88.55 (84.99; 91.35)	395	0.25 (0.222; 0.275)
23F	450	0.32 (0.306; 0.340)	94.65 (91.44; 97.86)	94.68 (91.63; 97.34)	345	0.31 (0.298; 0.329)

ECL, electrochemiluminescence; ROC, receiver operating characteristics; N, number of valid paired results from the ECL assays and World Health Organization reference enzyme-linked immunosorbent assay (ELISA) – for Deming regression, only values between the respective lower and upper limits of quantitation were used; CI, confidence interval; PCV7, 7-valent pneumococcal conjugate vaccine; PHiD-CV, non-typeable *Haemophilus influenzae* protein D conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; NA, not applicable: could not be calculated because too few values were $<0.35 \mu\text{g/mL}$ in the WHO reference ELISA (three for serotype 3, four for serotype 6A, none for serotype 19A), or, for Deming regression, due to a lack of observations in the threshold area (for 19A). Thresholds were calculated at three decimals and rounded here to two decimals.

portion of samples that were positive in the ECL assays was 3.24% when using $0.38 \mu\text{g/mL}$ as ECL threshold, 0.60% when using $0.34 \mu\text{g/mL}$ as ECL threshold and 1.11% when using $0.35 \mu\text{g/mL}$ as ECL threshold. We therefore considered a concentration of $0.35 \mu\text{g/mL}$ as the final threshold for the ECL assays.

3.3. Technical bridge between the ECL assays and the WHO reference ELISA

The qualitative concordance analyses were performed using a threshold of $0.35 \mu\text{g/mL}$ for both the ECL assays and the WHO reference ELISA. The overall serostatus agreement rates between the

ECL assays and the WHO reference ELISA ranged between 86.9% (serotype 6B) and 100% (serotype 19A) and were $\geq 90\%$ for 10 out of 13 (77%) PCV13 serotypes. Therefore, both acceptance criteria for the agreement between assays were met ($\geq 80\%$ for 100% of PCV13 serotypes and $\geq 90\%$ for $\geq 66\%$ of PCV13 serotypes) (Table 2). McNemar p-values showed statistically significant evidence of an imbalance in the distribution of discordant samples for all serotypes except 1, 3, and 6A: for serotypes 4, 5, 9V, and 18C, more samples tested positive ($\geq 0.35 \mu\text{g/mL}$) with the ECL assay and negative with the WHO reference ELISA than vice versa; for serotypes 6B, 7F, 14, 19F, and 23F, the opposite was observed (Table 2).

The GMRs calculated from pairs of concentration values obtained with the ECL assays and WHO reference ELISA falling between the LLOQs and ULOQs ranged between 0.85 (serotype 6B) and 1.29 (serotype 9V) for all PCV13 serotypes and were below 1.25 for 11 serotypes (85%) (Table 3). Therefore, both acceptance criteria for the quantitative part of the bridge (GMRs 0.67–1.50 for 100% of serotypes and 0.80–1.25 for $\geq 66\%$ of serotypes) were met. Results from the Deming regression analysis supported this outcome: the slopes and their 95% CIs ranged between 0.67 and 1.50 for 100% of serotypes and between 0.80 and 1.25 for 12 (92%, i.e., $\geq 66\%$) serotypes. Intercepts and slopes are shown in Table 3 and the corresponding Deming regression plots and Deming plots of the bias in Supplementary Fig. 1.

4. Discussion

Since the early 1980s, levels of IgG antibodies to PnPS in human serum samples have been quantified using serotype-specific ELISAs [30]. An international reference ELISA was established and, based on a correlation between antibody levels and protective efficacy of PCV7 against IPD, an anti-PnPS antibody concentration of $0.35 \mu\text{g/mL}$ has been used as a benchmark for non-inferiority when assessing new PCVs in clinical trials [10–12]. Given the importance of maintaining a link between PCV efficacy and serum IgG antibody concentrations measured in a particular assay, the WHO issued recommendations on the bridging of any new assay for anti-PnPS antibody quantification to the reference ELISA and on the establishment of assay-specific thresholds equivalent to the

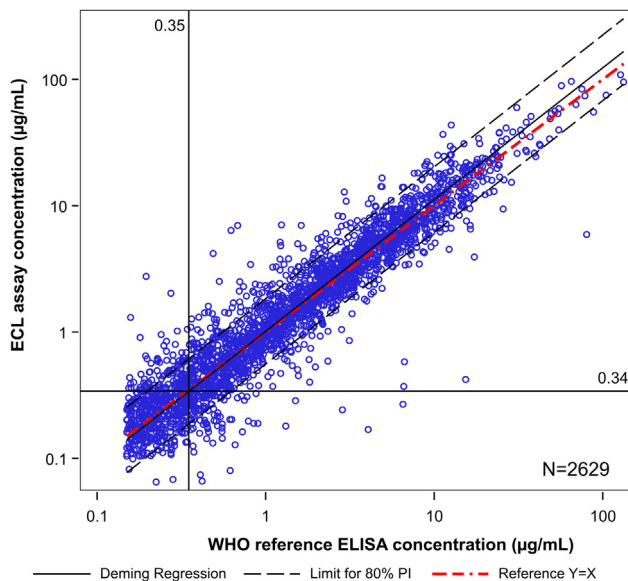


Fig. 2. Deming regression plotting ECL assay anti-PnPS concentrations in function of WHO reference ELISA anti-PnPS concentrations for the aggregated PCV7 serotypes. ECL, electrochemiluminescence; PnPS, pneumococcal capsular polysaccharide; WHO, World Health Organization; ELISA; enzyme-linked immunosorbent assay; PCV7, 7-valent pneumococcal conjugate vaccine; N, number of valid paired results from the ECL assays and WHO reference ELISA between the respective lower and upper limits of quantitation; PI, prediction interval.

Table 2

Qualitative concordance between the ECL assays and the WHO reference ELISA using the 0.35 µg/mL threshold in both assays.

Serotype	N	Concordant pairs, n		Discordant pairs, n		Agreement, %			McNemar p-value
		ELISA ≥ 0.35 ECL ≥ 0.35	ELISA < 0.35 ECL < 0.35	ELISA ≥ 0.35 ECL < 0.35	ELISA < 0.35 ECL ≥ 0.35	Overall	Positive	Negative	
1	190	82	99	5	4	95.3	94.3	96.1	1.0000
3	53	47	3	3	0	94.3	94.0	100.0	0.2500
4	451	264	151	3	33	92.0	98.9	82.1	<0.0001
5	188	94	70	3	21	87.2	96.9	76.9	0.0003
6A	53	47	4	2	0	96.2	95.9	100.0	0.5000
6B	451	254	138	55	4	86.9	82.2	97.2	<0.0001
7F	191	109	63	17	2	90.1	86.5	96.9	0.0007
9V	452	273	137	10	32	90.7	96.5	81.1	0.0009
14	451	386	48	13	4	96.2	96.7	92.3	0.0490
18C	449	250	158	5	36	90.9	98.0	81.4	<0.0001
19A	53	53	0	0	0	100.0	100.0	NA	NA
19F	445	347	46	46	6	88.3	88.3	88.5	<0.0001
23F	450	242	184	21	3	94.7	92.0	98.4	0.0003

ECL, electrochemiluminescence; WHO, World Health Organization; ELISA, enzyme-linked immunosorbent assay; N, number of valid paired results from the ECL assays and WHO reference ELISA; n, number of concordant or discordant pairs; NA, not applicable. Bold p-values indicate statistical significance ($p < 0.05$).

Table 3

Quantitative concordance between the ECL assays and the WHO reference ELISA.

Serotype	N	GMR (95% CI)	Deming regression	
			Intercept (95% CI)	Slope (95% CI)
1	139	0.95 (0.90; 1.00)	−0.013 (−0.033; 0.008)	1.091 (1.056; 1.127)
3	52	0.90 (0.82; 0.97)	−0.054 (−0.092; −0.017)	1.113 (1.005; 1.233)
4	379	1.24 (1.19; 1.28)	0.091 (0.076; 0.107)	1.079 (1.051; 1.108)
5	152	1.27 (1.21; 1.33)	0.090 (0.060; 0.121)	0.959 (0.890; 1.032)
6A	52	1.22 (1.03; 1.44)	0.059 (−0.026; 0.145)	1.153 (0.968; 1.379)
6B	371	0.85 (0.82; 0.89)	−0.078 (−0.096; −0.060)	1.144 (1.113; 1.177)
7F	158	0.91 (0.87; 0.95)	−0.048 (−0.067; −0.029)	1.106 (1.073; 1.140)
9V	381	1.29 (1.23; 1.36)	0.114 (0.090; 0.137)	1.073 (1.027; 1.122)
14	407	0.89 (0.86; 0.92)	−0.060 (−0.081; −0.039)	1.021 (0.993; 1.050)
18C	351	1.19 (1.14; 1.24)	0.077 (0.059; 0.095)	0.987 (0.955; 1.020)
19A	53	1.24 (1.13; 1.36)	0.177 (0.114; 0.240)	0.897 (0.835; 0.963)
19F	395	0.91 (0.86; 0.96)	−0.091 (−0.122; −0.061)	1.129 (1.081; 1.178)
23F	345	0.93 (0.89; 0.96)	−0.033 (−0.050; −0.016)	1.034 (1.003; 1.065)

ECL, electrochemiluminescence; WHO, World Health Organization; ELISA, enzyme-linked immunosorbent assay; N, number of valid paired results from the ECL assays and WHO reference ELISA between the respective lower and upper limits of quantitation; GMR, geometric mean of the individual ratios (ECL assay concentration over WHO reference ELISA concentration); CI, confidence interval.

0.35 µg/mL reference ELISA threshold [11]. Following this recommendation, GSK established a 22F-inhibition ELISA with a threshold of 0.2 µg/mL which was shown to correspond to a concentration of 0.35 µg/mL measured with the WHO ELISA without 22F pre-absorption [13,14] and was used during the clinical development of PHiD-CV [31].

Similarly, in the current study, we compared the newly developed, qualified, and validated multiplex pneumococcal ECL assays performed at GSK with the WHO reference ELISA performed at the ICH [27]. The two approaches we used (ROC-curve based method and Deming regression) both gave aggregated thresholds for the seven PCV7 serotypes close to 0.35 µg/mL (0.38 µg/mL and 0.34 µg/mL, respectively), with no relevant differences in serostatus agreement rates between assays when these three different thresholds were used. We therefore chose the 0.35 µg/mL threshold for subsequent concordance analyses between the ECL assays and the WHO reference ELISA and showed that antibody concentrations obtained with both assays were highly comparable. The predefined success criteria for serostatus agreement and for the geometric mean of individual ratios of antibody concentrations measured with the two assays were met. We did not observe a systematic trend for higher (or lower) anti-PnPS concentrations across serotypes when using the ECL assays compared to the WHO reference ELISA. For some serotypes, concentrations were lower while for others they were higher but GMRs were between 0.85 and

1.29 for the 13 PCV13 serotypes. In line with these results, during assay qualification, no systematic trend for lower or higher concentrations across serotypes was observed when comparing the anti-PnPS concentrations obtained with the ECL assays for the WHO quality control panel with their published concentrations using the WHO reference ELISA. A high concordance between the assays was also observed using the WHO quality control panel, with GMRs ranging between 0.70 and 1.25 across the PCV13 serotypes.

Others recently developed a multiplex ECL assay (also based on the MSD technology) to detect antibodies against the 15 PnPS included in a new investigational 15-valent PCV (containing PnPS for serotypes 22F and 33F in addition to the 13 PCV13 serotypes) [32]. Based on the thresholds (aggregated over the 15 PnPS) obtained from two different methods, a single ECL threshold value of 0.35 µg/mL for each of the 15 serotypes was recommended to assess serotype-specific antibody responses [32]. As such, these results are similar to the results we obtained with our ECL assays.

The originally established ELISA threshold of 0.35 µg/mL used for licensure of new PCVs against IPD was based on anti-PnPS IgG antibody concentrations and efficacy estimates aggregated for the seven PCV7 serotypes using pooled data from three IPD efficacy trials in infants vaccinated with PCV7 or PCV9 [10,12]. Because IPD rates are low, serotype-specific vaccine efficacy estimates from these trials were either not available or had too wide CIs to allow estimation of a protective antibody concentration by

serotype. Moreover, the licensure of PCV7 was based on the aggregated efficacy for the seven serotypes rather than serotype-specific efficacy. It was therefore deemed acceptable to estimate a single threshold applicable to all serotypes, using the simplifying assumption that the concentration of antibodies needed to confer protection was similar for all serotypes [10,12]. In line with this approach, we calculated a single threshold aggregated for the seven PCV7 serotypes for our ECL assays as our primary analysis. We also calculated individual thresholds for each of the 13 PCV13 serotypes which did not deviate more than 1.5-fold from the final 0.35 µg/mL ECL assay threshold for any of the serotypes. Serotype-specific ELISA thresholds have been published for the majority of PCV13 serotypes [33]. These range quite widely around the 0.35 µg/mL aggregate threshold but have not yet been incorporated into accepted guidelines for licensing. This emphasizes the importance of the 0.35 µg/mL aggregate threshold for the foreseeable future.

The original 0.35 µg/mL threshold concentration was estimated from reverse cumulative distribution curves (RCDCs) of antibody concentrations in vaccinated children as the concentration corresponding to the efficacy estimate [10,12]. RCDCs have also been used to derive the concentration equivalent to the 0.35 µg/mL WHO reference ELISA threshold for new assays or assay conditions [13,14,32]. In the current study, we used two different methods: an ROC curve-based approach and Deming regression. A draw-back of the RCDC approach is that it does not take into account that the same samples are tested in the new assay and the reference ELISA (i.e., matching of pairs). By contrast, the two methods we used to derive a threshold for the ECL assays do account for this. An advantage of using Deming regression rather than the ROC curve-based approach is that the former considers the continuous characteristic of the concentrations obtained with the WHO reference ELISA and takes into account measurement errors of both assays. We therefore consider Deming regression a good alternative method for estimating assay thresholds.

Multiplex ECL assays have several advantages over ELISA. The high speed of plate reading (<1 min per plate) and simultaneous measurement of antibodies to multiple PnPS—in contrast to ELISA which requires separate assays for each individual serotype—allow for an increased throughput and the use of lower sample volumes [15–19]. This is especially beneficial in infant vaccination studies with PCVs (currently containing up to 13 different PnPS) and co-administration of other vaccines, adding another 10 or more antigens to be tested in limited serum volumes. Our multiplex ECL assays allow addition of other PnPS and is therefore compatible with future generation PCVs containing more than 13 PnPS.

The ECL assay has a wider dynamic range than ELISA, meaning that low and high concentrations of antibodies can be measured with a single sample dilution, therefore minimizing sample retesting. A wider dynamic range has also been seen for Luminex-based multiplexed microsphere assays developed by several laboratories [15,16,19]. An advantage of the ECL assay compared to Luminex-based multiplex assays is that—similar to ELISA—direct binding of the PnPS to the surface of the ECL assay microplates through passive adsorption minimizes impact on antigenicity. This contrasts with the Luminex-based multiplex assays which require covalent binding of the PnPS to microspheres. Some of the methods (and chemical modifications) used for conjugation of the PnPS to the beads were shown to interfere with antigenicity of the PnPS, while with other conjugation methods serotype-specific epitopes appeared to remain intact [15,16,19,20].

In summary, the newly developed multiplex ECL assays which were validated for the 13 PCV13 PnPS offer a highly sensitive, robust, time- and serum volume-saving method for the detection and quantification of serotype-specific anti-PnPS antibodies in serum from infants and children. A concentration of 0.35 µg/mL

will be used as a threshold for each PCV13 serotype in future assessments of PCV immunogenicity in clinical trials.

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Author contributions

AB, DG, EM, FN, MF, SS and VV were involved in the design of the study. AEM, DF, MF, PDGDA, SL, and TJ performed the study and participated in the generation of the data. All authors participated in the analysis and interpretation of the data. All authors reviewed and commented critically on drafts of the manuscript for important intellectual content and gave final approval to submit for publication. All authors attest they meet the ICMJE criteria for authorship. The corresponding author had final responsibility to submit for publication. Drafts were developed by a professional publication writer according to the recommendations, documentation, and outline provided by the lead author.

Declaration of interests

The authors declare that they have no known competing financial interests

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Appendix A. Supplementary material

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