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2 standard reference serum 007sp
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Abstract

The pneumococcal ELISA reference standard serum, Lot 89SF, has been in use since 1990 and was replaced with a new reference standard serum, 007sp in 2013. This serum was generated under an FDA-approved clinical protocol, where 278 adult volunteers were immunized with the 23-valent unconjugated polysaccharide vaccine, Pneumovax II®, and a unit of blood was obtained twice within 120 days following immunization. Pooled serum was prepared from the plasma, filled at 6ml per vial and lyophilised. Five independent laboratories participated in bridging the serotype specific IgG assignments for 89SF to 007sp to establish equivalent reference values for 13 pneumococcal capsular serotypes (1,3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) using the WHO reference ELISA. A subsequent follow up study established equivalent reference values for an additional seven serotypes (8, 10A, 11A, 12F, 15B, 22F, 33F). In this study three laboratories assigned weight-based IgG concentrations in mcg/mL of serum to 007sp for four additional serotypes; 2, 9N, 17F and 20A. This study completes the assignment of serotypes in 89SF to 007sp. In addition, the IgG antibody assignments for a 12 member WHO QC serum panel were extended to cover the four additional serotypes. Agreement was excellent with a concordance correlation coefficient (r_c) > 0.996 when each laboratory was compared to the assigned values for the 12 WHO QC sera. The 007sp preparation has replaced 89SF as the pneumococcal reference standard. Sufficient quantities of 007sp are projected to be available for the next 25 years.

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47 **Introduction**

48 A *Streptococcus pneumoniae* Human Reference Serum, lot 89SF, greatly facilitated the standardization of ELISA
49 methodologies during a critical period when the first pneumococcal polysaccharide-conjugate vaccines were
50 being evaluated for licensure. The standard serum was used in serotype specific ELISAs designed to measure
51 IgG antibody specific for individual pneumococcal capsular polysaccharides. Serotype specific weight-based
52 values for IgG, IgA and IgM were originally derived for serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F for
53 Lot 89SF by Quateart, et al.[1]. Assignments for the additional serotypes in the 23-valent pneumococcal
54 polysaccharide vaccine were subsequently bridged from the assignments for the original 11 serotypes[2]. Due to
55 dwindling supplies of 89SF, a new reference standard serum, 007sp, was developed and described in 2011 [3].
56 This serum was generated under an FDA-approved clinical protocol, where 278 adult volunteers were
57 immunized with the 23-valent unconjugated polysaccharide vaccine, Pneumovax II®, and a unit of blood was
58 obtained twice within 120 days following immunization. Pooled serum was prepared from the plasma, filled at
59 6ml per vial and lyophilised. Five independent laboratories participated in bridging the serotype specific IgG
60 assignments for 89SF to the new reference serum, 007sp to establish equivalent reference values for 13
61 pneumococcal capsular serotypes (1,3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) using the WHO reference
62 ELISA[3] . This serum has replaced 89SF (which is no longer distributed) and been routinely used in pneumococcal
63 assays around the world for the past several years.
64 With the ongoing requirement to evaluate Pneumovax II® and the development of additional extended valency
65 conjugate vaccines, it has been imperative to assign values to 007sp for additional serotypes. In a three-centre
66 study we recently assigned to 007sp the IgG antibody values in mcg/mL to seven additional pneumococcal
67 serotypes (8, 10A, 11A, 12F, 15B, 22F, and 33F)[4]. This report describes the efforts undertaken by the same
68 three laboratories to establish the serotype specific IgG concentrations for 007sp to the final four remaining

serotypes in 89SF currently unassigned in 007sp (2, 9N, 17F, and 20A), and to assign values to a set of 12 existing World Health Organisation (WHO) Quality Control (QC) sera for the additional serotypes.

Results

To assess the consistency among the laboratories, the mean of the log IgG antibody concentrations of 007sp for each serotype (2, 9N, 17F, and 20A) was calculated for each laboratory and used to assess the level of agreement among the laboratories. There was a high level of agreement with the concordance correlation coefficient r_c exceeding 0.95 for all plots. For the same data, the Pearson correlation coefficient was ≥ 0.999, indicating excellent precision, and the accuracy coefficent (C_a) was ≥ 0.95 in each case. Analysis of variance (ANOVA) models were used to estimate IgG antibody concentrations for each of the serotypes in 007sp. Final point estimates and confidence intervals were obtained by back-transforming the estimated log-transformed concentrations and associated 95% confidence intervals. These estimated IgG antibody concentrations are the "assigned" values for each serotype (2, 9N, 17F, and 20A) in 007sp and are shown in Table 1. These values were derived by the double absorption of 007sp with both mono-substituted and di-substituted cell wall polysaccharide (CPS)[5-7], and thus in the future, when used as a reference standard serum, both standard and unknown test samples should be double absorbed. The IgG antibody concentrations assigned to 007sp compared to the original values assigned to 89SF are shown in Figure 1.

Serum IgG antibody concentrations against serotypes 2, 9N, 17F, and 20A were determined for the 12-member WHO QC serum panel using both 89SF and 007sp as the reference standards. Table 2 presents the assigned values for the QC serum panel (n≥27 for each estimate) while Figures 2 and 3 display the scatter plots and box

plots for the four serotypes analyzed. These plots illustrate the agreement of the four estimated assigned IgG values for 007sp compared to Lot 89SF for each WHO QC serum and serotype.

The scatter plots (Figure 2) show the high degree of agreement and correlation among the calculated (log) IgG concentrations for the panel of 12 WHO QC sera using 007sp (vertical scale) vs. Lot 89SF (horizontal scale) as reference standards. A perfect level of agreement would yield a straight line with slope of one and intercept at zero, and all data points cluster tightly about this line of identity. Laboratories 1 and 3, which both used automated liquid-handling robotics to perform the assays, showed a slightly lower degree of scatter around the line of identity compared to Laboratory 2, which used a manual assay process. The box plots (Figure 3) illustrate the deviation of the 007sp-based estimates from those obtained using Lot 89SF as reference standard for the 12 WHO QC sera. The IgG concentrations calculated using 007sp as reference standard are largely within two fold (1/2 – 2.0) of those calculated using lot 89SF as reference standard.

Table 3 presents the accuracy coefficient (C_a), Pearson correlation coefficient (r), and concordance correlation coefficient (r_c) which are measures of agreement between pairs of laboratories and between laboratories and consensus ELISA concentrations for the WHO QC sera. To form paired data between the labs for these comparisons, the serotype-specific replicate IgG antibody concentration values generated in each laboratory were replaced by a single predicted value obtained from a mixed-model analysis of variance. There was an exceptionally high degree of agreement with all values ≥ 0.99.

Discussion

In this study, we describe the assignment of IgG antibody concentrations in weight-based microgram per milliliter units to the human anti-pneumococcal standard reference serum 007sp and a panel of 12

pneumococcal QC sera for the final four additional serotypes originally calculated and assigned to 89SF. This new standard was developed in 2009/10 and was required to replace limited stocks of the original standard serum Lot 89SF. Assignment for additional serotypes are required as the original standard, Lot 89SF, which had values assigned for the 23 serotypes in Pneumovax II®, is no longer available (007sp is exclusively distributed via FDA). However studies evaluating Pneumovax II® are still undertaken and new conjugate vaccines are currently under development incorporating additional serotypes found in Pneumovax II®, but not in existing PCV's. Assignment of the weight-based antibody concentrations to human anti-pneumococcal standard reference serum 007sp was originally performed for the 13 serotypes represented in currently licensed conjugate vaccines (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F)[3]. Using established laboratories and a well characterized ELISA procedure [8,9] that was followed by all participating laboratories, it was possible to assign weight-based units to 007sp by running 007sp alongside a standard curve of 89SF and treating 007sp as the unknown. Very high levels of agreement between the participating laboratories for the weight-based units of IgG specific for 13 serotypes in 007sp were achieved. Having accepted concentration values for an existing standard has significantly simplified the assignment process. Subsequently we undertook a further assignment exercise utilizing the expertise of three laboratories. Values (mcg/ml) for IgG specific to seven additional serotypes (8, 10A, 11A, 12F, 15B, 22F, and 33F) were assigned to 007sp and the 12 QC sera described above[4]. As in the original assignment study, we have now assigned values (mcg/ml) for IgG specific for the final four serotypes in 89SF that remained unassigned (2, 9N, 17F and 20A). We were able to further validate the values obtained and the performance of 007sp as a standard during the process of assigning serotype specific IgG values (mcg/ml) to a panel of 12 WHO QC sera previously prepared from the sera of pneumococcal polysaccharide vaccinated adults. Concordance was high among laboratories (Table 3) and between results for laboratories and consensus ELISA concentrations. With the adherence to the uniform application of the WHO

ELISA[9] in the present study, we were able to achieve high levels of precision and accuracy in the values assigned to the additional four serotypes of 007sp and the WHO QC sera.

ANOVA mixed modeling is a flexible framework that allows estimation of ELISA concentrations for 007sp and the 12 WHO QC sera for each serotype by laboratory. These models may be used to compare and contrast results within and among laboratories. Random-effects ANOVA models allowed us to reduce the replicate measurements to a single predicted value which were then used to measure levels of consistency among the laboratories. While we were able to estimate serotype-specific concentrations for 007sp through a bridge to 89SF (Table 1), the actual ELISA concentrations for the WHO QC sera used in this study were unknown, so it was not possible to compare "true" values. The ANOVA mixed model provided a mechanism for estimating consensus values, which served as assigned values for these sera (Table 2).

Establishing a new reference serum for the pneumococcus was essential for ongoing efforts to evaluate new pneumococcal vaccines and to maintain the link with the original serology performed as part of the pivotal efficacy studies conducted prior to licensure. The high degree of agreement between the 007sp-based and lot 89SF-based estimates in the original assignment exercise[3] has inspired confidence in the validity of the 007sp assignments. In this follow on study, a similar high level of agreement has been observed.

The new standard, 007sp, now has assigned values for the 24 pneumococcal serotypes currently contained in licensed vaccines, is available in large quantities and should provide continuity for the foreseeable future. Its performance in ELISA suggests that it is unlikely to affect the operation of validated assays currently established in serology laboratories. Details of how to obtain 007sp and the QC sera are available at https://www.vaccine.uab.edu/.

Materials and Methods

Collection of Human Sera

The collection and processing of sera has been described in detail in a previous manuscript [3]. Briefly, 278 volunteers were vaccinated once with Pneumovax II®, and serum collected on two occasions post vaccination. Serological and virological testing showed sera to be free from Hepatitis B and C virus, syphilis and HIV. Sera from 262 volunteers were pooled and then aliquoted at 6 ml per vial and lyophilised while sera from the remaining 16 donors were separately aliquoted to create a new panel of individually calibrated sera for use in functional assays.

An existing WHO QC serum panel, established by D. Goldblatt (UCL Institute of Child Health) previously by immunizing adults with pneumococcal polysaccharide vaccine, and distributed by the National Institute for Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, United Kingdom), was supplied for assigning serotype-specific IgG assignments for the 12 QC serum panel members.

Laboratory Methods

Three laboratories participated in the assignment (In alphabetical order: Institute of Child Health, University College London, London, United Kingdom; Pfizer Vaccine Research and Development, Pearl River, NY; and Universitätsklinikum Erlangen Kinder- und Jugendklinik, Erlangen, Germany). Two of the three laboratories used liquid-handling robotics to perform various aspects of the ELISA while the other laboratory performed the assays by hand. The assignment of weight-based units followed the protocol established for the initial assignment, which can be found under the reference materials section at http://www.vaccine.uab.edu and mirrored the protocol used to assign values for an additional seven serotypes[4]. In the first phase of the study, serotype-specific IgG antibody assignments for four serotypes (2, 9N, 17F, and 20A) were established by calibrating Lot 007sp under double absorbent assay conditions against Lot 89SF under single absorbent conditions using the standardized pneumococcal reference ELISA (the "WHO ELISA")[8,9]. The ELISA protocol

followed by participating laboratories can be found at http://www.vaccine.uab.edu/ELISA%20Protocol.pdf. The only deviation from the WHO protocol is that double absorption of 007sp with cell wall polysaccharide (CPS)[5] was undertaken using two absorbents prepared from un-encapsulated S. *pneumoniae* mutant strains incorporating both mono- and di-substituted CPS[7,8] rather than CPS and purified 22F capsular polysaccharide. Lot 89SF had a value assigned for Serogroup 20, the serogroup included in Pneumovax 23®. This sugar has now been identified as serotype 20A[10] so this capsular polysaccharide and nomenclature has been used in this assignment exercise. Briefly, four independent sets of serial dilutions of lot 007sp (supplied by CBER, FDA) were made from four independent serum vials. The four sets of eight serial dilutions were run in duplicate as unknown samples on each ELISA plate in a 10-plate replicate series to generate approximately 40 data points per serotype for 007sp from each of the participating laboratories. Each plate also contained seven serial dilutions of lot 89SF run in duplicate and quality control serum. The ELISA procedure was carried out for each serotype, and the raw optical density measurements were sent to Pfizer's testing laboratories for analysis. In the second phase of the study, a panel of 12 existing WHO quality QC was assayed and quantified using both 007sp and 89SF as reference standards. Three WHO QC sera, as well as 007sp and 89SF, were run in duplicate on each ELISA plate yielding up to 10 independently determined QC values for each sample and serotype from each laboratory over a minimum of 5 days. The performance of 007sp was assessed by comparing calculated concentrations using 007sp to those using 89SF as the reference standard.

Statistical Analysis:

During each phase of the study and the selected repeated assays, there were about 40 determinations of IgG antibody concentrations for 007sp for each serotype from each laboratory. IgG antibody concentrations were estimated for the four serotypes using a linear mixed-effects analysis of variance (ANOVA) model. All models

were fit independently by serotype and included laboratory and batch as random effects. Confidence intervals (95% CI) were estimated by serotype, accounting for the variance components between the laboratories, between batches within a laboratory and residual variability. Data were analyzed after (common) log transformation of ELISA IgG concentrations. The means of the log concentrations for each serotype were calculated for each laboratory and used to assess agreement and precision among the three laboratories. Agreement is defined as the closeness of the (log) concentration between two laboratories for each of the four serotypes and is measured using Lin's concordance correlation coefficient (r_c), which is a combination of Lin's coefficient of accuracy (C_a)[11] and Pearson's correlation coefficient (r).

Once the four antigen-specific IgG concentration estimates for 007sp were finalized, IgG concentrations were determined for 12 samples from the WHO QC serum panel. Through the two phases of the study, each laboratory contributed up to ten IgG concentration estimates for each WHO QC sample for each serotype. The 12 WHO QC samples do not have known ELISA concentrations or assignments for serotypes 2, 9N, 17F and 20A, and hence, "consensus" ELISA IgG concentration values were estimated using an analysis-of-variance (ANOVA) mixed-effects model from the present data. Scatter plots and boxplots were employed to assess and evaluate the ability of the three laboratories to produce consistent estimates of antibody concentrations for each serotype in 007sp.

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223 receives research and contract support for serology from Merck, Sanofi Pasteur and GSK. DG participates in
224 occasional technical advisory boards and provides advice to GSK, Sanofi Pasteur and Merck and is a National
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Figure Legends:

Figure 1: Comparison of the original assigned values for four serotypes in 89SF with those assigned to 007sp.

Figure 2: Scatter plots showing the correlation among the derived concentrations for the panel of 12 WHO QC sera using 007sp (vertical scale) vs. 89SF (horizontal scale) as reference standards for the four serotypes analysed (N=>8 for each for the 12 QC serum from each laboratory).

Figure 3: Box plots illustrating the deviation of the 007sp estimates from those obtained using 89SF for the four serotypes of the panel of 12 WHO QC sera analysed (N≥8 for each QC serum from each laboratory; N_{total}≥108). In these plots, the box is defined by the 25th and 75th percentiles of the distribution; the line within the box represents the median or 50th percentile. Vertical lines extend to the most extreme observation that is less

than 1.5 times the interquartile range (75th to 25th percentiles), solid circles correspond to individual assay values which are progressively distant from the bulk of the data. Data above the horizontal line of 1 on the vertical axis indicates 007sp estimates are greater than estimates using Lot 89SF. On the vertical axis, 2 indicates a point where the 007sp estimate was twice the 89SF estimate. A value of 1/2 indicates the 89SF estimate was two times the 007sp estimate. Boxes centered on the horizontal line of 1 indicate a good agreement between the 007sp and 89SF estimates.

281

282 Table 1. Assigned IgG antibody concentrations (mcg/ml) for 007sp

Type	89SF ELISA IgG concn (mcg/mL)	007sp ELISA IgG concn (mcg/mL)	95% CI of 007sp IgG concn	n
2	12.24	24.63	(21.25, 28.55)	260
9N	7.77	7.03	(5.52, 8.94)	247
17F	1.75	8.51	(6.74, 10.73)	253
20A	8.73	10.47	(8.55, 12.81)	250

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285 Table 2. Assigned values for 12 pneumococcal WHO QC serum samples as determined with the new pneumococcal reference

286 standard 007sp

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WHO Calibration Serum	Assigned IgG value in mcg/mL (95% CI) for pneumococcal serotype: 2	9N	17F	20A
730	24.14 (21.59, 27.00)	5.43 (4.83, 6.12)	6.72 (6.02, 7.49)	11.55 (10.16, 13.12)
732	1.21 (1.08, 1.35)	2 (1.78, 2.26)	1.36 (1.22, 1.51)	6.17 (5.43, 7.01)
736	45.05 (40.16, 50.55)	1.66 (1.48, 1.87)	9.65 (8.65, 10.77)	0.93 (0.82, 1.06)
746	1.77 (1.58, 1.99)	8.05 (7.16, 9.06)	3.56 (3.19, 3.97)	1.56 (1.37, 1.77)
754	18.43 (16.45, 20.64)	16.02 (14.23, 18.02)	3.62 (3.25, 4.04)	3.44 (3.03, 3.91)
758	50.73 (44.88, 57.35)	3.12 (2.77, 3.51)	22.46 (19.98, 25.25)	5.76 (5.07, 6.55)
760	112.91 (100.64, 126.67)	10.01 (8.89, 11.26)	22.37 (20.05, 24.95)	12.79 (11.26, 14.54)
762	5.29 (4.72, 5.93)	0.88 (0.78, 0.98)	0.38 (0.34, 0.43)	29.56 (26.04, 33.56)
768	2.45 (2.19, 2.74)	8.68 (7.71, 9.77)	1.04 (0.93, 1.16)	17.48 (15.38, 19.87)
770	78.11 (69.62, 87.63)	13.44 (11.94, 15.12)	1.49 (1.34, 1.66)	167.72 (148.24, 189.75)
772	33.05 (29.41, 37.15)	3.06 (2.72, 3.44)	21.36 (19.11, 23.87)	34.56 (30.45, 39.22)
774	0.6 (0.53, 0.67)	1.82 (1.62, 2.05)	2.55 (2.29, 2.84)	1.48 (1.30, 1.68)

n = ≥27 for each estimate

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289

290 Table 3. Comparison of ELISA concentrations between laboratories and laboratory-to-consensus assigned values for WHO QC sera[a]
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		Value for Laboratory:		
Laboratories	Statistic	LAB 1	LAB 2	LAB 3
LAB 1 (N=48)	Accuracy Coef (C_a)[b]	1	1	1
LAB 1	Pearson CC (r)[b]	1	0.993	0.998
LAB 1	Concordance CC (r_c)[b]	1	0.993	0.998
	CCC 95% CI		(0.988, 0.996)	(0.996, 0.999)
LAB 2 (N=48)	Accuracy Coef (C_a)		1	1
LAB 2	Pearson CC (r)		1	0.994
LAB 2	Concordance CC (r_c)		1	0.994
	CCC 95% CI			(0.989, 0.997)
LAB 3 (N=48)	Accuracy Coef (C_a)			1
LAB 3	Pearson CC (r)			1
LAB 3	Concordance CC (r_c)			1
	CCC 95% CI			
Consensus Value (N=48)	Accuracy Coef (C_a)	1	1	1
	Pearson CC (r)	0.999	0.997	0.999
	Concordance CC (r_c)	0.999	0.997	0.999
	CCC 95% CI	(0.998, 0.999)	(0.995, 0.999)	(0.998, 0.999)

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293 [a] Consensus ELISA (log) concentrations were estimated within a serotype by use of a mixed-effects ANOVA model. Predicted ELISA
294 (log) concentrations were obtained for each laboratory by sample within a serotype for each of the replicate observations by use of
295 a mixed-effects ANOVA model. Values in parentheses are 95% confidence intervals.
296
297 [b] C_a, accuracy coefficient, r, Pearson correlation coefficient, r_c, concordance correlation coefficient