1 Title: Assignment of weight-based antibody units for four additional serotypes to a human anti-pneumococcal

2 standard reference serum 007sp

3

4 Running Title: Pneumococcal Reference Serum

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31 **Abstract**

32 The pneumococcal ELISA reference standard serum, Lot 89SF, has been in use since 1990 and was replaced with

33 a new reference standard serum, 007sp in 2013. This serum was generated under an FDA-approved clinical

34 protocol, where 278 adult volunteers were immunized with the 23-valent unconjugated polysaccharide vaccine,

35 Pneumovax II®, and a unit of blood was obtained twice within 120 days following immunization. Pooled serum

36 was prepared from the plasma, filled at 6ml per vial and lyophilised. Five independent laboratories participated

37 in bridging the serotype specific IgG assignments for 89SF to 007sp to establish equivalent reference values for

38 13 pneumococcal capsular serotypes (1,3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) using the WHO reference

39 ELISA. A subsequent follow up study established equivalent reference values for an additional seven serotypes

40 (8, 10A, 11A, 12F, 15B, 22F, 33F). In this study three laboratories assigned weight-based IgG concentrations in

41 mcg/mL of serum to 007sp for four additional serotypes; 2, 9N, 17F and 20A. This study completes the

42 assignment of serotypes in 89SF to 007sp. In addition, the IgG antibody assignments for a 12 member WHO QC

43 serum panel were extended to cover the four additional serotypes. Agreement was excellent with a

44 concordance correlation coefficient (r_c) > 0.996 when each laboratory was compared to the assigned values for

45 the 12 WHO QC sera. The 007sp preparation has replaced 89SF as the pneumococcal reference standard.

46 Sufficient quantities of 007sp are projected to be available for the next 25 years.

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

69   serotypes in 89SF currently unassigned in 007sp (2, 9N, 17F, and 20A), and to assign values to a set of 12

70   existing World Health Organisation (WHO) Quality Control (QC) sera for the additional serotypes.

71

72

73   **Results**

74   To assess the consistency among the laboratories, the mean of the log IgG antibody concentrations of 007sp for

75   each serotype (2, 9N, 17F, and 20A) was calculated for each laboratory and used to assess the level of

76   agreement among the laboratories. There was a high level of agreement with the concordance correlation

77   coefficient r_c exceeding 0.95 for all plots. For the same data, the Pearson correlation coefficient was ≥ 0.999,

78   indicating excellent precision, and the accuracy coeffient (C_a) was ≥ 0.95 in each case. Analysis of variance

79   (ANOVA) models were used to estimate IgG antibody concentrations for each of the serotypes in 007sp. Final

80   point estimates and confidence intervals were obtained by back-transforming the estimated log-transformed

81   concentrations and associated 95% confidence intervals. These estimated IgG antibody concentrations are the

82   "assigned" values for each serotype (2, 9N, 17F, and 20A) in 007sp and are shown in Table 1. These values were

83   derived by the double absorption of 007sp with both mono-substituted and di-substituted cell wall

84   polysaccharide (CPS)[5-7], and thus in the future, when used as a reference standard serum, both standard and

85   unknown test samples should be double absorbed. The IgG antibody concentrations assigned to 007sp

86   compared to the original values assigned to 89SF are shown in Figure 1.

87

88   Serum IgG antibody concentrations against serotypes 2, 9N, 17F, and 20A were determined for the 12-member

89   WHO QC serum panel using both 89SF and 007sp as the reference standards. Table 2 presents the assigned

90   values for the QC serum panel (n≥27 for each estimate) while Figures 2 and 3 display the scatter plots and box

91 plots for the four serotypes analyzed. These plots illustrate the agreement of the four estimated assigned IgG

92 values for 007sp compared to Lot 89SF for each WHO QC serum and serotype.

93 The scatter plots (Figure 2) show the high degree of agreement and correlation among the calculated (log) IgG

94 concentrations for the panel of 12 WHO QC sera using 007sp (vertical scale) vs. Lot 89SF (horizontal scale) as

95 reference standards. A perfect level of agreement would yield a straight line with slope of one and intercept at

96 zero, and all data points cluster tightly about this line of identity. Laboratories 1 and 3, which both used

97 automated liquid-handling robotics to perform the assays, showed a slightly lower degree of scatter around the

98 line of identity compared to Laboratory 2, which used a manual assay process. The box plots (Figure 3)

99 illustrate the deviation of the 007sp-based estimates from those obtained using Lot 89SF as reference standard

100 for the 12 WHO QC sera. The IgG concentrations calculated using 007sp as reference standard are largely within

101 two fold (1/2 – 2.0) of those calculated using lot 89SF as reference standard.

102

103 Table 3 presents the accuracy coefficient (C_a), Pearson correlation coefficient (r), and concordance correlation

104 coefficient (r_c) which are measures of agreement between pairs of laboratories and between laboratories and

105 consensus ELISA concentrations for the WHO QC sera. To form paired data between the labs for these

106 comparisons, the serotype-specific replicate IgG antibody concentration values generated in each laboratory

107 were replaced by a single predicted value obtained from a mixed-model analysis of variance. There was an

108 exceptionally high degree of agreement with all values ≥ 0.99.

109

110 **Discussion**

111 In this study, we describe the assignment of IgG antibody concentrations in weight-based microgram per

112 milliliter units to the human anti-pneumococcal standard reference serum 007sp and a panel of 12

113   pneumococcal QC sera for the final four additional serotypes originally calculated and assigned to 89SF. This

114   new standard was developed in 2009/10 and was required to replace limited stocks of the original standard

115   serum Lot 89SF. Assignment for additional serotypes are required as the original standard, Lot 89SF, which had

116   values assigned for the 23 serotypes in Pneumovax II®, is no longer available (007sp is exclusively distributed via

117   FDA). However studies evaluating Pneumovax II® are still undertaken and new conjugate vaccines are currently

118   under development incorporating additional serotypes found in Pneumovax II®, but not in existing PCV's.

119   Assignment of the weight-based antibody concentrations to human anti-pneumococcal standard reference

120   serum 007sp was originally performed for the 13 serotypes represented in currently licensed conjugate

121   vaccines (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F)[3]. Using established laboratories and a well

122   characterized ELISA procedure [8,9] that was followed by all participating laboratories, it was possible to assign

123   weight-based units to 007sp by running 007sp alongside a standard curve of 89SF and treating 007sp as the

124   unknown. Very high levels of agreement between the participating laboratories for the weight-based units of

125   IgG specific for 13 serotypes in 007sp were achieved. Having accepted concentration values for an existing

126   standard has significantly simplified the assignment process. Subsequently we undertook a further assignment

127   exercise utilizing the expertise of three laboratories. Values (mcg/ml) for IgG specific to seven additional

128   serotypes (8, 10A, 11A, 12F, 15B, 22F, and 33F) were assigned to 007sp and the 12 QC sera described above[4].

129   As in the original assignment study, we have now assigned values (mcg/ml) for IgG specific for the final four

130   serotypes in 89SF that remained unassigned (2, 9N, 17F and 20A). We were able to further validate the values

131   obtained and the performance of 007sp as a standard during the process of assigning serotype specific IgG

132   values (mcg/ml) to a panel of 12 WHO QC sera previously prepared from the sera of pneumococcal

133   polysaccharide vaccinated adults. Concordance was high among laboratories (Table 3) and between results for

134   laboratories and consensus ELISA concentrations. With the adherence to the uniform application of the WHO

135   ELISA[9] in the present study, we were able to achieve high levels of precision and accuracy in the values assigned

136   to the additional four serotypes of 007sp and the WHO QC sera.

137   ANOVA mixed modeling is a flexible framework that allows estimation of ELISA concentrations for 007sp and

138   the 12 WHO QC sera for each serotype by laboratory. These models may be used to compare and contrast

139   results within and among laboratories. Random-effects ANOVA models allowed us to reduce the replicate

140   measurements to a single predicted value which were then used to measure levels of consistency among the

141   laboratories. While we were able to estimate serotype-specific concentrations for 007sp through a bridge to

142   89SF (Table 1), the actual ELISA concentrations for the WHO QC sera used in this study were unknown, so it was

143   not possible to compare "true" values. The ANOVA mixed model provided a mechanism for estimating

144   consensus values, which served as assigned values for these sera (Table 2).

145   Establishing a new reference serum for the pneumococcus was essential for ongoing efforts to evaluate new

146   pneumococcal vaccines and to maintain the link with the original serology performed as part of the pivotal

147   efficacy studies conducted prior to licensure. The high degree of agreement between the 007sp-based and lot

148   89SF-based estimates in the original assignment exercise[3] has inspired confidence in the validity of the 007sp

149   assignments. In this follow on study, a similar high level of agreement has been observed.

150   The new standard, 007sp, now has assigned values for the 24 pneumococcal serotypes currently contained in

151   licensed vaccines, is available in large quantities and should provide continuity for the foreseeable future. Its

152   performance in ELISA suggests that it is unlikely to affect the operation of validated assays currently established

153   in serology laboratories. Details of how to obtain 007sp and the QC sera are available at

154   https://www.vaccine.uab.edu/.

155

156   **Materials and Methods**

157 *Collection of Human Sera*

158 The collection and processing of sera has been described in detail in a previous manuscript [3]. Briefly, 278

159 volunteers were vaccinated once with Pneumovax II®, and serum collected on two occasions post vaccination.

160 Serological and virological testing showed sera to be free from Hepatitis B and C virus, syphilis and HIV. Sera

161 from 262 volunteers were pooled and then aliquoted at 6 ml per vial and lyophilised while sera from the

162 remaining 16 donors were separately aliquoted to create a new panel of individually calibrated sera for use in

163 functional assays.

164 An existing WHO QC serum panel, established by D. Goldblatt (UCL Institute of Child Health) previously by

165 immunizing adults with pneumococcal polysaccharide vaccine, and distributed by the National Institute for

166 Biological Standards and Control (NIBSC; Potters Bar, Hertfordshire, United Kingdom), was supplied for

167 assigning serotype-specific IgG assignments for the 12 QC serum panel members.

168 *Laboratory Methods*

169 Three laboratories participated in the assignment (In alphabetical order: Institute of Child Health, University

170 College London, London, United Kingdom; Pfizer Vaccine Research and Development, Pearl River, NY; and

171 Universitätsklinikum Erlangen Kinder- und Jugendklinik, Erlangen, Germany). Two of the three laboratories used

172 liquid-handling robotics to perform various aspects of the ELISA while the other laboratory performed the

173 assays by hand. The assignment of weight-based units followed the protocol established for the initial

174 assignment, which can be found under the reference materials section at http://www.vaccine.uab.edu and

175 mirrored the protocol used to assign values for an additional seven serotypes[4]. In the first phase of the study,

176 serotype-specific IgG antibody assignments for four serotypes (2, 9N, 17F, and 20A) were established by

177 calibrating Lot 007sp under double absorbent assay conditions against Lot 89SF under single absorbent

178 conditions using the standardized pneumococcal reference ELISA (the "WHO ELISA")[8,9]. The ELISA protocol

179   followed by participating laboratories can be found at http://www.vaccine.uab.edu/ELISA%20Protocol.pdf. The

180   only deviation from the WHO protocol is that double absorption of 007sp with cell wall polysaccharide (CPS)[5]

181   was undertaken using two absorbents prepared from un-encapsulated S. *pneumoniae* mutant strains

182   incorporating both mono- and di-substituted CPS[7,8] rather than CPS and purified 22F capsular polysaccharide.

183   Lot 89SF had a value assigned for Serogroup 20, the serogroup included in Pneumovax 23®. This sugar has now

184   been identified as serotype 20A[10] so this capsular polysaccharide and nomenclature has been used in this

185   assignment exercise. Briefly, four independent sets of serial dilutions of lot 007sp (supplied by CBER, FDA) were

186   made from four independent serum vials. The four sets of eight serial dilutions were run in duplicate as

187   unknown samples on each ELISA plate in a 10-plate replicate series to generate approximately 40 data points

188   per serotype for 007sp from each of the participating laboratories. Each plate also contained seven serial

189   dilutions of lot 89SF run in duplicate and quality control serum. The ELISA procedure was carried out for each

190   serotype, and the raw optical density measurements were sent to Pfizer's testing laboratories for analysis.

191   In the second phase of the study, a panel of 12 existing WHO quality QC was assayed and quantified using both

192   007sp and 89SF as reference standards. Three WHO QC sera, as well as 007sp and 89SF, were run in duplicate

193   on each ELISA plate yielding up to 10 independently determined QC values for each sample and serotype from

194   each laboratory over a minimum of 5 days. The performance of 007sp was assessed by comparing calculated

195   concentrations using 007sp to those using 89SF as the reference standard.

196

197   **Statistical Analysis:**

198   During each phase of the study and the selected repeated assays, there were about 40 determinations of IgG

199   antibody concentrations for 007sp for each serotype from each laboratory. IgG antibody concentrations were

200   estimated for the four serotypes using a linear mixed-effects analysis of variance (ANOVA) model. All models

201   were fit independently by serotype and included laboratory and batch as random effects. Confidence intervals

202   (95% CI) were estimated by serotype, accounting for the variance components between the laboratories,

203   between batches within a laboratory and residual variability. Data were analyzed after (common) log

204   transformation of ELISA IgG concentrations. The means of the log concentrations for each serotype were

205   calculated for each laboratory and used to assess agreement and precision among the three laboratories.

206   Agreement is defined as the closeness of the (log) concentration between two laboratories for each of the four

207   serotypes and is measured using Lin's concordance correlation coefficient (r_c), which is a combination of Lin's

208   coefficient of accuracy (C_a)[11] and Pearson's correlation coefficient (r).

209

210   Once the four antigen-specific IgG concentration estimates for 007sp were finalized, IgG concentrations were

211   determined for 12 samples from the WHO QC serum panel. Through the two phases of the study, each

212   laboratory contributed up to ten IgG concentration estimates for each WHO QC sample for each serotype. The

213   12 WHO QC samples do not have known ELISA concentrations or assignments for serotypes 2, 9N, 17F and 20A,

214   and hence, "consensus" ELISA IgG concentration values were estimated using an analysis-of-variance (ANOVA)

215   mixed-effects model from the present data. Scatter plots and boxplots were employed to assess and evaluate

216   the ability of the three laboratories to produce consistent estimates of antibody concentrations for each

217   serotype in 007sp.

218

219   **Acknowledgements**

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261   Figure Legends:

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

264

265   Figure 2: Scatter plots showing the correlation among the derived concentrations for the panel of 12 WHO QC

266   sera using 007sp (vertical scale) vs. 89SF (horizontal scale) as reference standards for the four serotypes

267   analysed (N=>8 for each for the 12 QC serum from each laboratory).

268

269   Figure 3: Box plots illustrating the deviation of the 007sp estimates from those obtained using 89SF for the four

270   serotypes of the panel of 12 WHO QC sera analysed (N≥8 for each QC serum from each laboratory; $N_{total} \geq 108$).

271   In these plots, the box is defined by the 25th and 75th percentiles of the distribution; the line within the box

272   represents the median or 50th percentile. Vertical lines extend to the most extreme observation that is less

273   than 1.5 times the interquartile range (75th to 25th percentiles), solid circles correspond to individual assay

274   values which are progressively distant from the bulk of the data. Data above the horizontal line of 1 on the

275   vertical axis indicates 007sp estimates are greater than estimates using Lot 89SF. On the vertical axis, 2

276   indicates a point where the 007sp estimate was twice the 89SF estimate. A value of 1/2 indicates the 89SF

277   estimate was two times the 007sp estimate. Boxes centered on the horizontal line of 1 indicate a good

278   agreement between the 007sp and 89SF estimates.

279

280

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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284

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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	Assigned IgG value in mcg/mL (95% CI) for pneumococcal serotype:			
WHO Calibration Serum	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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Laboratories	Statistic	Value for Laboratory:		
		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)

292
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