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**Evaluation of a candidate 1<sup>st</sup> International Standard for anti-Pneumococcal  
sera 007sp**

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## **Summary**

To develop and evaluate the efficacy of pneumococcal vaccines it is important to have an accurate method for measuring the concentration of human antibodies directed against pneumococcal capsular polysaccharides (Pn PS). In 2000, representatives from academia, government, and industry met at the WHO in Geneva, Switzerland, and selected an enzyme-linked immunosorbent assay protocol for quantification of human IgG antibodies specific for *Streptococcus pneumoniae* capsular polysaccharides (Pn PS ELISA). In order to estimate the concentration of antibodies the reference serum 89SF was produced and antibody concentrations established. As quantities of 89SF are depleted, in order to maintain the link to clinical efficacy, a replacement was produced. Five laboratories from three countries participated in a collaborative study to assess the suitability and determine the pneumococcal serotype specific antibody content of a candidate International Standard serum preparation (007sp). The antibody concentrations of candidate standard were assigned by bridging the established standard 89SF using the standardised WHO Pn PS ELISA. Agreement was excellent with a concordance correlation coefficient ( $r_c$ )>0.93 when each laboratory was compared to the assigned values for 12 QC sera. On the basis of the results from this study, it is recommended that the candidate standard 007sp is established as an International Standard for the quantification of serotype specific anti-pneumococcal antibodies in sera.

## **Introduction**

*Streptococcus pneumoniae* Human Reference Serum, lot 89SF has been used as a standard serum in serotype specific ELISA designed to measure IgG antibody specific for individual pneumococcal capsular polysaccharides since 1990. 89SF is listed by the WHO expert committee on biological standardization in the technical report series outlining the serological evaluation and licensure of pneumococcal conjugate vaccines. In addition, 89SF is used as the standard in the WHO standardized ELISA for the quantitation of *Streptococcus pneumoniae* serotype specific IgG. This standard serum greatly facilitated the standardization of ELISA methodologies during a critical period when the first polysaccharide-conjugate vaccines were being evaluated for licensure. Lot 89SF consists of pooled, defibrinated plasma collected in the late 1980s from 17 individuals vaccinated with a 23-valent pneumococcal polysaccharide vaccine (Pnu-Imune, Lederle). The final volume of this collection was approximately 10 liters (2-ml/vial). Serotype specific weight based values for IgG, IgA and IgM were derived for serotypes by Quateart, et al. (1). Assignments for the additional serotypes in the 23-valent pneumococcal polysaccharide vaccine were subsequently bridged from the assignments for the original 11 serotypes (2). The Lot 89SF standard has been maintained and shipped to laboratories all over the world from the Center for Biologics and Evaluation Research (CBER), US Food and Drug Administration (FDA) at a rate of approximately 150 vials per year. In 2005, it was estimated that only two to five years supply of Lot 89SF remained. Since Lot 89SF was the reference standard used in the evaluation of the first pneumococcal conjugate vaccine to be licensed, a seven-valent conjugate (Prevnar™, Pfizer PCV7), the link to clinical efficacy would have been severed if stocks were depleted. Since comparable immune responses to PCV7 is a licensure approach for new pneumococcal conjugate vaccines, retaining the link to the original efficacy trials for PCV7 is critical.

A new *Streptococcus pneumoniae* Human Serum has been made with the aim to replace 89SF. Sera from volunteers were pooled, aliquoted and lyophilized for future ELISA standardization. This report describes the collaborative study undertaken to establish the serotype specific IgG concentrations for the new serum, in mg/L, designated 007sp and to validate its performance as Pn PS ELISA standard.

## Participants

Five laboratories participated in the study including the two World Health Organization Pneumococcal Serology Reference Laboratories at the Institute of Child Health, University College London, London, England and the Department of Pathology at the University of Alabama at Birmingham, Birmingham Alabama, USA. Three manufacturers also participated: Pfizer Vaccine Research, USA., GlaxoSmithKline Biologicals, Belgium., PPD Vaccines & Biologics Laboratory, USA on behalf of Merck Sharp & Dohme Corp. The participants were decided upon by the 007sp Working Group (see acknowledgements). Participants were assigned a random code number, not corresponding to the order of listing.

## Materials and Methods

### The candidate standard

Following written informed consent, 427 individuals were evaluated at the University of Iowa, and 287 healthy male and non-pregnant female volunteers between 18 and 45 years of age met eligibility requirements for this study (DMID Study Protocol number 06-0093). The study was approved by the University of Iowa IRB (Committee A). Volunteers were vaccinated once with 23 valent pneumococcal polysaccharide vaccine (Pneumovax II®), and returned 10-35 days following immunization, and 8 – 12 weeks following their first blood donation to donate one unit (500ml) of blood. Blood samples were processed in two steps using a “three bag” system. First, red blood cells were removed and the plasma transferred to a second bag where it was allowed to coagulate. The sera were collected in the third bag and bags were subjected to serological and virological testing. Using FDA licensed methods and in accordance with the requirements of directive 98/79, showed sera to be free from Hepatitis B and C virus, syphilis and HIV. The bags of serum was sent for pooling, filling and lyophilized under contract by Thermo Fisher (Thermo Fisher Scientific, Waltham, MA, USA).

Serum bags were opened and pooled in a sterile, pre chilled (2-8°C) jacketed tank. The sera were mixed gently, to avoid foaming, for 30 minutes. The blended serum was then passed through a succession of filters (1.0 micron, 0.45 micron, 0.2 micron) onto a sterile bio-processing bag. On the 21<sup>st</sup> of October 2008, Vials (25ml) were each filled with 6ml of serum. Vials were loaded onto pre-chilled (2-8°C) trays prior to lyophilisation. Initially, the shelf temperature of the freeze dryer was lowered to -50°C and held at this temperature for 7 hours. A vacuum of 75millitor was applied before the temperature was ramped to -10°C over 80 minutes and primary drying was continued over 30 hours. Final drying occurred at a temperature of 25°C for 24 hours with a reduced vacuum of 30mTorr. Vials were backfilled with dry nitrogen before stoppering. Stoppered vials were then crimped sealed and the final product labeled on the 28<sup>th</sup> October 2008 yielding 15,324 vials.

Microbial content was assayed following growth on TSA medium, no more than 5 cfu's per mL was confirmed for 3 samples, with no growth recorded. Residual moisture, as measured by Karl Fischer (Mitsubishi moisture meter), content is 0.811% (w/w) with a CV 2.1%, calculated using 3 vials. The mean mass of the fill was calculated, using 6 ampoules, to be 6.11g with a %CV 0.25. No data for the residual oxygen content or ampule integrity is presented in this report. There has been difficulty in acquiring all the filling data due to the death of Milan Blake the leader of this project but it is possible that it was not tested as part of the fill contract.

Retrospective analysis of oxygen content and ampule integrity will be conducted at NIBSC and data will be reported in the future. CEBR, USA ( $\approx 10,000$ ) and NIBSC, UK (5,000) will act as the custodians of the material which is being stored at  $-20^{\circ}\text{C}$ .

### **Study materials**

Participants in the collaborative study were sent four vials of the 007sp serum. Upon receipt, participants were asked to store the samples  $-20^{\circ}\text{C}$ . Prior to use in assays samples were to be reconstituted with 6ml sterile distilled water and kept at  $4^{\circ}\text{C}$  for up to 2 weeks, while completing the assays. If longer term storage of the samples was required, samples were aliquoted and stored at  $-20^{\circ}\text{C}$  until further use.

Additional reagents were sourced from a single place to provide greater consistency. Pneumococcal serotype polysaccharides were sourced from ATCC (American type culture collection, Rockville, USA), with the exception of serotype 6A which was supplied by Pfizer (Pfizer Vaccine Research, USA). Adsorbents cell wall polysaccharide (CWPS) was supplied from SII (Statens serum institut, Copenhagen, Denmark) and serotype 22F polysaccharide from ATCC). One vial of 89SF frozen preparation was supplied by CBER (FDA, USA). Secondary antibody was sourced from Southern Biotech (Alabama, USA). Microtitre plates were provided by Pfizer (Pfizer Vaccine Research, USA). Additional anti-pneumococcal QC sera were also supplied by NIBSC, UK (96/760, 96/762, 96/770, 96/772, 96/774, 96/776, 96/728, 96/732, 96/736, 96/746, 96/756 and 96/758)

### **Study design**

In the first phase participants were requested to assay the four vials of 007sp. This consisted of 8 serial dilutions, run in duplicate as unknown samples on each ELISA plate. Each plate was run in a 5-plate replicate series to generate a total of 20 data points per serotype for 007sp from each of the participating laboratories. Each plate also contained 7 serial dilutions of lot 89SF run in duplicate and a laboratory-specific QC serum. This was carried out for each serotype (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F). Raw optical density measurements from the five laboratories were sent to the CDC and antibody concentrations were derived using the CDC Program ELISA for Windows (Centers for Disease Control and Prevention, Atlanta, GA, USA). Biostatisticians at CDC, carried out basic quality control of the data and laboratories were requested to rerun selected assays that were deemed outside of quality control specifications.

In the second phase, to confirm an in use analyses of 007sp, laboratories were requested to assign antibody concentrations of 12 additional anti-pneumococcal QC sera from vaccinated individuals. Both 89SF and 007sp were used as standards. Agreement between the two could confirm the correct bridging of the values.

## **Assays for evaluation of the candidate standard**

### ***ELISA***

Serotype-specific IgG assignments were established by calibrating lot 007sp against lot 89SF using the standardized pneumococcal reference ELISA (the "WHO ELISA") (9). The selected protocol is as follows. Human serum samples are mixed before analysis with an absorbent containing C-polysaccharide (C-PS) and 22F capsular PS to neutralize antibody binding to C-PS and other common contaminants present in the Pn PS coating antigens. 22F PS is used because it is a rare serotype, not present in any conjugate vaccine, and is thought to contain

contaminating non-Pn PS epitopes. ELISA plates are coated with Pn PS by adsorbing individual Pn PS serotype antigens to micro-titer plates. Dilutions of absorbed human sera are then added to the ELISA plates. The serotype specific antibody bound to the ELISA plate is 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 is measured at 405 nm using an ELISA plate reader. By comparing the optical density of the sample wells to that of the standard (human anti-pneumococcal reference serum, lot 89SF) the level of antibody in the human serum can be calculated.

### **Stability Studies**

To determine the stability of the candidate 007sp standard, study of the candidate material was undertaken at NIBSC included an accelerated degradation and a stability study of the reconstituted material. The accelerated degradation study of the candidate standard (at temperatures of 4, 20, 37, 45 and 56°C), using a -20°C baseline was carried out on samples stored for 6, 12, 18 and 24 months. The samples were assessed using the ELISA and multiplex bead assay (3).

In an additional study, the candidate standard was reconstituted in 6ml of sterile distilled water and stored at -70°C, -20°C +4°C for 3 and 10 weeks. The pneumococcal serotype specific antibody content of the samples was determined using the multiplex bead assay.

### **Statistical Analysis**

Statistical analysis was carried out at CDC. Through this study there were at least 40 determinations of antibody concentrations for 007sp for each serotype from each laboratory. Antibody concentrations were estimated for the 13 serotypes using a linear mixed-effects analysis of variance (ANOVA) model. All models were fit independently by serotype and included laboratory and plate as random effects. Confidence intervals (95% CI) were estimated by serotype, accounting for the variance components between the laboratories, between plates within a laboratory and sample variability nested within plates and laboratory. Data were analyzed after natural 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 five laboratories. Agreement is defined as the closeness of the geometric means between two laboratories for each of the 13 serotypes and is measured using Lin's coefficient of accuracy ( $C_a$ ) (4). Precision measures how far a set of observations deviates from a straight line and is quantified using Pearson's correlation coefficient ( $r$ ). Lin's concordance correlation coefficient ( $r_c$ ), which is a combination of  $C_a$  and  $r$ , was employed to form a single statistic describing both agreement and precision.

For each serotype in 007sp, antibody concentrations estimated using ANOVA models adjusting for laboratory were obtained by back-transforming the estimated log-transformed concentration and associated 95% confidence interval. These concentrations served as the "assigned" values for each serotype in 007sp. Scatter plots and boxplots were employed to assess and evaluate the ability of the five laboratories to produce consistent estimates of antibody concentrations for each serotype in 007sp.

### **Results**

Quality control of the data submitted for analysis revealed the data to be of high quality and few were deemed outside of QC specifications leading to minimal re-testing. Mean of the log concentrations of 007sp for each serotype was calculated for each laboratory (Table 1) and used to assess the level of agreement among the laboratories. Figure 1 displays scatter plots between

all pairs of laboratories with the concordance correlation coefficient listed within each plot. The solid diagonal line indicates perfect agreement (slope=1; intercept=0). These statistics indicate a high level of agreement with  $r_c$  exceeding 0.97 for all plots. Data from all 5 laboratories was considered valid for all serotypes and no data was excluded. For these same data, the Pearson correlation coefficient was  $\geq 0.98$ , and the accuracy coefficient ( $C_a$ ) was  $\geq 0.99$  in each case. Analysis of variance (ANOVA) models were used to estimate antibody concentrations for each of the serotypes in 007sp, adjusting for laboratory. Variance components necessary for the calculation of confidence intervals were calculated adjusting for the replicate values within a plate and between plates within a laboratory. Final point estimates and confidence intervals were obtained by back-transforming the estimated log-transformed concentrations and associated 95% confidence intervals. These concentrations served as the “assigned” values for each serotype in 007sp and appear in Table 2. These values were derived following double adsorption of 007sp with cell wall polysaccharide (CPS) and polysaccharide 22F and thus in future, when used as a standard, both standard and unknown sera should be double adsorbed (Lot 89SF values were derived following single adsorption and thus the standard and unknown sera are dealt with differently in the current ELISA protocol). The values assigned to 007sp compared to the original values assigned to 89SF are shown in Figure 2.

Serum IgG ELISA concentrations were determined for the 12-member QC serum panel using both 89SF and 007sp as the reference standards. Figures 3 and 4 display the scatter plots and box plots for the first 7 serotypes (1, 3, 4, 5, 6A, 6B, and 7F) analysed and figures 5 and 6 show the same information for the remaining 6 serotypes (9V, 14, 18C, 19A, 19F, and 23F). These plots illustrate the agreement and precision of the 5 estimated assigned values for 007sp compared to Lot 89SF for each WHO QC serum and serotype. The scatter plots (figures 3 and 5) show the high degree of agreement and correlation among the calculated concentrations for the panel of 12 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 1 and intercept at 0. With rare exception, all data points cluster tightly about this line of identity. Lab 4’s estimated concentrations for serotype 3 were greater for 89SF than 007sp (figure 3). Lab 3 and 4 also reported slightly higher concentrations using 007sp for serotypes 19A and 14, respectively (figure 5).

The box plots (figures 4 and 6) illustrate the deviation of the 007sp-based estimates from those obtained using Lot 89SF as reference standard for the 12 QC sera. These plots offer more resolution than the scatter plots in that they relay more information regarding the deviation of the 007sp and Lot 89SF estimates. By and large, the concentrations calculated using 007sp as reference standard are within two fold (1/2 – 2.0) of those calculated using lot 89SF as reference standard. Notable exceptions include serotypes 6B and 19A for lab 5 and serotype 23F for lab 4.

Table 3 presents accuracy ( $C_a$ ), precision ( $r$ ), and concordance ( $r_c$ ) measures of agreement between pairs of laboratories and between laboratories and consensus ELISA concentrations for the QC sera. In order to form paired data between the labs for these comparisons, the five replicate concentration values 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 but one values  $\geq 0.90$ . In general, comparisons with lab 3 yielded the lowest measures of correlation and agreement. This is due to one concentration value for lab 3 (type 14) that is substantially greater than the values reported by the other 4 labs. If this value were removed, all statistics involving lab 3 improve. As an example, the  $r_c$  between labs 3 and 5 of 0.882 improves to 0.947 when this single value is deleted from the analysis.

## Stability Studies

The concentration of anti-pneumococcal serotype specific antibodies in a vial of 007sp was compared over time using the WHO ELISA and an in-house multiplex bead assay. Accelerated thermal degradation and reconstituted degradation were studied over a number of time points.

During the accelerated degradation studies, samples stored at 56°C became congealed and difficult to re-suspend and therefore these samples were not used. After 6 months antibody concentrations were established for 10 serotypes using a multiples bead assay (figure 7). Calculation of the predicted degradation after 6 months (table 4) showed good stability with an average loss of 0.035% per year across all serotypes. Serotypes 4 and 18C appeared to be least stable and showed a predicated annual loss of 0.072%. A more accurate prediction of degradation was made following analysis of 12 month samples by ELISA for serotypes 4 and 23F (table 4) which demonstrate a small annual loss of 1.34% and 0.84% respectively. Further prediction of degradation for all serotypes will be completed for 18 and 24 month samples.

Stability following reconstitution was monitored by comparison to a baseline sample stored at -70°C. After 3 weeks significant degradation was observed at 4, 20 and 37°C across all serotypes. The amount of serotype specific antibodies in the ampoules remained constant and no significant decrease was seen when stored at -20°C compared to -70°C after 10 weeks, across all serotypes (table 5).

## Discussion

Assignment of the weight based antibody units to human anti-pneumococcal standard reference serum Lot 89SF currently in use was defined by Quateart and colleagues in 1995 (6). They used the Zollinger method (10) to quantitate the amount of total antibody as well as IgA, IgM and IgG in the reference preparation. Briefly, an ELISA designed to capture immunoglobulin molecules in a known reference preparation was run side by side with a pneumococcal capsule specific ELISA and the signal obtained from the reference preparation capture of a known amount of IgG, IgA and IgM respectively, could be compared to the signal from the capsule specific ELISA (the "ELISA quantitation method"). This permitted quantitation of the amount of capsule specific IgG, IgA and IgM in Lot 89SF for 11 pneumococcal serotypes (1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). In 2004 this was extended through the assignment of weight-based antibody values for 13 additional PnPs serotypes (2, 6A, 8, 9N, 10A, 11A, 12F, 15B, 17F, 19A, 20, 22F, and 33F) and for the *S. pneumoniae* cell wall polysaccharide (C-Ps) (5) using an enzyme-linked immunosorbent assay (EIA)-based equivalence of absorbance method suggested by Concepcion and Frasch for cross-standardization (1). This method was also used to assign weight based IgG1 and IgG2 units for serotypes 3, 6B, 14, 19F and 23F in Lot 89S (8) and serotypes 1,4, 5, 7F, 9V and 18C (8).

In this study we describe the assignment of weight based units for 13 serotypes to a new Pneumococcal Serum (007sp) that has been established to replace dwindling stocks of 89SF. Having accepted concentration values for an existing standard has significantly simplified the assignment process. Using established laboratories and a well characterized ELISA procedure (4) that was followed by all participants, we were able 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.

These assignments were made using double absorptions (CPS and 22F PS), which will simplify the day-to-day running of the pneumococcal ELISA, as both unknowns and standards are double adsorbed. Previously 89SF had values assigned with single adsorption only (CPS) with the utility of 22F adsorption post dating the assignments (2), thus requiring the standard and unknown sera to be treated differently during each assay.

We were then able to further validate the values obtained and the performance of 007sp as a standard during the process of assigning serotype specific IgG values ( $\mu\text{g/ml}$ ) to a panel of 12 QC sera previously prepared from the sera of pneumococcal polysaccharide vaccinated adults. Our statistical analyses indicated a high level of agreement among the five laboratories participating in this study. Concordance was high among laboratories (Table 4) and between results for laboratories and consensus ELISA concentrations. Concordance ( $r_c$ ) is a combined measurement of agreement, i.e., it combines accuracy and precision and in this study was calculated across all serotypes and samples by laboratory. When each laboratory was compared to the consensus for the 12 QC sera,  $r_c > 0.93$  (Table 4). This indicates that with the adherence to the uniform application of the WHO ELISA (5) in the present study, we were able to achieve a level of precision and accuracy that should inspire confidence in the values assigned to the 13 serotypes of 007sp and the QC sera.

Establishing international standard for the pneumococcus is essential for ongoing efforts to evaluate new pneumococcal vaccines while maintaining the link with the original serology performed as part of the pivotal efficacy studies conducted prior to licensure. This link to the established  $\mu\text{g/ml}$  concentrations using 89SF in clinical studies is the predominant reason why IU was not assigned to this standard. Consistency of units is particularly important when considering the established threshold of protection of  $0.35\mu\text{g/ml}$ , as recommended by the WHO Technical Report Series 297 and also non inferiority of new relative to established vaccines. In general there is a high degree of agreement between the 007sp-based and Lot 89SF-based estimates. This inspires confidence in the validity of the 007sp assignments. The new standard, 007sp, is available in large quantities and should provide continuity for the foreseeable future. Its performance in ELISA suggests that it would not affect the operation of validated assays currently established in serology laboratories.

In this report the suitability of this candidate standard 007sp was investigated only for use with for use in the WHO pneumococcal ELISA. However, there is also potential to use 007sp as a standard for the opsonophagocytic assay (OPA) for quantification of functional anti pneumococcal antibodies was also consideration prior to production. Due to the larger volumes required for the OPA 007sp was filled at 6ml of sera per vial. The assignment of values for 007sp in the OPA is currently being perused and may possibly be submitted to ECBS in the future.

At a meeting in Emory Conference Centre, Atlanta, 14/01/2010 it was agreed and minuted by all participants of the *007sp Working Group* that these sera be submitted to ECBS as an international standard. The method, results and discussion of the proposal have been directly taken for a recently accepted paper in vaccine, which had been extensively reviewed and discussed by all participants of the *007sp Working Group* and therefore this report has not been circulated for comment.

## Stability and storage of the candidate standard

Accelerated degradation analysis of the candidate standard is ongoing. Initial results suggest that, in common with many serum standards, the preparation is stable at -20°C in a lyophilised state, with approximately 1% annual loss of antibody concentration. Supporting real time stability will be reported when it is available. The stability of 007sp will be closely monitored especially considering the use of the relatively large (25ml) stoppered vials. Upon reconstitution 007sp antibody concentrations were significantly depleted after 3 weeks at 4°C but remained unchanged after 10 weeks at -20°C. It is therefore recommended that once reconstituted the standard be stored at -20°C and that repeated freeze thaw cycles be avoided, although this is best practice it has not been scientifically tested. The exact shelf life of the reconstituted material will be determined in the future.

## Proposal

Based on the results obtained from the ELISA in this collaborative study, we propose that the *Streptococcus pneumoniae* Human Serum candidate standard 007sp is established as the 1st International Standard with a serotype specific antibody content as described in table 2 for potential use in assays for quantification of measuring the concentration of human antibodies by ELISA directed against pneumococcal capsular polysaccharides following immunization with pneumococcal vaccines.

## Acknowledgements

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Table 1: Mean (95% confidence interval) IgG antibody concentrations ( $\mu\text{g/ml}$ ) for 007sp for 13 serotypes from the 5 individual laboratories.

Type	Lab 1	Lab 2	Lab 3	Lab4	Lab 5
1	8.3954 (7.7869-9.0515)	8.8035 (8.1654-9.4914)	7.8783 (7.3073-8.4939)	8.3647 (7.7585-9.0184)	9.1132 (8.4527-9.8253)
3	1.4399 (1.3455-1.5409)	1.4570 (1.3614-1.5592)	1.5099 (1.4109-1.6159)	1.4742 (1.3776-1.5777)	1.3790 (1.2886-1.4757)
4	3.2300 (2.8566-3.6522)	3.2432 (2.8683-3.6670)	3.8553 (3.4096-4.3592)	2.9782 (2.6339-3.3675)	3.4147 (3.0200-3.8610)
5	7.1039 (6.6573-7.5804)	7.8199 (7.3283-8.3444)	7.9150 (7.4175-8.4459)	7.3342 (6.8732-7.8261)	7.4179 (6.9517-7.9155)
6A	3.8391 (3.6477-4.0405)	4.0116 (3.8116-4.2220)	4.0152 (3.8151-4.2258)	3.7850 (3.5963-3.9835)	4.0165 (3.8163-4.2272)
6B	8.8697 (7.434-10.582)	8.8541 (7.421-10.564)	7.3939 (6.197-8.822)	9.8105 (8.223-11.705)	10.6858 (8.957-12.749)
7F	8.2921 (8.1300-8.4574)	8.2947 (8.1325-8.4601)	8.3019 (8.1396-8.4674)	8.2987 (8.1364-8.4641)	8.3055 (8.1432-8.4712)
9V	6.8764 (6.4729-7.3050)	6.5622 (6.1771-6.9712)	6.1281 (5.7685-6.5101)	6.3652 (5.9917-6.7620)	6.2985 (5.9289-6.6911)
14	39.7773 (36.503-43.345)	39.8044 (36.528-43.375)	36.5734 (33.563-39.854)	34.4966 (31.657-37.591)	39.6001 (36.341-43.152)
18C	7.6051 (7.0846-8.1639)	7.7465 (7.2163-8.3157)	6.8823 (6.4112-7.3880)	7.3412 (6.8387-7.8806)	6.9835 (6.5054-7.4966)
19A	13.7338 (11.389-16.560)	13.1896 (10.938-15.904)	12.2990 (10.200-14.830)	12.8717 (10.675-15.521)	17.9317 (14.871-21.622)
19F	13.5858 (11.797-15.646)	12.8422 (11.151-14.789)	14.2861 (12.405-16.452)	15.8684 (13.779-18.274)	16.8068 (14.594-19.355)
23F	6.2371 (5.4570-7.1288)	6.4803 (5.6697-7.4067)	6.1644 (5.3934-7.0457)	4.9704 (4.3487-5.6810)	6.0420 (5.2862-6.9057)

Table 2: Assigned IgG antibody concentrations ( $\mu\text{g/ml}$ ) for 007sp

Type	IgG ELISA Concentration ( $\mu\text{g/ml}$ )	Lower 95% CI	Upper 95% CI	N
1	8.50	7.88	9.16	200
3	1.45	1.36	1.55	200
4	3.33	2.95	3.77	200
5	7.51	7.04	8.02	210
6A	3.93	3.74	4.14	200
6B	9.05	7.59	10.80	225
7F	8.30	8.14	8.46	200
9V	6.44	6.06	6.84	200
14	37.99	34.86	41.39	220
18C	7.30	6.80	7.84	205
19A	13.87	11.51	16.73	205
19F	14.61	12.68	16.82	200
23F	5.95	5.21	6.81	215

Table 3: Comparison of ELISA concentrations between laboratories and laboratory-to-consensus assigned values for QC sera<sup>a</sup>

	Statistic <sup>b</sup>	LAB 1	LAB 2	LAB 3	LAB 4	LAB 5
LAB 1 (N=156)	Accuracy (C <sub>a</sub> )	1.0	0.994	0.986	0.993	0.995
LAB 1	Precision (r)	1.0	0.989	0.931	0.995	0.982
LAB 1	CCC (r <sub>c</sub> )	1.0	0.982	0.918	0.989	0.977
	CCC 95% CI		(0.977, 0.986)	(0.892, 0.938)	(0.986, 0.991)	(0.969, 0.982)
LAB 2 (N=156)	Accuracy (C <sub>a</sub> )		1.0	0.998	1.000	0.978
LAB 2	Precision (r)		1.0	0.902	0.988	0.989
LAB 2	CCC (r <sub>c</sub> )		1.0	0.900	0.988	0.967
	CCC 95% CI			(0.866, 0.926)	(0.984, 0.991)	(0.960, 0.973)
LAB 3 (N=156)	Accuracy (C <sub>a</sub> )			1.0	0.998	0.964
LAB 3	Precision (r)			1.0	0.924	0.915
LAB 3	CCC (r <sub>c</sub> )			1.0	0.922	0.882
	CCC 95% CI				(0.894, 0.942)	(0.849, 0.908)
LAB 4 (N=156)	Accuracy (C <sub>a</sub> )				1.0	0.977
LAB 4	Precision (r)				1.0	0.983
LAB 4	CCC (r <sub>c</sub> )				1.0	0.961
	CCC 95% CI					(0.952, 0.968)
LAB 5 (N=156)	Accuracy (C <sub>a</sub> )					1.0
LAB 5	Precision (r)					1.0
LAB 5	CCC (r <sub>c</sub> )					1.0
Consensus Value (N=780)	Accuracy (C <sub>a</sub> )	1.000	0.995	0.990	0.995	0.993
	Precision (r)	0.994	0.982	0.944	0.991	0.985
	CCC (r <sub>c</sub> )	0.994	0.977	0.934	0.986	0.978
	CCC 95% CI	(0.993, 0.994)	(0.974, 0.980)	(0.926, 0.942)	(0.984, 0.987)	(0.975, 0.981)

<sup>a</sup> Consensus ELISA concentrations were estimated within a serotype by use of a random-effects ANOVA model. Predicted ELISA concentrations were obtained for each laboratory by sample within a serotype for each of the replicate observations by use of a random-effects ANOVA model. Values in parentheses are 95% confidence intervals.

<sup>b</sup> C<sub>a</sub>, accuracy, r, precision, r<sub>c</sub>, concordance correlation coefficient



Table 5: Mean and 95% confidence intervals of serotype specific antibodies to 10 serotypes, following reconstitution of the 007sp standard. A baseline samples was stored at -70°C and the 4°C sample was assayed after 3 weeks, -20°C after 10 weeks.

Type	4°C		-20°C		-70°C	
<b>1</b>	6.783	(6.056-7.598)	11.463	(10.624-12.368)	10.245	(9.349-11.227)
<b>4</b>	2.907	(2.619-3.227)	4.777	(4.512-5.057)	4.133	(3.764-4.538)
<b>5</b>	6.795	(6.155-7.502)	10.784	910.158-11.447)	9.349	(8.551-10.222)
<b>6B</b>	6.961	(6.176-7.846)	11.310	(10.581-12.089)	9.836	(8.920-10.846)
<b>7F</b>	7.991	(6.985-9.142)	11.232	910.482-12.035)	9.751	(8.908-10.675)
<b>9V</b>	5.304	(4.780-5.886)	8.808	(8.337-9.306)	7.740	(7.099-8.438)
<b>14</b>	31.788	(27.812-36.334)	52.528	(49.363-55.896)	45.745	(41.259-50.720)
<b>18C</b>	5.964	(5.439-6.541)	10.188	(9.491-10.938)	8.948	(8.200-9.764)
<b>19F</b>	11.354	(10.200-12.638)	19.481	(18.366-20.665)	17.486	(16.080-19.016)
<b>23F</b>	4.777	(4.385-5.203)	8.380	(7.728-9.087)	7.318	(6.817-7.856)

Figure 1: Scatter plots showing the correlation of antibody concentrations between laboratories for the 13 serotypes in 007sp with the log concentration of the values represented on the x and y axes. Each point represents the mean of at least 40 log-antibody concentrations for 007sp for each serotype from each laboratory. The concordance correlation coefficient ( $r_c$ ) is listed within each plot.

The solid diagonal line indicates perfect agreement (slope=1; intercept=0).

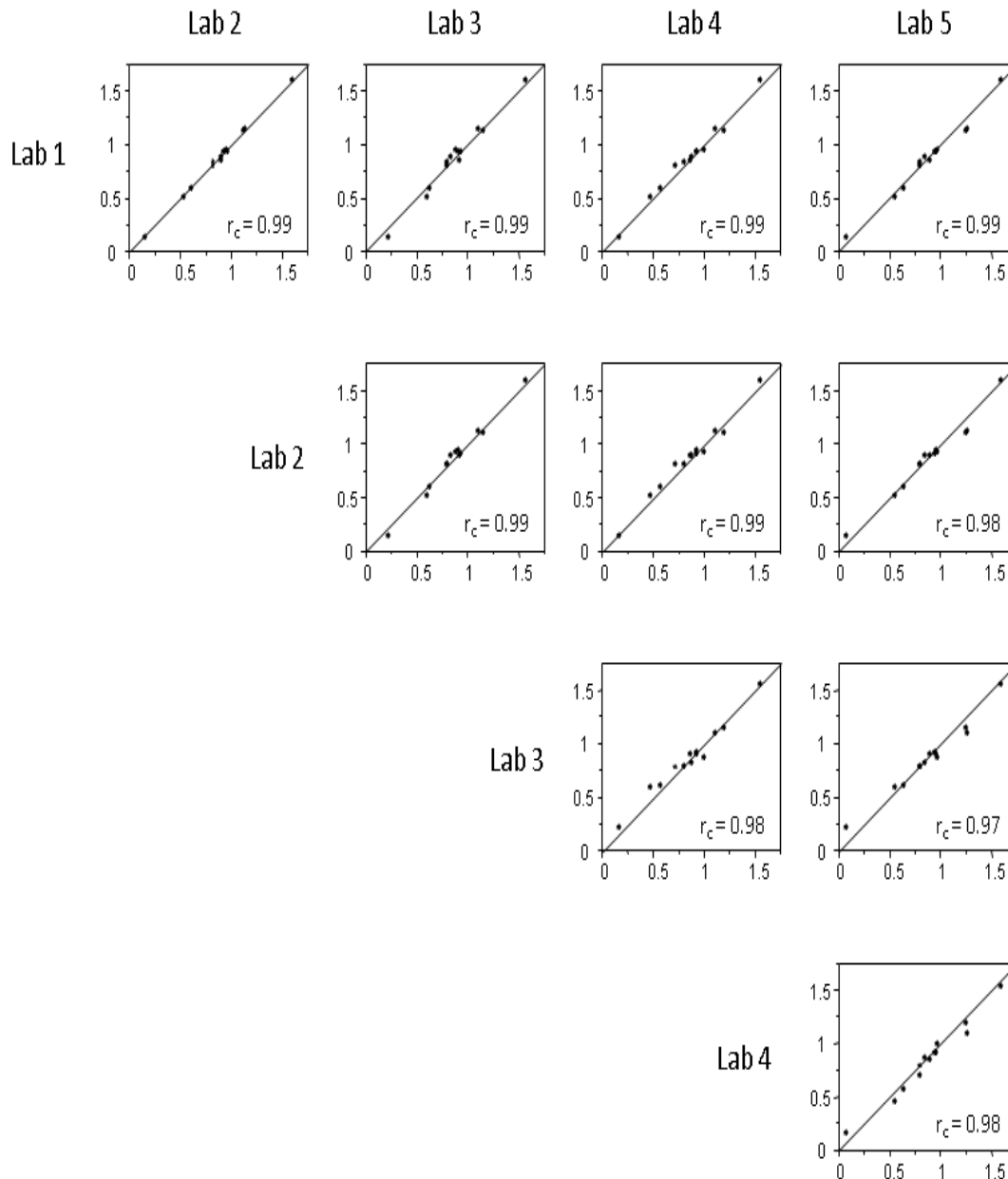


Figure 2: Comparison of the original assigned values for 13 serotypes in 89SF with those assigned to 007sp.

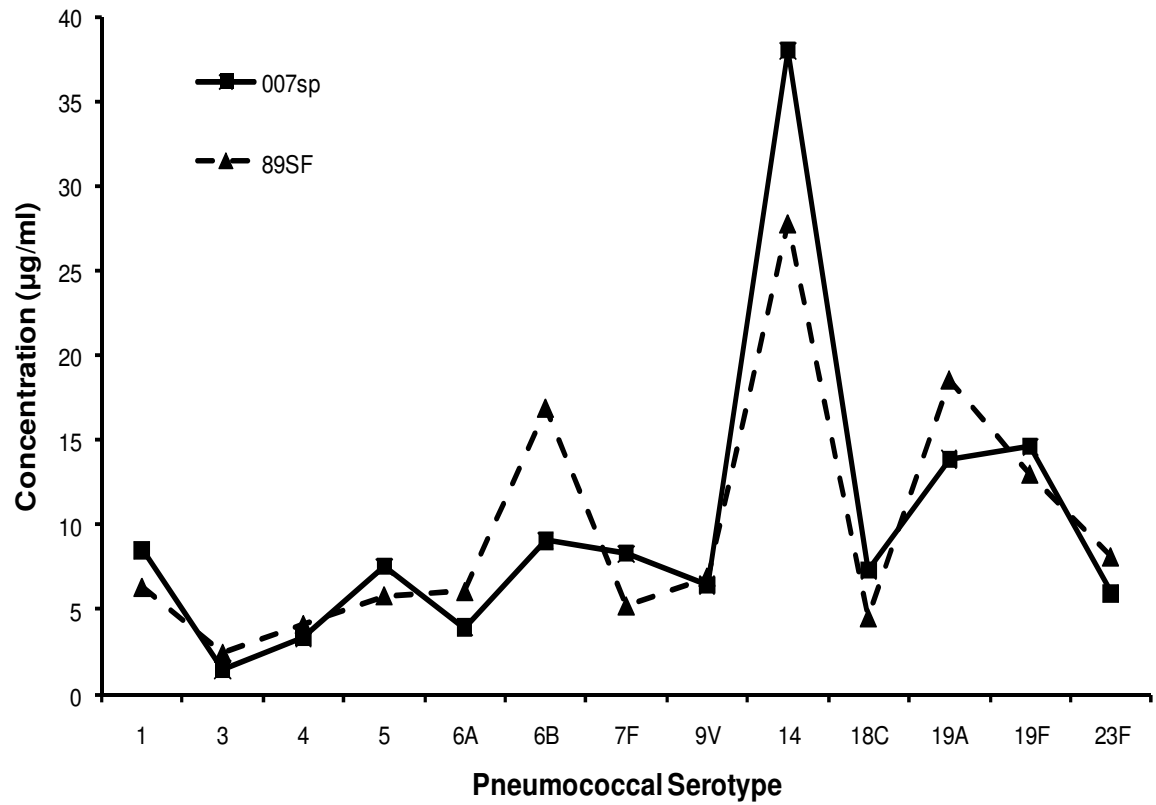


Figure 3: Scatter plots showing the correlation among the derived concentrations for the panel of 12 QC sera using 007sp (vertical scale) vs. 89SF (horizontal scale) as reference standards for the first 7 serotypes (1, 3, 4, 5, 6A, 6B, 7F) analysed (N=5 for each QC serum from each laboratory). The plots illustrate a high degree of agreement and correlation among the derived concentrations.

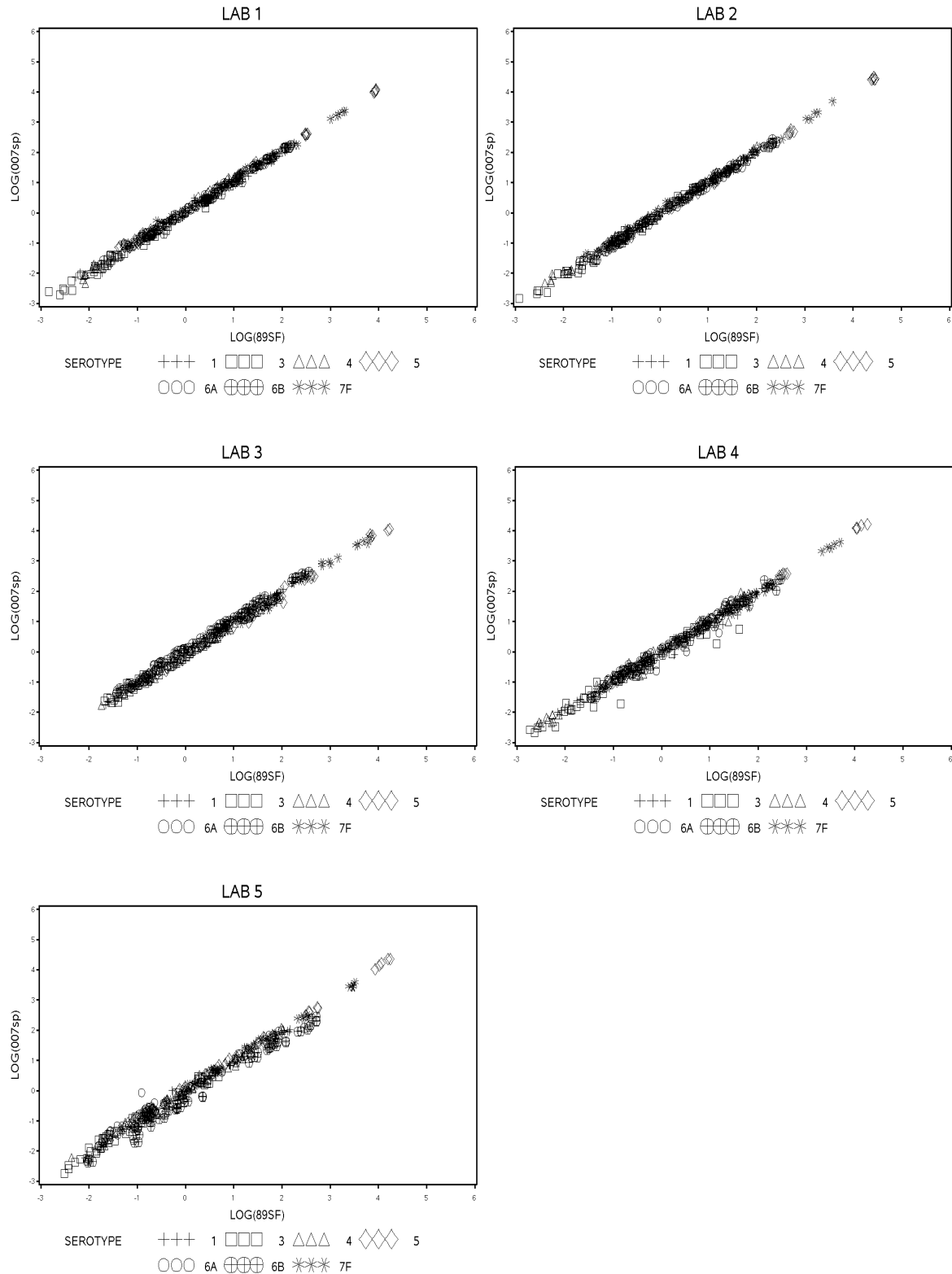


Figure 4: Box plots illustrating the deviation of the 007sp estimates from those obtained using 89SF for the first 7 serotypes (1, 3, 4, 5, 6A, 6B, 7F) of the panel of 12 WHO QC sera analysed (N=5 for each QC serum from each laboratory, total N=60). In these plots, the box is defined by the 25th and 75th percentiles of the distribution; the horizontal line within the box represents the median or 50th percentile, and the asterisk signifies the mean. Vertical lines extend to the most extreme observation that is less than 1.5 times the interquartile range (75th to 25th percentiles), diamonds and boxes correspond to individual assay values which are progressively distant from the mean. Data above the dotted horizontal line 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 0089SF estimate. A value of 1/4 indicates the 89SF estimate was four times the 007sp estimate. Boxes centered on the horizontal dotted line indicate a good agreement between the 007sp and 89SF estimates.

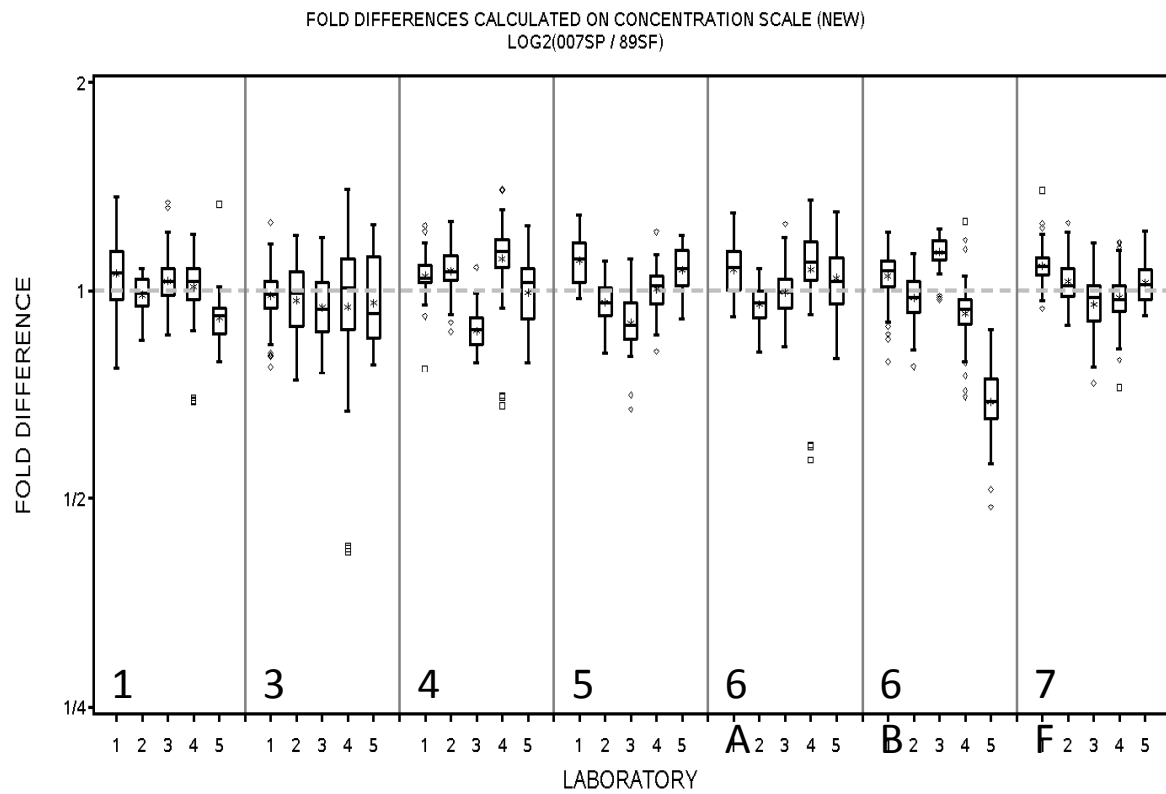


Figure 5: Scatter plots showing the correlation among the calculated concentrations using 007sp (vertical scale) vs. 89SF (horizontal scale) for the remaining 6 serotypes (9V, 14, 18C, 19A, 19F, 23F) analysed (N=5 for each QC serum from each laboratory). The plots illustrate a high degree of agreement and correlation among the calculated concentrations.

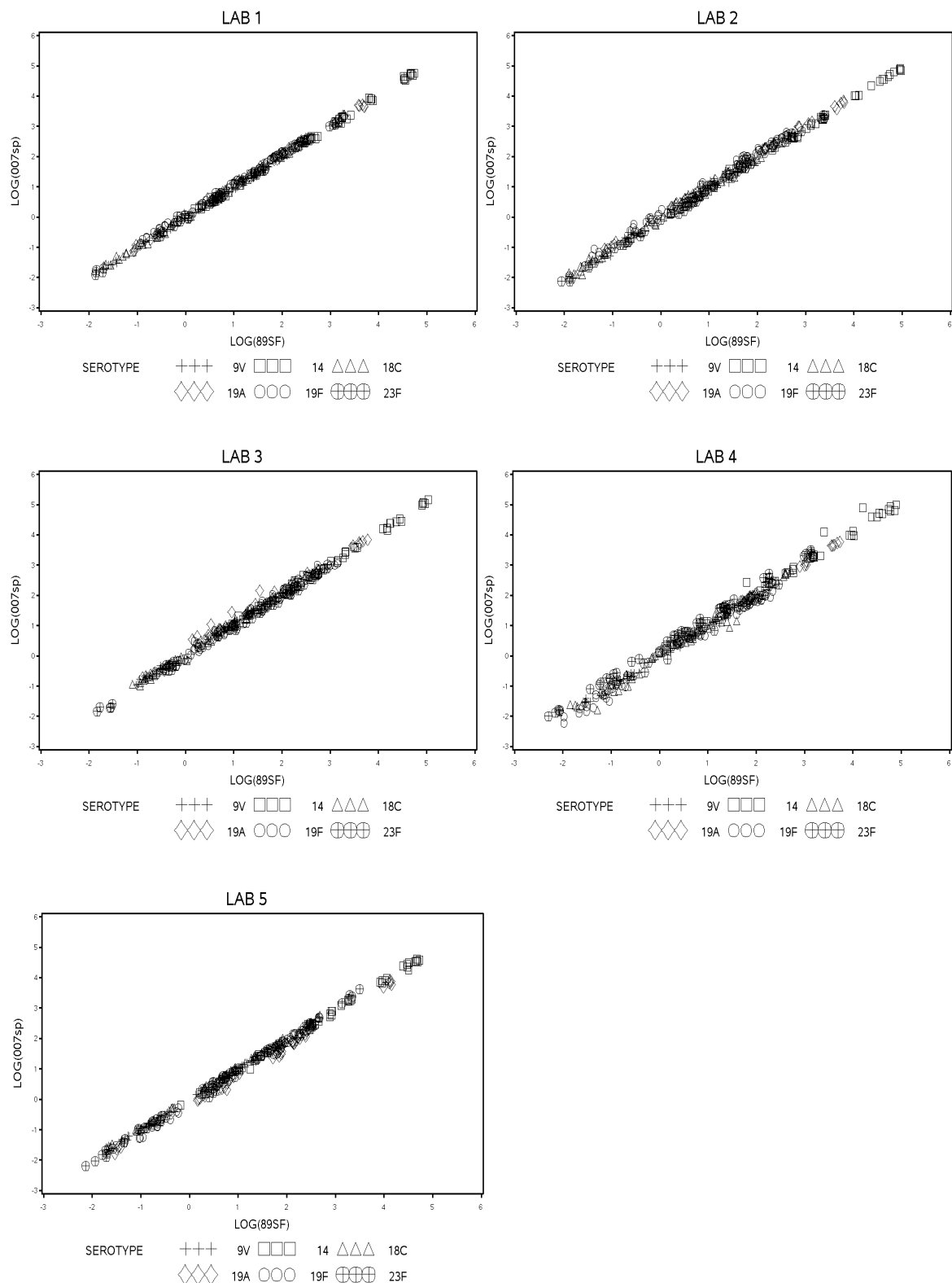


Figure 6: Box plots illustrating the deviation of the 007sp estimates from those obtained using 89SF for the remaining 6 serotypes (9V, 14, 18C, 19A, 19F, 23F) analysed (N=5 for each QC serum from each laboratory, total N=60). In these plots, the box is defined by the 25th and 75th percentiles of the distribution; the horizontal line within the box represents the median or 50th percentile, and the asterisk signifies the mean. Vertical lines extend to the most extreme observation that is less than 1.5 times the interquartile range (75th to 25th percentiles), diamonds and boxes correspond to individual assay values which are progressively distant from the mean. Data above the dotted horizontal line indicates 007sp estimates are greater than estimates using 89SF. On the vertical axis, 2 indicates a point where the 007sp estimate was twice the 89SF estimate. A value of  $\frac{1}{4}$  indicates the 89SF estimate was four times the 007sp estimate. Boxes centered on the horizontal dotted line indicate a good agreement between the 007sp and 89SF estimates.

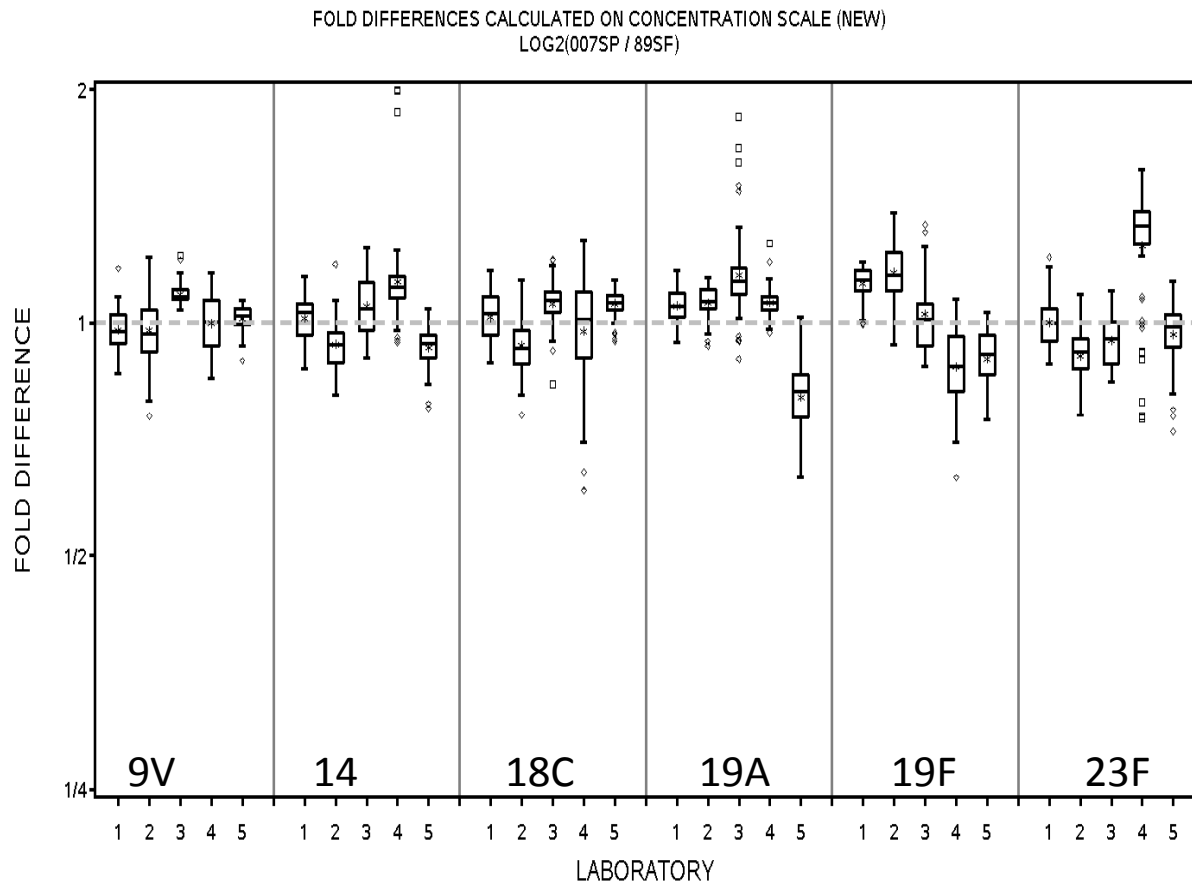


Figure 7: Thermal degradation samples after 6 months, showing relative serotype specific anti pneumococcal capsule antibody content of ampoules as a percentage of the content of the -20°C baseline, as determined by multiplex bead assay.

