Serotype-specific anti-pneumococcal IgG and immune competence: critical differences in interpretation criteria when different methods are used.

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Abstract (preferably no longer than 150 words)

Background: Serotype-specific antibody responses to pneumococcal polysaccharide are important in evaluating humoral immune function. Multiplex technologies allow simultaneous quantitation of multiple serotype-specific antibodies however there has been limited validation against the gold-standard ELISA and assay performance in the clinical setting has not been examined.

Methods and Materials: Pre- and post-immunization samples were analysed by both methods. The ability to correctly identify an adequate response to polysaccharide vaccine (as defined by current AAAAI guidelines) was determined.

Results: The xMAP Pneumo 14 multiplex assay correlated poorly with the ELISA, particularly for pre-immunization and infant samples. An adequate response to pneumococcal immunization was 'correctly' predicted by xMAP Pneumo for 21 of 26 (81%) adult pairs and 18 of 25 (72%) infant pairs. Seven of 25 infants and 4 of 26 adults were identified as having an inadequate response by ELISA and an adequate response by xMAP.

Conclusion: When applying current AAAAI guidelines, the xMAP Pneumo 14 assay does not allow reliable evaluation of antibody responses to polysaccharide antigens for the assessment of humoral immune competence. New guidelines for an adequate response should be established for new technologies when evaluating responses to polysaccharide vaccine in the clinical setting.

Key words: - list of up to 5 words

Pneumovax, Prevnar, immune response, multiplex, ELISA

Introduction

The assessment of serotype-specific antibody responses to pneumococcal polysaccharide vaccine is used routinely in the evaluation of humoral immune function and more particularly for the diagnosis of specific antibody deficiency. In addition, measurement of pneumococcal serotype-specific antibody levels in serum is used as the main indicator of vaccine immunogenicity in large-scale pneumococcal immunization programs. Although the functional activity or opsonophagocytic capacity of serotype-specific IgG provides strong correlation with vaccine efficacy, opsonophagocytic assays (OPA) have not been standardized internationally, and measurement of serotype-specific IgG by the standardized WHO ELISA is currently accepted as the 'gold standard' for evaluation of antibody responses to pneumococcal serotype [1]. However ELISAs require large sample volumes, are labour intensive, time consuming and rely heavily on operator expertise.

Multiplex technologies have the potential to significantly improve the efficiency of measuring anti-pneumococcal antibodies by allowing simultaneous quantitation of serotype-specific IgG to many serotypes in a single sample, thereby requiring smaller sample volume and offering increased throughput. Given these advantages, multiplex technology has been proposed as the platform of choice for high-throughput screening of pneumococcal antibody responses [2] and has been adopted by some laboratories for use in clinical practice. Whilst multiplex bead-based assays are reported to have good inter- and intra-assay reliability and precision when evaluations are performed using controls provided with the assay kits [2-5], concerns have been raised regarding assay specificity for measurement of IgG against some serotypes [2, 3, 5] and validation of assay performance for measurement of serotype-specific IgG in patient sera samples in the clinical setting has been limited [2]. More importantly, there has been no assessment of assay performance for evaluation of humoral immune function which involves an assessment of fold rise in serotype-specific IgG titre following immunization. This is particularly relevant since the multiplex platforms may be susceptible to non-specific detection of cross-reactive antibodies [6], and both pre-immunization and to a lesser extent post-immunization sera are known to contain high levels of non-specific cross-reactive IgG [7-9].

Based on a multicentre inter-laboratory study [10], the WHO has recommended that an alternative pneumococcal serotype-specific IgG assay would only be considered as an acceptable alternative to the WHO ELISA if at least 75% of sera measurements in the new assay fall within 40% of WHO ELISA assigned values for the 7 serotypes in Prevenar (PCV7). A recent comparison of 3 different multiplex bead assays found that none of these met WHO requirements [11].

In the current study we compared measurements of serotype-specific anti-pneumococcal IgG obtained using the multiplex Luminex[®] xMAP[®] Pneumo 14 assay with the gold standard WHO ELISA in sera samples from children and adults taken pre- and post- Prevenar[®] and Pneumovax[®] immunization.

Methods

Samples: 202 sera samples from the serum register in the Pneumococcal Laboratory (Allergy and Immune Disorders, MCRI, Melbourne) were included in the study. These comprised paired pre- and post-immunization sera from 50 infants immunized with 3 doses of PCV7 (6, 10 and 14 weeks), 25 post-immunization sera from infants immunized with 23vPPV at 12 months (no previous PCV7), paired pre- and post-immunization sera from 26 adults immunized with 23vPPV and 25 'stat/pre' sera from non-immunized adults, refer Table I. The healthy infant samples were obtained from the Fiji Pneumococcal Project [12], and adult samples were randomly selected from patient samples referred for evaluation of immune function to the Diagnostic Immunology Laboratory, Royal Children's Hospital, Melbourne. This study was approved by the University of Melbourne Human Research Ethics Committee and the Fiji National Research Ethics Review Committee (infant samples) and the Royal Children's Hospital Research Ethics Committee (adult samples). Serum concentrations of serotype-specific IgG against serotypes 1, 3, 4, 6B, 7F, 8, 9N, 9V, 12F, 14, 18C, 19A, 19F and 23F were measured by each method.

WHO ELISA: The ELISA method has been described elsewhere [13] and is based on WHO recommendations [1]. To remove non-specific antibodies samples were preabsorbed with cell wall polysaccharide (CWPS) at 10 µg/ml and serotype 22F at 30 µg/ml, before being applied to a precoated microtitre plate (one serotype per plate). Serotype-specific antibody was quantified by a HRP-conjugated anti-human IgG. An adequate antibody response to pneumococcal serotypes was defined according to the current AAAAI expert recommendations of a post-immunization titre ≥ 1.3 µg/ml or a 4 fold or greater rise between pre-immunization and post-immunization titres (to 50% or more of the serotypes tested for children aged 2-5 years and to 75% or more of serotypes tested for older children and adults). This definition was established using data from the WHO ELISA method [14].

Multiplex Bead Assay: The xMAP[®] Pneumo 14 (Pneumococcal Immunity Panel) was used according to the manufacturers' instructions with samples preabsorbed in Luminex buffer, comprising CWPS at 0.2 µg/ml and serotype 22F at 2 µg/ml.

Data analysis: Serotype-specific antibody concentrations were log (base 10) transformed to calculate Geometric Mean Concentration and 95% confidence intervals. Pair wise comparisons of serotype-specific GMC between method groups were performed for each serotype using two sample t-test and a p-value of <0.01 was considered statistically significant. Pearson's correlation coefficient (r) between methods was calculated for each serotype and each subgroup using the log transformed data.

Results

Comparison of geometric mean concentrations (GMC) of serotype-specific IgG measured by xMAP Pneumo 14 and ELISA

The serotype-specific IgG geometric mean concentrations (GMC) and 95% confidence intervals (95% CI) for each serotype and each patient sub-group are shown in Table II. The GMC were significantly higher for samples assayed by xMAP as compared to ELISA for the majority of serotypes. This was most marked in the 50 infant post-PCV7 samples where the GMC for IgG against serotypes 1, 3, 7F and 12F (all serotypes which are not in the 7-valent vaccine) as measured by xMAP were significantly higher, compared to measurements by ELISA. The GMC for serotype 19A measured by ELISA was higher than the GMC measured by xMAP for all patient sera groups except infant post-23vPPV sera.

The GMC derived by xMAP were generally closer to GMC derived by ELISA in post-23vPPV sera suggesting that specificity of antibody binding to xMAP beads was greatly improved for some serotypes following 23vPPV vaccination.

Correlation of serotype-specific IgG titres measured by xMAP Pneumo 14 and ELISA

The Pearson correlation, r , between the two methods was determined for each serotype and patient group. When all samples were included in the method comparison analysis, a correlation of $r > 0.7$ was demonstrated for 11 of 14 serotypes (refer Figure 1, supplementary table IV). However when analysed by patient group, the correlation between methods for infants pre- and post-PCV7 was $r < 0.7$ for 13 (of 14) serotypes in pre-PCV7 sera and 8 (of 14) serotypes in post-PCV7 sera. The correlation between the 2 methods improved when measuring serotype-specific IgG in infants post-23vPPV sera and in sera from the adult cohort.

Assessment of an adequate antibody response to polysaccharide antigens by the 2 methods

The current AAAAI expert guidelines for an adequate response to immunization, either a post titre with four-fold increase on baseline or a post titre $\geq 1.3 \mu\text{g/ml}$ (to 50% or more of the serotypes tested for children aged 2-5 years and to 75% or more of serotypes tested for older children and adults), were developed from ELISA data. For these guidelines to be used in assessment of an adequate response using results from a multiplex platform there should be a strong correlation between xMAP and ELISA. There were 26 adult paired samples pre and post 23vPPV immunization and 25 infant samples pre and post 23vPPV immunization. Applying the AAAAI guidelines, the response predicted by Luminex xMAP matched that predicted by the ELISA for 21 of 26 (81%) adult pairs and 18 of 25 (72%) infant pairs (Table III). Of note, 4 adults (of 26) were identified as having an inadequate response by ELISA and an adequate response by Luminex; and more importantly 7 of 25 infants were identified as having an inadequate response by ELISA and an adequate response by Luminex.

Discussion

We have conducted a comprehensive comparison of the xMAP and ELISA assays for measurement of 14 serotypes in serum samples from infants and adults obtained pre and post immunization with conjugated pneumococcal vaccine (PCV7) or polysaccharide pneumococcal vaccine. Previous studies comparing multiplex bead-based assays with ELISA have only validated measurement of serotype-specific IgG in the 12 QC sera from National Institute for Biological Standards and Control (NIBSC) [10] or a limited number of samples (often with no information on immunization status), or have only evaluated a small number of serotypes [2, 4, 11].

As has been reported previously [5, 11, 15] the GMC for the majority of serotypes was significantly higher when samples were assayed by xMAP as compared to ELISA. This was most marked for measurements in the infant post-PCV7 samples, with the GMC for some serotype-specific IgG being as much as 26 times greater when measured by xMAP than when assayed by WHO ELISA. Importantly, the xMAP assay revealed high titres of serotype-specific IgG in post-PCV7 immunization sera for serotypes not contained in the vaccine (1, 3, 7F, 9N and 12F). The WHO ELISA correctly indicated absent antibody to these serotypes which suggests that xMAP detects nonspecific binding of cross-reactive antibodies.

Although the clinical relevance of cross-reactive antibodies remains uncertain, it has been demonstrated that removal of such cross-reactive antibodies by pre-absorption with 22F and CWPS can significantly improve correlation between ELISA and OPA [16-18]. In this regard it is relevant to note that in the xMAP method, samples are pre-absorbed with serotype 22F and CWPS at concentrations of 2 µg/ml and 0.2 µg/ml respectively, which are up to 10-fold lower than the concentrations employed in the WHO ELISA method. We and others [13, 19] have shown that increasing the concentration of serotype 22F in the absorption buffer from 10 to 30 µg/ml can increase the specificity of the WHO ELISA, so increasing these concentrations in the xMAP method may improve binding specificity. To further investigate this, we compared measurements of serotype-specific IgG in 17 of the adult pre-immunization samples using the Luminex xMAP Pneumo 14 absorption buffer (serotype 22F 2 µg/ml and CWPS 0.2 µg/ml) and the WHO ELISA absorption buffer (serotype 22F 30 µg/ml and CWPS 10 µg/ml) in an ELISA assay. Serotype-specific IgG titres for serotypes 1, 4, 6, 9V, 12F, 14, 19A, 19F, 23F obtained using the WHO ELISA absorption buffer (containing higher concentrations of serotype 22F and CWPS) were 1.2 to 5-fold lower ($p < 0.01$ to $p < 0.0001$) than those obtained using the xMAP buffer (data not shown).

Concerns regarding the detection of non-specific cross reactive antibodies by multiplex assays have been reported previously [3, 6] and further studies to investigate the binding of cross reactive antibodies in the xMAP system would be warranted to confirm whether this may be contributing to the limited correlation with ELISA. Pickering et al [6] reported non-specific binding in a bead-based multiplex assay and showed that use of an alternative “StableGuard” buffer in the initial conjugation completely removed cross-reactive antibodies by efficiently blocking available sites on the microspheres.

The performance of xMAP was significantly enhanced in post-23vPPV immunization sera, suggesting that specificity of antibody binding to xMAP beads was greatly improved for some serotypes following 23vPPV vaccination, and consistent with previous studies showing lower levels of cross-reactive antibodies in post-immunization as compared to pre-immunization sera.

The strength of this validation analysis is that we have assessed the Luminex xMAP Pneumo 14 by comparing measurements of serotype-specific IgG against 14 serotypes in 202 clinical samples from infants

and adults, pre- and post-immunization with PCV7 or 23vPPV. Such a validation is of primary importance since it is in this clinical setting that the accurate and reliable measurement of serotype-specific IgG is essential.

Our findings show that results obtained by xMAP correlate poorly with ELISA data in pre-immunization and infant sera. Importantly, the xMAP performed poorly when used to assess antibody responses to polysaccharide antigens in the clinical setting using the current expert AAAAI guidelines. The current AAAAI expert guidelines for an adequate response to immunization as, either a post titre with four-fold increase on baseline or a post titre $\geq 1.3 \mu\text{g/ml}$ (to 50% or more of the serotypes tested for children aged 2-5 years and to 75% or more of the serotypes tested for older children and adults) were developed from ELISA data. The validity of directly applying these criteria to IgG titres obtained by the xMAP Pneumo 14 assay is uncertain. This approach would only be appropriate if a close correlation between the 2 methods could be established. The poor correlations between xMAP and ELISA presented in this study for both infant and adult sera samples, and previously reported for adult samples [2, 11], suggest that such an approach is not valid. Infant samples used in this study were collected from healthy 12 month old infants, 2 weeks post 23vPPV immunization. While it has been widely accepted that infants less than 2 years do not respond to polysaccharide vaccine [20-27], there is little evidence to support this and we have recently demonstrated that 12 month old infants can generate adequate serotype-specific IgG responses to the majority of serotypes in 23vPPV [28], with the functional capacity of these serotype-specific IgG responses confirmed [29]

Borgers *et al* [2] have suggested an alternative interpretation criterion to determine an adequate response to polysaccharide antigens using multiplex bead assays. They proposed that a post- immunization titre that was above the 5th percentile for post-immunization IgG titres amongst a healthy population could be used to identify a normal response to that serotype, and that a normal response to greater than 50% of serotypes tested could be deemed an adequate response. This approach correctly identified a number of antibody deficient subjects. Such an approach may be applicable to the xMAP Pneumo 14 since performance of this assay was greatly improved in post 23vPPV immunization samples. An alternative avenue to establish xMAP Pneumo 14 specific interpretation criteria for an adequate response could be to evaluate the correlation between xMAP and OPA, as it is possible that the xMAP may correlate differently to OPA compared to ELISA. Based on our data, it is likely that post-immunization sera, especially following 23vPPV, will correlate closely with OPA assessments, and an interpretation criteria that relies solely upon a post-immunization assessment would offer significant advantages in the clinical setting for evaluation of functional antibody responses.

In summary, this is the first and largest study evaluating the application of the xMAP Pneumo 14 multiplex assay for the quantitation of pneumococcal serotype-specific IgG in the clinical setting. It was demonstrated that this multiplex assay correlated poorly with the WHO ELISA in the clinical setting. If the current criteria for determination of an adequate response (derived from ELISA data) are applied, the xMAP Pneumo 14 assay does not allow reliable evaluation of functional antibody responses to polysaccharide antigens for the assessment of immune competence. Enhancement of the xMAP system with adequate absorption of cross-reactive antibodies and alternative criteria for assessment of functional antibody responses that are specific to the xMAP assay may improve the application of this assay in the clinical setting.

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Figure legend

Figure I. Serotype-specific IgG of 202 serum samples from 5 patient groups (□ infants Pre immunization, ✕ infants post-PCV7, ★ infants post-PPV23, ○ adults pre-PPV and ● adults post-PPV) measured by ELISA (x-axis) and Luminex X-MAP (y-axis).

Table I: Demographic characteristics of samples used in the validation.

Group	Sample size	Median age at blood collection	Days post immunization (median, interquartile range)
Infant unimmunized	50	1 year	-
Healthy infant post PCV7	50	19 weeks	28 (27 – 35 days)
Healthy infant post 23vPPV	25	1.1 year	14 (14 – 15 days)
Adult pre & stat samples	51	45 years	-
Adult post 23vPPV	26	49 years	43 (32 - 73 days)

Table II: Geometric Mean Concentration and 95% confidence intervals for pneumococcal serotype specific IgG measured by WHO ELISA and xMAP Pneumo 14 multiplex assay

	Infants Pre-PCV7 & 23vPPV (n=50)		Infants Post-PCV7 (n=50)		Infants Post-23vPPV (n=25)		Adults Stat or pre-23vPPV (n=51)		Adults Post-23vPPV (n=26)		All samples (n=202)	
	ELISA	xMAP	ELISA	xMAP	ELISA	ELISA	ELISA	xMAP	ELISA	xMAP	ELISA	xMAP
1	0.18 0.15-0.23	0.27* 0.23-0.32	0.07 0.06-0.08	1.85* 1.48-2.32	0.34 0.28-0.42	0.34 0.28-0.42	0.52 0.40-0.68	0.53 0.43-0.66	1.80 1.16-2.79	1.42 0.95-2.12	0.34 0.28-0.42	0.83* 0.71-0.97
3	0.31 0.21-0.45	0.26 0.19-0.36	0.09 0.07-0.13	0.94* 0.79-1.11	0.43 0.33-0.55	0.43 0.33-0.55	0.36 0.27-0.47	0.36 0.28-0.46	0.71 0.45-1.13	0.78 0.51-1.20	0.43 0.33-0.55	0.69* 0.57-0.84
4	0.08 0.07-0.10	0.11+ 0.10-0.12	5.18 4.09-6.56	6.27^ 4.74-8.30	0.62 0.48-0.79	0.62 0.48-0.79	0.29 0.23-0.37	0.22+ 0.17-0.28	0.57 0.39-0.85	0.49 0.32-0.74	0.62 0.48-0.79	0.63 0.49-0.82
6B	0.16 0.13-0.19	0.08* 0.06-0.09	0.92 0.70-1.22	2.35* 1.69-3.28	0.38 0.33-0.45	0.38 0.33-0.45	0.35 0.26-0.47	0.37 0.27-0.50	0.52 0.35-0.80	0.92^ 0.52-1.65	0.38 0.33-0.45	0.52* 0.41-0.65
7F	0.10 0.08-0.13	0.34* 0.28-0.43	0.05 0.04-0.05	0.38* 0.32-0.44	0.28 0.22-0.36	0.28 0.22-0.36	0.52 0.37-0.73	0.97* 0.72-1.31	2.63 1.61-4.31	2.84 1.66-4.85	0.28 0.22-0.36	0.82* 0.68-0.98
8	0.25 0.20-0.31	0.28 0.22-0.36	0.09 0.07-0.12	0.66* 0.58-0.75	0.48 0.38-0.62	0.48 0.38-0.62	0.48 0.35-0.67	0.77* 0.57-1.05	2.04 1.23-3.41	2.73 1.75-4.28	0.48 0.38-0.62	1.00* 0.81-1.20
9N	0.14 0.11-0.18	0.18^ 0.17-0.21	0.43 0.30-0.62	0.66^ 0.52-0.84	0.47 0.39-0.58	0.47 0.39-0.58	0.46 0.33-0.63	0.46 0.33-0.63	1.41 0.79-2.54	1.19 0.64-2.19	0.47 0.39-0.58	0.57+ 0.47-0.68
9V	0.09 0.08-0.12	0.16* 0.14-0.17	4.49 3.49-5.78	5.78^ 4.43-7.54	0.66 0.52-0.83	0.66 0.52-0.83	0.43 0.32-0.59	0.40 0.3-0.53	1.21 0.72-2.01	1.17 0.64-2.14	0.66 0.52-0.83	0.83* 0.66-1.00
12F	0.07 0.06-0.09	0.22 0.19-0.26	0.02 0.02-0.02	0.37* 0.30-0.44	0.10 0.08-0.12	0.10 0.08-0.12	0.18 0.14-0.23	0.37* 0.28-0.49	0.44 0.27-0.70	1.40* 0.87-2.26	0.10 0.08-0.12	0.49* 0.42-0.57
14	0.24 0.20-0.30	0.11* 0.08-0.15	2.52 1.57-4.05	4.41* 3.22-6.03	0.97 0.77-1.20	0.97 0.77-1.20	1.14 0.84-1.56	0.88 0.60-1.28	5.26 2.79-9.92	5.75 2.70-12.28	0.97 0.77-1.20	0.98 0.75-1.30
18C	0.06 0.05-0.07	0.08^ 0.07-0.10	4.80 3.69-6.24	7.01^ 4.98-9.86	0.63 0.49-0.82	0.63 0.49-0.82	0.40 0.29-0.57	0.45 0.31-0.66	1.22 0.74-2.01	1.30 0.67-2.50	0.63 0.49-0.82	0.81* 0.61-1.10
19A	0.45 0.38-0.53	0.14* 0.11-0.17	1.58 1.21-2.05	0.71* 0.60-0.85	1.30 1.10-1.60	1.30 1.10-1.60	2.42 1.84-3.18	1.42* 0.99-2.03	4.46 2.43-8.18	3.86 2.08-7.17	1.30 1.10-1.60	0.78* 0.63-0.96
19F	0.48 0.38-0.61	0.27* 0.23-0.32	4.68 3.73-5.88	10.18* 7.98-12.99	1.30 1.10-1.60	1.30 1.10-1.60	1.03 0.76-1.40	0.73^ 0.50-1.07	2.65 1.33-5.28	3.12 1.39-6.96	1.30 1.10-1.60	1.40 1.10-1.80
23F	0.22 0.17-0.27	0.11* 0.09-0.13	2.15 1.46-3.17	3.50* 2.46-4.99	0.61 0.50-0.74	0.61 0.50-0.74	0.46 0.35-0.61	0.28* 0.20-0.39	0.97 0.55-1.73	0.71 0.35-1.46	0.61 0.50-0.74	0.49+ 0.39-0.62

* p <0.0001 + p 0.001 to 0.0001 ^ p 0.01 to 0.001

Table III: Comparison of two methods, WHO ELISA and xMAP Pneumo 14 multiplex assay: Number of patients with an adequate response to immunization defined as post- titre $\geq 1.3 \mu\text{g/ml}$ or a post- titre 4 times baseline titre to more than 50% of the serotypes tested.

		Adult samples				Infant samples	
		xMAP				xMAP	
		YES	NO			YES	NO
ELISA	YES	6	1	ELISA	YES	17	0
	NO	4	15		NO	7	1

Figure I