Profiling avidity of antibodies elicited by vaccination using enzyme-linked immunosorbent assay-based elution – insights into a novel experimental and analytical approach

Bahaa Abu-Raya, MD,[1,2] Michelle L Giles, MD[3], Tobias R. Kollmann, MD, PhD, [2,4] Manish Sadarangani, BM BCh, DPhil,[1,2]

[1] Vaccine Evaluation Center, BC Children's Hospital, University of British Columbia, Vancouver, Canada

[2] Division of Infectious Diseases, Department of Pediatrics, University of British Columbia, Vancouver, BC, Canada

[3] Department of Obstetrics and Gynaecology, Monash University, Melbourne, Victoria, Australia

[4] Telethon Kids Institute, Perth, Australia

Corresponding author:
Bahaa Abu-Raya, MD
Vaccine Evaluation Center, BC Children's Hospital Research Institute,
University of British Columbia
950 West 28th Avenue, Rm A5-174 V5Z 4H4
Email: baburaya@bcchr.ubc.ca

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Introduction

Antibodies are evaluated by their quantity and quality. There are different techniques used to measure either the quantity or quality of these antibodies. Enzyme-linked immunosorbent assay (ELISA) assay is a commonly-used method and is based on formation of antigen-antibody complexes in a plate that are either directly detected and measured (direct ELISA) or following addition of a secondary antibody (indirect ELISA). Antibody affinity and avidity are measures of quality of antibodies. Affinity refers to the strength of a single interaction between antibodies and antigen, and avidity reflects the synergistic binding strength of antibodies to antigen through more than one point of interaction. Various laboratory techniques measure antibody avidity, to measure serologic responses to vaccination (e.g., pertussis[1], *Haemophilus influenzae* type b[2], *Streptococcus pneumoniae*[3], and measles virus[4]). Enzyme-linked immunosorbent assay (ELISA)-based elution assays are easy-to-perform and a commonly-used method that can be used in clinical and resource-limited laboratory settings.[5] Application of the ELISA technique to avidity measurements involves treating the antigen-antibody complexes with a bond-breaking agent (chaotrope) after which complexes resisting dissociation, at a specific chaotrope concentration, are quantified. Most studies that use this technique report avidity as a single relative avidity index (RAI) [1, 3, 6, 7], calculated as the ratio of antibody levels in samples treated and not treated with chaotrope.

The use of a fixed single concentration of chaotrope leads to an arbitrary separation of antibodies into either high avidity (i.e. antibodies resisting dissociation) or low avidity (i.e. antibodies eluted by the chaotrope). In reality, however, samples include antibodies with a wide (continuous) spectrum of avidity after vaccination. We have recently developed a novel approach to take this continuous avidity spectrum into

account. We here provide details of this easy to perform yet comprehensive laboratory methodology and the analytical approach using cord blood samples of women vaccinated or unvaccinated against pertussis in pregnancy and assessed for an anti-pertussis toxin (PT) IgG ELISA-based elution assay with ammonium thiocyanate as the chaotrope as an example[8]. Specifically, we aimed to use a gradient of concentrations of the chaotrope to: 1) confirm the need of a range of chaotrope concentrations to establish the avidity profile; 2) confirm that the avidity is not mainly driven by total antibody level; 3) and explore the distribution of avidity by vaccination status.

Laboratory analysis

Determination of the optimal range of bond-breaking agent (chaotrope)

In order to characterize the spectrum of antibody avidity in a sample, we used a range of concentrations of the chaotrope. To determine the optimal range, the assay was initially performed with a wide concentration gradient of chaotrope. The RAI achieved at each concentration was calculated (as above). Next, the lowest and the highest chaotrope concentrations of this range which provide helpful discrimination of antibody avidity were determined. The lowest concentration was the chaotrope concentration that achieved the highest RAIs that was still different from the RAIs achieved at the next lower concentration. Chaotrope concentrations below this lowest concentration are thus less discriminatory and were not used. The highest concentration of the chaotrope was the highest concentration which still yielded antibody levels above the lower levels of quantification (LLOQ) of the ELISA. In our initial experiments, the range of ammonium thiocyanate concentrations was 0.25

molar (M), 0.5M, 1M, 1.5M, 2M and 3M, while concentrations of 0.125M and 4M were rejected.

Sample selection

Samples not treated with the chaotrope or treated with the lowest concentration and yielded values lower than the ELISA's LLOQ were excluded from further analysis. Avidity cannot be reliably measured in sera with very low total antibody levels [6], or undetectable antibody levels following the addition of the lowest chaotrope concentration. Including such samples has the potential to introduce an error to the results, as these samples have undetectable antibody levels rather than low avidity antibodies.

Avidity indices

Different indices were calculated for each sample[8].

Fractional RAI: The RAI achieved at a specific chaotrope concentration and was calculated as the RAI at this specific concentration minus the RAI achieved at the next higher concentration of chaotrope (e.g., RAI_{2M}-RAI_{3M}=60%-40%=20%, where 2M and 3M represented the chaotrope concentration).

Total RAI: This reflected the weighted contribution of the fractional RAIs achieved at the different chaotrope concentrations. Higher weight was given to antibodies with higher avidity. To calculate this value, the order of the reaction between the different chaotrope concentrations and the RAIs was determined. Our data demonstrated high correlation between increasing ammonium thiocyanate concentration and decreasing RAI ($r=-0.88$, $p<0.001$), confirming a first-order kinetics within the range of chaotrope applied, a finding consistent with previous literature[9]. Thus, total RAI

was calculated by applying a factor to each fractional RAI corresponding to the respective concentration of ammonium thiocyanate (e.g. fractional RAI at 1.5M given a weight of 1.5).

As fractional and total RAI are relative measures, we calculated additional indices that incorporate antibody levels.

Fractional absolute avidity levels: The levels of antibodies that were still bound to the antigen at a specific chaotrope concentration. The fractional absolute levels of antibodies quantified at 0.25M, 0.5M, 1M, 1.5M, 2M, and 3M of chaotrope were classified as low, low-medium, medium, medium-high, high and very-high avidity antibodies, respectively. The levels of antibodies eluted by the lowest chaotrope concentration (0.25M) were classified as very-low avidity antibodies.

Total absolute avidity levels: This reflected the weighted contribution of the fractional absolute avidity levels. This index was calculated assuming a first order kinetics (as above) and combined both quality and quantity of antibodies into single measure.

Total antibody levels: antibody levels measured without treatment with chaotrope.

The logarithmic derivatives of total antibody levels, fractional and total absolute avidity levels were calculated and used for subsequent analyses.

Statistical analysis and results presentation

Confirming the need of a range of chaotrope concentrations to establish the avidity profile

To address this, the correlation between the different fractional absolute avidity levels of antibodies was tested. Specifically, a correlation matrix of Pearson's correlation coefficients of pairs of fractional absolute avidity levels achieved at two different chaotrope concentrations was performed. Statistical significance of each correlation coefficient of each paired comparison was set using Bonferroni correction to adjust for multiple comparisons. Overall, we did not find a high correlation between pairs of fractional absolute avidity levels achieved at two different chaotrope concentrations, to further confirm the need to use a range of chaotrope concentrations and to highlight the limitations of previous studies[1],[10] (Figure 1A).

Confirming that the avidity is not mainly driven by total antibody levels

To explore whether antibody levels influence the avidity, the correlation between the total antibody levels and the total RAI was tested. We did not find high correlation (Pearson's r=0.38) between total antibody levels and total RAI, suggesting that avidity is not driven by total antibody levels (Figure 1B).

Distribution of avidity by vaccination status

We previously have shown that vaccinated subjects had higher total RAI, total absolute avidity levels and fractional absolute levels of low–medium, medium, medium–high, high, and very-high avidity levels compared with unvaccinated subjects [8]. In order to further explore whether vaccinated and unvaccinated subjects differed by the quality and quantity of antibodies combined, density estimates of total absolute avidity levels was performed. We found that vaccination resulted in a shift of

148 the distribution of total absolute avidity levels, demonstrating that there are more
149 vaccinated subjects with higher avidity than unvaccinated subjects (Figure 2A).
150 **Clustering and separation of vaccinated and unvaccinated groups by avidity**
151 Using a heatmap, we previously have shown that vaccination was associated with
152 avidity profile consisting of high levels of high avidity antibodies [8]. To gain further
153 enhanced resolution on the results and to investigate whether it is possible to separate
154 vaccinated and unvaccinated subjects based on their avidity, dimensional reduction
155 method (principal component analysis) was applied. We found that the vaccinated and
156 unvaccinated groups could be separated based on their fractional absolute antibody
157 levels (Figure 2B).
158
159

Discussion:

We describe a laboratory and analytical approach that captures the continuous spectrum of avidity of antibodies, allowing more advanced analysis as compared to the traditional dichotomous avidity measurement. As avidity of antibodies is the result of ongoing affinity maturation and is a progressive process that increases over time after vaccination, such dichotomous approach clearly is suboptimal [11]. Utilizing our approach, we found that vaccination against pertussis resulted in increase of low–medium, medium, medium–high, high, and very-high avidity anti-PT antibodies. Previous studies that used 0.125M and 1.5M of chaotrope and a dichotomous approach to analysis reported that the RAI after pertussis vaccination was variable at 73.8% [1] and 46.9% [12], respectively. The presented analytical approach, as compared with the traditional approach, better reflects the biological process that happens after vaccination and allows using better statistical tools for data analysis. This approach can also be used in exploring the correlates of protection against infections as it incorporates both the quantity and quality of antibodies, and both these increase after vaccination and are likely relevant for protection. Altogether, it offers a new approach to evaluate the immunogenicity of vaccines that should be sought in future vaccine research. The use of RAI as the output of avidity studies following applying a fixed chaotrope concentration can still be useful in determining the timing of previous exposure to an antigen (e.g. the case of CMV infection in pregnancy).

The clinical relevance of avidity as proposed in our paper should be investigated. For other infections, e.g. meningococcal disease, function of antibodies may correlate with protection from infections [13]. In addition, as avidity maturation is an ongoing

process that increases in a time-dependent manner after vaccination, this approach can be applied to samples collected at different time points after vaccination.

Other laboratory techniques measure the avidity of antibodies including kinetic binding assays (e.g. surface plasma resonance, Bio-layer interferometry). In these assays, avidity is derived from the average of binding constants of antibodies to an antigen[14]. These techniques are laborious, more expensive, and require higher technical skills, thus limiting its applicability. In contrast, ELISA-based elution assays require smaller serum volumes, minimal technical skills, are cheaper, and thus can be adapted in resource-limited laboratory settings. Furthermore, measuring binding kinetics of antibodies is concentration-independent, which might pose a limitation when assessing samples with high-avidity but low total antibody levels, as the case of subjects remotely vaccinated. In such a case, testing by kinetic binding assays might be consistent with high avidity, which might lead to incorrect conclusions regarding the subjects' overall antigen-specific immunity. Future studies should directly compare ELISA-based elution assays and kinetic bindings assays to further investigate the advantages and disadvantages of both methods.

Figure titles and legends:

Figure 1: A) Correlation matrix of fractional absolute avidity levels of anti-pertussis toxin (PT) IgG achieved at different pairs of chaotrope concentrations based on Pearson's correlation coefficient test. Each box represents the correlation coefficient of pair of logged fractional absolute avidity levels of antibodies achieved at a pair of bond-breaking concentrations between all subjects by vaccination status (vaccinated [right], unvaccinated [left]). The boxes are colored according to the correlation coefficient from red (-1) to blue (+1) and the number represent the correlation coefficient rho value. Statistical significance of each correlation coefficient of each paired comparison was set using bonferroni correction. The X sign represents correlation coefficients that did not reach statistical significance, while absence of X sign represents statistical significance; **B)** Scatter plot of total anti-PT IgG levels and total relative avidity index of anti-PT IgG.

Figure 2: A) Distribution of total absolute avidity of anti-PT IgG by vaccination status. Kernel Density plot shows the total absolute avidity of antibodies in vaccinated versus unvaccinated subjects. The density curves were obtained using a Gaussian kernel. Part of the data presented this figure (unvaccinated group) was previously published[8]; **B)** Principal component analysis of the 7 fractional absolute anti-PT IgG levels by vaccination status. This principal component analysis shows each vaccination status as indicated by distinct colors. Each colored circle/triangle in space represents and individual avidity profile and similar avidity profiles are grouped more closely together in two-dimensional space. The principal components are ordered according to the amount of variance in the data they explain. The plot is based on principal component 1 and 2, which explains 53.9% and 17.4% of the total variance of the data, respectively.

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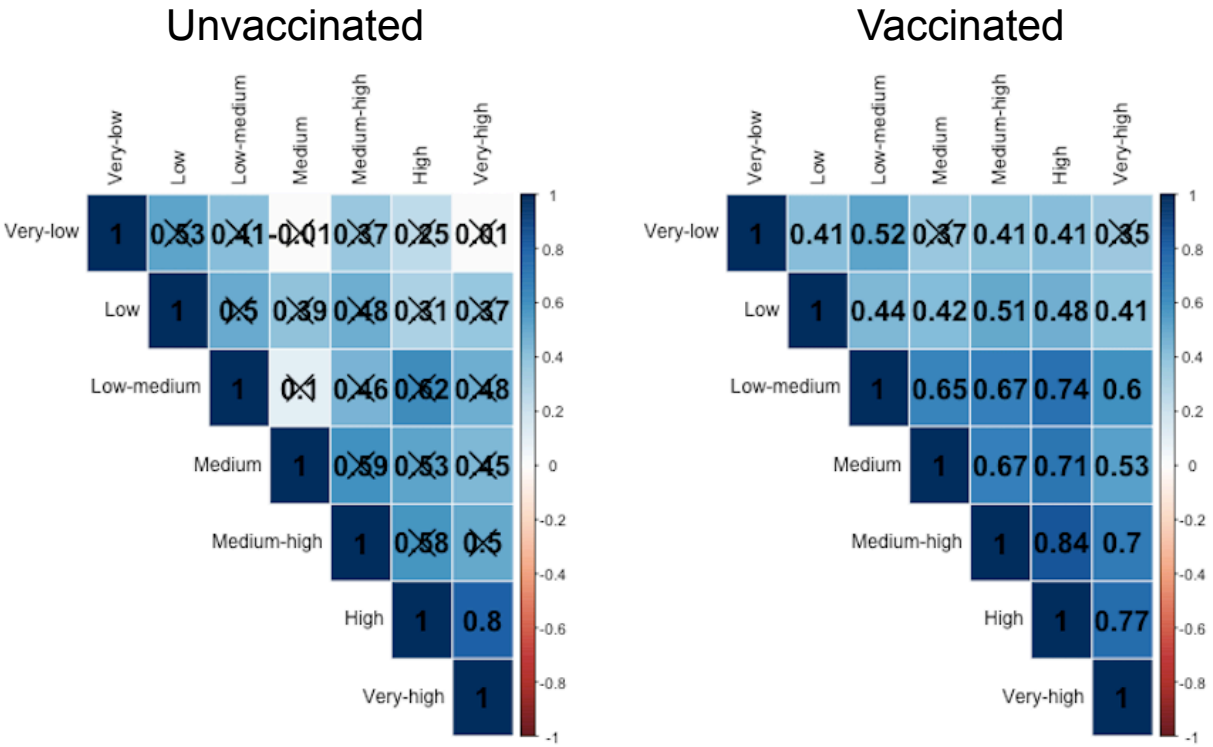
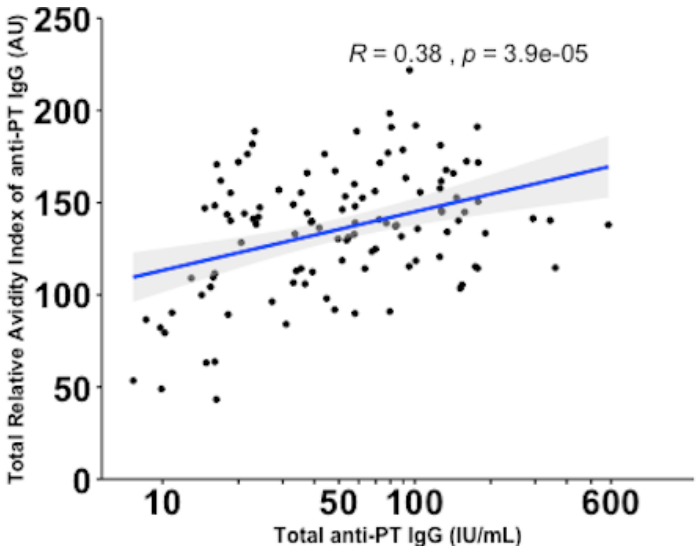
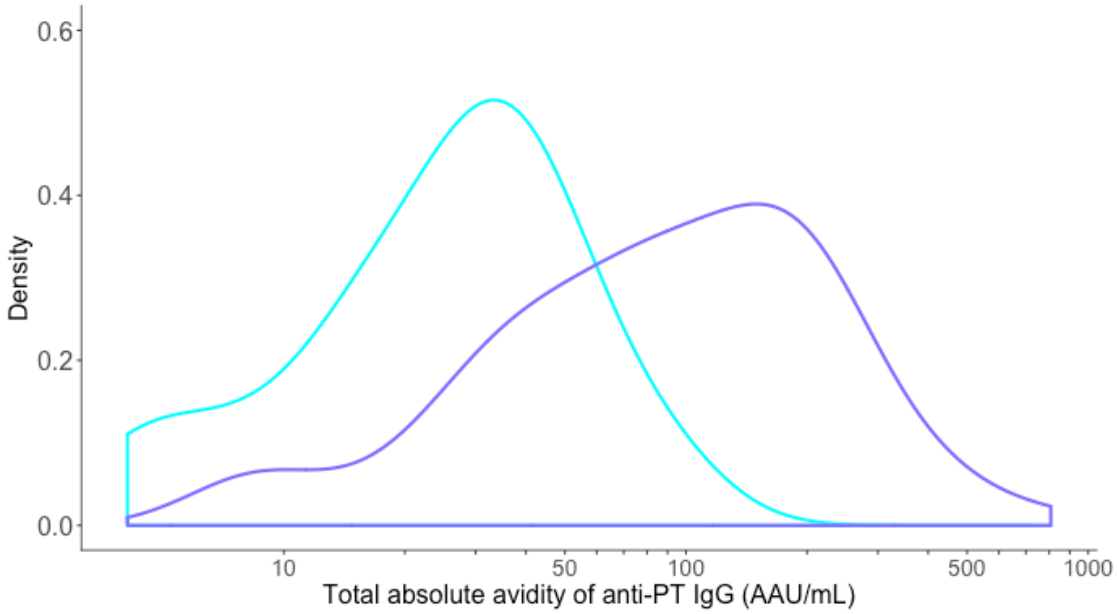
A


Unvaccinated
Vaccinated
Very-low
Low
Low-medium
Medium
Medium-high
High
Very-high
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
1 0.53 0.41 -0.01 0.37 0.25 0.01
1 0.5 0.39 0.48 0.31 0.37
1 0.1 0.46 0.62 0.48
1 0.59 0.53 0.45
1 0.58 0.5
1 0.8
1
1 0.41 0.52 0.37 0.41 0.41 0.35
1 0.44 0.42 0.51 0.48 0.41
1 0.65 0.67 0.74 0.6
1 0.67 0.71 0.53
1 0.84 0.7
1 0.77
1

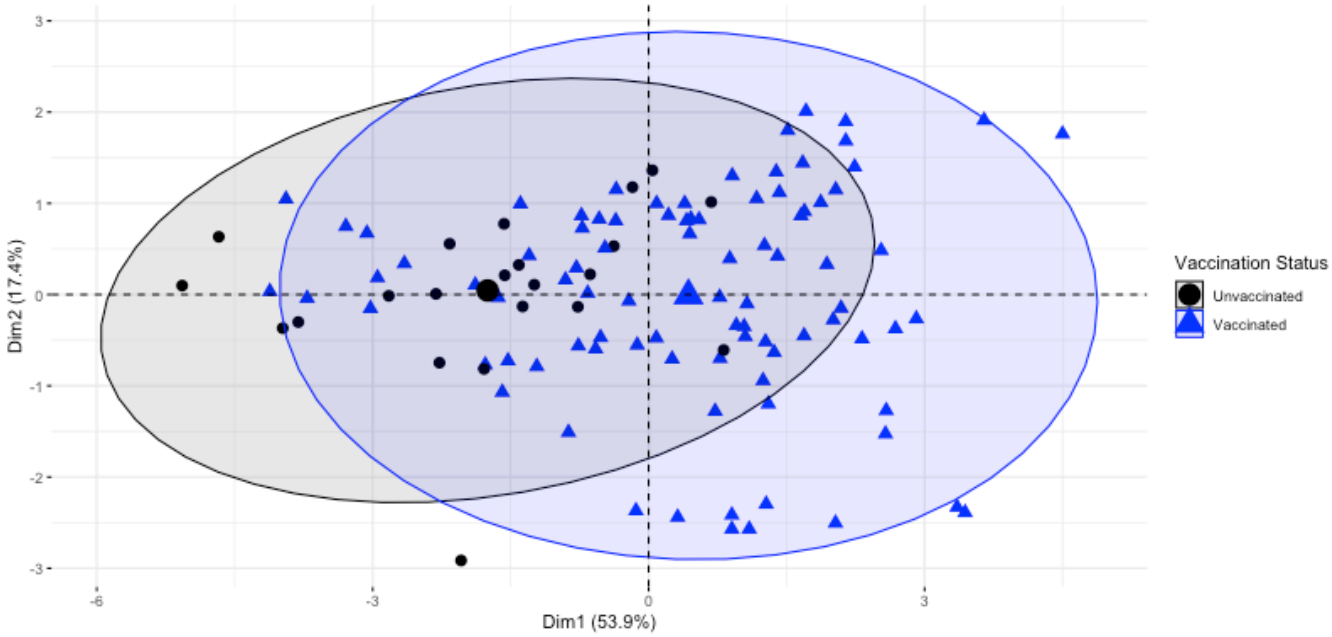
B


R = 0.38 , p = 3.9e-05
Total Relative Avidity Index of anti-PT IgG (AU)
250
200
150
100
50
0
10
50
100
600
Total anti-PT IgG (IU/mL)

A


0.6
0.4
0.2
0.0
Density
10
50
100
500
1000
Total absolute avidity of anti-PT IgG (AAU/mL)
Vaccination Status
Unvaccinated
Vaccinated

B


3
2
1
0
-1
-2
-3
Dim2 (17.4%)
-6
-3
0
3
Dim1 (53.9%)
Vaccination Status
Unvaccinated
Vaccinated