

# Measuring Antibody Avidity to Plasmodium Falciparum Merozoite Antigens Using a Multiplex Immunoassay Approach

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## Research

**Keywords:** Malaria, Plasmodium falciparum, antibodies, antibody avidity, multiplex immunoassay, methods study

**Posted Date:** April 13th, 2020

**DOI:** <https://doi.org/10.21203/rs.2.23647/v2>

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**Version of Record:** A version of this preprint was published at Malaria Journal on May 1st, 2020. See the published version at <https://doi.org/10.1186/s12936-020-03243-3>.

# Abstract

**Background:** Antibodies (Ab) play a significant role in immunity to *Plasmodium falciparum* malaria. Usually, following repeated exposure to pathogens, affinity maturation and clonal selection take place, resulting in increased antibody avidity. However, some studies suggest affinity maturation may not occur to malaria antigens in endemic areas. Information on development of antibody avidity is confusing and conflicting, in part, because different techniques have been used to measure avidity. Today, bead-based multiplex immunoassays (MIA) are routinely used to simultaneously quantitate antibody levels to multiple antigens. This study evaluated the feasibility of developing an avidity MIA with 5 merozoite antigens (AMA1, EBA-175, MSP1-42, MSP2, MSP3) that uses a single chaotropic concentration.

**Methods:** The most common ELISA protocols that used the chaotropic reagents guanidine HCl (GdHCl), urea, and ammonium thiocyanate (NH<sub>4</sub>SCN) were adapted to a multiplex MIA format. Then, different concentrations of chaotropes and incubation times were compared and results were expressed as an Avidity Index (AI), i.e., percentage of antibody remaining bound in the presence of chaotrope.

Experiments were conducted to i) identify the assay with the widest range of AI (discriminatory power), ii) determine the amount of chaotrope needed to release 50% of bound Ab using plasma from adults and infants, and iii) evaluate assay repeatability.

**Results:** Overall, 4M GdHCl and 8M urea were weaker chaotropes than 3M NH<sub>4</sub>SCN. For example, they failed to release significant amounts of Ab bound to MSP1-42 in adult plasma samples; whereas, a range of AI values was obtained with NH<sub>4</sub>SCN. Titration of NH<sub>4</sub>SCN revealed that 2M NH<sub>4</sub>SCN gave the widest range of AI for the 5 antigens. Binding studies using plasma from 40 adults and 57 one-year old infants in Cameroon showed that 2.1M ± 0.32 (mean ± SD) NH<sub>4</sub>SCN (adults) and 1.8M ± 0.23M (infants) released 50% of bound Ab from the merozoite antigens.

**Conclusions.** An avidity MIA is feasible for the 5 merozoite antigens that uses a single concentration (2M) of NH<sub>4</sub>SCN. The assay provides a simple method to quickly obtain information about Ab quantity and quality in the acquisition of immunity to malaria in endemic populations.

## Background

Antibodies (Ab) play a critical role in immunity to *Plasmodium falciparum*, primarily by blocking key epitopes on merozoites, preventing cytoadherence of infected erythrocytes, and enhancing phagocytosis. Antibody avidity (or functional affinity) is the net antigen-binding force of populations of Ab in sera [1]. Thus, studying antibody avidity provides insight into the extent of somatic mutation of immunoglobulin hypervariable regions and subsequent clonal selection. Considering the importance of Ab in immunity to malaria, including to merozoite antigens, surprisingly little is known about antibody avidity in naturally-infected individuals. Early studies showed that Ab avidity to an extract of *P. falciparum*-infected erythrocytes increased after a few infections in a low transmission region [2, 3]. However, in high transmission areas, most studies have found little or no increase in antibody avidity with age to

merozoite antigens, including MSP1, MSP2, MSP3 and EBA-175 [4–9], although an increase with age for AMA1 has been reported [4,5]. Thus, there is much to be learned about the role of Ab avidity in immunity to malaria.

A variety of protocols have been used to study antibody avidity. Although a few investigators have employed Plasma Magnetic Resonance or Bilayer Interferometry [4,7,10], most studies have used the ELISA format with different concentrations of chaotropic agents [2,3,5,6,8,9,11–17]. In these assays, results are often expressed as an Avidity Index (AI), defined as the amount (e.g., O.D.) of Ab remaining bound to an antigen in the presence of a chaotropic agent divided by amount of Ab bound in its absence multiplied by 100. Studies of avidity to merozoite antigens with plasma from naturally-infected humans have used a variety of chaotropes, including 0.5M, 2M, 4M, or 5M guanidine HCl (GdHCl) [5,6,9,11]; 8 M urea [2,14–17]; or 1M or 2.4M thiocyanate (SCN) [3,8]. Additional studies of other malarial antigens have used 3M NH<sub>4</sub>SCN for VAR2CSA [18,19] and 1M NH<sub>4</sub>SCN for volunteers immunized with the RTS/S vaccine [12,13]. A few studies, mainly laboratory-based, employed titration curves of urea or NH<sub>4</sub>SCN to determine amount of chaotrope needed to release 50% of bound Ab, usually in animal models vaccinated with malaria antigens [20,21]. Prior avidity studies have usually considered only 1 to 2 antigens, have not explained why the chaotrope was selected, or have compared results among various chaotropes for the same or multiple antigens. The use of different methodologies makes it impossible to compare results between studies.

Today, bead-based multiplex immunoassays (MIA) are commonly used to measure Ab to combinations of malarial antigens [22–26]. Currently, it is unclear if an avidity MIA with multiple malaria antigens can be developed that uses only one concentration of a chaotropic agent, since prior avidity studies of viral and bacterial antigens have found that different concentrations of salt were required to release 50% of bound Ab from different antigens [1,27]. Thus, this study explored the feasibility of an avidity MIA for 5 *P. falciparum* merozoite proteins (AMA1, EBA-175, MSP1-42, MSP2 and MSP3). The goal was to determine if accurate information could be obtained using a single concentration of only a single chaotrope. First, the most commonly used ELISA protocols for GdHCl, urea, and thiocyanate were adapted to the MIA format. These results allowed comparison of the amount of Ab released by the different chaotropes. Next, titration curves for GdHCl, urea and NH<sub>4</sub>SCN were examined; as well as, the length of incubation with different salt concentrations. Initial results revealed that 2M NH<sub>4</sub>SCN consistently gave the widest range of AI values. Subsequent experiments determined i) how 2M NH<sub>4</sub>SCN compared with the amount of NH<sub>4</sub>SCN required to release 50% of Ab bound to the antigens, ii) if the assay was appropriate for measuring avidity in infants with newly-developing malarial immunity as well as adults with high levels of immunity, and iii) the reproducibility of the avidity MIA. Overall, the combined results lead to the conclusion that 2M NH<sub>4</sub>SCN provides meaningful results in an avidity MIA for the 5 *P. falciparum* merozoite recombinant proteins evaluated.

## Methods And Materials

The Multiplex-Immunoassay: Details of the basic MIA using *P. falciparum* recombinant proteins was originally described in 2006 [22]. Characteristics of the recombinant proteins have also been detailed previously [22, 28] and include: AMA-1 (3D7), EBA-175, MSP1-42 (3D7), MSP2 (FcR3), and MSP3 (HB3). In brief, SeroMap beads (Luminex) with different spectral addresses were covalently coupled with saturating (i.e., optimal) amounts of recombinant proteins and pooled to create a 5-plex containing 2,000 beads of each antigen in 50 $\mu$ l. As reported previously, the 5-plex was compared with individual antigens (mono-plex) to ensure competition among antigens did not occur [22].

The antibody levels were determined by combining 50 $\mu$ l of plasma (diluted 1:300 with PBS plus 1% BSA, pH 7.2) with 50 $\mu$ l of the 5-plex bead mixture (total plasma dilution of 1:600) in filter microtiter plates. Following incubation at room temperature on a rotary shaker for 1 hour, beads were washed twice with PBS+0.05% Tween 20 and once with 1%BSA in PBS. Then, 100 $\mu$ l of secondary antibody (R-phycoerythrin-conjugated, Affini Pure F(ab')<sub>2</sub> fragment, Goat anti-human IgG Fc fragment specific, Jackson Immunoresearch, West Grove, PA) diluted to 2 $\mu$ g/ml in PBS-1% BSA was added to each well and incubated in the dark on a rotary shaker for 1 hr. Beads were washed, re-suspended in 100 $\mu$ l PBS-1% BSA, and 85 $\mu$ l of the microsphere suspension was analyzed using a Luminex M100 reader (Qiagen, Valencia, CA). The reader was programmed to read a minimum of 100 beads per spectral address, DD Gate 7500-15000. Results were expressed as median fluorescence intensity (MFI). The linear part of the Ab binding curve (MFI) was determined for each antigen and found to extend from ~1,000 to >20,000 MFI with r coefficients ranging between 0.913 and 0.995 (supplemental Fig. 1). The Basic Avidity MIA. The avidity MIA assay was performed as described above, with the inclusion of an additional step. After incubation with plasma, the bead-Ab complexes were washed and then incubated for 30 minutes with 100 $\mu$ l of PBS (no salt) or specified concentrations of different chaotropes (with salt). After washing, 100 $\mu$ l of R-phycoerythrin-conjugated goat-anti-human IgG (Fc $\gamma$  specific) was added to beads for 1 hr incubation. Percentage of high avidity Ab was calculated by the formula (AI = [MFI beads with salt] / [MFI beads with no salt] x100). Since AI evaluate strength of Ab binding and MFI indicate the amount of Ab, the two measures should be independent. Thus, in investigating the feasibility of an avidity MIA, it was important to confirm independence of MFI and AI. Results showed that similar AI were obtained when different dilutions of the same plasma sample was used (sFig. 2).

Plasma Samples. A collection of deidentified, archival, plasma samples from malaria endemic regions of Cameroon with high transmission were used [29,30]. Samples included: plasma from 5 selected adults with known Ab levels for the antigens; 40 adults residing in Ngali II (pregnant women, non-pregnant women, males) who had been repeatedly infected with malaria and had developed high malarial immunity, and 57 12-month-old infants living in Ngali II who had just begun developing immunity to malaria [30]. Two pools of plasma were used as positive controls (PC), with PC consisting of plasma samples pooled from adults living in Ngali II village (a high malaria transmission area), Cameroon and positive control #2 (PC#2) including plasma pooled from pregnant women living in the same village [29]. Negative controls (NC), used to establish a cut-off for positivity, included plasma samples from US individuals who had never been exposed to malaria.

Developing the Avidity MIA. The initial experiment adapted 3 ELISA protocols for measuring Ab avidity to the MIA format. The protocol employed 4M GdHCl for 10 minutes [5,11], 8M urea for 5-10 minutes [2] and 3M NH<sub>4</sub>SCN for 30 minutes [18,19]. Results are reported in Fig. 1. In the second set of experiments, 8M urea, and 1M, 2M and 3M NH<sub>4</sub>SCN were compared using the following conditions: 50µl of diluted plasma was incubated with 50µl Ag-coupled bead mixture for 60 minutes; washed three times; beads were re-suspended in 100µl of the chaotropic agent for 30 minutes; beads were washed and incubated with 100µl of PE-anti-human IgG for 60 minutes; washed and examined using a MicroChip 100 as described above (results in Fig. 2). In the third experiment, 2M, 4M and 8M GdHCl, 4M, 8M and 12M urea, and 1.5M, 3M and 4.5M NH<sub>4</sub>SCN were evaluated using the above protocol (results in supplemental Fig. 3). In the fourth experiment, the above protocol was followed except beads were incubated for either 15 or 30 minutes with the respective chaotropic agents to evaluate the effect of time of exposure to various concentrations of chaotrope (results in Fig. 3). To determine the amount of NH<sub>4</sub>SCN needed to remove 50% of bound Ab from the merozoite antigens, titrations curves of 0M, 1.5M, 3M and 4.5M NH<sub>4</sub>SCN were constructed (as described in the Results section) and the Forecast program was used to estimate the 50% concentration (results in Table 1). Finally, to evaluate the repeatability of the assay over time, archival data from 13 avidity assays, conducted over a 30-day period using MSP1-42, MSP2 and MSP3, were evaluated (results in Fig. 4).

Statistical Analysis. Antibody values were recorded as median fluorescence intensity (MFI). The avidity Index (AI) was calculated as the MFI of the sample treated with chaotrope divided by the MFI in the absence of chaotrope times 100. Results from replicate assays were expressed as means and standard deviations (SD). Median MFI and AI values for adults and infants were reported (not normally distributed). The estimated amount of salt needed to remove 50% of bound Ab was determined using the Forecast Program (Excel).

## Results

Comparison of the Three Different Chaotropes. In the initial experiment, ELISA protocols for 4M GdHCl, 8M urea and 3M NH<sub>4</sub>SCN were adapted to the MIA format (see Methods section for details). The avidity MIA included the malaria antigen 5-plex, the PC and 5 selected plasma samples with high Ab levels (Fig. 1). The PC was pooled from multiple adults and represents Ab in the “general population;” whereas, the 5 individual samples provided information about variation among different adults. Treatment of the PC with 4M GdHCl and 8M urea resulted in AI >80 for AMA1, EBA-175 and MSP1-42 (i.e., >80% of Ab remained bound); AI of 40 to 50 to MSP3; and 35 to 40 for MSP2 (Fig. 1). In contrast, treatment with 3 NH<sub>4</sub>SCN substantially reduced Ab binding with AI of 40 to AMA1, 50 to EBA-175, 15 to MSP2, and 15 to MSP3 (5), but AI >90 remained to MSP1-42. Thus, NH<sub>4</sub>SCN was a stronger chaotrope than the other two reagents for AMA1, EBA-175, MSP2, and MSP3.

As expected, a pattern similar to that with PC was observed among the 5 individuals, but substantial variation was also found. The 5 adults had different proportions of high avidity Ab (i.e., AI) for each of the

antigens. For example, after 3 NH<sub>4</sub>SCN treatment, person #1 had 92% high avidity Ab to MSP1-42, 87% to EBA-175, but only 5% to MSP3. Thus, it was possible for a person to have a high AI for one antigen and relatively fewer high avidity Ab to another antigen. Likewise, the AIs for MSP3 were generally lower than for the other antigens, except for individual #2 who had AIs over 80 for MSP3 (Fig. 1 E). Thus overall, AI for some antigens were higher than for others. In summary, the PC reflected the effect of combining plasma samples with different proportion of high avidity Ab, i.e., it didn't have the highest AIs, but rather the average of the combined samples. Additionally, significant variation in AIs was observed among individuals who had been exposed malaria all their lives.

Because of the relatively strong effect of 3M NH<sub>4</sub>SCN compared to GdHCl and 8M urea, a second experiment was conducted using the same plasma samples treated with 8M urea and a titration of NH<sub>4</sub>SCN (1M, 2M and 3M) (Fig. 2). In general, the effectiveness of 1M NH<sub>4</sub>SCN and 8M urea were similar for the 4 antigens (MSP1-42, EBA-175, MSP2 and AMA1), while the 3 concentrations of NH<sub>4</sub>SCN gave a nice titration, especially for AMA-1, EBA-175, and MSP-3, with most Ab remaining bound with 1M NH<sub>4</sub>SCN, intermediate amounts at 2M, and substantial amounts of Ab being removed with 3M. Overall, 2M NH<sub>4</sub>SCN provided the widest range of AIs (i.e., had the best discriminatory potential).

To further explore the influence of chaotrope concentration, one-half and twice the molar concentrations of 4M GdHCl, 8M urea and 3M NH<sub>4</sub>SCN were evaluated on MSP1, MSP2 and MSP3 as test antigens (Supplemental Figure 3). NH<sub>4</sub>SCN was the only chaotrope that dislodged Ab bound to MSP1, but at 3M and 4.5M released almost all Ab bound to MSP2 and MSP3. Urea remained the least stringent chaotrope, failing to release Ab from MSP1 at any concentration, and provided a weak dose-response with MSP2 and MSP3 at 8M and 12M (sFig. 3). In contrast, 4M and 8M GdHCl appeared to release almost all Ab bound to MSP2 and MSP3, but only 10-20% of Ab bound to MSP1 (sFig. 3). Overall, none of the concentrations of the 3 chaotropes improved the range of AI compared to 2M NH<sub>4</sub>SCN (Fig. 2).

Timing of Incubation with Chaotropes. Previous studies used various lengths of time for incubating antigen-Ab complexes with chaotropes, with some protocols simply washing bound antigen-Ab complexes with the salt solution whereas others incubated for 10, 15 or 30 minutes. The most common lengths were 10-15 and 30 minutes. In this experiment, Ab-antigen-bead complexes were incubated for 15 and 30 minutes with either 4M GdHCl, 8M urea or 2M NH<sub>4</sub>SCN (Fig. 3). When directly compared in this experiment, results showed that AIs were only slightly, but not significantly, lower after 30 compared to 15 minutes of incubation regardless of the chaotrope. In subsequent experiments, a 30-minute incubation period was used for convenience when conducting large multiple assays.

The Molar Amount of NH<sub>4</sub>SCN needed to Release 50% of Bound Ab. In this experiment, 40 plasma samples from adults living in the rural village of Ngali II were screened in the avidity MIA using 3 concentrations of NH<sub>4</sub>SCN (1.5M, 3.0M and 4.5M) and i) Ab prevalence, ii) levels and iii) for Ab-positive samples the amount of salt needed to release 50% of bound Ab was calculated (Table 1). Overall, 90-100% of adults had Ab to AMA1, EBA-175, MSP1 and MSP2, but only 68% had Ab to MSP3. Similar

median Ab levels (MFI) were detected to AMA1 and EBA-175 (16,799 and 13,815 MFI); equivalent median Ab levels to MSP1 and MSP2 (9,054 and 9,177); and lower levels of Ab to MSP3 (2,066 MFI). The molar concentration estimated to obtain AI of 50 ranged from 1.7 to 2.3M (Table 1), with a mean of  $2.1M \pm 0.32$  (mean  $\pm$  SD) for adults living in a malaria-endemic area. Thus, 2M  $NH_4SCN$  appeared to release about half of the Ab bound to the 5 antigens, regardless of Ab levels.

The above plasma samples were from adults who had been exposed to *P. falciparum* throughout their lives and, therefore, most likely had acquired a mature malarial humoral response over time. The question then became, would 2M  $NH_4SCN$  be appropriate for infants who were just beginning to acquire immunity? Therefore, 57 plasma samples were screened from infants who were 1 year of age (mean  $\pm$ SD:  $362 \pm 12$  days; range: 326-385 days) living in Ngali II, who had been followed since birth and had begun to make their own Ab after the decline of maternal antibodies. As expected, the majority of infants had Ab to AMA1 (72%), MSP1-42 (93%) and MSP2 (88%); with fewer infants having Ab to EBA-175 (49%) and MSP3 (37%). Ab levels were much lower in the infants to all of the antigens compared to adults (Table 1), but the amount of  $NH_4SCN$  needed to release 50% of bound Ab to the antigens was slightly lower, ranging from 1.6 to 2.1M (compared to 1.7 to 2.3 in adults; Table 1), with a mean of  $1.8M \pm 0.23M$ . Although lower in young children, assay conditions to release 50% of bound Ab did not differ drastically between infants and adults.

Reproducibility of the avidity MIA. In a separate study on Ab avidity, MSP1-42, MSP2 and MSP3 were included as antigens on 13 different plates run over a 30-day period. The protocol included incubating antigen-Ab for 60 minutes, then 30 minutes with 2M  $NH_4SCN$ , followed by 60 minutes with secondary Ab. The archival data provided information on day-to-day and plate-to-plate variation of the avidity MIA assay. Fig. 4 shows the variation in MFI and AI (mean  $\pm$  SD) for 13 replicate assays. Coefficient of Variation (CoV) for each of the AIs was smaller than the corresponding CoV for MFI, e.g., the MFI CoV for MSP1-42 with PC#2 was 32.2%; whereas, the AI CoV was 6.3% (Fig. 4 A and B). A few MFI values were outside of the mean  $\pm$  1 SD, but the corresponding AI remained within, demonstrating the independence of the two measures. Direct linear relationships with higher MFI ( $r = 0.898$ ) and AI ( $r = 0.936$ ) values and smaller CoV were detected (Fig. 4 G, H). Thus, repeating the avidity MIA using 2M  $NH_4SCN$  for 30 minutes resulted in similar avidity indexes.

## Discussion

Considering the importance of Ab in immunity to malaria and the extensive search for correlates for protection, it is surprising that Ab avidity has received relatively little attention. The exclusion method used in this study is a simple approach for measuring Ab avidity, whereby Ab-antigen complexes are treated with a chaotrope and the amount of Ab release is determined. Although the exact mechanism is unknown, it is hypothesized that the gap between antigens and bound Ab with good complementarity is tight enough to exclude the denaturing agent, unlike the gap in low avidity Abs [1]. As such, hydrophobic and ionic bonds stabilizing the complexes remain unbroken [31–33]. Since merozoite antigens have

multiple epitopes, each of which induces Ab with different binding-strengths (affinity), AI represent the overall percentage of Ab with sufficient complementarity to remain bound after exposure to a specific chaotrope. Accordingly, the term “high avidity antibodies” is defined by the concentration of chaotrope used. For example, in Figure 1A, plasma from Individual #3 would be described as having 82% and 75% high avidity Ab to AMA1 if 4M GdHCl and 8M were used, but only 8% high avidity to AMA1 in assays using 3M NH<sub>4</sub>SCN. Likewise, in Figure 2A, Individual #3 would be reported as having 95%, 30% and 8% high avidity Ab to AMA1 if 1M, 2M, or 3M NH<sub>4</sub>SCN were used. Since “High Avidity Ab” is defined by the concentration of chaotrope, it is difficult to compare results between studies and interpret results when different protocols are used.

Today, bead-based MIA are commonly used to measure Ab levels to malarial antigens [22–26]. The MIA format has many advantages, including the requirement for small amounts of antigen, speed, assaying >100 replicates (beads) instead of only a few wells, and being internally controlled since all antigens and reagents are present within the same well (e.g., if Ab are not detected for one antigen but are to others, then absence of Ab is not due to a technical error). For these reasons, having an avidity MIA using the exclusion method would allow researchers to quickly obtain quantitative data about Ab avidity. Based on the initial results, however, it was not clear if an avidity MIA using the 5 antigens and a single chaotrope was feasible.

Follow-up experiments sought to further characterize and refine the bead-based MIA using different concentrations of chaotropes (Fig. 2, and sFig.3) and different incubation periods (Fig. 3). Overall, 2M NH<sub>4</sub>SCN provided the widest range of AI for the 5 antigens. Since using a concentration of chaotrope that is in the center of the exclusion curve is desirable, 2M NH<sub>4</sub>SCN was compared with the actual molar amount of NH<sub>4</sub>SCN needed to release 50% of bound Ab to each of the 5 antigens (Table 1). Using plasma from 40 adults living in a highly endemic malaria region, mean molar concentrations of NH<sub>4</sub>SCN to release 50% of bound Ab ranged from 1.7M to 2.3M, with an average of  $2.1 \pm 0.32$ M for the 5 antigens (Table 2). Thus, 2M NH<sub>4</sub>SCN proved to be in the middle of the exclusion curves. Incubation with antigen-Ab complexes with 2M NH<sub>4</sub>SCN for 15 or 30 minutes didn't alter AIs significantly (Fig. 3), but the longer incubation period proved to be practical when running multiple plates. Using the avidity MIA, over 100 samples can be screened against the 5 or more antigens in a single afternoon with good reproducibility (Fig. 4). Since the assay provides information on both the amount (MFI) and the proportion of high avidity Ab (AI), data on both Ab quantity and quality is obtained in a single experiment. Without further experimentation, it is unclear if the protocol can be used for other malarial antigens, but results from this study provide a starting point for development of future avidity assays for other antigens.

In repeat experiments, AI values were more consistent than MFI (Fig. 4). In other words, AIs are technically more error-proof than raw MFIs. Technical and instrumental differences, for example due to pipetting errors or mis-calibration, result in variation of MFI when the same sample is used in multiple assays. However, the AI values are less affected since they measure the proportion of high binding antibodies. The wide dynamic range of MIA (e.g., 500 MFI to 25,000 MFI in this study) helps explain the very strong

association between AI and the coefficient of variation ( $r = 0.935$ ) (Fig. 4). Thus, these data support the feasibility of a simple, useful, repeatable avidity MIA for merozoite antigens.

Initially, it was hoped that by comparing the most commonly-used protocols in the same experiment, one might gain insight to help make comparisons and interpret data from previous studies. A conscientious search of the literature revealed that the 4 studies using 4M GdHCl focused on merozoite antigens, including AMA1 [5,6,9,11], MSP1 [5,6,9,11], MSP2 [9], MSP3 [5]. Whereas, the 5 studies that used 8M urea measured avidity to a schizont extract [2], *P. vivax* MSP1 and Pv Duffy Binding Protein [14,17], and Ab from individuals vaccinated with AMA1 and Pf25 [15,16]. In contrast, the 6 studies using SCN evaluated other malarial antigens including: a schizont-extract and 1M NH<sub>4</sub>SCN [3], EBA-175 using 2.4M NaSCN [8], the RTS/S vaccine and 1 M NH<sub>4</sub>SCN [12,13], and VAR2CSA with 3M NH<sub>4</sub>SCN [18,19]. Thus, it does not appear that any of these prior studies are similar enough to be directly compared with each other. Accordingly, comparisons can be made within the same study among different cohorts or treatment groups, but direct comparison between studies remains unfeasible. Variation in methodologies and antigens used in previous studies may explain why an inconsistent picture of changes in Ab avidity with age and the role of Ab avidity in protection from malaria exist.

Results from this study provide hints about maturation of the Ab response in individuals living in malaria endemic areas. First, individuals may have high AI to one antigen but low AI to another antigen. For example, in Figure 2, using 2M NH<sub>4</sub>SCN, Individual #1 AI of 90 to MSP1-42, 85 to EBA-175, 25 to AMA1, but only 8% to MSP3. This result indicates that affinity maturation does not occur at the same rate (or reach the same level), for all antigens. Second, at the population level, Ab avidity tends to be greater for some antigens than others. For example, in Figure 1, AI to MSP1-42 were high (>90 with 3M NH<sub>4</sub>SCN), but low AI to MSP2 and MSP3 (AI <15 for 3M NH<sub>4</sub>SCN). Thus, some antigens appear to induce affinity maturation better than others. Third, the amount of Ab was lower in infants than adults for the 5 merozoite antigens, requiring the use of 1:100 and 1:1000 dilutions of infant and adult plasma, respectively, for MFI to fall on the linear part of the binding curve. Interestingly, the amount of NH<sub>4</sub>SCN needed to release 50% of bound Ab was quite similar for Ab-positive infants and adults (Table 1), although lower amounts of chaotrope were required for infants for AMA1 and EBA-175, slightly lower for MSP1-42, but similar for MSP2 and MSP3 (Table 1). Studies in high transmission areas have reported little or no increase in Ab avidity with age for EBA-175, MSP1, MSP2, and MSP3[4–9]. Plasma used in this study was from infants and adults living in a rural village with perennial transmission who received an estimated 257 infectious mosquito bites/person/year [29,30]. Clearly, it would be interesting to investigate affinity maturation with age in this high transmission setting to determine if and when affinity maturation occurs. Overall, the use of 2M NH<sub>4</sub>SCN in future studies evaluating maturation of immunity from infancy to adulthood seems appropriate; whereas, investigators studying acquisition of immunity in young children might consider using 1.5M NH<sub>4</sub>SCN as the lower concentration will give a wider range of AI values.

## Conclusions

A multiplex bead-based avidity immunoassay is feasible for the merozoite antigens AMA1, EBA-175, MSP1-42, MSP2 and MSP3 that employs 2M of NH<sub>4</sub>SCN. Further studies are needed to determine if 2M NH<sub>4</sub>SCN is the best concentration of chaotrope for other malarial antigens. The assay is simple, measures avidity near the middle of the dynamic range, and AI are similar in repeated experiments. The assay provides a simple method to quickly obtain information about Ab quantity and quality in the acquisition of immunity to malaria in endemic populations.

## **Declarations**

### Ethical approvals and consent to participate

Approval for the original studies was obtained from the Ethics Committee, Cameroon and IRB at Georgetown University (1998-081). All participants gave written informed consent. The use of deidentified plasma samples for antibody studies was deemed exempt by the Committee on Human Subjects University of Hawai'i (adults: CHS #21891CHS, infants: CHS #21752).

### Consent for publication

Not applicable

### Availability of data and materials

Essentially all data used and/or analyzed in the current study are included in this publication. The data sets for individual experiments reported in this publication are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

The original study providing the archival plasma samples was supported by NIAID, NIH UOI AI43888; antibody analysis was funded, in part, by NIAID, NIH R21 AI105286; and STY was supported through a training grant from the Fogarty International Center (D43TW009074).

### Authors' contributions

NB, AK, and STY conceived the idea, NB, AK, WK and STY performed the laboratory experiments, analyzed the data, and assisted in writing the manuscript. RFGL and DWT conducted the initial study, supervised the study, analyzed the data and wrote the manuscript. All authors approved the final manuscript.

### Acknowledgements

We thank the entire staff at the Biotechnology Center, University of Yaoundé I, for their excellent technical support in conducting the field and laboratory studies. We are grateful to colleagues who provided the recombinant proteins: C. Long, Malaria Development Branch, NIAID, NIH for AMA-1 (3D7) and MSP1-42 (3D7); Science Applications International Corp., Fedrick, MD for EBA-175; R. Anders, La Trobe University, Victoria, Australia for MSP2; and J. Rayner, Wellcome Trust Sanger Institute, for MSP3 (HB3).

## Abbreviations

Ab antibody/antibodies

AI /AIs avidity index / avidity indexes

AMA1 apical merozoite antigen 1

CoV coefficient of variation

EBA-175 erythrocyte binding antigen -175

GdHCL guanidine HCl

IRB Institutional Review Board

MFI median fluorescence intensity

MIA bead-based, multiplex immunoassay

MSP merozoite surface protein

NC negative control

NH<sub>4</sub>SCN ammonium thiocyanate

SCN thiocyanate

SD standard deviation

PC positive control

PC#2 second (alternate) positive control

r value coefficient of correlation using simple linear regression

## References

1. Hedman K, Lappalainen M, Soderlund M, Hedman L. Avidity of IgG in serodiagnosis of infectious diseases. *Rev Med Microbiol.* 1993;4:123–9.

2. Leoratti FMS, Durlacher RR, Lacerda MVG, Alecrim MG, Ferreira AW, Sanchez MCA, et al. Pattern of humoral immune response to *Plasmodium falciparum* blood stages in individuals presenting different clinical expressions of malaria. *Malar J*. 2008;7:1–11.
3. Ferrerira MU, Kimura EK, de Souza JM, Katain AM. The isotype composition and avidity of naturally acquired anti-*Plasmodium falciparum* antibodies: Differential Patterns in Clinically Immune Africans and Amazonian Patients. *Am J Trop Med Hyg*. 1996;55:315–23.
4. Reddy S, Anders R, Beeson J, Farnert A, Kironde F, Berenzon S, et al. High avidity antibodies to *Plasmodium falciparum* merozoite antigens are associated with protection from malaria. *PLoS One*. 2012;7.
5. Ibison F, Olotu A, Muema DM, Mwacharo J, Ohuma E, Kimani D, et al. Lack of avidity maturation of merozoite antigen-specific antibodies with increasing exposure to *Plasmodium falciparum* amongst children and adults exposed to endemic malaria in Kenya. *PLoS One*. 2012;7:1–7.
6. Ssewanyana I, Arinaitwe E, Nankabirwa JI, Yeka A, Sullivan R, Kanya MR, et al. Avidity of anti-malarial antibodies inversely related to transmission intensity at three sites in Uganda. *Malar J*. *BioMed Central*; 2017;16:1–8.
7. Tijani MK, Reddy SB, Langer C, Beeson JG, Wahlgren M, Nwuba RI, et al. Factors influencing the induction of high affinity antibodies to *Plasmodium falciparum* merozoite antigens and how affinity changes over time. *Sci Rep [Internet]*. Springer US; 2018;8:1–12. Available from: <http://dx.doi.org/10.1038/s41598-018-27361-w>
8. Abagna HB, Acquah FK, Okonu R, Aryee NA, Theisen M, Amoah LE. Assessment of the quality and quantity of naturally induced antibody responses to EBA175RIII-V in Ghanaian children living in two communities with varying malaria transmission patterns. *Malar J [Internet]*. *BioMed Central*; 2018;17:1–11. Available from: <https://doi.org/10.1186/s12936-017-2167-3>
9. Akpogheneta OJ, Dunyo S, Pinder M, Conway DJ. Boosting antibody responses to *Plasmodium falciparum* merozoite antigens in children with highly seasonal exposure to infection. *Parasite Immunol*. 2010;32:296–304.
10. Dennison SM, Reichartz M, Seaton KE, Dutta S, Wille-Reece U, Hill AVS, et al. Qualified biolayer interferometry avidity measurements distinguish the heterogeneity of antibody interactions with *Plasmodium falciparum* circumsporozoite protein antigens. *J Immunol*. 2018;201:1315–26.
11. Wipasa J, Suphavilai C, Okell LC, Cook J, Corran PH, Thaikla K, et al. Long-lived antibody and B cell memory responses to the human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax*. *PLoS Pathog*. 2010;6.
12. Olotu A, Clement F, Jongert E, Vekemans J, Njuguna P, Ndungu FM, et al. Avidity of anti-circumsporozoite antibodies following vaccination with RTS,S/AS01E in young children. *PLoS One*. 2014;9:1–15.
13. Ajua A, Lell B, Agnandji ST, Asante KP, Owusu-Agyei S, Mwangoka G, et al. The effect of immunization schedule with the malaria vaccine candidate RTS,S/AS01E on protective efficacy and anti-circumsporozoite protein antibody avidity in African infants. *Malar J*. 2015;14:1–6.

14. Zakeri S, Babaeekhou L, Mehrizi AA, Abbasi M, Djadid ND. Antibody responses and avidity of naturally acquired anti-*Plasmodium vivax* duffy binding protein (PvDBP) antibodies in individuals from an area with unstable malaria transmission. *Am J Trop Med Hyg.* 2011;84:944–50.
15. Berry AA, Gottlieb ER, Kouriba B, Diarra I, Thera MA, Dutta S, et al. Immunoglobulin G subclass and antibody avidity responses in Malian children immunized with *Plasmodium falciparum* apical membrane antigen 1 vaccine candidate FMP2.1/AS02 A. *Malar J* [Internet]. BioMed Central; 2019;18:1–10. Available from: <https://doi.org/10.1186/s12936-019-2637-x>
16. Patra K, Li F, Carter D, Gregory A, Baga S, Reed S, et al. Alga-produced malaria transmission-blocking vaccine candidate Pfs25 formulated with a human use-compatible potent adjuvant induces high-affinity antibodies that block *Plasmodium falciparum* infection of mosquitoes. *Infect Immun.* 2015;83:1799–808.
17. Mehrizi AA, Zakeri S, Salmanian AH, Sanati MH, Djadid ND. IgG subclasses pattern and high-avidity antibody to the C-terminal region of merozoite surface protein 1 of *Plasmodium vivax* in an unstable hypoendemic region in Iran. *Acta Trop.* 2009;112:1–7.
18. Tutterrow Y Lo, Salanti A, Avril M, Smith JD, Pagano IS, Ako S, et al. High Avidity Antibodies to full-length VAR2CSA correlate with absence of placental malaria. *PLoS One.* 2012;7.
19. Babakhanyan A, Fang R, Wey A, Salanti A, Sama G, Efundem C, et al. Comparison of the specificity of antibodies to VAR2CSA in Cameroonian multigravidae with and without placental malaria: A retrospective case-control study. *Malar J.* 2015;14.
20. Lew AM, Anders RF, Edwards SJ, Langford CJ. Comparison of antibody avidity and titre elicited by peptide as a protein conjugate or as expressed in vaccinia. *Immunology.* 1988;65:311–4.
21. Miura K, Deng B, Wu Y, Zhou L, Pham T, Diouf A, et al. ELISA units, IgG subclass ratio and avidity determined functional activity of mouse anti-Pfs230 antibodies judged by a standard membrane-feeding assay with *Plasmodium falciparum*. *Vaccine.* 2019;37:2073–8.
22. Fouda GG, Leke RFG, Long C, Druilhe P, Zhou A, Taylor DW, et al. Multiplex assay for simultaneous measurement of antibodies to multiple *Plasmodium falciparum* antigens. *Clin Vaccine Immunol.* 2006;13:1307–13.
23. Priest JW, Plucinski MM, Huber CS, Rogier E, Mao B, Gregory CJ, et al. Specificity of the IgG antibody response to *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale* MSP1 19 subunit proteins in multiplexed serologic assays. *Malar J* [Internet]. BioMed Central; 2018;17:1–20. Available from: <https://doi.org/10.1186/s12936-018-2566-0>
24. Ondigo BN, Park GS, Gose SO, Ho BM, Ochola LA, Ayodo GO, et al. Standardization and validation of a cytometric bead assay to assess antibodies to multiple *Plasmodium falciparum* recombinant antigens. *Malar J.* 2012;11:1–17.
25. Ubillos I, Campo JJ, Jiménez A, Dobaño C. Development of a high-throughput flexible quantitative suspension array assay for IgG against multiple *Plasmodium falciparum* antigens. *Malar J* [Internet]. BioMed Central; 2018;17:1–15. Available from: <https://doi.org/10.1186/s12936-018-2365-7>

26. Kerkhof K, Canier L, Kim S, Heng S, Sochantha T, Sovannaroeth S, et al. Implementation and application of a multiplex assay to detect malaria-specific antibodies: a promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas. *Malar J. BioMed Central*; 2015;14:1–14.
27. Stenger R, Smits M, Kuipers B, Kessen S, Boog C, van Els A. Fast, antigen-saving multiplex immunoassay to determine levels and avidity of mouse serum antibodies to Pertussis, Diphtheria, and Tetanus antigens. *Clin Vaccine Immunol.* 2011;18:595–603.
28. Fang R, Wey A, Bobbili NK, Leke RFG, Taylor DW, Chen JJ. An analytical approach to reduce between-plate variation in multiplex assays that measure antibodies to *Plasmodium falciparum* antigens. *Malar J.* 2017;16.
29. Leke RFG, Bioga JD, Zhou J, Fouda GG, Leke RJI, Tchinda V, et al. Longitudinal studies of *Plasmodium falciparum* malaria in pregnant women living in a rural Cameroonian village with high perennial transmission. *Am J Trop Med Hyg.* 2010;83:996–1004.
30. Tassi Yunga S, Fouda GG, Sama G, Ngu JB, Leke RGF, Taylor DW. Increased susceptibility to *Plasmodium falciparum* in infants is associated with low, not high, placental malaria parasitemia. *Sci Rep [Internet]. Springer US*; 2018;8:1–10. Available from: <http://dx.doi.org/10.1038/s41598-017-18574-6>
31. Nozaki Y, Tanford C. The solubility of amino acids and related compounds in aqueous urea. *J Biol Chem.* 1963;238:4074–81.
32. Kamoun P. Denaturation of globular proteins by urea: breakdown of hydrogen or hydrophobic bonds? *TIBS.* 1988;13:424–415.
33. Macdonald R, Hosking C, Jones C. The measurement of relative antibody affinity by ELISA using thiocyanate elution. *J Immunol Methods Immunol methods.* 1988;106:191–4.

## Table

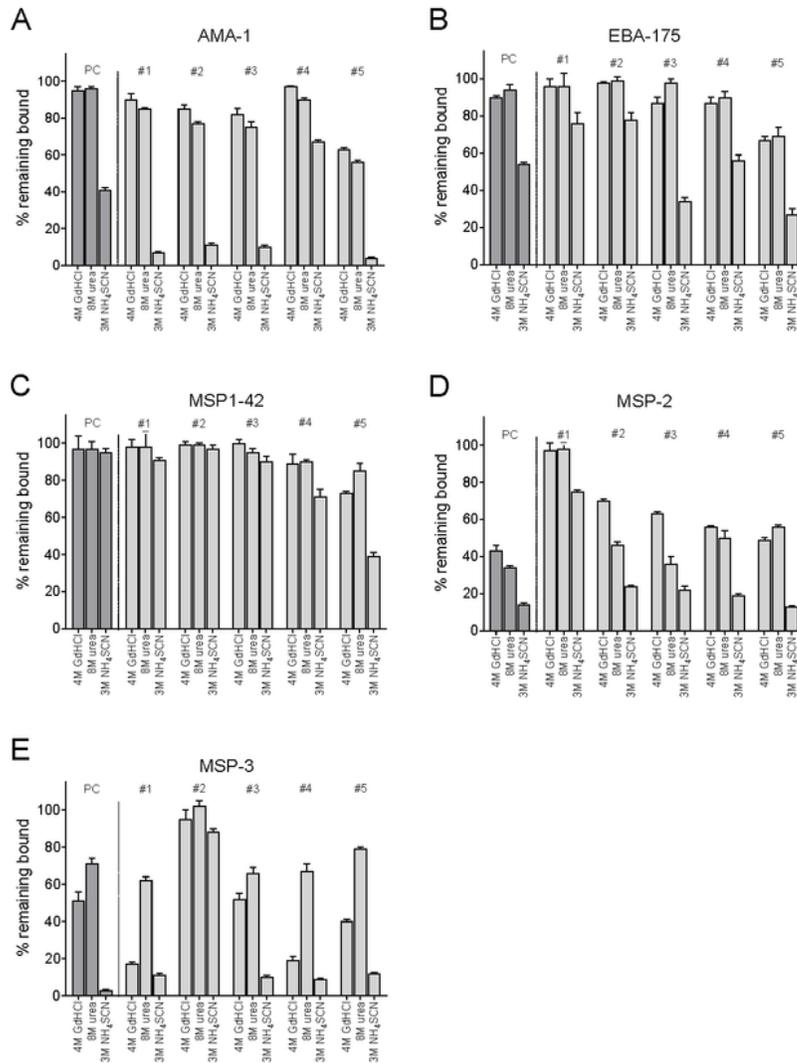
Table 1: Molar Amount of  $\text{NH}_4\text{SCN}$  needed to Release 50% of Bound Antibody

N = 40 Adults	AMA1	EBA-175	MSP1-42	MSP2	MSP3
% Ab positive	100 (40/40)	90 (36/40)	100 (40/40)	98 (39/40)	68 (27/40)
Median MFI (95% CI)* (1:1,000 dilution)	16,799 (13,176, 18,855)	13,815 (11,189, 17,637)	9,054 (6,907, 14,221)	9,177 (5,309, 11,151)	2,066 (1,287, 3,477)
Molars of chaotrope to release 50% Ab bound (mean $\pm$ SD)	2.31M $\pm$ 0.44	2.25 $\pm$ 0.33	2.32 $\pm$ 0.82	1.71 $\pm$ 0.22	1.70 $\pm$ 1.72
n= 57 1-year old infants	AMA1	EBA-175	MSP1-42	MSP2	MSP3
% Ab positive	72 (41/57)	49 (28/57)	93 53/57	88 (50/57)	37 (21/57)
Median MFI (95% CI)* (1:100 dilution)	9,664 (5,717, 13,906)	1,094 (673, 1,584)	8,974 (4,365, 11,808)	5,536 (2,335, 9,276)	1,247 (750, 1,653)
Molars of chaotrope to release 50% Ab bound Ab	1.78M $\pm$ 0.27	2.0 $\pm$ 0.50	2.1 $\pm$ 0.59	1.6 $\pm$ 1.1	1.6 $\pm$ 0.12

\*95% Confidence Interval (CI) of the median based on Ab-positive samples

## Figures

Figure 1



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Figure 1

Comparison of Avidity Indexes for 5 Merozoite Antigens using 3 different Chaotropic Agents. Antigen-coupled beads were incubated with a 1:600 dilution of the positive control (PC) and plasma from adults (#1 - #5) living in a high malaria transmission area in Cameroon. After incubation, Ab-bound beads were incubated with 4M GdHCl for 10 minutes, 8 M urea for 10 minutes, or 3M NH<sub>4</sub>SCN for 30 minutes. Results show mean AI values (i.e., the percentage of Ab remaining bound after treatment with salt) ± SD

for 3 replicate samples. Antigens included: AMA1 (3D7), EBA-175, MSP1 (3D7), MSP2 (FC27) and MSP3 (HB3). Baseline (no salt) MFI values [mean  $\pm$  SD] for the 6 plasma samples (PC + 5 adults) were as follows: A) AMA1= 23,677 $\pm$ 1079 MFI; B) EBA-175=22,002 $\pm$ 1573 MFI; C) MSP1= 11,196 $\pm$ 1405 MFI; D) MSP2= 5,702 $\pm$ 2282; and E) MSP3= 4,128 $\pm$ 1232 MFI.

Figure 2:

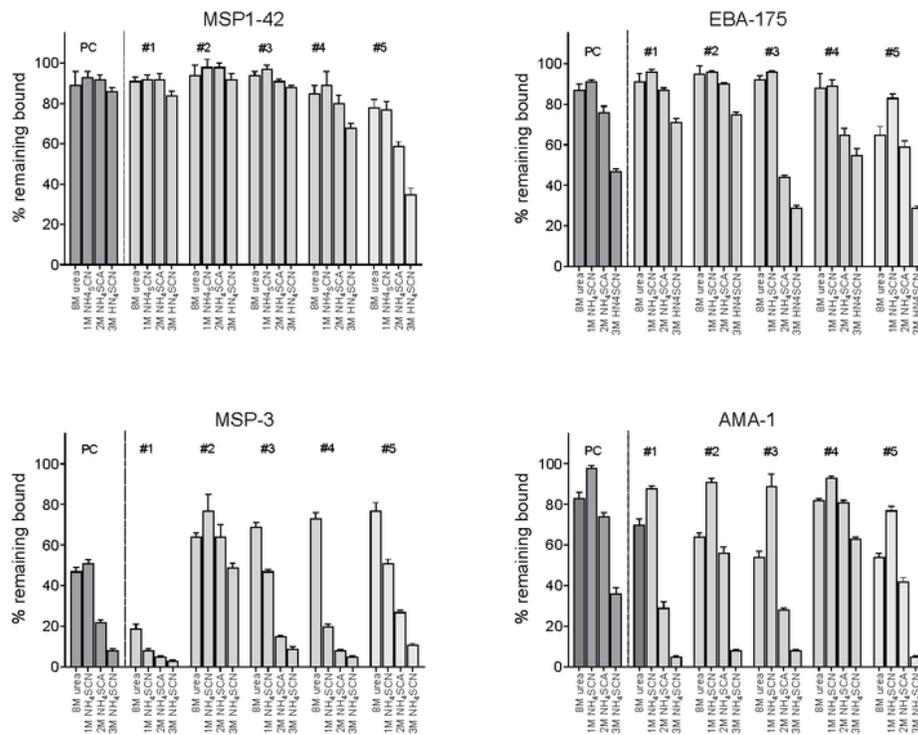
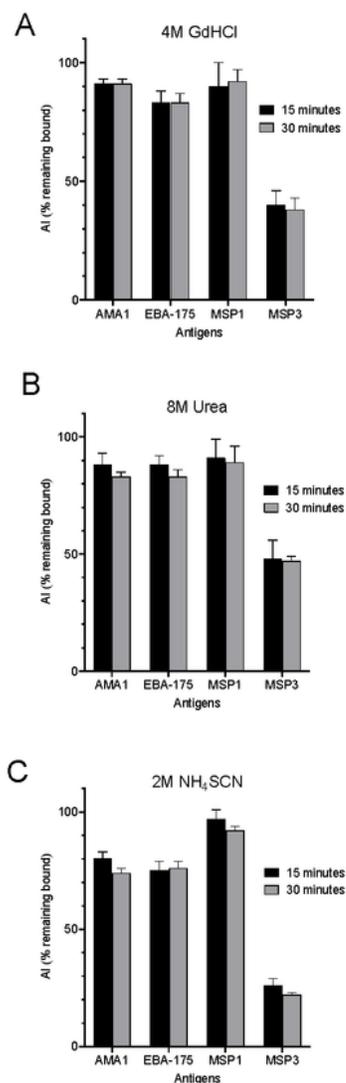


Figure 2

Avidity Indexes after Treatment with 8M urea and 1M, 2M and 3M NH<sub>4</sub>SCN. The same PC and samples from 5 individual adults used in Figure 1 were re-tested in this experiment. Following incubation of plasma with the antigen-coupled beads, the complexes were incubated with 8M urea or 1M, 2M and 3M or NH<sub>4</sub>SCN for 30 minutes and amount of Ab remaining bound (AI) were calculated. Error bars represent the mean  $\pm$  one SD for 3 replicates. Baseline MFI values [mean  $\pm$  SD based on 15 replicates in the absence of salt] were as follows: A) AMA-1 23,958 $\pm$ 98 MFI; B) EBA-175: 22,413 $\pm$ 166; C) MSP1 3D7: 10,169 $\pm$ 208 MFI; D) MSP3: 2,122 $\pm$ 82MFI. OPTIONAL INSERT: Comparison of data in Fig. 1 and 2 show that the majority of mean AI have overlapping SD; however, a few differences are seen, especially with 8M urea for AMA1 and MSP3 that are due to inter-assay variation.

Figure 3:



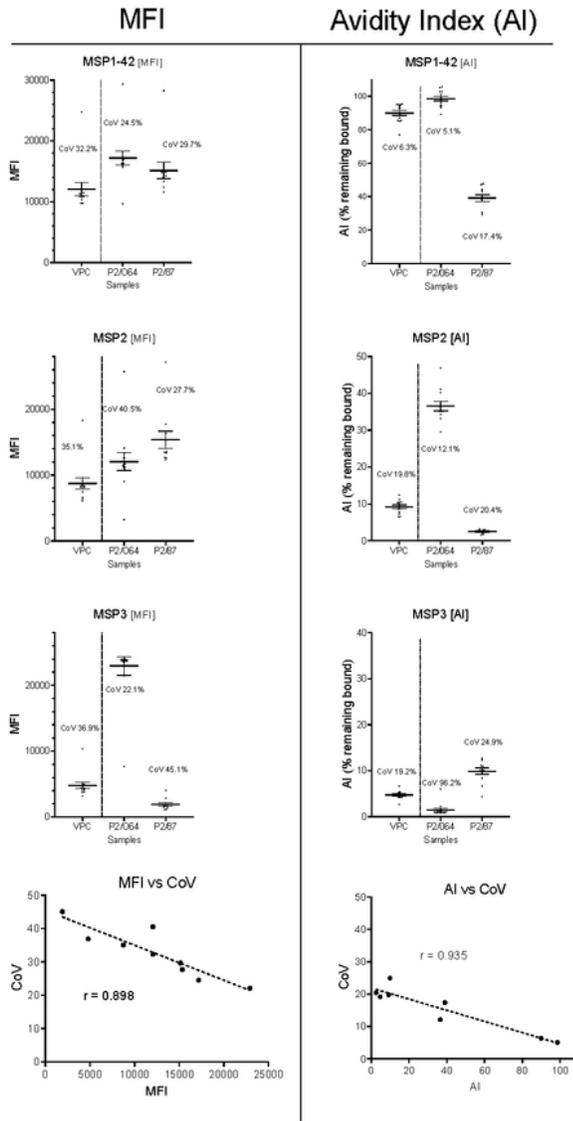
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### Figure 3

Comparison of the Length of Incubation with the Chaotrope. Antigen-coupled beads were incubated with the pooled Positive Control (PC) for 60 minutes. Beads were then washed, incubated with 4M GdHCl, 8M urea, or 1M, 2M or 3M NH<sub>4</sub>SCN for either 15 or 30 minutes at RT on a rotary shaker. Following washing and incubation with R-phycoerythrin-labeled, Goat anti-human IgG (Fc fragment) for 60 minutes, MFI were recorded and AI calculated. Error bars represent mean  $\pm$  SD based on 3 replicates. Baseline MFI values

[mean  $\pm$  SD MFI for 30 replicates in the absence of salt] were as follows: AMA-1 23,955 $\pm$ 133 MFI; EBA-175 22,473 $\pm$ 587; MSP1 (3D7) 10,042 $\pm$ 333 MFI; and MSP3: 1,990 $\pm$ 155 MFI.

Figure 4.



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Figure 4

Variation among Replicates Assays. A second Positive-Control (PC#2) and plasma from two adults (P2/064, P2/87) were used as positive controls on 13 plates in a separate avidity study conducted over a

30-day period. Horizontal bars represent means and SD for 13 replicates. Bottom 2 figures are based on the 9 samples shown in the figures above; trendline represents a simple linear regression.

## Supplementary Files

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