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Cross-reactivity of two SARS-CoV-2 serological assays in a malaria-endemic setting

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Abstract

Background: Accurate SARS-CoV-2 serological assays are critical for COVID-19 serosurveillance. However, previous studies have indicated possible cross-reactivity of these assays, including in malaria-endemic areas.

Methods: We tested 213 well-characterized pre-pandemic samples from Nigeria using two SARS-CoV-2 serological assays: Abbott Architect IgG and Euroimmun NCP IgG assay, both targeting SARS-CoV-2 nucleocapsid protein. To assess antibody binding strength, an avidity assay was performed on these samples and on plasma from SARS-CoV-2 PCR-positive persons.

Results: Thirteen (6.1%) of 212 samples run on the Abbott assay and 38 (17.8%) of 213 run on the Euroimmun assay were positive. Anti-*Plasmodium* IgG levels were significantly higher among false-positives for both Abbott and Euroimmun; no association was found with active *P. falciparum* infection. An avidity assay using various concentratIons of urea wash in the Euroimmun assay reduced loosely-bound IgG: of 37 positive/borderline pre-pandemic samples, 46%, 86%, 89%, and 97% became negative using 2M, 4M, 5M, and 8M urea washes, respectively. The wash slightly reduced avidity of antibodies from SARS-CoV-2 patients within 28 days of PCR confirmation; thereafter avidity increased for all urea concentrations except 8M.

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Conclusions: This validation found moderate to substantial cross-reactivity on two SARS-CoV-2 serological assays using samples from a malaria-endemic setting. A simple urea wash appeared to alleviate issues of cross-reactivity.

Introduction

The COVID-19 pandemic has led to more than 100 million confirmed cases and more than 2.2 million deaths from COVID-19 globally as of early February 2021 (1). However, with mild or asymptomatic disease presentations (2) and access to SARS-CoV-2 molecular and antigen testing still limited in many places, cumulative infections may be underestimated. Serological assays that detect antibodies can be useful for understanding the true extent of SARS-CoV-2 exposure in a population (3, 4). A multitude of rapid and laboratory-based SARS-CoV-2 serological assays have been developed since the begining of the pandemic: as of early February 2021, 65 SARS-CoV-2 serological tests have received emergency use authorization (EUA) from the United States Food & Drug Administration (5).

In addition to manufacturer validation results, results from independent validations of SARS-CoV-2 immunoassay performance are becoming increasingly available (6-9). An important concern in development of SARS-CoV-2 serologic assays is to ensure that measured antibody responses are specific to SARS-CoV-2 infection in the human host. High specificity becomes even more relevant when seropositivity levels are low in a population (10-12), as even small declines in test specificity can lead to large proportions of false-positive serological tests.

Most independent validations of SARS-CoV-2 serological assays have used samples from Chinese, European, or North American COVID-19 cases and negative (typically pre-2020)

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controls (7, 13-15). A concern for certain geographical areas is cross-reactivity to endemic pathogens that were not included in validation studies. Previous serological studies for Zika (16), dengue (17), and HIV (18) have shown false positive results from persons exposed to malaria parasites, though the mechanisms for these false positive test results have not been fully elucidated.

A recent study found false positive SARS-CoV-2 serology tests with four commercially-available IgG ELISA kits in samples from Nigeria and Ghana, but not in samples from Madagascar, Germany, Columbia, or Lao People's Democratic Republic (19). Data from Benin showed that approximately 25% of 60 samples from patients with acute malaria in 2019 had positive SARS-CoV-2 serological results (20).

An urgent need exists for specific SARS-CoV-2 serologic assays appropriate for a wide variety of settings; accuracy of such assays in the context of other endemic infectious diseases needs to be carefully assessed. Here, we present results from laboratory testing of two commercially-available SARS-CoV-2 serological assays. These assays were performed on a well-characterized panel of Nigerian samples collected in 2018, as well as on samples from SARS-CoV-2 PCR-positive patients from 2020. The prevalence of false positive serological test results was investigated to determine any association with malaria infection and antibody levels. Strength of IgG binding from false-positive and true-positive test results was examined.

Materials and Methods

Specimens tested

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De-identified samples from Nigeria's national biorepository at the National Reference Laboratory (NRL) that were initially collected as part of the 2018 Nigeria HIV/AIDS Indicator and Impact Survey (NAIIS) (21) were tested for SARS-CoV-2 antibodies. Whole blood was collected from participants and for those consenting, stored as plasma at NRL at -80^{o}C. Through the Nigeria Multi-disease Serologic Surveillance of Stored Specimens (NMS4) project (22), these samples had been tested for presence of malaria antigens, and IgG against a variety of endemic pathogens in Nigeria (22, 23). The multiplex bead assay (MBA) for IgG against a panel of infectious and vaccine-preventable diseases was performed on the MAGPIX platform as described previously (23-25) with a serum dilution of approximately 1:400. The multiplex malaria antigen detection assay was also performed on the MAGPIX platform as described previously (26, 27) at a whole blood dilution of 1:40. All assays were performed at the NRL (Nigeria Centre for Disease Control, NCDC) in Abuja, Nigeria.

For SARS-CoV-2 serology, we sampled 107 children <15 years old and 106 adults >15 years old (Table 1). Approximately half of samples were intentionally selected based on histidine-rich protein 2 (HRP2) antigen positivity indicating current or recent infection with *Plasmodium falciparum.* Of HRP2 antigen positives, one-third had low-positive, one-third medium-positive, and one-third high-positive malaria antigen values, based on antegenemia tertiles.

We also tested plasma samples from 32 SARS-CoV-2 PCR-positive patients in Lagos that had been collected at various time points since PCR confirmation.

Laboratory methods for SARS-CoV-2 serological assays

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Two commercially available SARS-CoV-2 IgG assays were assessed using the pre-pandemic samples. The Euroimmun Anti-SARS-CoV-2 NCP ELISA (IgG) (Euroimmun Medizinische Labordiagnostika, Lübeck, Germany) assay detects IgG antibodies against SARS-CoV-2 nucleocapsid (NCP) protein. The automated Abbott Architect Plus i2000sr Analyzer (Abbott, Illinois, USA) and SARS-CoV-2 IgG kit is a method for detecting IgG antibodies against the SARS-CoV-2 NCP. Both tests were performed according to manufacturer recommendations and also using an avidity assay with a urea wash (see Supplementary materials).

Samples were initially run at the Center for Human Virology and Genomics (CHVG), at the Nigerian Institute of Medical Research (NIMR) in Lagos, Nigeria. To examine inter-laboratory variations, the Euroimmun and Abbott tests were also run on additional sample aliquots at the NRL.

Statistical analyses

Log-transformed antibody and malaria antigen values were compared among (true) negative and (false) positive pre-pandemic samples using the Wilcoxon rank-sum test for non-normally distributed data. Given the exploratory nature of the analyses and the relatively large number of comparisons (n=78), we used the Benjamini-Hochberg adjustment and a false discovery rate of 10% to define which comparisons were statistically significant (28). Agreement among test results from the two laboratories was measured with kappa statistics for categorical test outcomes (e.g., positive, negative, borderline), and signed-rank non-parametric tests to compare optical density ratios on the same test samples. Significant differences among avidity index means were determined by two-sided t-tests using unequal variances. Sensitivity and

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specificity confidence limits were calculated using binomial exact formulas. Stata 16.0 (College Station, Texas) and Microsoft Excel were used for analyses.

Ethical approval

Written informed consent for future testing of collected blood samples was provided by participants during NAIIS data collection. Written consent was obtained from SARS-CoV-2-positive patients for plasma collection and storage for future testing. This cross-reactivity evaluation was approved by the National Health Research Ethics Committee of Nigeria (NHREC) (protocol number NHREC/01/01/2007-31/08/2020) and by the US Centers for Disease Control and Prevention.

Results

Of 213 pre-pandemic samples from the 2018 NAIIS, the median age was 14 years (inter-quartile range: 10 years, 23 years) and 127 (60.1%) were from females (Table 1). In total, 107 (50.2%) were positive for *P. falciparum* HRP2 antigen, indicating current/recent malaria infection, and 139 (65.3%) were seropositive for glurp, 162 (76.1%) were seropositive for pfama1, and 193 (90.6%) were seropositive for pfmsp1. All 213 samples were tested with the Euroimmun assay, and 212 with the Abbott assay (one sample had insufficient volume for the Abbott assay). Twenty Euroimmun results were borderline after the first run and were repeated. Two Abbott tests had invalid results after the first run and were repeated.

Test specificity

For the pre-pandemic samples, the Abbott test had two (0.9%) invalid results after two test runs, 197 (92.9%) negative, and 13 (6.1%) positive results (Table 2). The Euroimmun had seven

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(3.3%) borderline results after repeating, 168 (78.9%) negative results, and 38 (17.8%) positive results. All but two of the 13 positive Abbott results were also positive on Euroimmun, while the remaining two were negative (Table 3). Excluding invalid and borderline results, specificity was 81.6% (95% Confidence Interval (CI): 75.6%, 86.3%) for Euroimmun and 93.8% for Abbott (95% CI: 89.6%, 96.4%) assays. Using a sequential algorithm (both tests negative), specificity was 94.6% (95% CI: 90.5%, 97.0%).

Inter-laboratory results agreement

For pre-pandemic samples, there was moderate to strong agreement (29) between the Euroimmun and the Abbott assay results from tests run at NIMR and at NRL, with kappa statistics of 0.6220 for Euroimmun (0.7655 if borderline results excluded) and 0.8621 for Abbott (Supplementary Tables 1 and 2). Tests run at NIMR had on average higher OD ratios for both Euroimmun and Abbott compared to tests run at NRL (p-value for sign-rank test <0.001 for both).

Relationships between positive SARS-CoV-2 serological tests and levels of malaria and other antibodies in pre-pandemic samples

Levels of malaria antibodies were significantly higher for pre-pandemic samples with positive SARS-CoV-2 antibody test results for five of nine malaria IgG targets: PfCSP, glurp (Euroimmun only), Pfama1 (Euroimmun only), pmmsp1, and pomsp-1 (Figure 1 and Supplementary Table 3). There was no significant association with pfmsp1, pvmsp1, hrp2 or lsa1 malaria IgG antibodies. In assessing active malaria infection, no significant association was observed with presence or levels of any of the four malaria antigen targets (Supplemental Figure 1).

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For either Abbott or Euroimmun, but not for both, positive SARS-CoV-2 serological results had significantly higher antibodies for several other pathogens included in the NMS4 multiplex bead-based assay, including lymphatic filariasis (Abbott assay), onchocerciasis (Abbott), syphilis/yaws (Euroimmun), cysticercosis (Abbott), and taeniasis (Abbott) (Supplemental Table 3).

Avidity assay for pre-pandemic samples with SARS-CoV-2 IgG assay

Forty pre-pandemic samples (32 positive, 5 borderline, and 3 negative by Euroimmun assay) were run using four concentrations of urea wash (Figure 2A). The three negative samples remained negative and the five borderline samples became negative at all four urea concentrations. Of the 32 positive samples, 11, 3, 1, and 0 remained positive and 9, 2, 3, and 1 became borderline using the 2M, 4M, 5M, and 8M washes, respectively (Supplemental Figure 2). Of these initial 32 positives, 12 (38%) , 27 (84%), 28 (88%), and 31 (97%) became negative using the 2M, 4M, 5M, and 8M washes, respectively. For all pre-pandemic samples, the OD ratio to calibrator (Figure 2A and Supplemental Figure 3A) and avidity index (AI) (Figure 2B) steadily decreased with increasing urea concentrations. Though the 2M urea wash had only a slight effect on amount of retained anti-NCP IgG (median AI: 71.5%), the more stringent 4M (median AI: 31.0%), 5M (median AI: 18.1), and 8M (median AI: 11.7%) removed the vast majority of cross-binding IgG antibodies in pre-pandemic samples.

Avidity assay for samples from SARS-CoV-2 PCR-positive persons with SARS-CoV-2 IgG assay

Using 32 samples from patients testing positive for SARS-CoV-2 by PCR, OD ratios decreased at higher urea concentrations, but this was dependent on time since PCR positivity (Figure 3 and

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Supplemental Figure 3B). Persons with samples collected <28 days after a PCR positive test showed a decrease in OD ratio with increasing urea concentrations (Figure 3A). However, samples collected ≥28 days after a positive PCR largely retained the OD ratio through the 2, 4 and 5M urea washes before substantially dropping off at the 8M wash (Figure 3A). This was reflected in the strength of IgG binding, with significant differences in AIs for all urea wash concentrations for samples <14 days versus ≥28 days post-PCR positivity (Figure 3B). Using the more stringent 5M and 8M urea washes, samples collected 14-27 days post-PCR positivity had AIs significantly lower than those collected at ≥28 days. For samples collected ≥28 days post-PCR positivity, median AIs were largely similar at urea concentrations ≤5M (2M, 129.6%; 4M 109.2%; 5M, 89.9%; 8M, 21.2%), but dropped quickly for samples collected 14–28 days post PCR positivity (2M, 65.4%; 4M 38.4%; 5M, 20.6%; 8M, 5.1%). Positive associations were observed between time since PCR positivity and AI at all urea concentrations, but correlations were not strong (Supplemental Figure 4).

Level of anti-NCP IgG versus strength of binding

For both the pre-pandemic and the SARS-CoV-2 PCR positive sample sets, a general negative trend was observed between total amount of anti-NCP IgG detected (by OD ratio to calibrator) and AI for different urea wash concentrations (Supplemental Figure 5), but these trends showed high variability. The OD to calibrator ratio was significantly higher at all urea washes for SARS-CoV-2 PCR positives versus pre-pandemic samples; differences in AIs between these sample sets were only seen for 4 and 5M urea washes (Supplemental Figures 6A & 6B).

Sensitivity and specificity of Euroimmun NCP assay with different concentration of urea

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Combining the pre-pandemic and the SARS-CoV-2 PCR-positive panels to examine effects of various concentrations of a urea wash step on test performance, sensitivity decreased with increasing concentrations of urea (to 12.5% at 8M) while specificity increased (to 100% at 8M) (Table 4). Samples collected ≥14 days post PCR did not show as sharp declines in sensitivity; samples collected ≥28 days post PCR retained 100% sensitivity up to 5M, at which sensitivity dropped to 83.3% (Table 4).

Discussion

Our results from this highly-endemic malaria setting showed a high level of false positive results with the Euroimmun NCP SARS-CoV-2 serological assay (17.8%), and lower levels with the Abbott Architect assay (6.1%) – both yielding specificity levels below the WHO-recommended 97% for SARS-CoV-2 serological assays (30). Though active malaria infection was not associated with reduced specificity of these two assays, levels of anti-*Plasmodium* IgGs against multiple malaria antigen targets were significantly higher in false positive samples versus true negatives. The IgGs leading to false positive serological results were found to be weakly-bound to the SARS-CoV-2 antigens, and most were removed with low concentrations of the protein denaturant urea. No significant correlation was seen between the level of cross-binding IgG and the strength of IgG binding, suggesting that these IgGs that are binding SARS-CoV-2 antigens are not due to a true affinity maturation process. Importantly, a relatively simple urea wash step during the Euroimmun assay improved assay specificity.

The 93.8% specificity we found from this Nigerian sample set with the Abbott Architect is lower than estimates from previous evaluations, including 99.6% reported by the manufacturer using a panel of pre-COVID-19 samples and samples from patients with other respiratory illnesses

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(total n=1,070) (31) and 99.6% by an independent evaluation using 1,099 pre-pandemic samples (32). The specificity of 81.6% on the Euroimmun NCP assay we found was substantially lower than the manufacturer-reported specificity of 99.8% using pre-COVID-19 panels from Germany, the United States, and China, including some samples positive for influenza, Epstein-Barr virus, and rheumatoid factor-positive samples (n=1,140) (33).

Our study is the first to demonstrate an association between SARS-CoV-2 antibody cross-reactivity and existing malaria antibodies. Previous specificity experiments with SARS-CoV-2 serological assays have typically included samples from non-malaria-endemic areas positive for autoimmune diseases, other human coronaviruses, Epstein-Barr virus, cytomegalovirus, and other respiratory pathogens, and most have shown low to no cross-reactivity (13, 34, 35). However, a recent study found much higher levels of cross-reactivity to SARS-CoV-2 (primarily to the NCP) among pre-pandemic samples from Tanzania and Zambia compared to those from the United States; given that the cross-reactive samples also showed strong reactivity against other human coronaviruses, the authors concluded that exposure to other coronaviruses may induce cross-reactive antibodies against SARS-CoV-2 in sub-Saharan Africa (36). However, seasonal coronaviruses are not unique to the African continent, and the lower specificity of SARS-CoV-2 serological assays with African samples may have been due to other factors as well.

Findings from previous studies using samples from Benin, Nigeria, and Ghana have led to speculation that malaria may contribute to cross-binding antibodies or other humoral factors (19, 20). An additional study in the malaria high-endemic country of Gabon found that 32 of 135 (23.7%) samples from 2014 were positive for SARS-CoV-2 antibodies using a NCP antigen

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serological assay, although authors acknowledge that the cause of the cross-reactivity cannot be isolated with certainty (37). Our current study found anti-*Plasmodium* IgG levels to be significantly higher in samples with false positive SARS-CoV-2 results compared to those with negative results. This was found for 5 of 9 *Plasmodium* antigen targets in our malaria panel, and encompassed three malaria parasite species: *P. falciparum*, *P. malariae*, and *P. ovale*. This significant association held true for 3 of the 5 of these targets (PfCSP, PmMSP1, PoMSP1) for both Euroimmun NCP and Abbot assays. Although levels of several NTD antibodies were higher in the samples with false-positive Abbott SARS-CoV-2 results, levels of only one NTD antibody, to syphilis/yaws, were significantly higher in samples with Euroimmun NCP false-positive results, and no NTD antibodies were higher for both tests; thus NTDs might be a less likely contributor to SARS-CoV-2 cross-reactivity than malaria. In addition, previous immunological studies support that malaria antibodies cross-reactive with other pathogens could arise from polyclonal and atypical B cell populations promoted during malaria infection (38, 39).

Our current study evaluated the strength of IgG cross-binding to SARS-CoV-2 antigens that elicit these false-positive results. Using relatively low concentrations of 2M, 4M, and 5M of the protein denaturant urea (typically 6M or 8M is used to remove loosely-bound IgG (40, 41)), most borderline or false-positive pre-pandemic samples were recategorized as negative. A more stringent 8M concentration was found to also substantially reduce binding of actual SARS-CoV-2 antibodies, leading to many false negatives. Given sensitivity–specificity trade-offs with increasing urea concentration, the 4M wash appeared to yield promising results, especially for samples taken ≥28 days post-PCR confirmation. The finding of strong IgG binding post-28 days

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283 exposure suggests that reliable results can be obtained for population serostudies for SARS-
284 CoV-2 IgG that do not enroll many individuals with recent COVID-19. These findings are
285 consistent with previous studies using avidity assays for SARS-CoV-2 serology, with clear
286 increases in IgG avidity as time from exposure increases (42, 43).
287
288 An avidity assay is not specific for malaria IgG; any weak-binding of IgG would be removed by
289 this process. Regardless of the exact mechanisms contributing to cross-reactivity on SARS-CoV-2
290 serological assays, the relatively simple urea wash step holds potential to mitigate this problem
291 of false positives on NCP-based assays. This might be especially important for samples from
292 sub-Saharan Africa or other malaria endemic areas.
293
294 A major limitation of our study is that the pre-pandemic samples had not been tested for
295 presence of antibodies to other human coronaviruses. Evidence suggesting some cross-
296 reactivity of SARS-CoV-2 serological tests with malaria was found, but it cannot be ruled out
297 that the primary cause of cross-reactivity is exposure to other human coronaviruses, which may
298 be more prevalent in sub-Saharan Africa versus other parts of the world. Another limitation of
299 our study was the modest inter-laboratory agreement for the Euroimmun test results, possibly
300 due to different laboratory equipment for this open-system assay. It is important to note that
301 the variability was primarily with low-positive, borderline, or negative samples that have lower
302 ODs and are thus more sensitive to change with minor OD variation; additionally, the
303 Euroimmun result is a ratio of two OD values and therefore has more potential for variability
304 than a raw signal. The agreement between the Abbott test results was strong, even though

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NIMR used the Abbott Architect Plus i2000sr Analyzer while NRL used the Abbott Architect i1000sr Analyzer; chemiluminescent assays are known to perform more reliably than ELISA spectrophotometers.

Our study indicated substantial cross-reactivity to two commercial, SARS-CoV-2 IgG serological assays targeting the NCP antigen using Nigerian plasma samples from 2018. Cross-reactive samples had significantly higher levels of malaria antibodies, although it is unclear whether this is directly responsible for false positive results. Use of a simple urea wash appeared to substantially reduce cross-reactivity and should be considered when testing samples from malaria-endemic regions using SARS-CoV-2 ELISA platforms.

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Conflict of interest: The authors declare no conflicts of interest.

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Tables and Figures

Table 1. Characteristics of individuals and samples from the 2018 Nigeria HIV/AIDS Indicator and Impact Survey (n=213)

Age (years)		
Median [IQR]	14	[10,23]
<5	5	(2.4%)
5 - 9	42	(19.7%)
10-14	60	(28.2%)
15 - 19	30	(14.1%)
20 - 24	24	(11.3%)
25 - 29	14	(6.6%)
30-34	11	(5.2%)
35-39	10	(4.7%)
40-44	15	(7.0%)
45 - 60	2	(0.9%)
Sex		
Female	127	(60.1%)
Malaria		
Positive*	107	(50.2%)

*Based on HRP2 antigen positivity from a bead-based immunoassay

IQR = interquartile range

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Table 2. Results of two SARS-CoV-2 serological assays on selected samples from the 2018 Nigeria HIV/AIDS Indicator and Impact Survey

	Abbott	Euroimmun
	n=212*	n=213
Invalid**	2 (0.9%)	N/A
Borderline***	N/A	7 (3.3%)
Negative	197 (92.9%)	168 (78.9%)
Positive	13 (6.1%)	38 (17.8%)

*One sample had insufficient volume to be tested with the Abbott assay

**Two samples had invalid results after two runs with the Abbott assay

***Twenty samples were borderline initially on Euroimmun, and seven remained borderline after a repeat test.

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Table 3. Combinations of Abbott and Euroimmun SARS-CoV-2 assay results on selected samples from the 2018 Nigeria HIV/AIDS Indicator and Impact Survey

Abbott	Euroimmun	N	(%)
-	-	165	(77.8%)
-	+	25	(11.8%)
+	+	11	(5.2%)
-	Borderline*	7	(3.3%)
+	-	2	(0.9%)
Invalid**	+	1	(0.5%)
Invalid**	-	1	(0.5%)
Total		212	(100%)

* Borderline assay result defined as a second borderline response after a first borderline value according to Euroimmun.

** Invalid assay result defined by Abbott analyzer.

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515 Table 4. Sensitivity and specificity estimates when including urea wash for Euroimmun assay

	Sensitivity, % (95% CI)			Specificity, % (95% CI)
	All SARS-CoV-2+ (n=32)	≥14d SARS-CoV-2+ (n=22)	≥28d SARS-CoV-2+ (n=12)	(n=207)**
No wash	96.9 (89.1, 100*)	95.5 (72.2, 99.9)	100.0 (73.5, 100*)	84.1 (78.4, 88.9)
2M urea	78.1 (60.0, 90.7)	81.8 (59.7, 94.8)	100.0 (73.5, 100*)	94.4 (90.3, 97.2)
4M urea	62.5 (43.7, 78.9)	72.7 (49.8, 89.3)	100.0 (73.5, 100*)	98.5 (95.7, 99.7)
5M urea	53.1 (34.7, 70.9)	63.6 (40.7, 82.8)	83.3 (51.6, 97.9)	99.5 (97.3, 100.0)
8M urea	12.5 (3.5, 29.0)	18.2 (5.2, 40.3)	33.3 (9.9, 65.1)	100.0 (98.2, 100*)

Note: Sensitivity calculated from SARS-CoV-2 PCR+ panel and specificity calculated from pre-pandemic samples.

* One-sided, 97.5% confidence interval.

** Those false positive and borderline samples not able to run with all urea wash concentrations were subtracted from numerator and denominator; samples with persistent borderline results were excluded from analysis.

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Figure 1. Levels of anti-*Plamsodium* IgG antibodies by SARS-CoV-2 antibody test result for Euroimmun (n=168 for negative, n=38 for positive) and Abbott (n=197 negative, n=13 positive) for pre-pandemic samples (2018 Nigeria HIV/AIDS Indicator and Impact Survey). Plots display five anti-malaria IgG antibodies significantly associated with SARS-CoV-2 IgG positivity (A) and four not significantly associated (B). Boxes shows interquartile range (IQR), lines displaying median, and whiskers extending 1.5x above and below IQR. Markers display values outside if 1.5x IQR. NCP: nucleocapsid protein; MFI: median fluorescent intensity.

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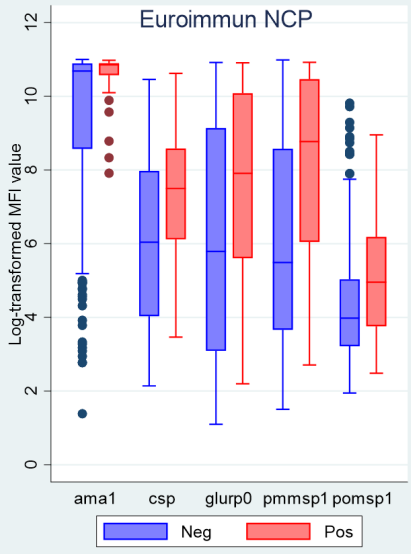
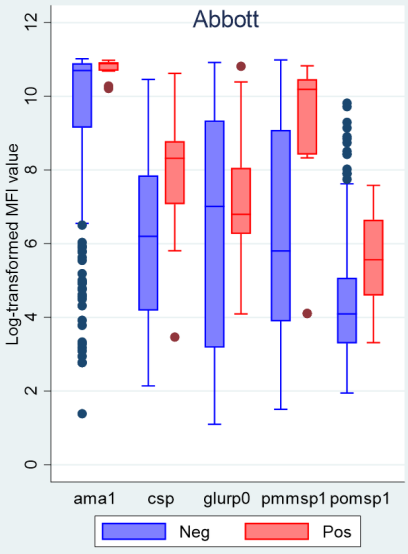
(A)





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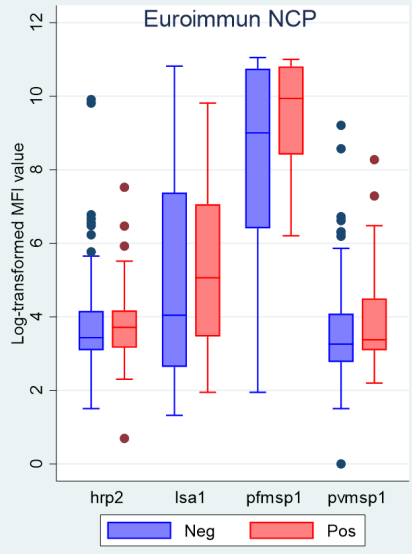

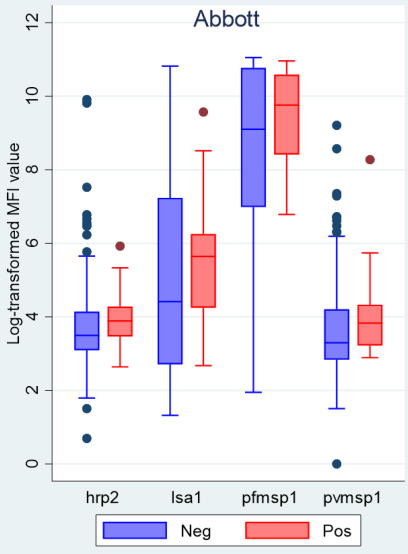

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Figure 2: Test results with or without urea-based avidity assays at 2M, 4M, 5M, and 8M with the Euroimmun NCP assay for 40 pre-pandemic samples (2018 Nigeria HIV/AIDS Indicator and Impact Survey). The panel includes samples with positive (n=32), borderline (n= 5), and negative (n=3) calls. (A) (A) Boxplots display OD to calibrator ratios for all samples at each wash. (B) Avidity index for all samples at different molarities of urea wash. Grey hash line displays an avidity index of 100% which would represent no loss of IgG signal. For (A) and (B), boxes show interquartile range (IQR), lines displaying median, X symbol showing mean, and whiskers extending 1.5x above and below IQR. Markers display values outside if 1.5x IQR.

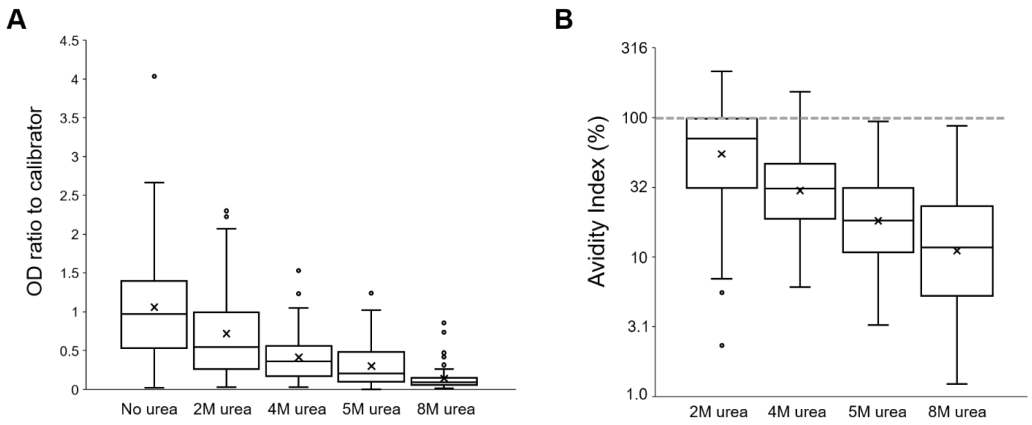

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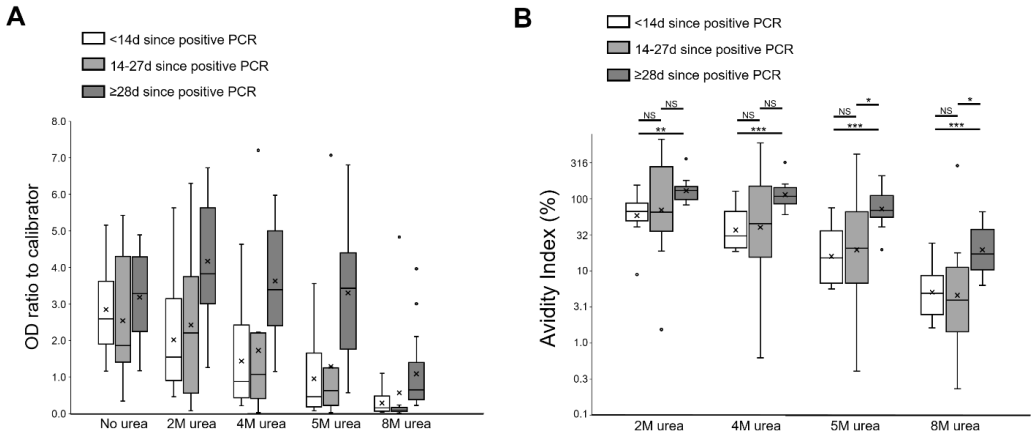
Figure 3. Test results with or without urea-based avidity assays at 2M, 4M, 5M, and 8M with the Euroimmun NCP assay for 32 samples from persons with previous SARS-CoV-2 positive PCR. Each plot has three categories indicating persons testing SARS-CoV-2 PCR positive less than 14 days prior to sample collection, between 14 and 27 days prior, and 28 days or greater. (A) Boxplots display OD to calibrator ratios for all samples at each wash. (B) Avidity index for all samples at each molarity of urea wash with statistically-significant differences indicated: NS, not significant; *p<0.05; **p<0.01; ***p<0.001. For (A) and (B), boxes show interquartile range (IQR), lines displaying median, X symbol showing mean, and whiskers extending 1.5x above and below IQR. Markers display values outside if 1.5x IQR.



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Supplementary Materials

Antibody and malaria antigen multiplex testing

For the pre-pandemic samples from 2018 NAIIS, the multiplex bead assay (MBA) for IgG against a panel of infectious and vaccine-preventable diseases was performed on the MAGPIX platform as described previously (1-3) with a serum dilution of approximately 1:400. The multiplex malaria antigen detection assay was also performed on the MAGPIX platform as described previously (4, 5) at a whole blood dilution of 1:40. All assays were performed at NRL. Malaria HRP2 antigen and antibody positivity for various *Plasmodium falciparum* antibodies (pfmsp1, pfama1, and glurp) was determined by using finite mixture models and defining a seropositive threshold for each as the mean plus two standard deviations from the distribution of the assumed seronegative population.

The Euroimmun NCP assay protocol consisted of sample plating, then incubation for 60 minutes at 37 °C. Two positive control wells, two negative control wells, and two calibrator control wells were included on each plate. A first wash step was done, followed by the addition of the enzyme horseradish peroxidase (HRP)-conjugated anti-human IgG, and then a second incubation for 30 minutes at room temperature (18-25°C). Wells were washed a second time, and a chromogen substrate solution was added. Following a third incubation at room temperature for 30 minutes, the reaction was stopped. After shaking the micro plate, the resultant absorbance was read on a microplate reader at 450 nanometer (nm) with reference at 650 nm.

Assay results are expressed as a ratio, calculated by dividing the ELISA optical densities (OD) of the sample by those of an internal calibrator provided with the test kit. A ratio <0.8 is considered negative, ≥0.8 to <1.1 borderline, and ≥1.1 positive. Borderline tests were repeated a second time and the second result taken as final.

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The Abbott Architect CMIA assay results are expressed as the specimen result in relative light units from the chemiluminescent reaction divided by the average of three internal calibrator replicates; if the resulting ratio is <1.40, the specimen is considered negative, and if ≥1.40, positive. Any results deemed invalid by the analyzer were repeated a second time and the second result taken as final.

To determine the binding strength for IgG in cross-reactive samples, an avidity assay was conducted by introducing a urea wash step of various concentrations (2M and 8M, initially, then 4M and 5M) between sample incubation and detection antibody incubation on samples that were either borderline or positive, plus additional negative samples using the Euroimmun assay protocol. To determine the effect of the urea wash on true positive samples, it was also run on plasma collected from patients testing positive for SARS-CoV-2 at various time points post PCR confirmation. The urea wash step could not be used in the closed-system platform Abbott analyzer. By incubating with a denaturing agent, the urea wash would remove loosely-bound antibodies to the SARS-CoV-2 antigen target. The avidity assay for cross-reactive samples was performed by plating samples on a microplate and incubating for 60 minutes at 37 °C, then washing them. Diluted urea in phosphate buffered saline (PBS, 100μL) was added to all sample wells except control after the first Euroimmun wash. The plate was incubated for 10 minutes and washed prior to conducting the steps outlined above. This procedure was initially done twice, once with 2M urea and once with 8M urea concentrations, and then conducted with additional aliquots from the same samples with 4M and 5M urea wash at the NRL. An avidity index was calculated for each sample by the formula: (OD ratio to calibrator for urea exposed)/(OD ratio to calibrator for non-urea exposed) x 100%.

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Supplementary Tables and Figures

Supplementary Table 1. Agreement between Euroimmun SARS-CoV-2 serological tests conducted at NIMR and at NRL for pre-pandemic samples (2018 NAIIS)

		NRL Euroimmun NCP results			
		Borderline	Negative	Positive	Total
NIMR Euroimmun NCP results	Borderline	2	2	3	7
	Negative	7	154	6	167
	Positive	4	7	27	38
	Total	13	163	36	212

Note: kappa = 0.6220 for all results; kappa = 0.7655 if borderline results excluded.

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Supplementary Table 2. Agreement between Abbott SARS-CoV-2 serological tests conducted at NIMR and at NRL for pre-pandemic samples (2018 NAIIS)

		NRL Abbott results		
		Negative	Positive	Total
NIMR Abbott results	Negative	196	3	199
	Positive	0	10	10
	Total	196	13	209

Note: kappa = 0.8621.

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Supplementary Table 3. Relationship between antibody log values to a panel of other infectious diseases and false positivity on the Euroimmun and Abbott tests

Disease	Pathogen	Antigen	p-value from Wilcoxon rank sum test for Euroimmun	Notes (when p-value significant)	p-value from Wilcoxon rank sum test for Abbott	Notes (when p-value significant)
Malaria (minimum panel of species-specific targets)	*Plasmodium falciparum*	Pf MSP1-19	0.0349		0.4082	
		Hrp2	0.169		0.3400	
		Glurp	0.0119	Ab values higher in positives	0.3423	
		Csp	0.0037	Ab values higher in positives	0.0056	
		Ama1	0.0112	Ab values higher in positives	0.0302	
		Isa	0.1595		0.1479	
	Plasmodium malariae	Pm MSP1-19	0.0002	Ab values higher in positives	0.0026	Ab values higher in positives
	Plasmodium ovale	Po MSP1-19	0.0059	Ab values higher in positives	0.0042	Ab values higher in positives
	Plasmodium vivax	Pv MSP1-19	0.0766		0.1333	
Lymphatic filariasis	*Wuchereria bancrofti*	Wb123	0.1228		0.0116	Ab values higher in positives
		Bm14	0.4666		0.0337	
		Bm33	0.2483		0.0327	
Onchocerciasis	*Onchocerca volvulus*	OV-16	0.1235		0.0041	Ab values higher in positives
		OV-33	0.0529		0.0147	Ab values higher in positives
Schistosomiasis	*Schistosoma spp.*	SEA	0.476		0.3681	
Strongyloidiasis	*Strongyloides stercoralis*	NIE	0.0443		0.7326	

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Trachoma	*Chlamydia trachoma*	Pgp3	0.0467		0.0296	
Syphilis/yaws	*Treponema pallidum*	r-p17	0.0815		0.1291	
		TmpA	0.0012	Ab values higher in positives	0.1602	
Cysticercosis	*Taenia solium*	T24H	0.5689		0.0106	Ab values higher in positives
Taeniasis	*Taenia solium*	rES33	0.1379		0.0189	Ab values higher in positives
Measles	Measles virus	Whole virus	0.3724		0.596	
Rubella	Rubella virus	Whole virus	0.7898		0.6838	
Diphtheria	*Corynebacterium diphtheria*	Diphtheria toxoid	0.4613		0.4082	
Tetanus	*Clostridium tetani*	Tetanus toxoid	0.5259		0.5155	
Campylobacteriosis (*C. jenuni)*	*Campylobacter jejuni*	campy 18	0.8957		0.8746	
		campy 39	0.7689		0.7937	
Cholera	*Vibrio cholerae*	Cholera	0.0007	Ab values higher in negatives	0.1076	
ETEC infection	*Enterotoxigenic Escherichia coli I (ETEC)*	labile toxin β subunit	0.0013	Ab values higher in negatives	0.9831	
Cryptosporidiosis	*Cryptosporidium parvum*	Cp17	0.0727		0.0637	
		Cp23	0.2166		0.1139	
Toxoplasmosis	*Toxoplasma gondii*	SAG2	0.0863		0.1917	
Giardiasis	*Giardia lamblia*	VSP3	0.2246		0.6646	
Salmonellosis	*Salmonella enterica serotype typhimurium*	SalB	0.1824		0.8987	
	Salmonella enterica serotype enteritidis	SalD	0.3756		0.2944	

Note: grey shading indicates positive statistical significance after accounting for a false discovery rate of 10%.

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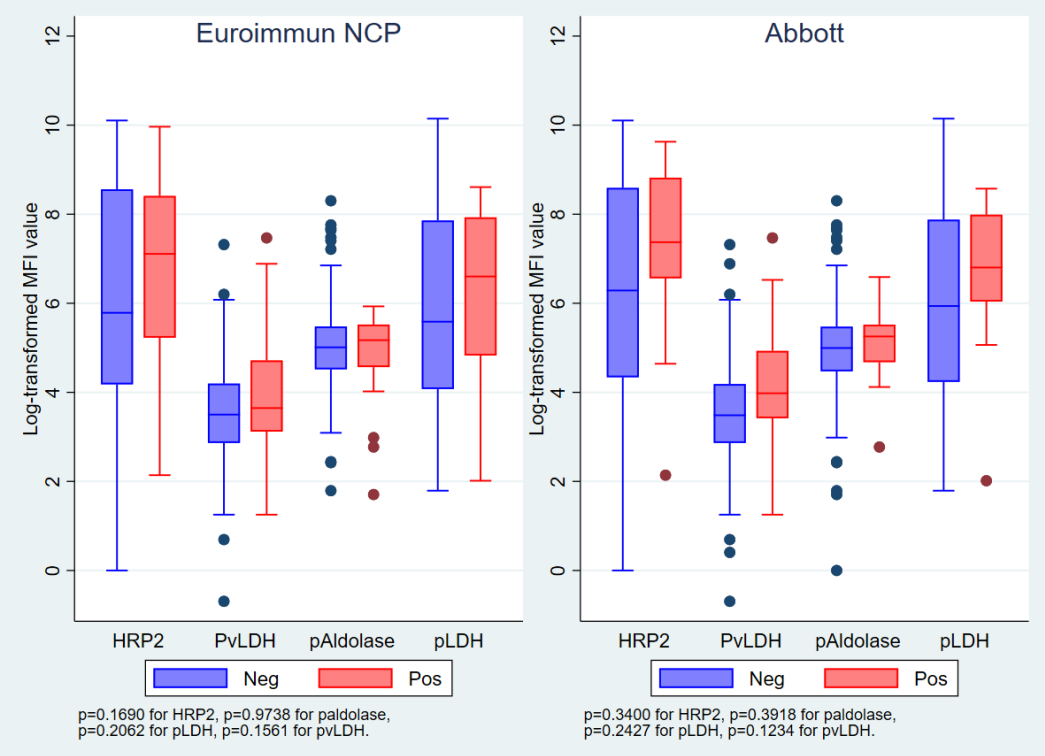
Supplementary Figure 1. Levels of *Plasmodium* antigens for pre-pandemic samples (2018 NAIIS) as determined by malaria antigen detection assay for: Euroimmun (n=168 for negative, n=38 for positive), and Abbott (n=197 negative, n=13 positive) SARS-CoV-2 antibody test result. Boxes shows interquartile range (IQR), lines displaying median, and whiskers extending 1.5x above and below IQR. Markers display values outside if 1.5x IQR.



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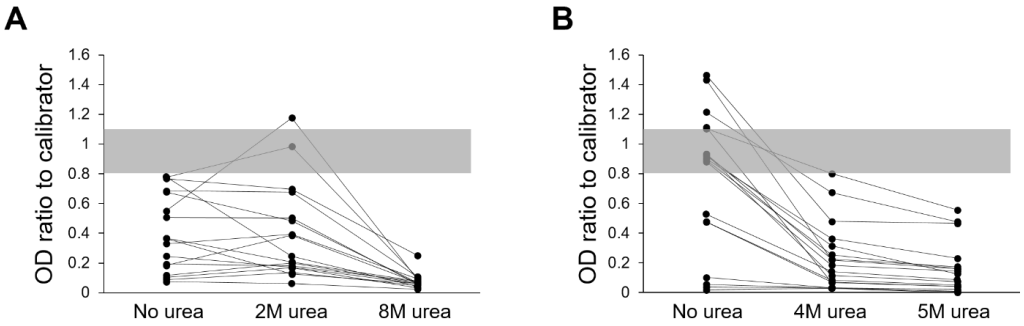
Supplemental Figure 2. Removal of weakly-bound IgG from pre-pandemic samples (2018 NAIIS) after incubation with protein denaturant urea for samples with inadequate volume to test for all four concentrations: 2, 4, 5, 8M urea. (A) The optical density (OD) to plate calibrator ratio for samples with 2M and 8M washes only (n=17). (B) The optical density (OD) to plate calibrator ratio for samples with 4M and 5M washes only (n=18). For both plots, the ratio range from 0.8 to 1.1 is highlighted grey and would elicit a 'borderline' call, with 'positive' samples above, and 'negative' samples below.



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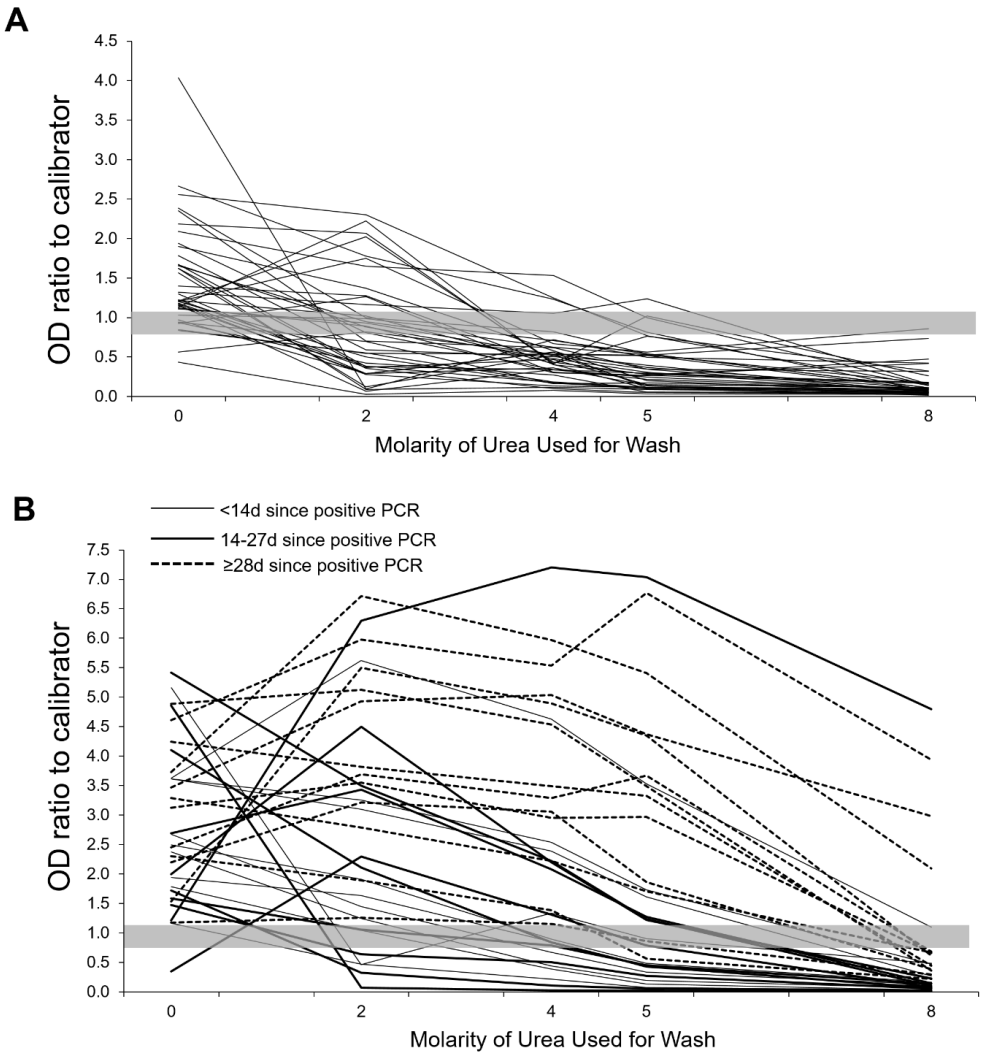
Supplemental Figure 3. The optical density (OD) to plate calibrator ratio for all individual samples with or without urea-based avidity assays at 2M, 4M, 5M, and 8M with the Euroimmun NCP assay for 40 pre-pandemic samples (2018 Nigeria HIV/AIDS Indicator and Impact Survey) (A) and for 32 samples from persons with previous SARS-CoV-2 positive PCR (B). For both (A) and (B), the optical density (OD) to plate calibrator ratio for all individual samples by molarity of urea wash. The ratio range from 0.8 to 1.1 is highlighted grey and would elicit a 'borderline' call, with 'positive' samples above, and 'negative' samples below.



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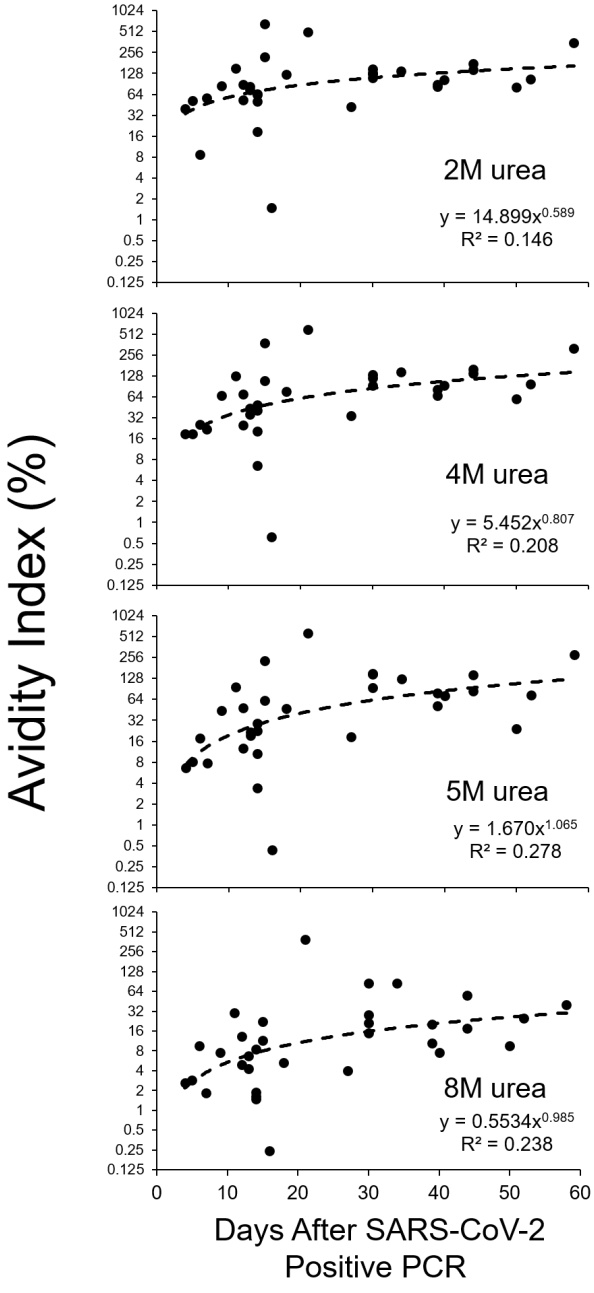
Supplemental Figure 4. Association between time since positive SARS-CoV-2 PCR and IgG avidity index. Plots show results for 2, 4, 5, and 8M urea avidity experiments. For each plot, x-axis displays when sample was collected from an individual after a positive PCR result, and y-axis displays avidity index.



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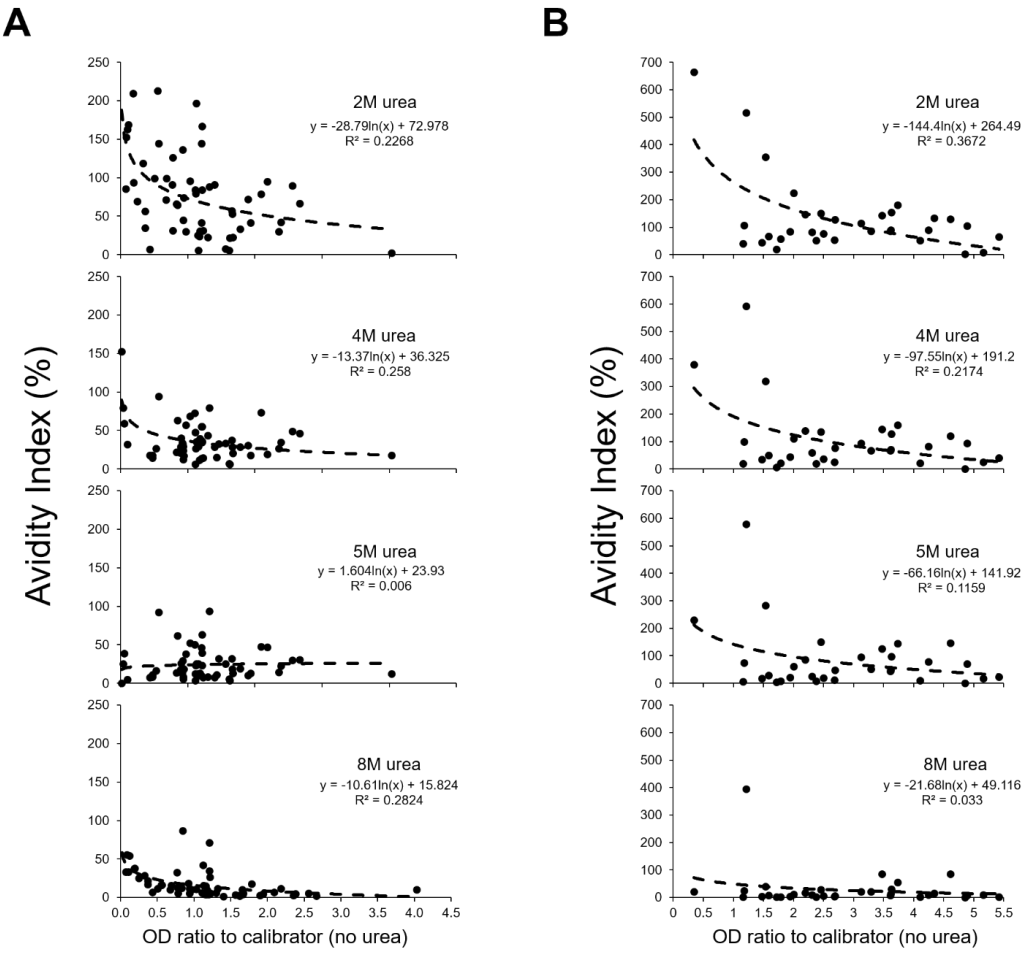
Supplemental Figure 5. Correlation between optical density (OD) ratio to calibrator and avidity index (AI) by different urea wash concentrations. (A) OD ratio versus AI for pre-pandemic samples (2018 NAIIS). (B) OD ratio versus AI for samples from with positive SARS-CoV-2 PCR. For each plot, regression line is displayed as hashed line with regression estimates.



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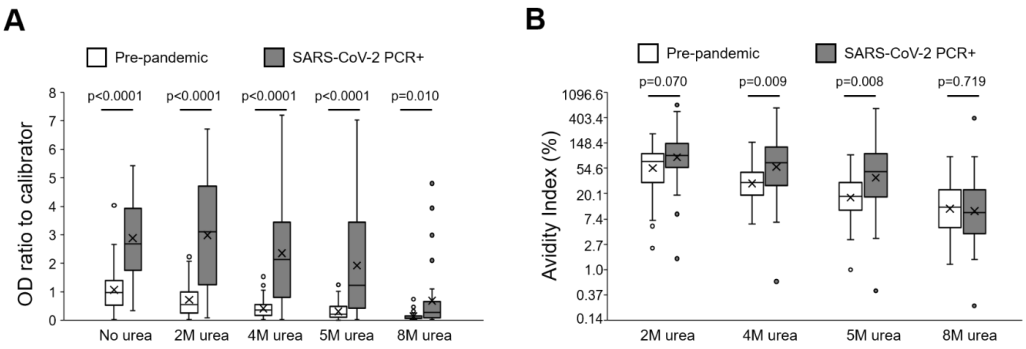
Supplemental Figure 6. Differences in absolute quantity of IgG and avidity indices between pre-pandemic and SARS-CoV-2 PCR positive sample sets.



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