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Severe Acute Respiratory Syndrome Coronavirus 2 Seroassay Performance and Optimization in a Population With High Background Reactivity in Mali

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Background. False positivity may hinder the utility of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) serological tests in sub-Saharan Africa.

Methods. From 312 Malian samples collected before 2020, we measured antibodies to the commonly tested SARS-CoV-2 antigens and 4 other betacoronaviruses by enzyme-linked immunosorbent assay (ELISA). In a subset of samples, we assessed antibodies to a panel of *Plasmodium falciparum* antigens by suspension bead array and functional antiviral activity by SARS-CoV-2 pseudovirus neutralization assay. We then evaluated the performance of an ELISA using SARS-CoV-2 spike protein and receptor-binding domain developed in the United States using Malian positive and negative control samples. To optimize test performance, we compared single- and 2-antigen approaches using existing assay cutoffs and population-specific cutoffs.

Results. Background reactivity to SARS-CoV-2 antigens was common in prepandemic Malian samples. The SARS-CoV-2 reactivity varied between communities, increased with age, and correlated negligibly/weakly with other betacoronavirus and *P falciparum* antibodies. No prepandemic samples demonstrated functional activity. Regardless of the cutoffs applied, test specificity improved using a 2-antigen approach. Test performance was optimal using a 2-antigen assay with population-specific cutoffs (sensitivity, 73.9% [95% confidence interval {CI}, 51.6–89.8]; specificity, 99.4% [95% CI, 97.7–99.9]).

Conclusions. We have addressed the problem of SARS-CoV-2 seroassay performance in Africa by using a 2-antigen assay with cutoffs defined by performance in the target population.

Keywords. Africa; COVID-19; malaria; SARS-CoV-2; serology.

Although multiple serological tools have been developed to monitor severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spread, there is no international standard, and the optimal test may vary with the target population. Assays measuring SARS-CoV-2 antibodies typically assess 1 of 3 viral antigens: spike protein, nucleocapsid protein (NCP), or the receptor binding domain (RBD), a fragment of spike protein mediating viral adhesion [1, 2]. Most assays are licensed under emergency use authorization and have not been qualified in populations where demographics and exposure histories differ significantly from the original assay validation population in high-income countries. As a result, regions most in need of reliable serological tools due to limited access to gold-standard molecular diagnostics may be disproportionately affected by uncertain assay performance. This is particularly relevant in sub-Saharan Africa, where high rates of false positivity have been described for multiple serological assays, conceivably due to cross-reactivity with other coronaviruses or other endemic infections, including malaria [3–8].

In the setting of high background reactivity and uncertain population seroprevalence, particular attention must be given to test specificity. This includes selection of the most appropriate antigen targets and consideration of combination antigen testing [9]. Understanding the pattern and degree of background reactivity in the target population must be the primary aim of assay qualification. Understanding the nature and causes of this reactivity are important secondary aims. These objectives must be considered within the limitations of locally available laboratory infrastructure and time pressure to provide reliable public health data.

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MATERIALS AND METHODS

To select an optimal serological assay for population serosurveillance in Mali, we evaluated Malian samples collected before 2020 for background immunoglobulin (Ig)G reactivity to commonly tested SARS-CoV-2 antigens and characterized background reactivity by testing for antibodies to other betacoronaviruses, a panel of *Plasmodium falciparum* antigens, and in vitro SARS-CoV-2 pseudovirus neutralizing activity. We subsequently evaluated the performance of an assay protocol implemented for population serosurveillance in the United States [10] using Malian negative control (prepandemic) and positive control (convalescent) samples and assessed the performance of different assay configurations by comparing single- and 2-antigen approaches using Malian population-specific cutoffs (Figure 1).

Enzyme-Linked Immunosorbent Assay

A semiautomated enzyme-linked immunosorbent assay (ELISA) procedure developed by National Institute of Biomedical Imaging and Bioengineering/National Institute of Health (NIH) [10, 11] was used to test for IgG antibodies to SARS-COV-2 spike protein, RBD, and NCP, and the spike proteins of SARS-CoV-1, Middle East respiratory syndrome coronavirus (MERS-CoV), OC43, and HKU1 in Malian samples at Laboratory of Malaria Immunology and Vaccinology (LMIV)/National Institute of Allergy and Infectious Diseases (NIAID).

Enzyme-linked immunosorbent assays were performed as previously described [10]. In brief, 100 µL of a single-antigen suspension in 1× phosphate-buffered saline (PBS) was added to each well of a 96-well Immulon 4 HBX ELISA plate and allowed to coat overnight at 4°C for 16 hours. Antigen suspension concentrations were as follows: SARS-CoV-2 spike protein 1 µg/mL; RBD 2 µg/mL; NCP 1 µg/mL; and other betacoronavirus spike proteins 1 µg/mL. Wells were washed 3 times with 300 µL 1× PBS + 0.05% Tween 20 by automated plate washer followed by blocking for 2 hours at room temperature with 200 µL 1× PBS + 0.05% Tween 20 + 5% nonfat skim milk. After blocking, wells were washed again 3 times. Heat-inactivated plasma samples were diluted at 1:400 in blocking buffer and 100 µL of sample was added to wells. Positive, negative, and blank controls were included on all plates. Positive controls were monoclonal antibody dilutions of the neutralizing antibody CR3022 (10 µL of 500, 250, 125, 62.5, 31.3 ng/mL, LMIV internal construct). Negative controls were pooled prepandemic plasma from Malian children. Samples were incubated for 1 hour at room temperature, then washed 3 times with 300 µL wash buffer. One hundred microliters of Goat Anti-Human IgG (H+L) Cross-Adsorbed secondary antibody, horseradish peroxidase ([HRP] Thermo Fisher) diluted at 1:4000 in blocking buffer was added to wells, incubated for 1 hour at room temperature, then washed 3 times with 300 µL wash buffer. One hundred microliters of 1-Step Ultra TMB Substrate (Thermo Fisher) was added, and the plate was incubated for 10 minutes before the addition of 1N sulfuric acid stop solution (Thermo Fisher). Absorbance was read at 450 nm and 650 nm on the Spectramax M3 microplate reader (Molecular Devices, San Jose, CA). Absorbance values (optical density [OD]) at 650 nm were subtracted from A450 to remove background signal.

Plate variability was minimized by confirming consistency in absolute absorbance values for blanks (OD <0.10), negative controls (OD <0.25), and positive controls (highest concentration OD >3.50, decreasing with dilution). Plates with control results outside of the acceptable range were repeated. Test variability was addressed by testing all samples and controls in duplicate. Discordant duplicate results (coefficient of variation >20%) were repeated until concordant. Process qualification and assay optimization was conducted before transfer to the Malaria Research and Training Center (MRTC)/Department of the Epidemiology of Parasitic Diseases (DEAP) Immunology Laboratory in Bamako, Mali. Successful assay transfer was confirmed using identical matched positive control dilutions.

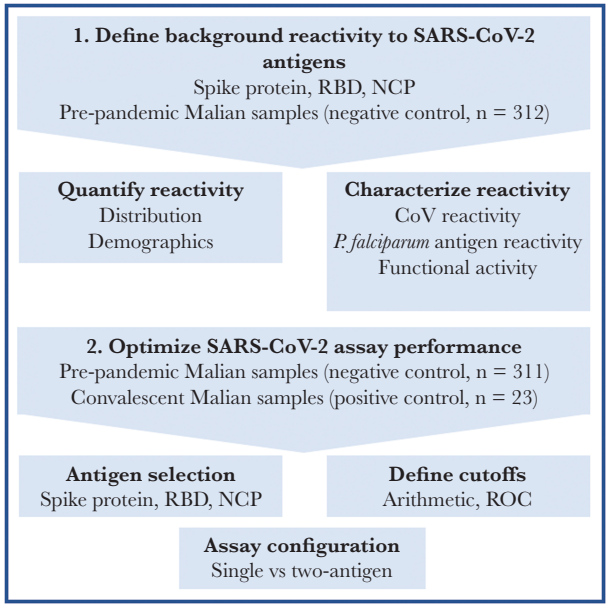

Figure 1. Study flow chart. CoV, coronavirus; NCP, nucleocapsid protein; *P falciparum*, *Plasmodium falciparum*; RBD, receptor binding domain; ROC, receiver operator characteristic; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Plasma Samples

Prepandemic negative control samples collected from Malian individuals (n = 312) were obtained from studies at 4 MRTC sites engaged in malaria research conducted in collaboration with NIAID/NIH. The sites were as follows: Sotuba, an urban population of healthy adults; Bancoumana, a rural population of healthy adults; Ouelessebougou, a rural population of women in their childbearing years; and Kalifabougou, a rural population of all ages. Rural sites experience high rates of seasonal

malaria infection (Supplementary Material: methods). All prepandemic samples were collected during NIH-sponsored studies that were approved by the Malian University of Science, Techniques, and Technologies of Bamako (USTTB) Faculty of Medicine, Pharmacy and Odontostomatology (FMPOS) human research ethics committee and the NIAID/NIH institutional review board and were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines (ClinicalTrials.gov Identifier NCT02942277, NCT03952650, NCT03989102, and NCT01322581).

Positive control samples were collected from convalescent polymerase chain reaction (PCR)-confirmed Malian individuals (n = 23) after discharge from Point G Hospital. At the time of collection, local management guidelines mandated hospital-based isolation of all confirmed cases, irrespective of severity. Individuals were contacted to return for blood sample collection and to disclose relevant clinical history. Convalescent samples were collected as part of a public health surveillance activity in collaboration with the Malian Ministry of Health coronavirus disease 2019 (COVID-19) Coordination Unit and with the approval of the USTTB FMOS-Faculté de Pharmacie (FAPH) ethics committee (No. 2020/114/CE/FMOS/FAPH).

Severe Acute Respiratory Syndrome Coronavirus 2 Antigens and Other Betacoronavirus Spike Proteins

The SARS-CoV-2 full-length spike protein (VRC-SARS-CoV-2 S-2P-3C-His8-Strep2x2), RBD protein (Ragon-SARS-CoV-2 S-RBD(319-529)-3C-His8-SBP), and the full-length spike proteins for the other betacoronaviruses SARS-CoV-1, MERS-CoV, OC43, and HKU1 were produced as previously described [11–13]. Full-length SARS-CoV-2 nucleocapsid protein was prepared at LMIV/NIAID, Bethesda (Supplementary Material: methods).

Plasmodium falciparum Antigen Suspension Bead Array

A subset of prepandemic negative control samples from Malian adults were tested for antibodies to panel of *P falciparum* antigens at LMIV/NIAID, Bethesda, by suspension bead array using the Luminex MAGPIX platform as previously described [14, 15]. The panel of 11 recombinant antigens tested represent serological markers of both historical and recent malaria exposure with quantitative results expressed as normalized median fluorescence intensity for each antigen.

Pseudovirus Neutralization Assay

To understand the nature of background SARS-CoV-2 antigen reactivity in Mali, functional activity was assessed in a subset of samples by pseudovirus neutralization assay. The SARS-CoV-2 spike glycoprotein (Sgp) neutralization was measured by flow cytometry neutralization assay using pseudotyped VSVdG-EGFP-SARS2-Sgp and BHK-21-ACE2 cells at Laboratory of Viral Diseases/NIAID, Bethesda (Supplementary Material: methods).

Data Analysis

To understand SARS-COV-2 antigen reactivity profiles in prepandemic samples, ELISA absorbance signal distributions were assessed for normality using the Anderson-Darling test, and normalization was attempted using the Tukey ladder of powers. Median assay absorbance values for each antigen were calculated. Dispersion was reported using interquartile range (IQR) and coefficient of variation, outliers were reported using the Tukey's box plot method, and shape of distribution was reported using skew and excess kurtosis. Antigens with the lowest absolute reactivity and lowest dispersion were considered most desirable for use in assay development. Differences in SARS-CoV-2 antigen and other betacoronavirus reactivity between geographical sites and age groups (<10, 10–17, ≥18 years) were assessed using the Kruskal-Wallis test. A $P < .05$ was considered significant after adjustment for multiple comparisons using the Holm-Sidak method. As a proof of concept, background reactivity to SARS-CoV-2 antigens in Malian samples was compared with background reactivity in samples from US adults using the Mann-Whitney *U* test (Supplementary Material: methods). Potential cross-recognition between SARS-CoV-2 antigens and other betacoronavirus spike proteins was examined by linear correlation. This is consistent with methodology used to assess for cross-reactivity in US negative control samples and permits comparison between these populations [11]. The SARS-CoV-2 antigen and *P falciparum* antigen reactivity was assessed by Spearman correlation. Correlations were classed as negligible $r < 0.3$, weak $0.3 < r < 0.5$, moderate $0.5 < r < 0.7$, or strong >0.7 [16].

The performance of a 2-antigen assay developed for use in the US population [10] was assessed using Malian negative (n = 311) and positive control samples (n = 23). This assay has been previously used for large-scale serosurveillance [17]. The sensitivity, specificity, and positive predictive value across a range of population seroprevalences were calculated as measures of test performance with 95% confidence intervals (CIs). To evaluate different approaches to improve assay performance, single- and 2-antigen configurations were assessed using a range of population-specific cutoffs. Population-specific cutoffs were developed using 2 methods. First, arithmetic thresholds of 2, 3, and 4 standard deviations above the mean of the negative control cohort were used. This approach was used in the original development of the assay for surveys in the United States [10]. Second, receiver operating characteristic (ROC) curves were generated from the positive and negative control cohorts for spike and RBD, and cutoffs for each antigen were selected based on optimizing the Youden Index [18, 19]. Measures of test performance were calculated for each of the different assay optimization approaches. Data were analyzed using Microsoft Excel and GraphPad Prism 9.

Data Availability Statement

Deidentified data collected for this study may be made available to others after approval of a signed data access agreement.

RESULTS

A total of 312 negative control samples collected between 2017 and 2019 were available from 4 study sites (Supplementary Table 1). Some sample volumes were insufficient to test all antigens (SARS-COV-2 spike protein n = 312; RBD n = 311; NCP n = 233; spike proteins of SARS-CoV-1, MERS-CoV, OC43, and HKU1 n = 248; and *P falciparum* antigens n = 67). A total of 23 positive control samples were available from adults in Bamako with a history of PCR-confirmed COVID-19 (Supplementary Table 2, Supplementary Figure 1). The time between diagnosis by PCR and sample collection for ELISA ranged between 27 and 270 days. Disease severity ranged from asymptomatic to critical using World Health Organization (WHO) stratification criteria [20]. Almost half of subjects (10 of 23) were asymptomatic or paucisymptomatic.

Defining Background Reactivity to Severe Acute Respiratory Syndrome Coronavirus 2 and Other Betacoronaviruses in Mali

Malian negative control (prepandemic) samples demonstrated marked reactivity to SARS-CoV-2 antigens (Figure 2). Nucleocapsid protein demonstrated the highest absolute absorbance values and greatest dispersion (median, spike protein 0.220 [IQR, 0.148–0.344], RBD 0.172 [IQR, 0.100–0.298], NCP 0.415 [IQR, 0.232–0.841]). The distribution of background reactivity for each antigen was asymmetrical and demonstrated a positive skew and kurtosis for each antigen. Transformation using Tukey's ladder of powers did not reliably normalize results. In a proof-of-concept comparison with US samples (n = 20), Malian samples demonstrated greater reactivity to all antigens and a different pattern of distribution (Supplementary Material: methods, Supplementary Figure 2). The degree of pre-existing reactivity observed was thought likely to affect the performance of serological assays. Based on the pronounced background reactivity to NCP, this antigen construct was not considered appropriate for use in Mali.

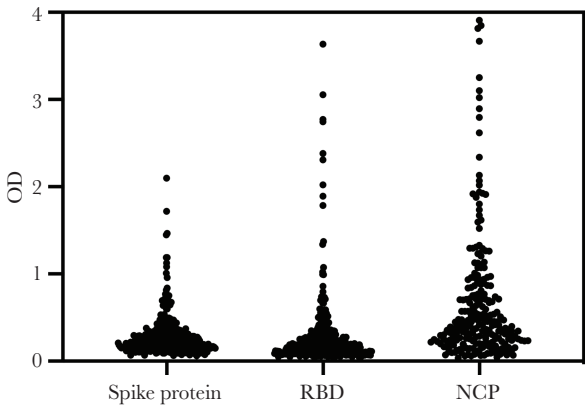

Figure 2. Severe acute respiratory syndrome coronavirus 2 antigen immunoglobulin G reactivity by enzyme-linked immunosorbent assay in coronavirus disease 2019-naive samples Malian samples: spike protein (n = 311), receptor binding domain ([RBD] n = 312), nucleocapsid protein ([NCP] n = 233). OD, optical density.

Pre-existing reactivity to SARS-CoV-2 antigens varied according to the site of collection (Kruskal-Wallis test, $P = .0006$ for spike protein and RBD, $P = .0238$ for NCP) (Figure 3). Reactivity was highest in samples from women in their reproductive years at the Ouelessebougou site. Samples from the Ouelessebougou site were collected at the same time of year as samples from the Bancoumana and Kalifabougou sites, reducing the potential impact of seasonal variation (Supplementary Table 1). In samples from Kalifabougou that included children and adults (n = 100), reactivity to all SARS-CoV-2 antigens increased with age group (Kruskal-Wallis test, $P = .0006$ for spike protein, RBD, and NCP) (Figure 4).

Similarly, reactivity to other betacoronavirus spike proteins (n = 233) differed between sites and was highest in samples from Ouelessebougou (Kruskal-Wallis test, $P < .0008$ for SARS-CoV-1, MERS-CoV, OC43, and HKU1) (Supplementary Figure 3). In samples from Kalifabougou, reactivity to the common cold coronaviruses OC43 and HKU1 increased with age group (Kruskal-Wallis test, $P < .0008$ for each). Age-related reactivity was also observed for SARS-CoV-1 and MERS-CoV but was less pronounced (Kruskal-Wallis test, SARS-CoV-1 $P = .0725$, MERS-CoV $P = .0034$).

Although the patterns of site and age-related reactivity were similar for SARS-CoV-2 antigens and other betacoronavirus spike proteins, linear correlations in assay absorbance values were modest (Figure 5A). In contrast, a strong correlation was observed between the common cold betacoronaviruses OC43 and HKU1 (Pearson r = 0.728, $P < .0001$), which share a high degree of sequence homology and may elicit serological cross-reactivity [11, 21]. The SARS-CoV-2 spike and RBD reactivity correlated minimally (Pearson r = 0.22, $P = .0006$), despite RBD being a subunit of the whole spike protein.

To determine whether SARS-CoV-2 spike protein or RBD ELISA reactivity in Malian prepandemic samples was functionally active, a subset was tested by SARS-CoV-2 pseudovirus neutralization assay (n = 89; Sotuba n = 59, Bancoumana n = 14, Ouelessebougou n = 16). No functional activity was observed at the lowest dilution for any of the Malian samples, including samples with high assay absorbance signal to RBD, the major target of the antibody neutralization response (Supplementary Figure 5A and B). In contrast, positive control US convalescent samples (9 of 10) demonstrated neutralizing activity comparable to the neutralizing potency of recombinant α-RBD monoclonal antibody and neutralizing activity ($\log_{10}$ half-maximal inhibitory concentration [IC_{50}]) were strongly correlated with spike protein and RBD OD values (spike protein: Pearson r = 0.895, $P = .0011$; RBD: Pearson r = 0.841, $P = .0045$) (Supplementary Figure 5C).

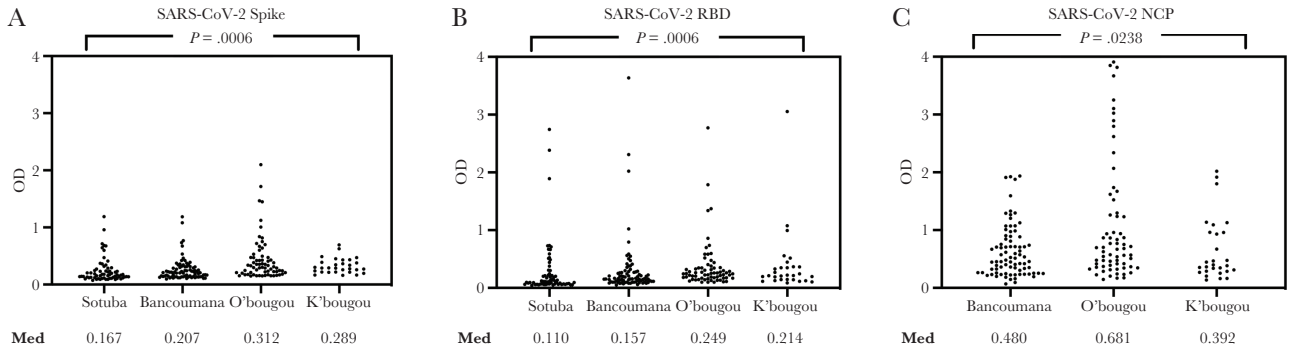

Figure 3. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigen immunoglobulin G reactivity by site in coronavirus disease 2019-naive Malian samples (≥18 years only). (A) Spike protein (B) receptor binding domain (RBD), (C) nucleocapsid protein (NCP). Groups compared using Kruskal-Wallis test. *P* values corrected adjusted multiple comparisons using the Holm-Sidak method. K'bougou, Kalifabougou site; Med, median optical density (OD) value; O'bougou, Ouelessebougou site.

Severe Acute Respiratory Syndrome Coronavirus 2 and *Plasmodium falciparum* Antigen Reactivity

A subset of prepandemic negative control samples was tested for antibodies to a panel of 11 *P falciparum* antigens by suspension bead array (n = 13 Sotuba, n = 54 Bancoumana) to assess the correlation between malaria-specific antibodies and SARS-CoV-2 background reactivity. The SARS-CoV-2 ELISA absorbance values and *P falciparum* antigen median fluorescence indices demonstrated negligible correlation (Figure 5B). No substantial relationship was observed between any of the SARS-CoV-2 antigens and *P falciparum* antigens from multiple lifecycle stages and reflective of short-lived and long-lived serological response postmalaria.

Optimizing Severe Acute Respiratory Syndrome Coronavirus 2 Assay Performance in Mali

To establish the clinical effect of high background reactivity, the performance of the US-developed 2-antigen assay was assessed using Malian negative control samples (n = 311) and positive control samples (n = 23). This assay requires both spike protein and RBD assay absorbance values above US population-derived cutoffs to classify a positive test. In Malian samples, the test sensitivity was 78.3% (95% CI, 56.3–92.5) and specificity was 97.4% (95% CI, 95.0–98.9). In the US population, the estimated sensitivity and specificity of this assay are 100% (95% CI, 92.9–100) and 100% (95% CI, 98.8–100) (Figure 6) [10]. At a hypothetical seroprevalence of 1%, the positive predictive value (PPV) of the test was 23.5% (95% CI, 13.0–38.6), at 10% the PPV was 77.2% (95% CI, 62.3–87.4), and at 30% the PPV was 92.9% (95% CI, 86.4–96.4). As a result, the existing assay approach and cutoffs were not considered suitable for use in Mali where there are limited molecular diagnostic data and an uncertain pretest probability.

To contrast the effect of single- and 2-antigen configurations on test performance, the false positivity rate in Malian negative control samples was calculated using US-derived cutoffs. The false positive rate was 2.6% (8 of 311) using a 2-antigen configuration, 6.8% (21 of 311) using a single-antigen spike, and 23.4% (73 of 312) using single-antigen RBD. In the positive control cohort, 78.3% (18 of 23) were seropositive for both spike protein and RBD. This represented a modest reduction in sensitivity compared with a single-antigen approach, where

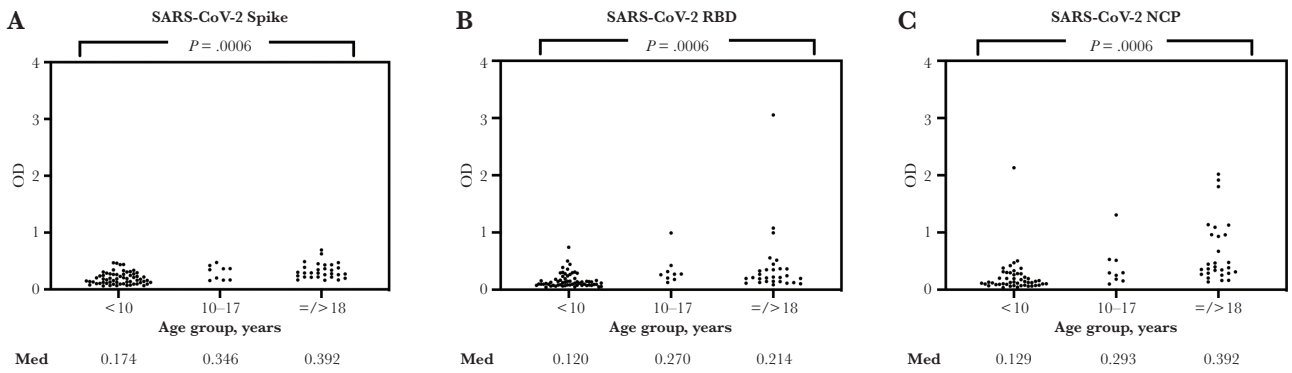

Figure 4. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigen immunoglobulin G reactivity by age group in coronavirus disease 2019-naive Malian samples (<10, 10–17, ≥18 years, Kalifabougou site). (A) Spike protein (B) receptor binding domain (RBD), (C) nucleocapsid protein (NCP). Groups compared using Kruskal-Wallis test. *P* values corrected for multiple comparisons using the Holm-Sidak method. K'bougou, Kalifabougou site; Med, median optical density (OD) value.

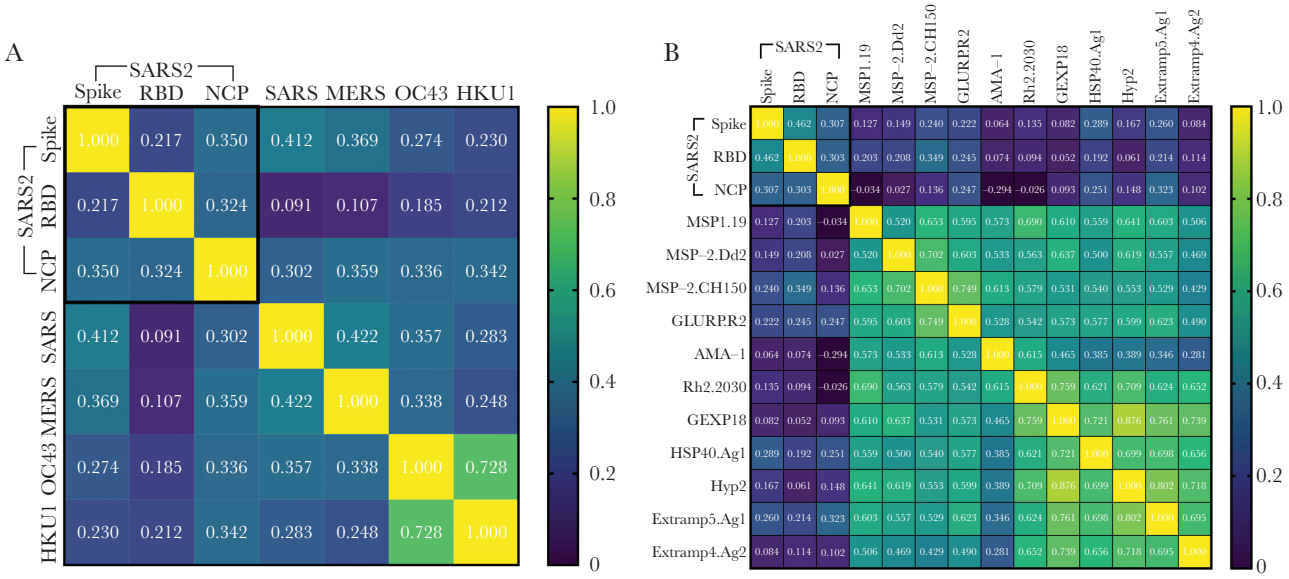

Figure 5. Correlation matrix of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) antigen immunoglobulin G reactivity in coronavirus disease 2019-naive Malian samples with (A) 4 other betacoronavirus spike proteins (Pearson r). (B) A panel of 11 *Plasmodium falciparum* antigens (Spearman r). *Plasmodium falciparum* antigen panel: MSP1.19, merozoite surface, long-lived antibody response; MSP-2.Dd2, merozoite surface; MSP-2.CH150, merozoite surface; GLURP.R2, merozoite, long-lived antibody response; AMA-1, sporozoite/merozoite, long-lived antibody response; Rh2.2030, merozoite, short-lived antibody response; GEXP18, gametocyte, short-lived antibody response; HSP40.Ag1, infected erythrocyte/gametocyte, short-lived antibody response; Hyp2, infected erythrocyte/parasitophorous vacuole membrane; Etramp5.Ag1, infected erythrocyte/parasitophorous vacuole membrane, short-lived antibody response; Etramp4.Ag2, infected erythrocyte/parasitophorous vacuole membrane. MERS, Middle East respiratory syndrome coronavirus; NCP, nucleocapsid protein; RBD, receptor binding domain; SARS, SARS-CoV-1; SARS2, SARS-CoV-2.

single-antigen spike protein and RBD sensitivities were both 82.6% (19 of 23).

To improve test performance, several population-specific cutoffs chiefly targeting improvements in specificity were assessed (Table 1). Cutoffs selected using the optimized Youden Index from ROC curves generated from the positive and negative control cohorts were not associated with this reduction in sensitivity (spike protein: area under the curve [AUC] 0.896, Youden Index 0.781; RBD: AUC 0.867, Youden Index 0.728) (Supplementary Figure 6). The ROC curve-derived cutoffs delivered a sensitivity of 73.9% (95% CI, 51.6–89.8) and specificity of 99.4% (95% CI, 97.7–99.9), representing a modest improvement in specificity compared with the US population-derived cutoffs. At a hypothetical seroprevalence of 1%, the PPV of this test was 53.7% (95% CI, 22.2–82.5), at 10% the PPV was 92.7% (95% CI, 75.9–98.1), and at 30% the PPV was 98.0% (95% CI, 92.4–99.5).

DISCUSSION

If serological surveillance is to be useful in the COVID-19 response, test selection and validation in the target population will be critical. Our findings highlight (1) the need for care in SARS-CoV-2 assay selection and interpretation and (2) the challenge of assay implementation in sub-Saharan Africa.

Background reactivity to SARS-CoV-2 antigens was common, varied geographically, and increased with age group. There was limited correlation between SARS-CoV-2 antigen reactivity and other betacoronavirus reactivity in Malian samples, similar to the pattern reported in prepandemic US samples [11]. The SARS-CoV-1 and MERS-CoV antibodies may result in SARS-CoV-2 cross-reactivity, whereas common cold coronaviruses are thought to have minimal cross-reactivity [11, 21]. Although MERS-CoV circulates in camel

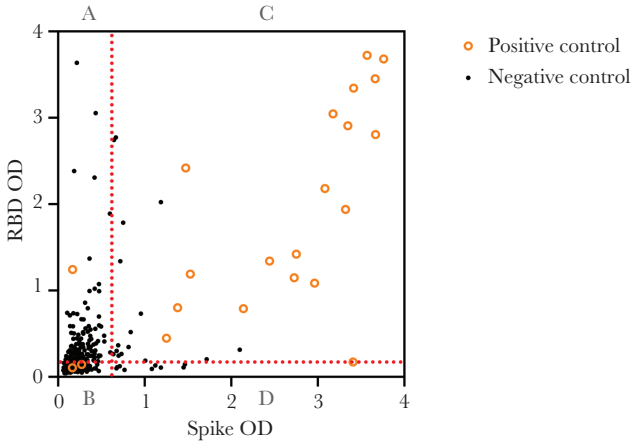

Figure 6. The performance of US cutoffs in Malian positive control (n = 23) and negative control (n = 311) samples. OD, optical density; RBD, receptor binding domain. Dotted lines represent US assay cutoffs for spike protein: 0.674 and RBD: 0.306 [10]. Quadrant A (spike negative, RBD positive): positive control: 1/23, negative control: 65/311. Quadrant B (spike negative, RBD negative): positive control: 3/23, negative control: 225/311. Quadrant C (spike positive, RBD positive): positive control: 18/23, negative control: 8/311. Quadrant D (spike positive, RBD negative): positive control: 1/23, negative control: 13/311.

Table 1. Performance Characteristics of Single- and 2-Antigen Approaches Using US and Population-Specific Cutoffs in Malian Positive Control and Negative Control Samples

Configuration	US Cutoffs	Arithmetic Malian Cutoffs (Mean + 2 SD)	Arithmetic Malian Cutoffs (Mean + 3 SD)	Arithmetic Malian Cutoffs (Mean + 4 SD)	ROC Curve Malian Cutoffs
Single antigen: spike					
Threshold (OD)	0.674	0.791	1.041	1.291	0.743
Sensitivity (95% CI)	82.6% (61.2–95.1)	82.6% (61.2–95.1)	82.6% (61.2–95.1)	78.3% (56.3–92.5)	82.6% (61.2–95.1)
Specificity (95% CI)	92.9% (89.5–95.5)	96.1% (93.4–98.0)	97.4% (95.0–98.9)	98.7% (96.7–99.7)	95.5% (92.6–97.5)
Single antigen: RBD					
Threshold (OD)	0.306	1.183	1.625	2.067	0.766
Sensitivity (95% CI)	82.6% (61.2–95.1)	60.9% (38.5–80.3)	43.5% (23.2–65.5)	39.1% (19.7–61.5)	78.3% (56.3–92.5)
Specificity (95% CI)	76.5% (71.4–81.1)	96.5% (93.8–98.2)	97.1% (94.6–98.7)	98.1% (95.9–99.3)	94.5% (91.4–96.8)
Two-antigen: Spike and RBD					
Sensitivity (95% CI)	78.3% (56.3–92.5)	56.5% (34.5–76.8)	43.5% (23.2–65.5)	39.1% (19.7–61.5)	73.9% (51.6–89.8)
Specificity (95% CI)	97.4% (95.0–98.9)	99.7% (98.2–100)	99.7% (98.2–100)	100% (98.8–100)	99.4% (97.7–99.9)

Abbreviations: CI, confidence interval; OD, optical density; RBD, receptor binding domain; ROC, receiver operating characteristic; SD, standard deviation.

populations in Northern Mali [22], absolute assay absorbance values to MERS-CoV spike protein were similar to SARS-CoV-1 and SARS-CoV-2, suggesting that there is limited MERS-CoV exposure in our population. Furthermore, there was no substantial correlation between SARS-CoV-2 background reactivity and *P falciparum* antibodies in our population. Other cumulative exposures and nonspecific binding or artefact may contribute to SARS-CoV-2 antigen background reactivity in Mali.

Nonfunctional antibodies binding complex glycans on the SARS-CoV-2 spike protein have been associated with recent malaria infection (falciparum and non-falciparum) [7], and low-avidity SARS-CoV-2 NCP background reactivity has also been associated with some *Plasmodium* antibodies (and antibodies to other neglected tropical diseases) [6]. This highlights the importance of test validation in malaria-endemic populations and suggests there is not a single straightforward mechanism for cumulative background reactivity in these populations.

Due to high background reactivity in Malian samples, single-antigen tests may not offer sufficient specificity for use in serosurveillance unless very high cutoffs are applied. Although a threshold of several standard deviations above the mean of the negative control cohort is commonly used to establish preliminary cutoffs in single-antigen assays, these values are prohibitively high in Mali. In contrast, a 2-antigen assay configuration may offer a useful approach. In Mali, SARS-CoV-2 spike protein and RBD appear to be preferred antigens compared with NCP due to the potential for correlation with neutralizing activity [23, 24], higher background reactivity absorbance values observed in NCP ELISA of COVID-19-naive Malian samples, and the lower specificity of NCP as an antigen reported in other populations [25–27]. In a 2-antigen configuration, cutoffs of 2 standard deviations above the mean of the negative control cohort, which was considered the optimal method in the US population [10], markedly reduced test sensitivity, whereas population-specific cutoffs selected using the Youden Index improved test performance.

This study has several limitations. First, the positive control cohort was small, and some cases had minimal SARS-CoV-2 antibody reactivity. Convalescent samples from patients with PCR-confirmed COVID-19 were difficult to obtain in Mali due to the limited number of cases and absence of routine follow-up blood sampling. Limited reactivity in some donors from the positive control cohort may be related to the mild nature of most cases included (17% asymptomatic, 57% mild, 9% moderate-severe, 13% critical based on WHO stratification criteria) [20] and resulted in an overlap between positive and negative control cohorts. Although use of such a positive control cohort dominated by paucisymptomatic or asymptomatic individuals adversely affects the apparent performance of the test, this population likely

reflects a community serosurveillance sample and highlights the importance of well considered cutoffs. Second, only a single construct of each SARS-CoV-2 antigen was tested in this study and better performing options may become available. To rapidly establish a test for use in Mali and provide timely data for public health use, only antigens that were available in sufficient quantity for large scale use were assessed here. Assay evaluation using new antigen constructs is underway to help further improve test specificity. Finally, the impact of seasonal malaria has not been specifically assessed in our cohort. Although no specifically cross-reactive *P falciparum* antibodies were identified, it is likely that intense seasonal malaria may contribute to the cumulative background reactivity seen in the negative control cohort. In this study, background reactivity has been characterized based on demography, cross-reactivity with other betacoronaviruses, antibodies to a panel of *P falciparum* antigens, and functional activity. Any other effects of malaria on the assay have been offset by including samples from areas with varying endemicity (Supplementary Material: methods) and correcting for background reactivity irrespective of the underlying cause. Overall, despite the limitations to this study, the pattern and degree of background reactivity in Mali has been sufficiently described to help optimize SARS-CoV-2 serological testing approaches.

CONCLUSIONS

This methodical evaluation of serological assay options and the adaptation of approaches for use in Mali has yielded an optimized test that is well validated and makes use of existing laboratory infrastructure. Although increased background reactivity to SARS-CoV-2 antigens must be acknowledged, this reactivity may be largely offset through the use of a 2-antigen assay and adaptation of assay cutoffs to suit the local population. Although assay characteristics may be further improved in the future, this thorough understanding of test performance will provide reassurance for community seroprevalence estimates in Mali [28].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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