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## **Cross-reactivity of SARS-CoV-2 Laboratory Diagnostics to Endemic Diseases in Africa: a Diagnostic Accuracy Study**

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## **Background**

Serology is a great tool to assess the level of immunity against SARS-CoV-2 in settings with limited access to molecular diagnostics. However, African populations displays a particular immunological profile with massive circulation of infectious agents from different aetiologies that can affect assays performance.

## **Methods**

We evaluated the OMEGA Diagnostics COVID-19 ELISA-IgG and the ID Screen® SARS-CoV-2-N IgG Indirect in Senegal using a panel of 636 blood samples covering several African-endemic diseases and healthy donors to determine test sensitivity and specificity. The sensitivity panel of sera includes 461 serum samples collected from 91 patients hospitalized for COVID-19 disease. COVID-19 cases were confirmed by qRT-PCR and samples were collected on an interval of three days until viral clearance. In addition, 272 sera obtained from COVID-19 negative individuals were selected from a well-documented biobank of sera collected before the COVID-19 outbreak.

## **Finding**

High-cross reactivity have been found in individuals with a history of exposure to Chikungunya, HIV, malaria (*Plasmodium falciparum*), rheumatoid factor as well as healthy donors with respective specificities of 55%, 41.8%, 70%, 70% and 75%. ELISA experiments with commercial assays targeting either SARS-CoV-2 Nucleocapsid protein and Spike 2 protein or nucleocapsid protein only suggest that cross-reactivity might be directed against Spike 2 protein and not Nucleocapsid protein. Further samples characterisation reveals that anti-malaria IgG is the leading cause of such poor specificities, but exposure to other diseases contributed as well.

## **Interpretation**

We anticipate that COVID-19 seroprevalence can be biased if assays are not contextualized. Since malaria is endemic in African settings, we propose that a particular attention must be given in serological surveillance of COVID-19 or anti-SARS-CoV-2 antibodies quantification as vaccines are being rolled out.

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## Introduction

Six weeks after the first COVID-19 confirmed cases detected in Wuhan on 31 December 2019, SARS-CoV-2 virus spread to Egypt and all over the African continent.<sup>1,2</sup> As of June 7<sup>th</sup> 2021, in Senegal which declared its first COVID-19 case on March 2<sup>nd</sup>, 41,713 cases have been confirmed with 1,148 deaths reported.<sup>3</sup> As noted in many African countries, the number of COVID-19 cases is lower in comparison to other affected areas around the world. However, given the limited access to PCR in hard-to-reach areas, the COVID-19 burden among African populations is considered underestimated.<sup>4</sup> Several studies in subgroups in Malawi, Kenya and Nigeria have reported discrepancies between number of reported COVID-19 cases and SARS-CoV-2 seroprevalence. The specificity of the SARS-CoV-2 IgG ELISA described in these studies or given by the manufacturers were respectively 97%, 99% and not described.<sup>5-7</sup> On a larger study scale in six districts in Zambia, an estimated 454,708 SARS-CoV-2 infections occurred between July 4 and July 27, 2020 compared with 4,917 laboratory confirmed cases reported in official statistics.<sup>8</sup> The study reveals that only 8.2% of people with positive IgG ELISA test (specificity of 99%) were aware of their positive status before testing. On the other hand, studies have shown high prevalence of COVID-19 in pre-pandemic samples, hypothesizing that there might have been circulation of SARS-CoV-2 earlier, or cross-reactivity with other coronaviruses.<sup>9,10</sup>

These findings show that sero-epidemiological studies are needed to better document SARS-CoV-2 transmission and the disease burden among African populations. Moreover, with Africa's largest-ever immunization drive currently being underway with COVID-19 vaccines having now been given in almost all African countries, investigating accurately African populations serostatus is more than needed in order to frame vaccine delivery and public health policies related to the pandemic.<sup>11</sup>

However, attention must be given to the deployment of COVID-19 serology diagnostics in African countries where malaria is endemic. In fact, it has been shown that the Abbott Architect and Euroimmun NCP SARS-CoV-2 IgG assays targeting the SARS-CoV-2 Nucleoprotein (NP) cross-react with anti-*Plasmodium falciparum* IgG with respective cross-reactivity of 6.1% and 17.8%.<sup>12</sup> Cross-reactivities between 1) Euroimmun ELISAs that rely on SARS-CoV-2 Nucleocapsid antigen (IgG) and S1 subunit (IgG and IgA) and 2) SCoV-2 Detect IgG ELISA from InBios that uses S1 as substrate revealed a  $\leq 25\%$  false positivity rate amongst a control group likely due to unspecific immune responses elicited by acute malaria.<sup>13</sup> More recently, it has been demonstrated that individuals from malaria endemic regions showed cross-reactivity to sialic acid on N-linked glycans of SARS-CoV-2 S protein.<sup>14</sup>

## METHODS

### Specimens collection

Following the manufacturer's protocol, a total of 636 serum samples was tested in duplicate. The sensitivity panel of sera includes 461 serum samples collected from 91 patients hospitalized in Fann hospital following a standard treatment for COVID-19 disease according to the national guidelines in Senegal. COVID-19 cases were confirmed by RT-PCR and samples were collected on an interval of three days until viral clearance confirmed with two successive PCR negative results. In addition, 272 sera obtained from COVID-19 negative individuals were

selected from a well-documented biobank of sera collected before the COVID-19 outbreak (before September 2019). This specificity panel was constituted of samples positive for malaria (PCR and IgG), dengue (PCR, IgM and IgG), yellow fever (IgM and IgG), Zika (IgM and IgG), Chikungunya (IgM and IgG), Influenza A/B (PCR), HIV (total antibodies) or rheumatoid arthritis (agglutination) conditions and samples tested negative for the same diseases and Crimean Congo haemorrhagic fever, West Nile fever encephalitis and Rift Valley fever by both qPCR and ELISA. Plaque Reduction Neutralization (PRNT) assays have been performed on samples positive for flavivirus by ELISA.

#### SARS-CoV-2 RNA extraction and characterization by RT-PCR

The initial extraction steps were carried out in a BSL-3 laboratory. Total RNA was extracted from 200 µl of nasopharyngeal swab using a QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's specifications. RNA were eluted with 60 µl nuclease-free water and immediately used for testing. An inactivation step was added: briefly after addition of the lysis buffer, the mixture was heated at 65°C during 30 min for viral inactivation.

For the SARS-CoV-2 diagnostic, four protocols were used: the Berlin protocol along with the TIB MOLBIOL kits which targeted the *E*, *N* and *RdRP* genes, the HKU protocol targeting the *N* and *RdRP* genes, and the protocol proposed by the Unité des Virus Emergents in Marseille (distributed by the European Virus Archive, EVAg) targeting the *E* gene.<sup>15,16</sup> All protocols were validated using the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen, Darmstadt, Germany) in a final volume of 20 µl including 5 µl of extracted RNA. The cycling conditions for all protocols were as follow: 50°C for 15 min and 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

According to IPD algorithm during the early stages of the COVID-19 pandemic, a sample was tested simultaneously with the TIB MOLBIOL targets (*E* and *RdRP*) and the Marseille Target (*E* gene). If necessary, the Berlin protocol (*E*, *N* and *RdRP*) or HKU protocols were used sequentially for additional confirmation of SARS-CoV-2 positive samples.

#### Influenza A/B samples characterization

RNA extraction was performed from 200 µL of each sample using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Each RNA sample was eluted with 100 µL nuclease-free water. One-step real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using the ABI 7500 platform according to the CDC protocol for the identification of influenza A (H1pdm09 and H3) and B viruses (courtesy of the US Centers for Disease Control and Prevention, Atlanta, GA). The reagents (probes, primers, enzymes) and positive control material were kindly provided by the CDC. Total reaction volumes were 20 µL, containing 1 µL AgPath-ID TM one-step quantitative RT-PCR kit (Thermo Fisher Scientific, USA), 5 µL H<sub>2</sub>O, 12.5 µL 2× buffer, 0.5 µL each primer and probe (0.8 µM forward primer [40 µM], 0.8 µM reverse primer [40 µM], and 0.2 µM probe [10 µM]), and 5 µL purified RNA. Each RNA sample was tested for the presence of RNAs derived from three genes: RNase P was targeted as a measure of the extraction procedure to assess the specimen content of human RNA and lack of reaction inhibitors, the matrix gene segment was for the identification of influenza A viruses, and the nucleoprotein gene was for the identification of influenza B viruses. A second real-time RT-PCR, using the same reagent proportions, was performed for the subtyping of influenza A viruses using

primers targeting hemagglutinin genes of H1pdm09 and H3 subtypes discrimination. Reverse transcription was performed at 50°C for 3 minutes and terminated at 95°C for 2 minutes. PCR was assessed after 45 cycles of 95°C for 15 seconds and 55°C for 30 seconds.

#### Samples characterization with the OMEGA Diagnostics COVID-19 IgG ELISA

The OMEGA Diagnostics COVID-19 IgG uses Indirect IgG ELISA (Cambridgeshire, United Kingdom) to quantitatively detect human IgG antibodies specific to SARS-CoV-2. The micro ELISA plate is pre-coated with purified SARS-CoV-2 Spike 2 protein antigen, after adding samples to wells, the specific IgG antibody present in the samples will bind with the pre-coated SARS-CoV-2 Spike protein antigen immobilized on the plate throughout an incubation of 30 min at room temperature. After plate washing to remove unbound proteins present in the sample, anti-human IgG antibodies conjugated to Horseradish Peroxidase (HRP) are added as secondary antibody and the plate incubated during 30 minutes. Following the second incubation, unbound secondary antibodies are removed by washing with the kit wash buffer, and 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate is added to each well. Only the wells that contain specific human IgG will appear blue in colour. The reaction is terminated by the addition of stop solution (H<sub>2</sub>SO<sub>4</sub>) and the colour turns yellow. The optical density (OD) is measured using a spectrophotometer (Multiskan™ FC Microplate Photometer) at the wavelength of 450 nm. A colorimetric readout indicates the degree of antibody binding.

The optical density (OD) below the calibrator indicates that the sample is negative. Samples with optical densities at least 10% higher than the calibrator are considered positive. Samples with OD between the cut-off value and the upper limit (calibrator value+10% of the calibrator value) will be considered intermediate.

#### Samples analysis with the ID Screen® SARS-CoV-2-N IgG Indirect ELISA

The ID Screen® SARS-CoV-2-N IgG Indirect ELISA (IDVet, Innovative Diagnostic, Grabels, France) is intended to detect IgG directed against the SARS-CoV-2 Nucleocapsid protein (NP).<sup>17</sup> Sera, diluted at 1/20, were analyzed in duplicates according to the recommendations of the suppliers. The optical density (OD) was measured at the wavelength of 450 nm (iMark BIORAD Microplate Reader). For each sample, the "Sample/Positive control" (S/P) ratio was expressed as a percentage (S/P%):  $S/P\% = (OD_{\text{sample}} - OD_{\text{nc}}) / (OD_{\text{pc}} - OD_{\text{nc}}) * 100$ . Samples presenting a  $S/P\% \leq 30\%$  are considered negative, between 30% and 40%: doubtful and  $\geq 40\%$ : positive.

#### *Plasmodium falciparum* samples characterization

##### **a. Antigen preparation and ELISA assay**

Crude schizont antigens from *Plasmodium falciparum* 0703 field-adapted strain were prepared from in vitro continuous culture on O+ erythrocytes in RPMI medium containing 0.5% Albumax.<sup>18</sup> Schizonts stage parasites were harvested and lysed in three volumes of sterile distilled water and stored into aliquots in liquid nitrogen.<sup>19</sup> The ELISA assay was performed as previously described.<sup>20</sup> The 0703 crude extract (iRBC) was set to 1/3000 dilution by a dose-effect assay using positive control sera. Maxisorp plates (Nunc, Roskilde, Denmark, Ref 439454) were coated with 100 µl of diluted crude extracts antigen in phosphate-buffered saline (DPBS 1X, pH 7.4). Non-infected red blood cell extracts (RBC) were also tested for non-specific binding. Plasma samples were diluted at 1/200 in DPBS-0.05% Tween-1% skimmed milk, and tested in duplicate. A pool of sera from malaria-immune African adults and a pool of sera from malaria-naive European individuals were included in each assay

as positive and negative controls, respectively. The absorbance (A) was measured at 450 nm (iMark BIORAD Microplate Reader) and results were expressed as followed:  $A_{\text{sample}} = A_{\text{iRBC}} - A_{\text{RBC}}$ . Finite mixture models, assuming two underlying distributions of “negative” (unexposed) and “positive” (exposed) individuals, were created from log-transformed MFI values to determine seropositivity cut-off. Finite mixture models were fit with the flexmix package in R version 3.5.1 (Comprehensive R Archive Network, Vienna, Austria).

**b. Detection by PCR**

DNA were extracted in the field from 140  $\mu\text{l}$  of serum sample using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) according to the manufacturer’s recommendations. Real-time multiplex transcriptase–polymerase chain reaction tests were performed on the SmartCycler device (Cepheid, Sunnyvale, California, United States), using the LightMix® Modular Plasmodium typing (TIL MOLBIOL, Berlin, Germany) kits containing pre-mixed primers and probes for the simultaneous detection and differentiation of Plasmodium species. Results were analysed according to the manufacturer’s recommendations. Runs were valid if results generated for all controls (positive and negative) were correct. Samples were considered positive if there was an amplification curve with a crossing point value within the defined cut-off (crossing point < 39), equivocal if the crossing point value was higher than the cut-off and negative if there was no amplification.

Rheumatoid factor samples characterization

Positive rheumatoid factor samples were tested with the Biosystems Rheumatoid Factor Slide test (Ref 31014). According to manufacturer’s instructions for use, 50 $\mu\text{l}$  of serum sample is deposited on predefined circular circles on the card. Two drops of agglutination reagent are later added and mixed with the serum samples, the card is allowed to mix shake at 100rpm for 2 minutes. The card is then observed under the microscope for the presence of agglutination. All positive samples should develop and agglutination and therefore have titers  $\geq 30$  IU/ml.

HIV samples characterization

Tests are performed with the HIV Ag/Ab Combo assay on the ARCHITECT SYSTEM.

The ARCHITECT HIV Ag/Ab Combo assay for use on the ARCHITECT iSystem is a two-step immunoassay to determine the presence of HIV-1 p24 antigen, antibodies to HIV-1 (group M and group O), and antibodies to HIV-2 in human serum or plasma using CMIA technology with flexible assay protocols, referred to as Chemiflex. In the first step, sample, ARCHITECT i Wash Buffer, assay diluent, and paramagnetic microparticles are combined. HIV-1 p24 antigen and HIV-1/HIV-2 antibodies present in the sample bind to the HIV-1/HIV-2 antigen and HIV-1 p24 monoclonal (mouse) antibody coated microparticles. After washing, the bound HIV-1 p24 antigen and HIV-1/HIV-2 antibodies bind to the acridinium-labelled conjugates. Following another wash cycle, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (RLU). A relationship exists between the amount of HIV antigen and antibodies in the sample and the RLU detected by the ARCHITECT iSystem optics. The presence or absence of HIV-1 p24 antigen or HIV-1/HIV-2 antibodies in the specimen is determined by comparing the chemiluminescent signal in the reaction to the cut-off signal determined from an ARCHITECT HIV Ag/Ab Combo calibration. Specimens with signal to cut-off (S/CO) values greater than or equal to 1.00 are considered reactive for HIV-1 p24 antigen or HIV-1/HIV-2 antibodies. Specimens with S/CO values less than 1.00 are considered nonreactive for HIV-1 p24 antigen and HIV-1/HIV-2 antibodies.

## Arbovirus samples characterization

### **a.** Differential arboviral nucleic acid testing by RT-PCR

Acute sera samples were tested using real-time RT-PCR. RNA was extracted from 140 µl of serum sample using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) according to the manufacturer's recommendations. Then, a one-step real-time PCR assay was performed on the ABI7500 instrument (Applied Biosystem) using the Quantitect Probe Kit (Qiagen, Heiden, Germany). The RT-PCR reaction was performed in a final volume of 25 µl containing 10 µL of extracted RNA, 10 µL of buffer (2 X QuantiTect Probe), 1.25 µL of each primer, 0.5 µL of probe, and 0.2 µL of RT-PCR Master Mix Quantitect. Thermal cycling parameters were as follows: reverse transcription for 10 minutes at 50.0°C; 15 minutes at 95.0°C for reverse transcriptase inactivation and DNA polymerase activation followed by 45 amplification cycles of 15 second at 95.0°C and 1 minute at 60°C (annealing-extension step). Details for primers and probe can be found in Bob *et al.* 2017.<sup>21</sup>

### **b.** *IgM ELISA tests*

Briefly, IgM antibodies were captured with goat anti-human IgM antibodies (KPL, USA). ZIKV, DENV, CHIKV and YFV antigens were added and specific binding was demonstrated by using a ZIKV, DENV, CHIKV and YFV mouse hyper immune ascitic fluid as primary antibodies and as secondary antibody an anti-mouse IgG horseradish peroxidase-labelled conjugate (Cappel, USA). Sera were considered as positive sample if the optical density was > 0.20 above the negative control antigen and the ratio (R) between the sample and the negative serum control was > 2.

### **c.** *IgG ELISA tests*

For IgG positive samples, and indirect ELISA was used. Briefly, specific ZIKV antigen was captured by mouse hyper immune ascitic fluids then the samples were added and specific binding was revealed by an anti-Human IgG horseradish peroxidase conjugated (KPL, USA).

Sera were considered as positive sample if the optical density was > 0.20 above the negative control antigen and the ratio (R) between the sample and the negative serum control was >2.

### **d.** Seroneutralization tests

Arboviruses ELISA IgM and IgG positives samples were analyzed for flavivirus-neutralizing antibodies using plaque reduction neutralization tests (PRNT) as described by De Madrid and Porterfield, 1969.<sup>22</sup> Briefly, twofold dilutions of sera starting at 1:10 were mixed with equal volumes of medium containing viral reference strains (and incubated for 1 hour at 37°C. Serum/virus mixtures were then used to infect Vero cells monolayers in 24 well plates. After 1 hour at 37°C, cells were covered with DMEM containing 2% FBS and 0.4% carboxymethyl cellulose and incubated during 4 days for ZIKAV, YFV and 6 days for DENV Neutralizing antibody titers were determined by using a PRNT with a 90% cut-off value and a sample was classified as positive if the titer was  $\geq 10$ .

## Statistical analysis

All statistical analyzes are performed using R.4.1.0 software (2021-05-18). A sample is considered positive when the OD value is greater than the cut-off. Different cut-offs were computed using the ROC curve method. For each graph, AUC and 95% CI are provided to assess the goodness of fit.



The performance of the tests is evaluated using two indicators: the sensitivity (agreement between the positive results) and the specificity (agreement between the negative results). For each of these indicators, 95% confidence interval is provided for statistical significance.

Pearson Chi-squared test is performed for testing the independence between two categorical variables. Pearson's correlation test is performed when the two variables are quantitative. Each test can be concluded using the p-value. The p value < 0.05 (or <0.10) was considered significant.

## Results

The two enzyme-linked immunosorbent assays for IgG antibodies were the OMEGA Diagnostics COVID-19 ELISA-IgG which uses the SARS-CoV-2 NP and Spike 2 (S2) as antigen and the ID Screen® SARS-CoV-2-N IgG Indirect assay which uses the SARS-CoV-2 NP as antigen. Following the manufacturer's protocol, a total of 636 serum samples was tested in duplicate to contextualize the assay performance in West African settings. The sensitivity panel of sera includes 461 serum samples collected from 91 patients hospitalized in Fann hospital for COVID-19 disease according to the national guidelines in Senegal. COVID-19 cases were confirmed by qRT-PCR and samples were collected on an interval of three days until viral clearance confirmed with two successive PCR negative results. In addition, 272 sera obtained from COVID-19 negative individuals were selected from a well-documented biobank of sera collected before the COVID-19 outbreak (before September 2019). This specificity panel was constituted of samples positive for malaria (PCR and IgG), dengue (PCR, IgM and IgG), yellow fever (IgM and IgG), Zika (IgM and IgG), Chikungunya (IgM and IgG), Influenza A/B (PCR), HIV (total antibodies) or rheumatoid arthritis (agglutination) conditions and samples tested negative for the same diseases and also Crimean Congo haemorrhagic fever, West Nile fever encephalitis and Rift Valley fever.

The OMEGA COVID-19 ELISA-IgG reported in its instruction for use an overall sensitivity of 88% and a specificity of 97%. In this study, the OMEGA Diagnostics COVID-19 IgG ELISA test shows globally a sensitivity of 81.1% [95% CI:77.3, 84.6] (Figure 1A). Sensitivity was then calculated per days post onset of symptoms (DPO) and the highest value was reached when sampling occurred after 21 days post onset of symptoms (DPO) with a value of 98.0% [95% CI: 89.1, 99.9] (Figure 1B). The specificity of this assay using samples negative for the different conditions as previously described was 75% [95% CI:50.9, 91.3]. The overall extent of the cross-reactivity was 66.9% [95% CI:61.0, 72.5] (Figure 1A) with high cross-reactivities with Chikungunya IgG samples (55% [95% CI:31.5, 76.9]), HIV total antibody positive samples (41.8% [95% CI:29.8-54.5]), malaria IgG samples (70% [95% CI:53.5, 83.4]), and rheumatoid factor (70% [95% CI:50.6, 85.3]) (Table 1).

Leveraging on the existing literature and given the lower specificity with past exposure to *Plasmodium falciparum* compared to active infection (70% versus 80%) (Table 1), we investigated the role of anti-*Plasmodium falciparum* IgG in the lower specificities obtained with the OMEGA Diagnostics COVID-19 IgG ELISA. The results reveal that among the 272 COVID-19 negative samples used in the specificity study, 182 were false positive for SARS-CoV-2 IgG (182/272; 66.5%) and that 106/182; 58.2% were positive for anti-*P. falciparum* IgG (supplementary Table 1). This reveals a correlation between past exposure of malaria and the SARS-CoV-2 IgG ELISA specificity ( $X^2=7.17$ , degree of freedom= 2, p-value= 0.023). The distribution of anti-*P. falciparum* IgG per tested conditions with associated specificities is represented in Table 1. Based on the level of immunity against malaria,

samples data can be categorized in three distinct groups: a first group of conditions displaying a concentration of anti-*P. falciparum* IgG  $\leq 50\%$ , a second group with a percentage comprised between ]50-100[ and a third group with a 100% percentage of anti-*P. falciparum* IgG (Figure 2).

The first group (Figure 1A) includes HIV, rheumatoid factor, Zika IgM, Influenza A/B, Dengue virus and dengue IgM with respective specificities and anti-*P. falciparum* IgG percentage of 41.8% [95% CI: 29.8, 54.5] and 32/67; 47.7%, 75% [95% CI: 50.9, 91.3] and 17/20 ;85%, 75.0% [95% CI: 19.4, 99.4] and 1/4; 25.0%, 82.2% [95% CI: 67.9, 92.0] and 20/45; 44%, 80% [95% CI: 44.4, 97.5] and 2/10; 20%, 100% [95% CI: 29.2, 100] and 0/3; 0%. In this group, a Pearson correlation test shows that high percentage of anti-*P. falciparum* IgG is associated with low specificity against SARS-CoV-2 NP/S2 antigens ( $r = -76.2$  [90% CI: -96.0, -5.0] p-value = 0.08).

The second group (Figure 1B) includes Chikungunya IgG, healthy donors, Zika IgG, yellow fever IgM and Chikungunya IgM with respective specificities and anti-*P. falciparum* IgG percentage of 55% [95% CI: 31.5, 76.9] and 18/20; 90.0%, 75.0% [95% CI: 50.9, 91.3] and 17/20 ;85%, 80.0% [95% CI: 28.4, 99.5] and 4/5; 80.0%, 80.0% [95% CI: 28.4, 99.5] and 4/5; 80.0%, 100% [95% CI: 47.8, 100] and 3/5; 60.0%. In this group, a Pearson correlation test shows that high percentage of anti-*P. falciparum* IgG is associated with low specificity against SARS-CoV-2 NP/S2 antigens ( $r = -93.6$  [90% CI: -99.0, -49.4] p-value = 0.02).

The third group (Figure 1C) contains acute malaria, yellow fever IgG and dengue IgG with respective specificities of 77.8% [95% CI: 40.0, 97.2], 80.0% [95% CI: 28.4, 99.5], 100% [95% CI: 39.8, 100]. All samples were 100% positive to anti-*P. falciparum* IgG (9/9, 5/5 and 4/4) and a Pearson correlation test reveals that there is no significant correlation between the number of anti-*P. falciparum* IgG per condition with specificity against SARS-CoV-2 NP/S2 antigens ( $r = -68.9$  [90% CI: -98.6, -66.3] p-value = 0.31). The data indicates that the presence of anti-*P. falciparum* does not necessarily lead to a specificity loss against SARS-CoV-2 antigens, suggesting that cross-reactivity to malaria does not occur in all samples with post-exposure to *P. falciparum*. We hypothesized that cross-reactivity is detectable at a certain concentration of anti-*P. falciparum* IgG or that another factor is competing for binding to the antibody to SARS-CoV-2 NP or S2.

To test whether the observed cross-reactivities were related to the S2 or NP, we tested a subset of pre-pandemic samples randomly selected per condition from the COVID-19 negative samples using the ID Screen® SARS-CoV-2-N IgG Indirect that targets SARS-CoV-2 NP and compared the results against the OMEGA COVID-19 IgG that targets both NP and S2. For the 152 samples, a global specificity of 97.4% [95% CI: 93.4, 99.3] and 79.6% [95% CI: 72.3, 85.7] and a global sensitivity of 64.7% [95% CI: 55.4, 73.2] and 75.6% [95% CI: 66.9, 83.0] ( $N=119$ ) were obtained with ID Screen® SARS-CoV-2-N IgG Indirect and OMEGA COVID-19 IgG respectively (Figure 3). Specificities per condition for both ELISAs are detailed in supplementary Table 2. These data suggest that S2 protein is more prone to cross-reactivity to anti-*P. falciparum* compared to NP.

Finally, we sought to determine the optimal threshold for the OMEGA COVID-19 IgG ELISA that would give the best balance between sensitivity ( $N=461$ ) and specificity ( $N=272$ ). The manufacturer cut-off gave a sensitivity and specificity of 81.1% [95% CI: 77.3, 84.6] and 67.5% [95% CI: 61.6, 73.0] ( $N=272$ ). By recalculating the assay performance through ROC analysis, we observed that a cut-off of 0.559 or 0.650 or 0.755 gave respective sensitivities and specificities of 80.3% [95% CI: 76.3, 83.8] and 79.8% [95% CI: 74.6, 84.4], 74.2% [95% CI: 69.9, 78.1] and 88.8% [95% CI: 84.5, 92.3], 68.3% [95% CI: 63.9, 72.6] and 94.9% [95% CI: 91.7, 97.2] (Figure

4, Table 2). All specificities were greater than 80.0% at a cut-off of 0.650 except for HIV (76.1%). Specificity maxima were reached at 0.755 with values  $\geq 90\%$  except for HIV (89.6%) (Table 2). Since anti-*Plasmodium falciparum* IgG cross-reactivity to SARS-CoV-2 IgG ELISA is easier to clean up by ROC analysis in comparison to anti-HIV antibodies cross-reactivities to SARS-CoV-2 IgG ELISA, it is plausible that HIV also plays a role in the overall loss of specificity on top of malaria.

## Discussion

We evaluated the performance of the OMEGA COVID-19 IgG ELISA using a well-characterized sensitivity panel of COVID-19 samples of PCR positive followed up patients and a deep and unique specificity panel with pre-pandemic samples for several diseases. We showed that overall sensitivity was 81.1% with a maximum peak of 98% reached beyond 21 days following onset of symptoms, aligning with the finding that a substantial proportion of COVID-19 patients requires 3-6 weeks to develop anti-SARS-CoV-2 IgG.<sup>23</sup> Interestingly, overall specificity was 66.9% driven by cross-reactivities of Chikungunya IgG, HIV, *Plasmodium falciparum* IgG, healthy donors' samples and rheumatoid factor with specificities below 80% threshold for the OMEGA COVID-19 IgG ELISA. A deeper characterization of non-malaria cross-reactive samples revealed that *Plasmodium falciparum* IgG level can be as high as 90% and that such presence is correlated with reduced specificity of the COVID-19 IgG ELISA. However, data also suggest that cross-reactivity is not solely attributable to malaria and that other factors such as HIV might play a role in competing with SARS-CoV-2 antigens. By running a comparison of COVID-19 IgG ELISA on random subset of pre-pandemic samples between NP and S2-coated plate (OMEGA Diagnostics) versus NP only (ID Screen®), we noticed that specificities were respectively 79.6% and 97.4% and cross-reactivity with malaria samples only appeared with the mixture of NP and S2 antigens. These results suggest that SARS-CoV-2 IgG ELISA targeting S2 antigens are more prone to cross-reactivity with malaria samples. We also notice that high specificity is counterbalanced with reduced sensitivity (*i.e.* 64.7% for ID Screen® versus 75.6% for OMEGA Diagnostics) but also as seen with ROC analysis to improve the specificity of the OMEGA Diagnostics assay. Here, three cut-offs were displayed with a highest specificity of 94.9% at 0.755 but with a low sensitivity of 68.3%. Globally, we noted with these analyses that the higher the specificity, the lower the sensitivity, demonstrating that COVID-19 IgG serology implementation in Africa requires a combination of knowledge about COVID-19 prevalence but also on circulation of potentially cross-reactive diseases. In other words, these results highlight the need for thorough evaluation of COVID-19 diagnostics in African population that display a different immunological profile. A combination of both ELISA Tests with a screening using OMEGA Diagnostics assay following by a validation of positive samples on ID Screen® test can be proposed to thaw the lack of OMEGA Diagnostics assay specificity. Yet, to fully understand the reasons behind observed cross-reactivities, further characterizations of all cross-reactive samples against other circulating coronaviruses and respiratory viruses should be carried out. Another limitation of the study is with the limited number of samples for certain diseases, preventing an accurate conclusion of the impact of such diseases on COVID-19 IgG serology. Also, the lack of purified recombinant S2 proteins does not allow to study the determinism of its cross-reactivity with anti-*P. falciparum* IgG and potentially other diseases such as HIV. ELISA plates coated with S1 protein should also be included to study broadly cross-reactivity.

Since seroprevalence studies an important tool to precisely assess the burden of COVID-19 in African population with limited molecular capacity, we urge stringent evaluations of serological assays in African settings should be performed by national laboratories prior to deployment, ideally working more closely with manufacturers to design, develop, and validate assays prior to deployment and launch for use in Africa. A biobank of potentially cross-reactive samples and a positive control panel where multiple positives are needed to be considered a true positive result should be made available to any country undergoing serostatus monitoring. Molecular interactions between cross-reactants should also be investigated and avidity washes with urea to remove low-affinity binding also explored by national research laboratories.<sup>12</sup>

### **Ethical Statement**

Pre-COVID-19 samples for malaria (PCR), dengue, yellow fever, Zika, Chikungunya, Influenza A/B, HIV, rheumatoid arthritis and samples tested negative for the same diseases and also Crimean Congo haemorrhagic fever, West Nile fever encephalitis and Rift Valley fever were part of national public health surveillance program of the Senegalese Ministry of Social Action and Health performed in collaboration with Institut Pasteur de Dakar. Therefore, consultation with ethics committee was not required. Pre-COVID-19 samples for malaria endemic areas were from a longitudinal cohort survey performed in Dielmo village and approved by the Senegalese National Ethics Committee for Research in Health (reference number 00000007/MSAS/CNERS/Sec 26 January 2021). Samples from COVID-19 RT-PCR positive patients were obtained from a multicentre cohort survey approved by the Senegalese National Ethics Committee for Research in Health (reference number 00000068/MSAS/CNERS/Sec, 10 April 2020).

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### **Role of the funding source**

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. CTD, ON, IVW and AAS had full access to all the data in the study. The corresponding author had final responsibility for the decision to submit for publication.

### **Author contributions**

Conceptualization and supervision: CTD, ON, IVW and AAS

Funding acquisition: JF, AAS

Investigation and methodology: ON, FD, BT, RF, BD, FT, KD, VPMC, AB, AMN

Formal analysis: CT, MD, CL, JF, OF, RWP

Data curation: CTD, ON, CT, JF, RWK and IVW

Writing-original draft: CTD, ON, AKB and IVW

All authors contributed to data interpretation, critically reviewed the first draft and approved the final version of the manuscript, and agreed to be accountable for the work. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

### Declaration of interest

JRAF was an employee of Mologic Ltd, which was the development partner of one of the ELISAs adopted in this study. The remaining authors declare no competing interest.

### Data availability

All data are available in the main text or the Supplementary Materials and are available from the corresponding authors upon reasonable request.

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## Research in context

### *Evidence before this study*

Cross-reactivity of malaria with flaviviruses and HIV serology has been extensively described in the scientific literature. Leveraging on these findings, a particular attention must be given when deploying COVID-19 serology in malaria endemic countries. We reviewed the literature available on COVID-19 serology findings up to and including July 18, 2021. For this, we searched PubMed and Google Scholar databases with no language restrictions using the search terms “COVID-19”, “SARS-CoV-2”, “serology”, “malaria”, and “cross-reactivity”.

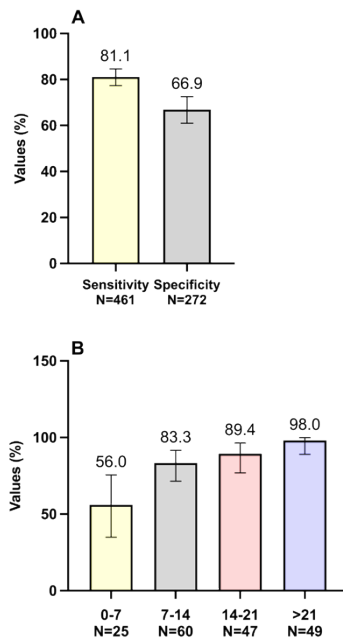
### *Added value of this study*

In this study, we identify a high degree of cross-reactivity (66.9%) to SARS-CoV-2 proteins in pre-pandemic samples in Senegal. These antibody responses were highest in individuals with IgG indicating past exposure to malaria, CHIKV, HIV and rheumatoid factor. We tested 636 serum samples to contextualize the assay performance in West African settings. Importantly, a sensitivity panel including 461 COVID PCR-confirmed convalescent patient samples, and 272 sera obtained from COVID-19 negative individuals selected from a well-documented pre-pandemic biobank representing samples positive for malaria (PCR and IgG), dengue (PCR, IgM and IgG), yellow fever (IgM and IgG), Zika (IgM and IgG), Chikungunya (IgM and IgG), Influenza A/B (PCR), HIV (total antibodies) or rheumatoid arthritis (agglutination). We believe this panel represents the most comprehensive specificity panel tested to date for SARS-CoV-2 serological tests.

### *Implications of all the available evidence*

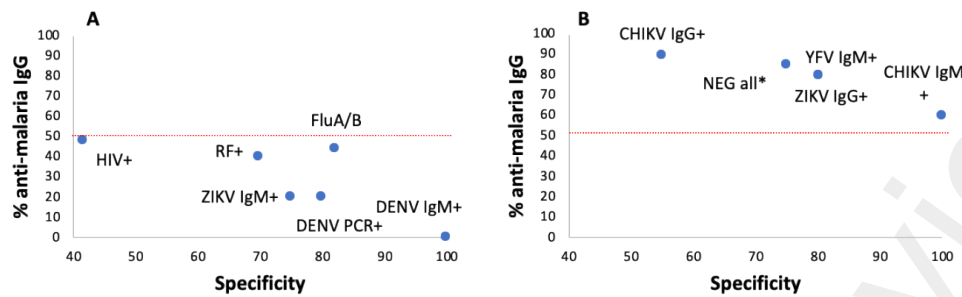
Sero-epidemiological studies are needed to better document SARS-CoV-2 transmission, disease burden and immunization among African populations. This study highlights the need for thorough evaluation of COVID-19 diagnostics in African settings that should be performed by national laboratories prior to deployment, ideally working more closely with manufacturers to design, develop, and validate assays prior to deployment and launch for use in Africa.

**FIGURE 1**



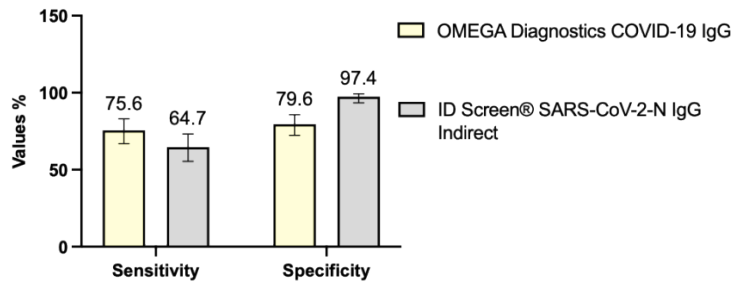
**Figure 1 : Global performance of the OMEGA Diagnostics COVID-19 IgG ELISA. A)** Sensitivity (in yellow) was 81.1% [95% CI:77.3-84.6] and was calculated on 461 samples from 91 followed up patients positive to SARS-CoV-2 by PCR. Sampling occurred each 3 days following PCR confirmation until viral clearance from nasopharyngeal swabs. Specificity (in grey) was 66.9% [95% CI:61.0-72.5] calculated on 272 COVID-19 pre-pandemic samples including Chikungunya, dengue, yellow fever, Zika, malaria, HIV, flu, rheumatoid factor and samples from healthy donors. **B)** Sensitivity was calculated per days post onset (DPO) of COVID-19 symptoms. It was 56.0% [95% CI:34.9-75.6] ( $N=25$ ) between 0-7 DPO, 83.3% [95% CI:71.5-91.7] between 7-14 DPO ( $N=60$ ), 89.4% [95% CI:76.9-96.5] ( $N=47$ ) between 14-21 DPO and 98% [95% CI:89.1-99.9] ( $N=49$ ) beyond 21 DPO.

**FIGURE 2**



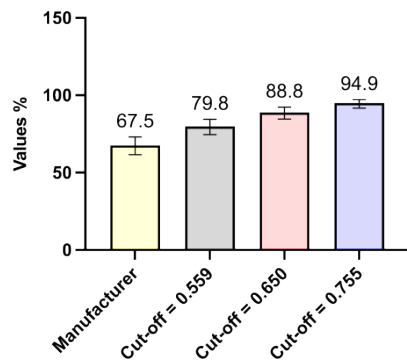
**Figure 2: Classification of samples according to specificity and percentage of malaria presence.** Correlation between the level of immunity against *P. falciparum* and specificity against the OMEGA Diagnostics COVID-19 IgG ELISA has been determined by a Pearson correlation test with a confidence interval of 90%. The red dotted line represents the 50% threshold of anti-*P. falciparum* percentage. **A.** Repartition of the anti-*P. falciparum* IgG percentage of HIV, rheumatoid factor, Zika IgM, acute dengue, FluA/B and dengue IgM according to their respective specificity against the OMEGA Diagnostics COVID-19 IgG ELISA. Each condition displays an anti-*P. falciparum* IgG percentage  $\leq 50\%$ . **B.** Repartition of anti-*P. falciparum* IgG percentage of Chikungunya IgG, Neg all\* (samples negative for the different conditions included in this study and Crimean Congo haemorrhagic fever, West Nile fever encephalitis and Rift Valley fever) according to their respective specificity against the OMEGA Diagnostics COVID-19 IgG ELISA. Each condition displays an anti-*P. falciparum* IgG percentage between [50-100].

FIGURE 3



**Figure 3 : Specificity comparison between ID Screen SARS-CoV-2-N IgG Indirect ELISA versus OMEGA Diagnostics COVID-19 IgG ELISA.** Specificities have been calculated on 152 samples including Chikungunya IgG (N=8), Chikungunya IgM (N=2), dengue IgG (N=4), dengue IgM (N=2), acute dengue (N=10), fluA/B (N=20), HIV (N=20), malaria IgG (N=40), acute malaria (N=7), negative yellow fever, Dengue, West Nile, chikungunya, zika, rift valley fever and Crimean Congo haemorrhagic fevers (N=10) by RT-PCR and ELISA IgM/IgG, rheumatoid factor (N=19), yellow fever IgG (N=4), yellow fever IgM (N=1), Zika IgG (N=4), Zika IgM (N=1). Values are given with a 95% confidence interval.

**FIGURE 4**



**Figure 4 : Specificity of the OMEGA Diagnostics COVID-19 IgG ELISA following ROC curves analysis.** Specificities are calculated according to OMEGA Diagnostics COVID-19 IgG ELISA manufacturer cut-off (in yellow) or ROC curve-derived cut-off on the challenge panel (N=272). Specificities values were 67.5% [95% CI:61.6-73.0] according to the manufacturer's analysis instructions, 79.8% [95% CI:74.6-84.4] for a cut-off of 0.559 (in grey), 88.8% [95% CI:84.5-92.3] for a cut-off of 0.650 (in pink) and 94.9% [95% CI:91.7-97.2] for a cut-off of 0.755 (in purple).

**TABLE 1**

Conditions	Specificity (95% CI)	% malaria positive samples
CHIKV IgG+	55.0 (31.5, 76.9) N=20	18/20; 90.0
CHIKV IgM+	100 (47.8, 100) N=5	3/5; 60.0
DENV IgG+	100 (39.8, 100) N=4	4/4; 100
DENV IgM+	100 (47.8, 100) N=3	0/3; 0.0
DENV PCR+	80.0 (44.4, 97.5) N=10	2/10; 20.0
Influenza A/B+	82.2 (67.9, 92.0) N=45	20/45; 44.4
HIV+	41.8 (29.8, 54.5) N=67	32/67; 47.7
Malaria IgG+	70.0 (53.5, 83.4) N=40	40/40; 100
Malaria PCR+	80.0 (44.4, 97.5) N=9	9/9; 100
NEG all*	75.0 (50.9, 91.3) N=20	17/20; 85.0
Rheumatoid factor+	70.0 (50.6, 85.3) N=30	12/30; 40.0
YFV IgG+	80.0 (28.4, 99.5) N=5	5/5; 100
YFV IgM+	80.0 (28.4, 99.5) N=5	4/5; 80.0
ZIKV IgG+	80.0 (28.4, 99.5) N=5	4/5; 80.0
ZIKV IgM+	80.0 (28.4, 99.5) N=4	1/4; 25.0

**Table 1 : Specificity of the OMEGA COVID-19 IgG ELISA against the different conditions.** NEG all\* samples are tested negative for the following diseases: Yellow fever, Dengue, West Nile, Chikungunya, Zika, Rift v-Valley fever and Crimean Congo haemorrhagic fevers in RT-PCR and ELISA IgM/IgG. Specificities have been calculated with a 95% confidence interval. Percentage of malaria positive samples represent the number of *anti-P. falciparum* per condition out of the total number per samples per condition.

**TABLE 2**

Conditions	Specificity (95% CI) (N=272)				Total
	Cut-off manufacturer	Cut-off 0.559	Cut-off 0.650	Cut-off 0.755	
CHIKV IgG+	55.0 (31.5, 76.9)	75.0 (50.9, 91.3)	90.0 (68.3, 98.8)	95.0 (75.1, 99.9)	N=20
CHIKV IgM+	100 (47.8, 100)	100 (47.8, 100)	100 (47.8, 100)	100 (47.8, 100)	N=5
DENV IgG+	100 (39.8, 100)	100 (39.8, 100)	100 (39.8, 100)	100 (39.8, 100)	N=4
DENV IgM+	100 (47.8, 100)	100 (29.2, 100)	100 (29.2, 100)	100 (29.2, 100)	N=3
DENV PCR+	80.0 (44.4, 97.5)	90.0 (55.0, 99.7)	100 (69.2, 100)	100 (69.2, 100)	N=10
Influenza A/B+	82.2 (67.9, 92.0)	93.3 (81.7, 98.6)	95.6 (84.9, 99.5)	100 (92.1, 100)	N=45
HIV+	41.8 (29.8, 54.5)	61.2 (48.5, 72.9)	76.1 (64.1, 85.7)	89.6 (79.7, 95.7)	N=67
Malaria IgG+	70.0 (53.5, 83.4)	80.0 (64.4, 90.9)	90.0 (76.3, 97.2)	92.5 (79.6, 98.4)	N=40
Malaria PCR+	80.0 (44.4, 97.5)	77.8 (40.0, 97.2)	89.9 (51.8, 99.7)	100 (66.4, 100)	N=9
NEG all*	75.0 (50.9, 91.3)	80.0 (56.3, 94.3)	95.0 (75.1, 99.9)	95.0 (75.1, 99.9)	N=20
Rheumatoid factor+	70.0 (50.6, 85.3)	83.3 (65.3, 94.4)	83.3 (65.3, 94.4)	93.3 (77.9, 92.2)	N=30
YFV IgG+	80.0 (28.4, 99.5)	80.0 (28.4, 99.5)	100 (47.8, 100)	100 (47.8, 100)	N=5
YFV IgM+	80.0 (28.4, 99.5)	100 (47.8, 100)	100 (47.8, 100)	100 (47.8, 100)	N=5
ZIKV IgG+	80.0 (28.4, 99.5)	100 (47.8, 100)	100 (47.8, 100)	100 (47.8, 100)	N=5
ZIKV IgM+	80.0 (28.4, 99.5)	75.0 (19.4, 99.4)	100 (39.8, 100)	100 (39.8, 100)	N=4

**Table 2: Evolution of specificity values per conditions following different ELISA cut-off.** NEG all\* samples are tested negative for the following diseases: Yellow fever, Dengue, West Nile, Chikungunya, Zika, Rift Valley fever and Crimean Congo haemorrhagic fevers in RT-PCR and ELISA IgM/IgG.