

SARS-CoV-2 Cross-Reactivity in Prepandemic Serum from Rural Malaria-Infected Persons, Cambodia

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Inhabitants of the Greater Mekong Subregion in Cambodia are exposed to pathogens that might influence serologic cross-reactivity with severe acute respiratory syndrome coronavirus 2. A prepandemic serosurvey of 528 malaria-infected persons demonstrated higher-than-expected positivity of nonneutralizing IgG to spike and receptor-binding domain antigens. These findings could affect interpretation of large-scale serosurveys.

Serosurveys for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the Greater Mekong Subregion (GMS) of Cambodia have been limited to those screening healthcare workers in 2 urban hospital-based settings (1,2). These antibody-based studies are necessary to determine at-risk populations and direct disease containment measures; however, before informing public health decisions, serologic assays require careful, country-specific calibration because several regions report fluctuating results or high background reactivity in different populations (3–5). This variability might be attributable to myriad serologic assays, the hypothesized cross-reactivity from common cold-type respiratory coronaviruses (6), previous *Plasmodium* infections (7,8; S. Lapidus et al., unpub. data, <https://www.medrxiv.org/content/10.1101/2021.05.10.21256855v1>), or previously uncharacterized betacoronaviruses in wildlife popu-

lations in the rural GMS (9–11). Although many serologic SARS-CoV-2 investigations are in progress, considering how pathogen diversity in the GMS might influence estimations of SARS-CoV-2 seroprevalence is prudent.

The Study

We tested serum or plasma samples collected from 528 malaria-infected persons in Cambodia during 2005–2011 (before SARS-CoV-2 emerged in 2019) for IgG reactive to SARS-CoV-2 spike and receptor-binding domain (RBD) proteins by using ELISA (12,13). We used de-identified, anonymized serum or plasma samples biobanked after malaria research studies (NCT00341003, NCT00663546, and NCT01350856, approved by the National Institute of Allergy and Infectious Diseases and the National Ethics Committee on Human Research in Cambodia) for this retrospective study.

Because 6 other coronaviruses (OC43, HKU1, 229E, NL63, severe acute respiratory syndrome coronavirus 1 [SARS-CoV-1], and Middle East respiratory syndrome coronavirus) possess structural proteins capable of infecting humans, we selected highly specific ELISAs for the SARS-CoV-2 structural proteins (12,13). Compared with other coronaviruses, SARS-CoV-2 shows varying levels of spike protein sequence homology; levels are highest for SARS-CoV-1 (76% identity, 87% similarity) and lowest for the common cold coronavirus HKU1 (29% identity, 40% similarity) (12). Reactivity to both spike and RBD antigens above cutoff values is required for a positive test with reported sensitivity of 100% (95% CI 92.9%–100%) and specificity of 100% (95% CI 98.8%–100%) (12,13). Prepandemic samples had levels above the set cutoffs for SARS-CoV-2 spike and RBD antigens (Figure 1) varying from 4.4% to 13.8% positivity to both SARS-CoV-2 spike and RBD depending on which cutoff

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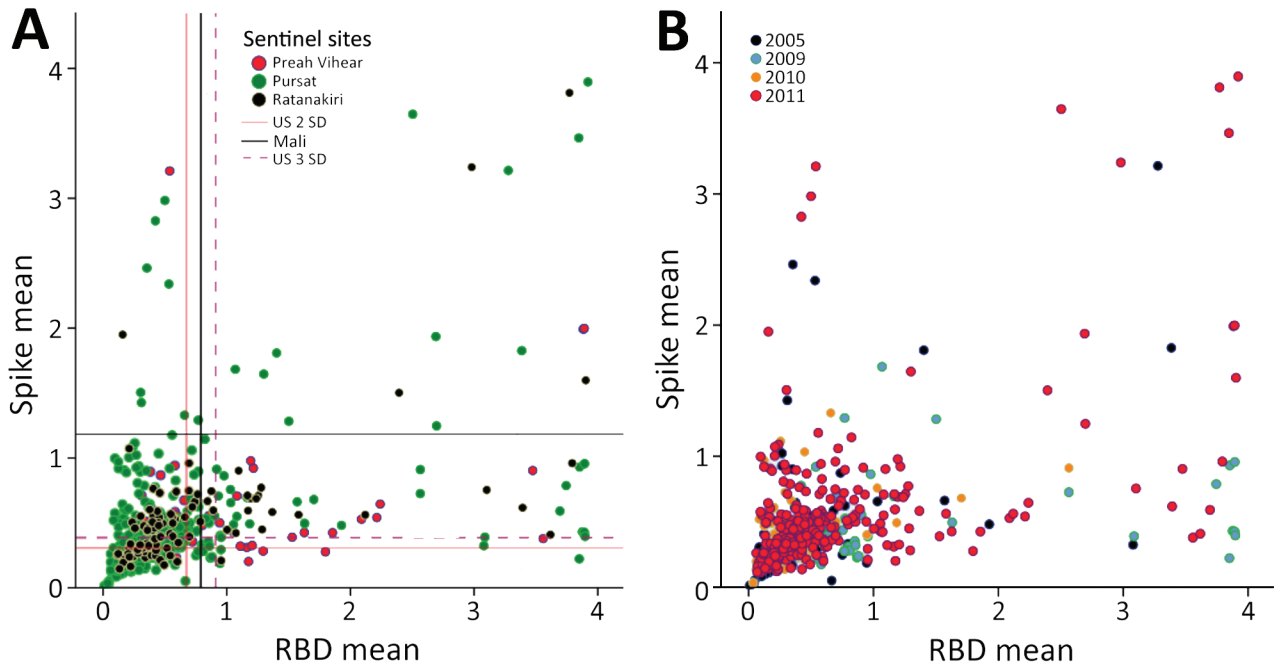


Figure 1. Mean antibody intensity in arbitrary ELISA units to spike and RBD in serum samples from prepandemic, malaria-positive rural persons in Cambodia, 2005–2011. A) Provinces indicated by color: Preah Vihear (pink), Pursat (green), Ratanakiri (black). B) Years indicated by color: 2005 (purple), 2009 (turquoise), 2010 (orange), and 2011 (pink). RBD, receptor binding domain.

values (calibrated for the Mali or US populations) were used for this assay (4,12,13) (Table; Figure 1; Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/28/2/21-1725-App1.pdf>).

To test whether the higher-than-expected positivity was an artifact of our in-house ELISA, we tested a subset of samples with a commercially validated SARS-CoV-2 Spike S1-RBD IgG ELISA Detection Kit (Genscript, <https://www.genscript.com>). Of the 24 persons who were seronegative by in-house assay and 11 who were seropositive by in-house assay, 18 tested negative and 9 tested positive by the commercial test, yielding an overall concordance of 77.1% between assays (Appendix Table 2). This inconsistency might be explained by the stringency of the in-house assay that tests both spike and RBD versus the commercial kit

that tests for RBD only; nevertheless, higher-than-expected positivity was observed in both assays. Because common cold coronaviruses do circulate in Cambodia, but no cases of SARS-CoV-1 or Middle East respiratory syndrome have been documented, we tested a subset of the cohort for IgG to HKU1 and OC43. Reactivity between subjects was comparable despite SARS-CoV-2 serostatus (Figure 2, panel A).

We further tested 289 samples to assess whether a relationship existed between antibodies to *Plasmodium* spp. and SARS-CoV-2 proteins by using 2 known malarial antigens: *Plasmodium falciparum* apical membrane antigen 1 (AMA-1), which is highly immunogenic and an indicator of parasite exposure, and *P. falciparum* Pfs25 protein (Pfs25), which is poorly immunogenic and expressed only during

Table. SARS-CoV-2 ELISA results by cutoff values in prepandemic serum samples from rural malaria-infected persons in 3 Cambodia provinces, 2005–2011*

Province	Year	Total	No. positive by 2 SDs	No. positive by 3 SDs	No. positive, Mali
Preah Vihear	2011	81	12 (15)	6 (7)	5 (6)
	2005	80	8 (10)	4 (5)	3 (4)
	2009	76	12 (16)	6 (8)	3 (0.9)
	2010	81	5 (6)	3 (4)	1 (0.3)
	2011	110	17 (15.5)	12 (11)	6 (5.4)
	Subtotal	347	42 (12)	25 (7)	13 (3.7)
Ratanakiri	2011	100	19 (19)	6 (6)	5 (5)
Total	All	528	73 (13.8)	37 (7)	23 (4.4)

*Values are no. (%) except as indicated. Using United States arbitrary ELISA unit cutoffs of 2 SDs for spike (0.674) and receptor binding domain (RBD) (0.306); United States 3 SDs for spike (0.910) and RBD (0.387); and Mali cutoff for spike (0.791) and RBD (1.183). SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

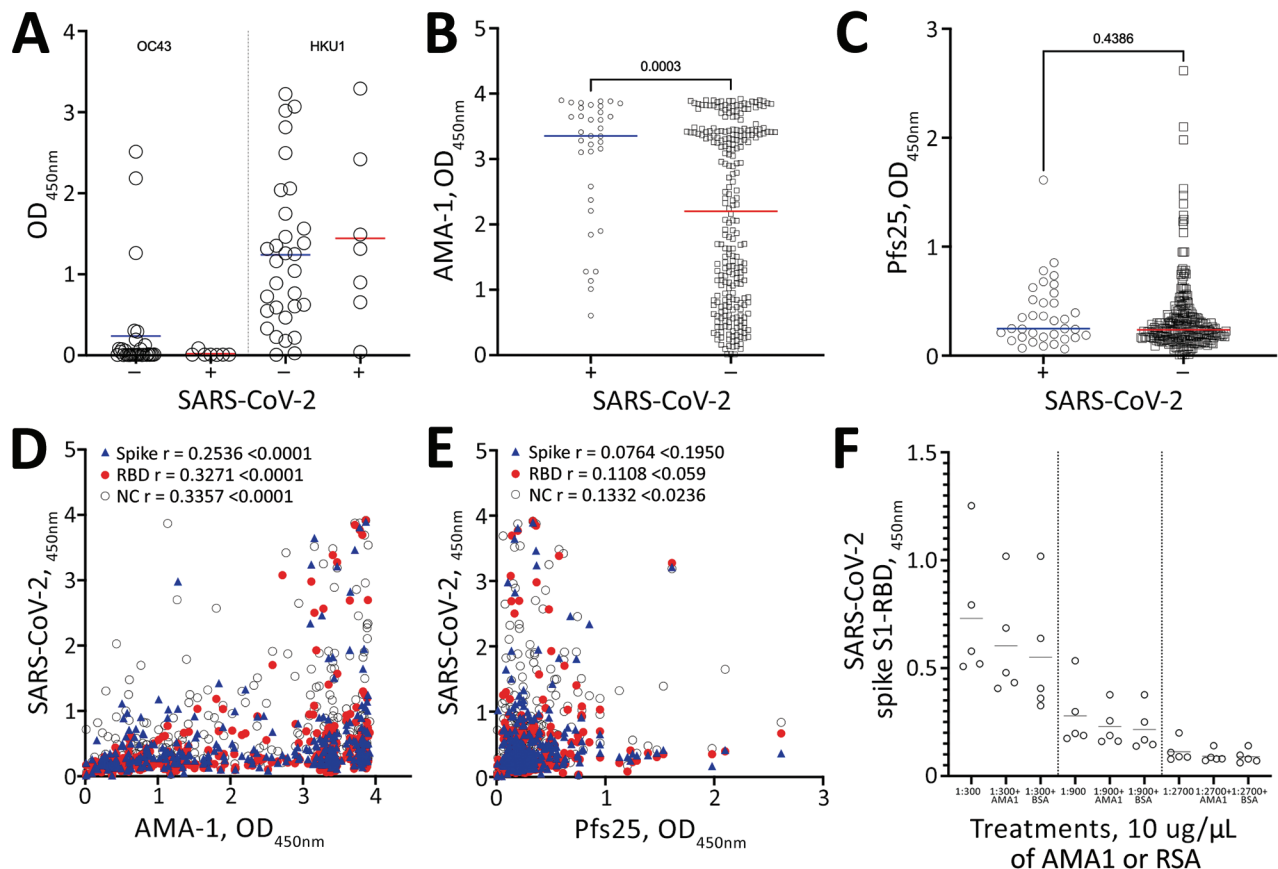


Figure 2. Mean antibody levels in pre-pandemic serum samples from malaria-positive rural persons in Cambodia, 2005–2011, to A) common cold OC43 and HKU1 viruses, B) *Plasmodium falciparum* AMA-1 and C) *P. falciparum* Pfs25 protein by SARS-CoV-2 serosurvey statuses. D–E) Correlation of mean IgG levels of AMA-1 and Pfs25 against Spike (blue triangles), RBD (red circles) and NC (open circles) IgG levels in pre-pandemic serum samples from malaria-positive rural persons in Cambodia. F) OD levels of RBD protein after preincubation of serum samples with 10mg/mL of AMA-1 or BSA. AMA-1, apical membrane antigen 1; BSA, bovine serum albumin; NC, nucleocapsid; OD, optical density; RBD, receptor binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

the mosquito stages of parasite development (4) (Figure 2, panels B–E). Of note, when we grouped samples by SARS-CoV-2 serostatus, we detected significantly higher levels of AMA-1 antibodies in SARS-CoV-2-seropositive persons than seronegative persons (mean AMA-1 antibody level 3.0 vs. 2.1; $p = 0.0003$) (Figure 2, panel B). As expected, no difference was seen in antibody levels to Pfs25 with regard to SARS-CoV-2 seropositivity (Figure 2, panel C). A weak but statistically significant positive correlation was detected between spike and RBD with AMA-1 IgG (Figure 2, panel D). This finding corroborates recent observations that higher SARS-CoV-2 seroreactivity by ELISA or rapid tests is detected in persons from malaria-endemic areas, expanding previous observations to include Southeast Asia (7,8; S. Lapidus et al., unpub. data). We also evaluated samples for seroreactivity against the nucleocapsid protein that also positively correlated with

the AMA-1 IgG. Only nucleocapsid antibodies were weakly correlated with Pfs25 antibodies, which reinforces the argument for nonspecific nucleocapsid reactivity (Figure 2, panel E). Preincubation with 10 mg/mL of AMA-1 or bovine serum albumin had no notable effect on reactivity to SARS-CoV-2 spike S1-RBD (Figure 2, panel F). Therefore, *Plasmodium* spp. exposure might contribute to SARS-CoV-2 malaria-related background reactivity. This reactivity could be attributed to immune responses to other *Plasmodium* spp. proteins, polyclonal B cell activation during infection, or interaction with the sialic acid moiety on N-linked glycans of the SARS-CoV-2 spike protein (7; S. Lapidus et al., unpub. data). Of note, SARS-CoV-2 spike proteins used in the assays were produced in HEK293 mammalian cells and likely have comparable glycosylation patterns. Elsewhere, malaria-induced cross-reactivity in pre-pandemic samples from malaria-experienced persons from

Africa was mitigated by the modification of 2 commercial assays to add a urea wash (S. Lapidus et al., unpub. data).

To elucidate the functionality of the detected antibodies, we took a subset ($n = 21$) of the samples with the highest reactivity to SARS-CoV-2 total IgG and performed neutralization assays (Appendix Figure). No neutralizing activity was identified despite high levels of antibodies reacting to both spike and RBD proteins. Identical results were obtained by using a surrogate virus neutralization test targeting RBD interaction with the host cell receptor ACE2 (Genscript) (Appendix Table 3) (14). Both SARS-CoV-2 infection and vaccination can trigger high levels of nonneutralizing antibodies, whereas neutralizing antibodies aimed primarily at the RBD seem to wane faster and remain at low titers (14). Plausibly, the cross-reactive nonfunctional antibodies to SARS-CoV-2 were raised during an infection by *Plasmodium* spp. (S. Lapidus et al., unpub. data), but we cannot discard the hypothesis that nonneutralizing SARS-CoV-2-reactive antibodies in prepandemic serum samples might be linked to the ability of betacoronaviruses to evade immune recognition because of their complex surfaces (14,15). A limitation in understanding the assays' specificity is the lack of prepandemic samples from non-malaria-endemic areas and from present-day confirmed SARS-CoV-2 convalescent samples in Cambodia.

Conclusions

We found in a widely used, highly specific, and validated ELISA that $\approx 4\%$ – 14% of prepandemic serum samples from malaria-infected persons in Cambodia were positive for nonneutralizing antibodies to SARS-CoV-2 spike and RBD antigens by using various standardized optical density cutoff values (4,12,13). We noted a relationship between increased SARS-CoV-2 seroreactivity and antimalarial humoral immunity, which was also recently shown in Africa (S. Lapidus et al., unpub. data). The plausibility of regular spillover events, or simply increased exposure to uncharacterized betacoronaviruses, as a reason for SARS-CoV-2 cross-reactivity is also increased in settings at high risk for zoonotic disease transmission because of agricultural and dietary practices such as bat guano collection and consumption of wild meats (9–11). Given that 50%–80% of GMS residents are classified as rural, careful calibration of serologic assays targeting SARS-CoV-2 will be necessary in national and subnational serosurveys. Although neutralization assays with live virus are often considered the standard because of their specificity, they are cost-prohibitive

for large-scale serosurveys. The use of competition ELISA assays such as surrogate virus neutralization tests targeting the RBD-ACE2 blockade might be an attractive option for populations at high risk for zoonotic exposures in resource-scarce settings without Biosafety Level 3 facilities.

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References

1. Song LH, Hoan NX, Bang MH, Kreamsner PG, Velavan TP. Viral and serological testing of SARS-CoV-2 among health care workers and patients in Vietnam. *Lancet Reg Health West Pac*. 2021;8:100113. <https://doi.org/10.1016/j.lanwpc.2021.100113>
2. Nopsopon T, Pongpirul K, Chotirosniramit K, Jakaew W, Kaewwjit C, Kanchana S, et al. Seroprevalence of hospital staff in a province with zero COVID-19 cases. *PLoS One*. 2021;16:e0238088. <https://doi.org/10.1371/journal.pone.0238088>
3. Uyoga S, Adetifa IMO, Karanja HK, Nyagwange J, Tuju J, Wanjiku P, et al. Seroprevalence of anti-SARS-CoV-2 IgG antibodies in Kenyan blood donors. *Science*. 2021;371:79–82. <https://doi.org/10.1126/science.abe1916>
4. Sagara I, Woodford J, Dicko A, Zeguime A, Doucoure M, Kwan J, et al. Rapidly increasing SARS-CoV-2 seroprevalence and limited clinical disease in 3 Malian communities: a prospective cohort study. *Clin Infect Dis*. 2021 Jun 29 [Epub ahead of print]. <https://doi.org/10.1093/cid/ciab589>
5. Murhekar MV, Bhatnagar T, Selvaraju S, Saravanakumar V, Thangaraj JWV, Shah N, et al.; ICMR Serosurveillance Group. SARS-CoV-2 antibody seroprevalence in India, August–September, 2020: findings from the second nationwide household serosurvey. *Lancet Glob Health*. 2021;9:e257–66. [https://doi.org/10.1016/S2214-109X\(20\)30544-1](https://doi.org/10.1016/S2214-109X(20)30544-1)
6. Ng KW, Faulkner N, Cornish GH, Rosa A, Harvey R, Hussain S, et al. Preexisting and de novo humoral immunity to SARS-CoV-2 in humans. *Science*. 2020;370:1339–43. <https://doi.org/10.1126/science.abe1107>
7. Steinhart LC, Ige F, Iriemenam NC, Greby SM, Hamada Y, Uwandu M, et al. Cross-reactivity of two SARS-CoV-2 serological assays in a setting where malaria is endemic. *J Clin Microbiol*. 2021;59:e0051421. <https://doi.org/10.1128/JCM.00514-21>

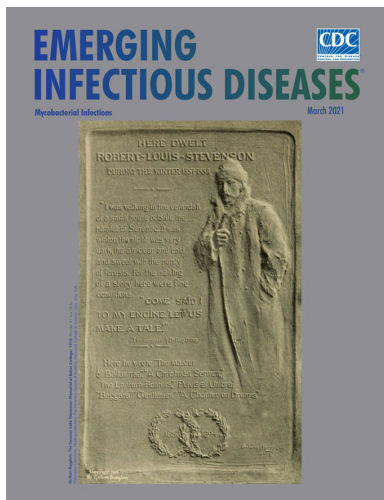
8. Vanroye F, Bossche DVD, Brosius I, Tack B, Esbroeck MV, Jacobs J. COVID-19 antibody detecting rapid diagnostic tests show high cross-reactivity when challenged with pre-pandemic malaria, schistosomiasis and Dengue samples. *Diagnostics (Basel)*. 2021;11:1163. <https://doi.org/10.3390/diagnostics11071163>
9. Delaune D, Hul V, Karlsson EA, Hassanin A, Ou TP, Baidaliuk A, et al. A novel SARS-CoV-2 related coronavirus in bats from Cambodia. *Nat Commun*. 2021;12:6563. <https://doi.org/10.1038/s41467-021-26809-4>
10. Wacharapluesadee S, Tan CW, Maneern P, Duengkae P, Zhu F, Joyjinda Y, et al. Evidence for SARS-CoV-2 related coronaviruses circulating in bats and pangolins in Southeast Asia. [Erratum in: *Nat Commun*. 2021;12:1430]. *Nat Commun*. 2021;12:972. <https://doi.org/10.1038/s41467-021-21240-1>
11. Huong NQ, Nga NTT, Long NV, Luu BD, Latine A, Pruvot M, et al. Coronavirus testing indicates transmission risk increases along wildlife supply chains for human consumption in Viet Nam, 2013–2014. *PLoS One*. 2020;15:e0237129. <https://doi.org/10.1371/journal.pone.0237129>
12. Klumpp-Thomas C, Kalish H, Drew M, Hunsberger S, Snead K, Fay MP, et al. Standardization of ELISA protocols for serosurveys of the SARS-CoV-2 pandemic using clinical and at-home blood sampling. *Nat Commun*. 2021;12:113. <https://doi.org/10.1038/s41467-020-20383-x>
13. Hicks J, Klumpp-Thomas C, Kalish H, Shunmugavel A, Mehalko J, Denson J-P, et al. Serologic cross-reactivity of SARS-CoV-2 with endemic and seasonal betacoronaviruses. *J Clin Immunol*. 2021;41:906–13. <https://doi.org/10.1007/s10875-021-00997-6>
14. Tan CW, Chia WN, Qin X, Liu P, Chen MI-C, Tiu C, et al. A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2-spike protein-protein interaction. *Nat Biotechnol*. 2020;38:1073–8. <https://doi.org/10.1038/s41587-020-0631-z>
15. Bachmann MF, Mohsen MO, Zha L, Vogel M, Speiser DE. SARS-CoV-2 structural features may explain limited neutralizing-antibody responses. *NPJ Vaccines*. 2021;6:2. <https://doi.org/10.1038/s41541-020-00264-6>

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SARS-CoV-2 Cross-Reactivity in Prepandemic Serum from Rural Malaria-Infected Persons, Cambodia

Appendix

Appendix Table 1. SARS-CoV-2 seroreactivity in rural malaria-experienced persons by site, year, sex, and age, Cambodia*

Site	Samples	Year	Male	Female	<10 y	10–17 y	>18 y
Preah Vihear	81 (15)	2011	51 (9.7)	30 (5.7)	7 (1.6)	27 (6.0)	47 (10.5)
Pursat	347 (66)	2005 2009 2010 2011	261 (49.4)	86 (16.3)	20 (4.5)	71 (15.9)	256 (57.3)
Ratanakiri†	100 (19)	2011	11 (2.1)	8 (1.5)	6 (1.3)	7 (1.6)	6 (1.3)
Total	528	528	323 (61.2)	124 (23.5)	33 (7.4)	105 (23.5)	309 (69.1)

*All values are no. (%) unless otherwise indicated. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

†81 samples were missing data on sex and age.

Appendix Table 2. Seropositivity status by 2 SARS-CoV-2 IgG assays in rural malaria-experienced persons, Cambodia*

Participant no.	Test 1 Spike / RBD 2 SD cutoff	Mean RBD OD (450nm)	Mean Spike OD (450nm)	Test 2 Genscript / Spike S1-RBD		Mean (450nm)	Mean (450nm)/ Cutoff (0.12) >1
				Cutoff†	Mean (450nm)		
1	Negative	0.2157	0.2637	Negative	0.0000	0.0000	
2	Negative	0.0813	0.1241	Negative	0.0005	0.0041	
3	Negative	0.0847	0.3078	Negative	0.0220	0.1812	
4	Negative	0.0480	0.0532	Negative	0.0077	0.0630	
5	Negative	0.0854	0.5203	Negative	0.0081	0.0663	
6	Negative	0.0616	0.1983	Negative	0.0221	0.1820	
7	Negative	0.0613	0.1358	Positive	0.2514	2.0708	
8	Negative	0.0369	0.0361	Negative	0.0215	0.1767	
9	Positive	3.7444	0.7881	Positive	1.0889	8.9691	
10	Negative	0.1912	0.2379	Negative	0.0112	0.0923	
11	Negative	3.8750	0.4312	Positive	0.6211	5.1161	
12	Negative	3.8917	0.4280	Positive	0.6598	5.4349	
13	Negative	3.8971	0.3964	Positive	0.7262	5.9815	
14	Negative	3.0869	0.3910	Positive	0.4061	3.3451	
15	Positive	3.8931	0.9565	Negative	0.0825	0.6796	
16	Positive	3.8532	0.9306	Negative	0.0925	0.7615	
17	Positive	2.5646	0.7263	Positive	0.2392	1.9703	
18	Positive	3.8824	1.9920	Positive	0.4771	3.9296	
19	Positive	3.4743	0.9039	Positive	0.5926	4.8814	
20	Negative	2.2409	0.6454	Positive	0.2270	1.8694	
21	Positive	3.8911	1.9976	Positive	0.6885	5.6713	
22	Negative	0.2088	0.2645	Negative	0.0517	0.4255	
23	Negative	0.2221	0.1648	Negative	0.0071	0.0585	
24	Negative	0.1635	0.1754	Negative	0.0089	0.0729	
25	Negative	0.2032	0.2628	Negative	0.0428	0.3526	
26	Positive	3.9031	1.5984	Positive	1.3099	10.7900	
27	Negative	0.2109	1.0731	Negative	0.1066	0.8777	
28	Negative	0.2123	0.2391	Negative	0.0813	0.6697	
29	Negative	0.1325	0.1466	Negative	0.0050	0.0412	
30	Positive	3.7932	0.9603	Positive	0.6937	5.7142	
31	Positive	2.3932	1.5027	Positive	0.2116	1.7430	
32	Negative	0.2703	0.3878	Negative	0.1121	0.9234	
33	Negative	0.2216	0.3375	Negative	0.0015	0.0124	

Participant no.	Test 1 Spike / RBD 2 SD cutoff	Mean RBD OD (450nm)	Mean Spike OD (450nm)	Test 2 Genscript / Spike S1-RBD		Mean (450nm)	Mean (450nm)/ Cutoff (0.12) >1
				Cutoff†	Mean (450nm)		
34	Negative	3.6172	0.4099	Negative	0.0972	0.8007	
35	Positive	3.1024	0.7545	Positive	0.6504	5.3575	
Cutoff					0.1215		
Negative control				Negative		0.0014	
Positive control				Positive		2.0219	

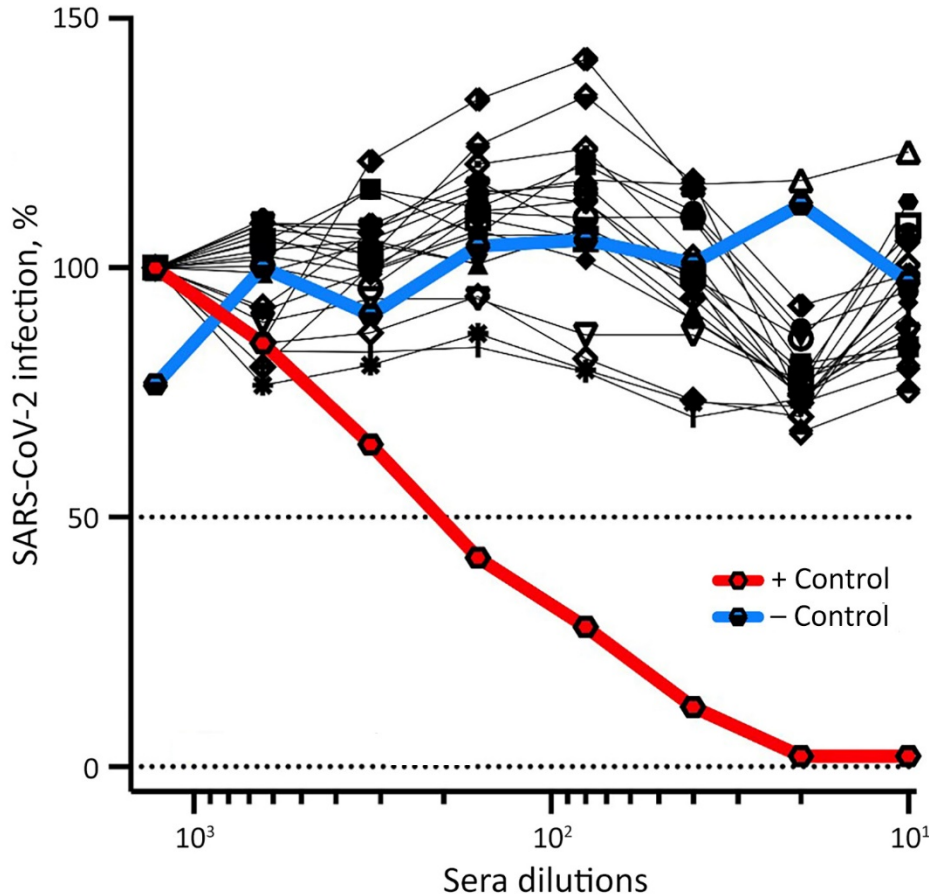
*RBD, receptor binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

†Positives for test 2 genscript = mean OD 450/cutoff (0.12) >1.

Appendix Table 3. SARS-CoV-2 surrogate virus neutralization test results in rural malaria-experienced persons, Cambodia*

Participant no.	Mean OD sVNT assay	Mean/ Positive CTRL	(1-Mean/Positive CTRL)	% neutralizing capacity	Detectable SARS-CoV-2 neutralizing antibody	Mean RBD	Mean Spike	Spike/RBD seropositive
2	2.9837	1.3814	-0.3814	-38.14	NO	0.0813	0.1241	Negative
3	3.0597	1.4166	-0.4166	-41.66	NO	0.0847	0.3078	Negative
4	3.0404	1.4076	-0.4076	-40.76	NO	0.048	0.0532	Negative
5	2.8734	1.3303	-0.3303	-33.03	NO	0.08545	0.5203	Negative
6	2.9994	1.3886	-0.3886	-38.86	NO	0.06165	0.1983	Negative
7	3.0158	1.3962	-0.3962	-39.62	NO	0.0613	0.1358	Negative
8	2.8516	1.3202	-0.3202	-32.02	NO	0.03685	0.0361	Negative
9	2.2910	1.0607	-0.0607	-6.07	NO	3.74435	0.7881	POSITIVE
10	2.8065	1.2993	-0.2993	-29.93	NO	0.1912	0.2379	Negative
11	2.2671	1.0496	-0.0496	-4.96	NO	3.87495	0.4312	Negative
12	2.3722	1.0982	-0.0982	-9.82	NO	3.8917	0.428	Negative
13	2.5024	1.1585	-0.1585	-15.85	NO	3.8971	0.3964	Negative
14	2.5129	1.1634	-0.1634	-16.34	NO	3.08685	0.391	Negative
15	2.4956	1.1554	-0.1554	-15.54	NO	3.89305	0.9565	POSITIVE
16	2.5930	1.2005	-0.2005	-20.05	NO	3.85315	0.9306	POSITIVE
17	2.2649	1.0486	-0.0486	-4.86	NO	2.56455	0.7263	POSITIVE
18	2.5655	1.1878	-0.1878	-18.78	NO	3.8824	1.992	POSITIVE
19	2.2631	1.0477	-0.0477	-4.77	NO	3.47425	0.9039	POSITIVE
20	2.4560	1.1371	-0.1371	-13.71	NO	2.2409	0.6454	Negative
21	1.8539	0.8583	0.1417	14.17	NO	3.8911	1.9976	POSITIVE
22	2.8783	1.3326	-0.3326	-33.26	NO	0.2088	0.2645	Negative
23	2.8563	1.3224	-0.3224	-32.24	NO	0.2221	0.1648	Negative
24	2.6625	1.2326	-0.2326	-23.26	NO	0.1635	0.1754	Negative
25	2.7740	1.2843	-0.2843	-28.43	NO	0.20315	0.2628	Negative
26	2.4614	1.1396	-0.1396	-13.96	NO	3.90305	1.5984	POSITIVE
27	2.8388	1.3143	-0.3143	-31.43	NO	0.21085	1.0731	Negative
28	2.7149	1.2569	-0.2569	-25.69	NO	0.2123	0.2391	Negative
29	2.6724	1.2373	-0.2373	-23.73	NO	0.13245	0.1466	Negative
30	2.2980	1.0639	-0.0639	-6.39	NO	3.79315	0.9603	POSITIVE
31	1.9825	0.9178	0.0822	8.22	NO	2.39315	1.5027	POSITIVE
32	2.1600	1.0000	0.0000	0.00	NO	0.27025	0.3878	Negative
33	2.8539	1.3213	-0.3213	-32.13	NO	0.22155	0.3375	Negative
34	2.4904	1.1530	-0.1530	-15.30	NO	3.6172	0.4099	Negative
35	2.3534	1.0895	-0.0895	-8.95	NO	3.10235	0.7545	POSITIVE
Neg. CTRL	2.5788	1.1939	-0.1939		NO			
Pos. CTRL	0.0863	0.0399	0.9601	96.01	YES			
SARS-CoV-2 neutralizing antibody standard serial dilution								
156.25	1.1358	0.5258	0.4742	47.42	YES			
312.5	0.5059	0.2342	0.7658	76.58	YES			
625	0.1636	0.0757	0.9243	92.43	YES			
1250	0.0687	0.0318	0.9682	96.82	YES			
2500	0.0490	0.0227	0.9773	97.73	YES			
5000	0.0465	0.0215	0.9785	97.85	YES			
10000	0.0517	0.0239	0.9761	97.61	YES			

*SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sVNT, surrogate virus neutralization test.



Appendix Figure. Microneutralization of SARS-CoV-2 in serum samples from pre-pandemic malaria-experienced rural persons, Cambodia. Twenty-one SARS-CoV-2 ELISA-positive Cambodian serum samples, negative controls (serum samples from US patients in 2014), and positive controls (serum samples from US patients who died of SARS-CoV-2 infection in 2020) were heat-inactivated (56°C, 1hr), serially 2-fold diluted (1:10 to 1:1,280) in OptiPRO SFM (catalog no. 12309-019, ThermoFisher Scientific, <https://www.thermofisher.com>) supplemented with 2mM L-Glutamine (catalog no. 25030-081, ThermoFisher Scientific) and 1x Antibiotic-Antimycotic (catalog no. 15240062, ThermoFisher Scientific). Diluted serum samples were mixed with an equal volume of SARS-CoV-2 diluted to 200 TCID₅₀/25µl (USA-WA1/2020, catalog no. NR-52281, BEI Resources, <https://www.beiresources.org>) and incubated at room temperature for 1 hour. Fifty micro liter of the virus-plasma mixture was added in triplicate to Vero cells grown in a 96-well plate and incubated for 3 days at 37°C in a humidified incubator with 5% CO₂. After incubation, media was removed and 200µL of 10% neutral buffered formalin was added and incubated at room temperature for 30 min to inactivate the virus and fix the cells. After incubation, the plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS), and 1:4,000 diluted SARS-CoV-2 nucleocapsid antibody (catalog no. 40143-R001, Sino Biological, <https://www.sinobiological.com>) was added to each well (50 µL per well). After incubation (room temperature, 1hr), the plates were washed 3 times, and 1:10,000 diluted horseradish peroxidase-conjugated anti-rabbit IgG antibody (catalog no.

32460, ThermoFisher Scientific) was added (100 μ l per well), and the plates were incubated at room temperature for 1 hr. The plates were then washed 6 times followed by 30 min of room temperature incubation with horseradish peroxidase substrate solution (100 μ L per well) prepared by adding a 10-mg o-phenylenediamine dihydrochloride tablet (catalog no. P8287, MilliporeSigma, <https://www.sigmaaldrich.com>) to 20 mL of phosphate citrate buffer preparation (catalog no. P4922, MilliporeSigma). The reaction was stopped by adding 1 M sulfuric acid (100 μ L per well), and the optical density was measured at 492 nm (OD₄₉₂). To calculate percent infection, the optical density obtained with the lowest amount of serum (1:1,280) was used to set 100% infection for each serum sample. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.