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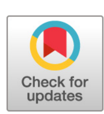

COVID-19 Serology at Population Scale: SARS-CoV-2-Specific Antibody Responses in Saliva

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Nora Pisanic and Pranay R. Randad contributed equally. Order was determined on the following basis: N.P. developed the first draft of the manuscript, led laboratory analyses, and performed data analyses; P.R.R. provided critical input on the manuscript, led laboratory analyses, and performed data analyses.

ABSTRACT Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the cause of an ongoing pandemic that has infected over 36 million and killed over 1 million people. Informed implementation of government public health policies depends on accurate data on SARS-CoV-2 immunity at a population scale. We hypothesized that detection of SARS-CoV-2 salivary antibodies could serve as a noninvasive alternative to serological testing for monitoring of SARS-CoV-2 infection and seropositivity at a population scale. We developed a multiplex SARS-CoV-2 antibody immunoassay based on Luminex technology that comprised 12 CoV antigens, mostly derived from SARS-CoV-2 nucleocapsid (N) and spike (S). Saliva and sera collected from confirmed coronavirus disease 2019 (COVID-19) cases and from the pre-COVID-19 era were tested for IgG, IgA, and IgM to the antigen panel. Matched saliva and serum IgG responses ($n = 28$) were significantly correlated. The salivary anti-N IgG response resulted in the highest sensitivity (100%), exhibiting a positive response in 24/24 reverse transcription-PCR (RT-PCR)-confirmed COVID-19 cases sampled at >14 days post-symptom onset (DPSO), whereas the salivary anti-receptor binding domain (RBD) IgG response yielded 100% specificity. Temporal kinetics of IgG in saliva were consistent with those observed in blood and indicated that most individuals seroconvert at around 10 DPSO. Algorithms employing a combination of the IgG responses to N and S antigens result in high diagnostic accuracy (100%) by as early as 10 DPSO. These results support the use of saliva-based antibody testing as a noninvasive and scalable alternative to blood-based antibody testing.

KEYWORDS SARS-CoV-2, COVID-19, saliva, oral fluid, serology, antibody test, multiplex, diagnostics, immunoserology

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The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused >36 million COVID-19 cases and >1 million deaths as of October 2020, involving all populated continents (1). There is a critical need to perform broad-scale population-based testing to improve COVID-19 prevention and control efforts, understand transmission dynamics, and track herd immunity (2, 3).

Saliva offers several advantages as a diagnostic medium in that it is a noninvasive, painless, safe, and convenient specimen. Whereas some consider phlebotomy specimens to be too invasive and uncomfortable, saliva sampling is widely accepted, particularly among vulnerable or difficult-to-reach populations, and could facilitate home-based self-collection (4–6). If saliva can support measurements of both the presence of SARS-CoV-2 RNA (7) as well as antibodies against SARS-CoV-2 (8, 9), this sample type could provide an important opportunity to monitor individual and population-level SARS-CoV-2 transmission, infection, and seropositivity at granular spatial and temporal scales.

We have previously demonstrated the utility of saliva-based antibody testing for the diagnosis, surveillance, and study of infection by multiple viral pathogens (10, 11). Saliva harvested from the space between the gums and the teeth (gingival crevicular fluid [GCF], referred to here as "saliva") has a composition resembling that of serum, including being enriched with antibodies (10–14). Development of high-performing antibody assays to detect prior infection with SARS-CoV-2 was identified as a critical priority early in the COVID-19 pandemic response (2, 3). We hypothesize that saliva-based antibody testing can fill this critical gap and provide a pivotal capacity for combating the COVID-19 pandemic.

The objectives of this study were to (i) develop and validate a multiplex bead-based immunoassay for the detection of SARS-CoV-2-specific IgG, IgA, and IgM responses; (ii) evaluate the assay performance using saliva compared to using serum specimens and identify SARS-CoV-2 antigens that could result in high sensitivity and specificity to identify antibody responses to prior SARS-CoV-2 infection; and (iii) describe the antibody kinetics in saliva compared to those in serum by the time since the onset of COVID-19 symptoms.

MATERIALS AND METHODS

Study design. Saliva and serum samples were provided by collaborators from Emory University from patients in three settings: (i) PCR-confirmed COVID-19 cases while admitted to the hospital, (ii) confirmed COVID-19 cases who we invited to donate specimens after recovering from their acute illness, and (iii) PCR-tested patients with symptoms consistent with COVID-19 being tested at an ambulatory testing center who donated specimens at the time of testing and/or at a follow-up convalescent-phase research visit. Collaborators at Johns Hopkins University provided (i) serum samples from PCR-tested patients presenting with COVID-19-like symptoms such as fever, cough, and dyspnea who were recruited in both inpatient and outpatient clinical care sites and (ii) negative saliva and serum samples collected prior to the COVID-19 pandemic. Participants provided verbal and/or written informed consent and provided saliva and blood specimens for analysis. Whenever possible, remnant clinical blood specimens were used. Only deidentified serum or plasma and saliva aliquots with limited metadata (days post-symptom onset [DPSO] and SARS-CoV-2 reverse transcription-PCR [RT-PCR] status [ever positive or negative]) were shared for this study. Participation in these studies was voluntary, and the study protocols have been approved by the respective Institutional Review Boards (IRBs).

Saliva and blood sample collection. Saliva samples were collected by instructing participants to gently brush their gum line with an Oracol S14 saliva collection device (Malvern Medical Developments, UK) for 1 to 2 min or until saturation. This saliva collection method specifically harvests GCF, which is enriched with primarily IgG antibody derived from serum (13). The saturated sponge was then inserted into the storage tube, capped, and stored at 4°C until processing whenever possible. Saliva was separated from the Oracol S14 swabs by centrifugation (10 min at 1,500 × *g*) and transferred into the attached 2-ml cryovial. Samples collected since the onset of the COVID-19 pandemic were heat inactivated at 60°C for 30 min prior to analysis; prepandemic saliva samples were not heat inactivated. Blood samples were collected into ACD (acid, citrate, dextrose) or serum separator tubes (SSTs) and processed according to each clinical laboratory's procedure. Plasma/serum was also heat inactivated, if collected after the onset of the COVID-19 pandemic, at 60°C for 30 min, aliquoted into 2-ml cryovials, and stored at ≤20°C until analysis.

Multiplex magnetic microparticle ("bead")-based SARS-CoV-2 immunoassay. Ten SARS-CoV-2 antigens were obtained commercially or from collaborators at the Icahn School of Medicine at Mount Sinai (15). These included four SARS-CoV-2 receptor binding domain (RBD) proteins, one ectodomain

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(ECD) protein containing the S1 and S2 subunits of the spike protein, two S1 subunits, one S2 subunit, and two N proteins (see Table S1 in the supplemental material). Each SARS-CoV-2 antigen, along with one SARS-CoV-1 antigen (Native Antigen Company [NAC] SARS 2002 N) and one human coronavirus 229E (hCoV-229E) antigen (Sino Biological hCoV-229E ECD), was covalently coupled to magnetic microparticles (MagPlex microspheres; Luminex) as described previously (10, 11). Along with a control bead conjugated with bovine serum albumin (BSA), the multiplex panel included 13 bead sets. Coupling of antigens to beads was confirmed using commercial primary antibodies against the antigen or against the tag (e.g., anti-His_6 tag antibody), followed by a species-specific R-phycoerythrin (PE)-labeled antibody, and was considered successful if the median fluorescence intensity (MFI) (arbitrary units) reached >10,000 at 1 μg/ml of antigen-specific antibody (except for the BSA-conjugated bead set). Saliva was centrifuged (5 min at 20,000 × *g* at 20°C), and 10 μl of the supernatant was added to 40 μl of assay buffer (phosphate-buffered saline with 0.05% Tween 20 [PBST], 0.02% sodium azide, and 1% BSA) containing 1,500 beads of each bead set per microplate well. The plate was incubated at room temperature for 1 h on a plate shaker at 500 rpm. Beads were washed twice with 200 μl PBST; 50 μl of PE-labeled anti-human IgG, IgA, or IgM diluted 1:100 in assay buffer was added; and the plate was incubated again for 1 h on a plate shaker at 500 rpm. Beads were washed as described above and then suspended in 100 μl assay buffer. Finally, the MFI of each bead set was measured on a Bio-Plex immunoassay instrument (Bio-Rad Laboratories, Hercules, CA). The same protocol was used for serum and plasma samples, except that these samples were tested at a final dilution of 1:1,000 in bead mix and assay buffer compared to a final dilution of 1:5 for saliva. At least two blanks (assay buffer) were included on each plate for background fluorescence subtraction. We first tested all samples for IgG and then used any remaining volume of saliva to test for IgA and IgM. For this reason, not all samples were tested for all antibody isotypes. A subset of saliva samples was tested in duplicate and in a masked fashion to determine intra- and interassay variability.

Statistical analysis. The median MFI derived from the BSA bead set was subtracted from each blank-subtracted antigen-specific MFI signal for each sample to account for nonspecific binding of antibodies to beads without antigen. The average MFI was used for samples that were tested more than once. A Wilcoxon-Mann-Whitney test was used to compare the antibody responses between samples collected during early (0 to 14 DPSO) and late (>14 DPSO) convalescent phases and negative samples for each antigen in the multiplex assay. The average intra- and interassay variabilities were evaluated by determining the standard deviation and percent coefficient of variation (CV%) of subsets of 47 and 49 samples, respectively. Pearson's correlation was used to determine the correlation between antigen-specific IgG, IgA, and IgM MFI in matched saliva and serum/plasma samples collected from the same person at the same time point. The average MFIs of saliva samples from known uninfected individuals (pre-COVID-19 only, $n = 90$) plus 3 standard deviations for each antigen-specific IgG, IgA, and IgM were used to establish cutoff values to discriminate negative from positive samples. The corresponding procedure was used for serum samples. Because the prior hCoV infection status for saliva and serum samples was not known, MFI cutoff values were not calculated for anti-Sino Biological hCoV-229E ECD IgG, IgA, and IgM. The sensitivity and specificity for detecting samples from confirmed RT-PCR-positive individuals (positive samples) and for samples obtained from individuals prior to the COVID-19 pandemic and individuals who tested RT-PCR negative and were clinically confirmed to not have been infected with SARS-CoV-2 (negative samples) were determined for each antigen/isotype pair (IgG, IgM, and IgA) in saliva and serum. We applied an algorithm that defined a sample as positive if it was determined to be reactive to GenScript N and at least one of the RBD antigens or spike ECD. This was informed by the fact that none of the negative samples were positive for both GenScript N and at least one of the RBD or ECD antigens at the same time. Locally weighted regression (locally estimated scatterplot smoothing [LOESS]) was used to visualize and compare the temporal kinetics of saliva and serum antigen-specific IgG, IgA, and IgM responses among individuals with RT-PCR-confirmed prior SARS-CoV-2 infection after symptom onset.

Ethical statement. This study has been approved by the Johns Hopkins Bloomberg School of Public Health Institutional Review Board (IRB) (IRB no. IRB00012253), the Johns Hopkins Medicine IRB (IRB no. IRB00247886), and the Emory University IRB (IRB no. 00110683 and 00022371).

RESULTS

Saliva and serum samples. A total of 33 saliva samples and 204 serum samples were collected from 33 and 59 individuals, respectively, with RT-PCR-confirmed prior SARS-CoV-2 infection. A total of 134 saliva and 118 serum samples were collected from participants in prepandemic cohort studies (16) (from 2012 to early 2019) or from participants of ongoing studies (2 saliva and 6 serum samples) who had no clinical history consistent with COVID-19 and tested negative for COVID-19 by RT-PCR (Table 1).

SARS-CoV-2 antigen-specific IgG, IgA, and IgM cutoff values. Saliva from 138 individuals (167 samples) and serum from 171 individuals (322 samples) were tested with the multiplex assay. Cutoffs to discriminate between positive and negative samples and comprehensive data on ranges, medians, means, and standard deviations for the binding of each antibody isotype to each antigen, stratified by specimen type and DPSO, are provided in Table S2 in the supplemental material. Saliva and serum collected at >14 DPSO had significantly elevated IgG levels (MFI) against all SARS-CoV-2

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TABLE 1 Saliva and serum samples used for assay development and characterization

Sample type	Saliva		Serum	
	No. (%) of participants	No. (%) of samples	No. (%) of participants	No. (%) of samples
All samples	138 (100)	167 (100)	171 (100)	322 (100)
SARS-CoV-2 PCR positive	33 (24)	33 (20)	59 (35)	204 (63)
SARS-CoV-2 PCR negative	105 (76)	134 (80)	112 (65)	118 (37)
Matched samples	28 (100)	28 (100)		
SARS-CoV-2 PCR positive	22 (79)	22 (79)		
SARS-CoV-2 PCR negative	6 (21)	6 (21)		

antigens compared to negative saliva samples. Serum collected at >14 DPSO also had significantly elevated IgA and IgM levels (MFI) against all SARS-CoV-2 antigens compared to negative sera, which was not the case for saliva.

Correlation between saliva and serum SARS-CoV-2-specific IgG, IgA, and IgM. Twenty-eight participants provided matched saliva and serum samples collected during the same visit (6 negative samples and 22 RT-PCR-confirmed SARS-CoV-2 infection samples). Two saliva samples were depleted after testing for IgG, resulting in 28 matched saliva and serum samples tested for IgG and 26 matched saliva and serum samples tested for IgA and IgM. IgG levels in matched saliva and serum samples were significantly correlated for all SARS-CoV-2 and SARS-CoV-1 antigens (Fig. 1). IgA levels in matched samples were modestly correlated, with significance detected for only a subset of antigens: GenScript N, Sino Biological N, Sino Biological ECD, GenScript S1, and NAC SARS 2002 N (Fig. 2). IgM levels in matched saliva and serum samples were also significantly correlated for all SARS-CoV-2 and SARS-CoV-1 antigens, although the correlation was weaker than for IgG (Fig. 3).

Sensitivity and specificity. In saliva and serum, the sensitivity for the detection of prior SARS-CoV-2 infection increased when salivary IgG was measured at >10 to 14 DPSO (Fig. 4 and Fig. S1). Interestingly, the same two antigens (GenScript N and Mt. Sinai RBD) resulted in the highest sensitivity and specificity, respectively, when measuring IgG in both saliva and serum. Most RBD antigens yielded very high sensitivity in serum but were more variable in saliva (Fig. 4). Serum and salivary IgA to SARS-CoV-2 also increased with time since symptom onset. Increases in serum IgM were detectable for some antigens such as RBD. The assay sensitivity for detecting salivary IgA and IgM to SARS-CoV-2 was low. The specificity for the classification of pre- and post-COVID negative saliva and serum samples correctly ranged from 98% to 100% for SARS-CoV-2 IgG, with RBD antigen (Mt. Sinai) exhibiting the highest specificity in both specimen types. The specificity for IgA or IgM detection was overall slightly lower than that in the IgG assay.

Several algorithms involving the immune response to multiple antigens simultaneously resulted in the correct classification of all positive (only >14 DPSO samples) and negative saliva and serum samples. Examples include defining a sample as positive when the salivary IgG level is above the cutoff for at least one N and at least one RBD antigen.

Kinetics of SARS-CoV-2-specific IgG, IgA, and IgM responses in serum compared to saliva. The kinetics of antigen-specific IgG (Fig. 5), IgA, and IgM (Fig. S2) responses in serum and saliva are shown with isotype-specific cutoffs. The kinetics and magnitude of the antigen-specific IgG and IgA responses in saliva generally correlate with those observed in serum. IgG and IgM seroconversion in serum seems to occur simultaneously. Even though the kinetics of SARS-CoV-2-specific IgA and IgG levels are similar in saliva, the sensitivity for IgA detection is low. IgM levels in saliva were lower than those in serum and generally remained just above or below the cutoff for most antigens in the multiplex assay. Importantly, salivary SARS-CoV-2-specific IgG consistently crosses the cutoff at around 10 DPSO, mimicking seroconversion in serum. At ≥10 DPSO, the sensitivity for detecting recent SARS-CoV-2 infection by IgG in saliva remained high (28/28 above the cutoff).

Reactivity of antibodies with SARS-CoV-1 and hCoV proteins following SARS-CoV-2 infection. We sought to evaluate reactivity to SARS-CoV-1 and hCoV proteins in

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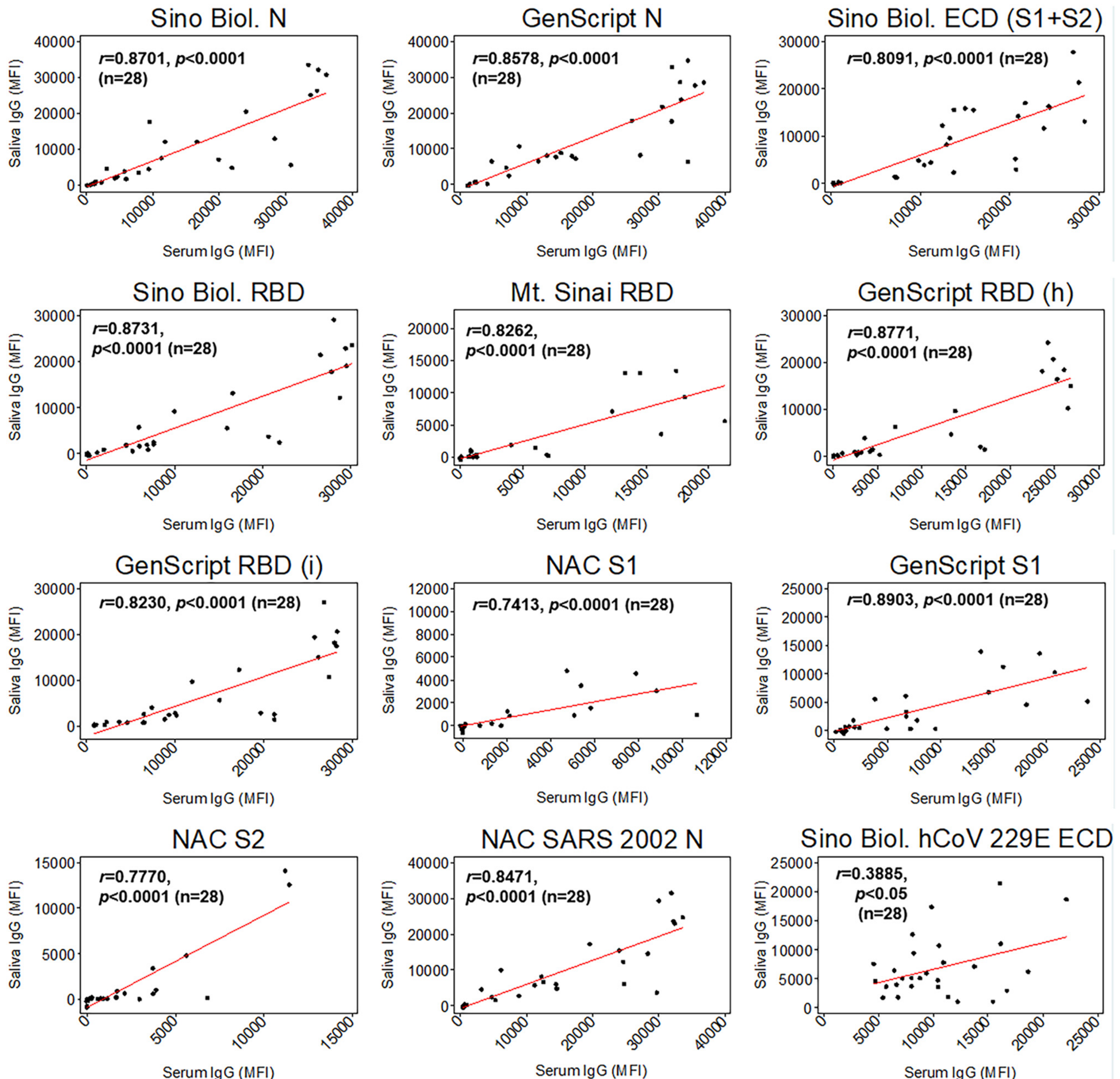

FIG 1 Correlation between matched SARS-CoV-2-specific IgG responses in saliva and serum ($n = 28$). The Pearson correlation coefficient is provided for IgG responses to each antigen. Sino Biol., Sino Biological; NAC, Native Antigen Company; N, nucleocapsid protein; ECD, ectodomain; S1/S2, S1 or S2 subunit of the spike protein; RBD, receptor binding domain; (h), produced in human cells; (i), produced in insect cells; MFI, median fluorescence intensity.

samples from COVID-19 cases. All convalescent-phase salivary IgG (>14 DPSO) (24/24; 100%) reacted with the SARS-CoV-1 N antigen (NAC SARS 2002 N). Similarly, 98% of convalescent-phase sera contained SARS-CoV-1 N-reactive IgG and IgA (Fig. 4). The median salivary IgG and IgA levels and serum IgG, IgA, and IgM levels to NAC SARS 2002 N were significantly elevated in COVID-19 convalescent-phase samples compared to negative samples, indicating that SARS-CoV-2 infection elicits antibodies that cross-react with SARS-CoV-1 antigens (Table S2). The MFIs for saliva and serum IgG and IgA to Sino Biological hCoV-229E ECD were high in the majority of samples regardless of timing or SARS-CoV-2 infection history (Fig. 5 and Table S2), which is consistent with frequent human exposure to hCoVs such as 229E. Interestingly, antibody levels were significantly higher in samples collected at >14 DPSO than in negative samples,

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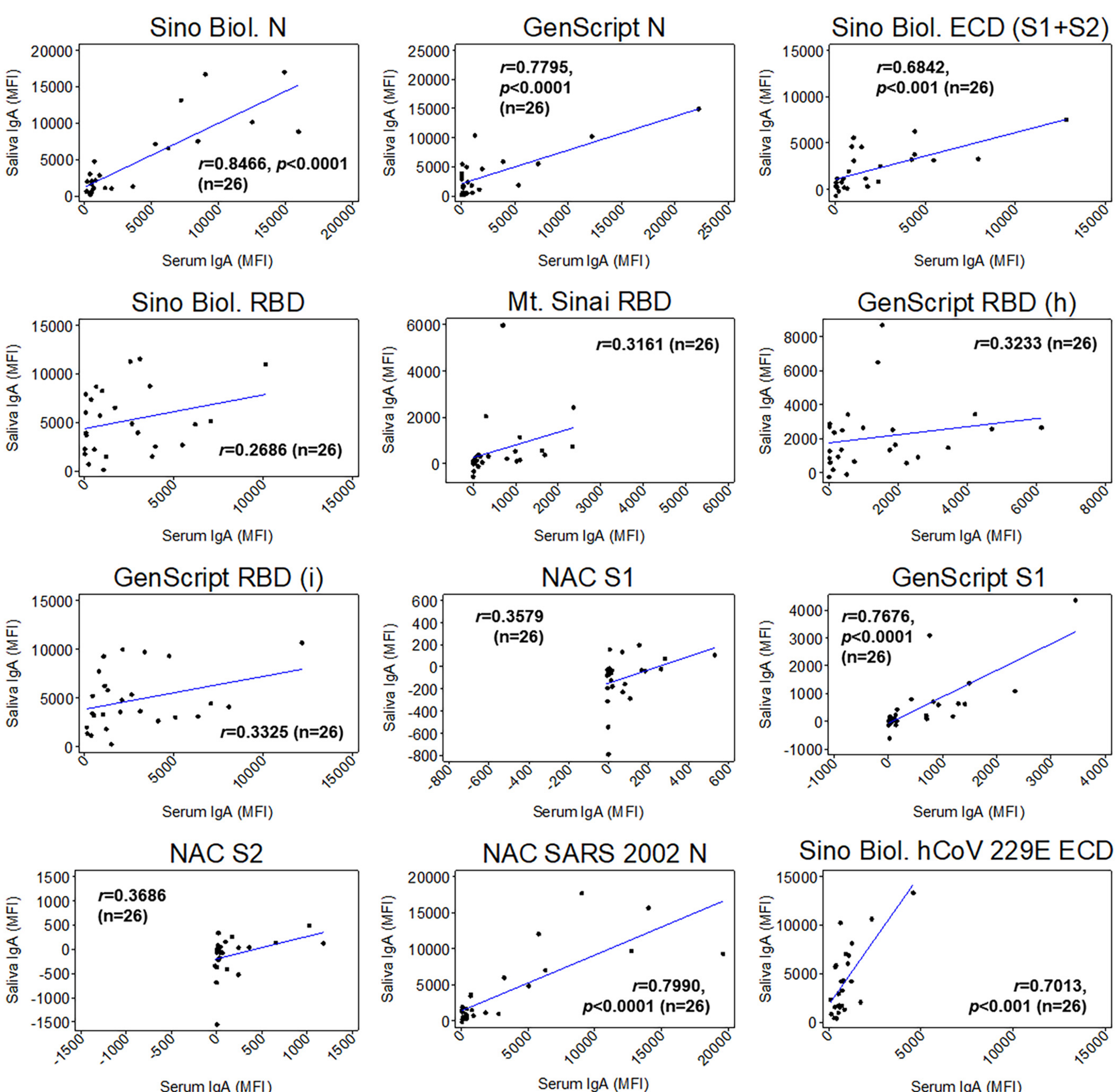

FIG 2 Correlation between matched salivary and serum SARS-CoV-2-specific IgA responses ($n = 26$). The Pearson correlation coefficient is provided for IgA responses to each antigen. *P* values are provided for statistically significant correlations only ($P < 0.05$). Sino Biol., Sino Biological; NAC, Native Antigen Company; N, nucleocapsid protein; ECD, ectodomain; S1/S2, S1 or S2 subunit of the spike protein; RBD, receptor binding domain; (h), produced in human cells; (i), produced in insect cells; MFI, median fluorescence intensity.

suggesting some amount of boosting of antibodies to hCoV-229E by SARS-CoV-2 infection (Table S2).

Intra- and interassay variability. The average intra-assay variability ranged from 5% to 12% (CV%), and the interassay variability ranged from 12% to 22% (CV%) (Table S3).

DISCUSSION

Our results testing matched specimens demonstrate that salivary SARS-CoV-2-specific IgG levels reflect those in serum. We also demonstrated that the kinetics of IgG

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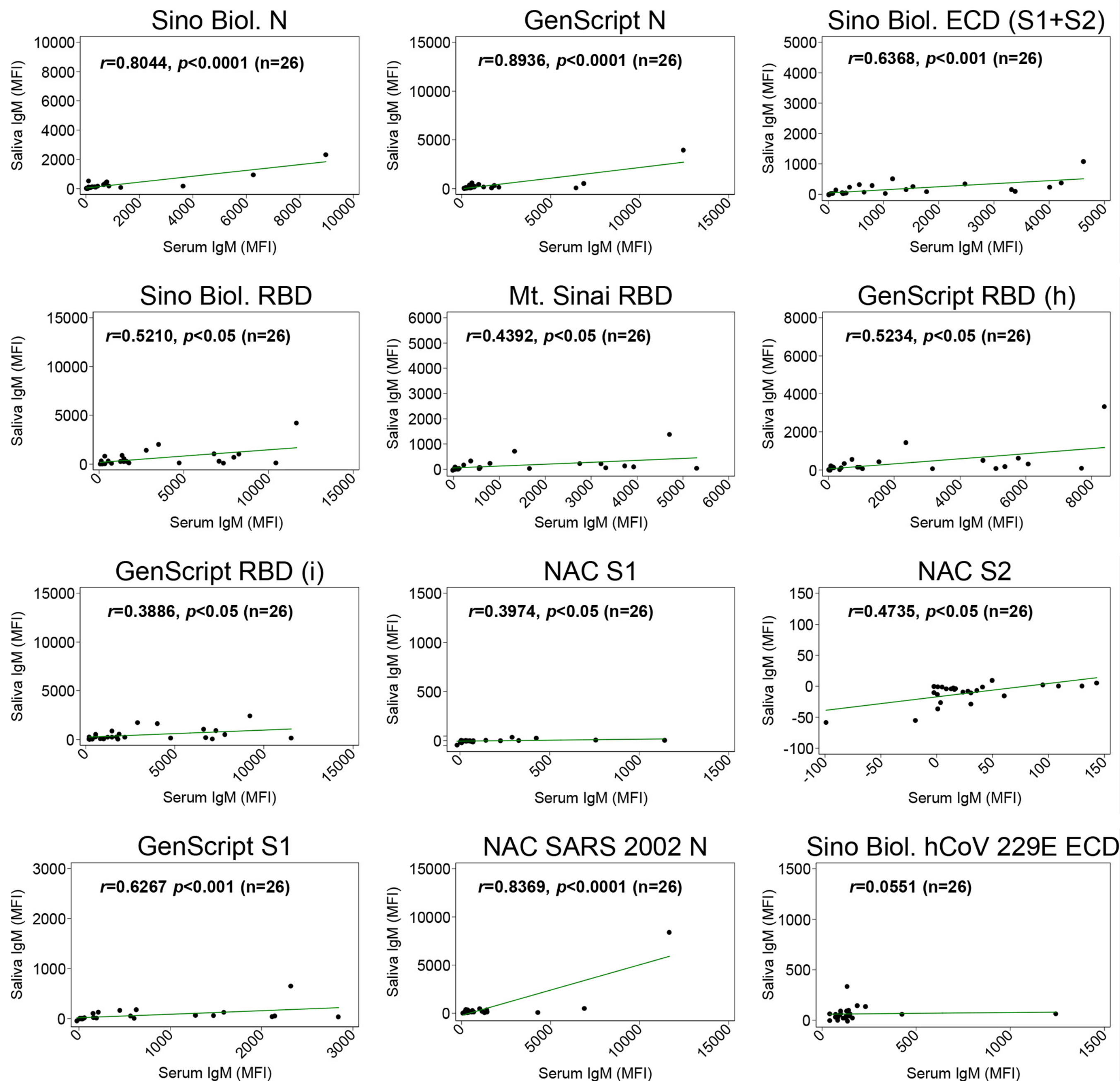

FIG 3 Correlation between matched salivary and serum SARS-CoV-2-specific IgM responses ($n = 26$). The Pearson correlation coefficient is provided for IgM responses to each antigen. *P* values are provided for statistically significant correlations only ($P < 0.05$). Sino Biol., Sino Biological; NAC, Native Antigen Company; N, nucleocapsid protein; ECD, ectodomain; S1/S2, S1 or S2 subunit of the spike protein; RBD, receptor binding domain; (h), produced in human cells; (i), produced in insect cells; MFI, median fluorescence intensity.

responses in saliva are consistent with those in serum and indicate that most individuals seroconvert at approximately 10 DPSO. Thus, saliva-based assays can be used to detect prior SARS-CoV-2 infection with excellent sensitivity and specificity and represent a practical, noninvasive alternative to blood for COVID-19 antibody testing. Additional specimen testing would be needed to precisely define assay sensitivity at early (<14 DPSO) and later (>3 months) time points. These findings represent a major advance in addressing an urgent need in the COVID-19 pandemic response as a saliva-based multiplex immunoassay could accomplish large-scale SARS-CoV-2 "serosurveillance." Because saliva can be transported at ambient temperatures and even

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a) Saliva

	Salivary IgG				Salivary IgA				Salivary IgM			
	Sensitivity			Specificity	Sensitivity			Specificity	Sensitivity			Specificity
	Days after symptom onset				Days after symptom onset				Days after symptom onset			
Group	**1-7**	**>7-14**	**>14**	**Negative**	**1-7**	**>7-14**	**>14**	**Negative**	**1-7**	**>7-14**	**>14**	**Negative**
N	3	6	24	134	2	2	22	83	2	2	22	84
GenScript N	67%	67%	100%	99%	50%	0%	50%	96%	0%	0%	5%	98%
Sino Biol. N	0%	67%	88%	99%	0%	100%	36%	96%	0%	0%	18%	98%
Sino Biol. ECD (S1+S2)	33%	67%	96%	99%	50%	50%	59%	96%	0%	0%	5%	99%
Sino Biol. RBD	0%	83%	100%	98%	0%	0%	23%	99%	0%	50%	27%	98%
Mt. Sinai RBD	0%	83%	92%	100%	0%	0%	27%	99%	0%	50%	32%	98%
GenScript RBD (h)	0%	50%	92%	99%	0%	0%	18%	99%	0%	50%	27%	98%
GenScript RBD (i)	0%	50%	88%	99%	0%	0%	23%	96%	0%	50%	14%	96%
NAC S1	0%	33%	67%	99%	0%	0%	18%	99%	0%	0%	5%	98%
GenScript S1	0%	33%	46%	98%	0%	50%	41%	99%	0%	50%	64%	99%
NAC S2	0%	67%	54%	99%	0%	0%	14%	98%	0%	0%	0%	99%
NAC SARS 2002 N	33%	67%	100%	99%	50%	0%	45%	98%	0%	0%	5%	96%
Algorithm[a]	33%	67%	100%	100%	50%	0%	45%	98%	0%	0%	5%	100%

b) Serum

	Serum IgG				Serum IgA				Serum IgM			
	Sensitivity			Specificity	Sensitivity			Specificity	Sensitivity			Specificity
	Days after symptom onset				Days after symptom onset				Days after symptom onset			
Group	**1-7**	**>7-14**	**>14**	**Negative**	**1-7**	**>7-14**	**>14**	**Negative**	**1-7**	**>7-14**	**>14**	**Negative**
N	60	93	49	118	60	93	49	112	60	93	49	112
GenScript N	15%	66%	100%	99%	40%	80%	96%	93%	7%	28%	31%	97%
Sino Biol. N	8%	53%	90%	99%	23%	58%	80%	99%	5%	35%	67%	98%
Sino Biol. ECD (S1+S2)	5%	70%	100%	99%	20%	80%	96%	96%	12%	68%	78%	97%
Sino Biol. RBD	13%	74%	98%	98%	32%	78%	96%	98%	8%	66%	78%	97%
Mt. Sinai RBD	13%	74%	98%	100%	30%	81%	98%	99%	20%	84%	90%	99%
GenScript RBD (h)	13%	74%	98%	99%	32%	83%	98%	96%	20%	80%	90%	97%
GenScript RBD (i)	3%	56%	92%	99%	25%	74%	96%	97%	12%	69%	86%	95%
NAC S1	3%	52%	86%	99%	10%	45%	80%	98%	2%	17%	29%	99%
GenScript S1	0%	4%	43%	98%	0%	6%	22%	98%	8%	69%	84%	98%
NAC S2	13%	63%	88%	99%	17%	65%	88%	97%	2%	26%	22%	99%
NAC SARS 2002 N	13%	65%	98%	99%	38%	85%	98%	99%	10%	39%	39%	97%
Algorithm[a]	13%	67%	100%	100%	28%	81%	98%	98%	10%	39%	39%	100%

Note: Cutoffs to discriminate positive from negative samples were calculated as follows: Mean (pre-COVID-19 specimens) + 3 standard deviations. [a]Positive for GenScript N and at least one RBD antigen.

FIG 4 Assay sensitivity and specificity for each SARS-CoV-2 antigen and antibody isotype using saliva (a) and serum (b). Samples collected from individuals with RT-PCR-confirmed prior SARS-CoV-2 infection are stratified by time since symptom onset. Darker shades of green indicate higher and darker shades of red indicate lower sensitivity and specificity. Sino Biol., Sino Biological; NAC, Native Antigen Company; N, nucleocapsid protein; ECD, ectodomain; S1/S2, S1 or S2 subunit of the spike protein; RBD, receptor binding domain; (h), produced in human cells; (i), produced in insect cells.

self-collected at home (14), salivary antibody tests could greatly increase the capacity for testing in a variety of settings (e.g., in schools, workplaces, the community, and resource-limited settings) and could clarify population seropositivity and susceptibility to SARS-CoV-2. Because IgG was frequently detectable at day 10 after COVID-19 symptom onset, salivary antibody testing could be diagnostically useful at the time of clinical presentation. Interestingly, molecular testing for SARS-CoV-2 in saliva may perform better than nasopharyngeal (NP) sampling (7), and the U.S. FDA recently granted emergency-use authorization (EUA) approval for a mail-in saliva-based SARS-CoV-2 test (17). Thus, it is feasible that a single saliva sample could provide both molecular and serological data on current and prior SARS-CoV-2 infection status and accelerate goals for nationwide population-level surveillance.

Our kinetics analysis of antibody responses in saliva following COVID-19 symptom onset revealed a congruence with those observed in serum. In both saliva and serum, IgG appeared before or at approximately the same time as IgM, consistent with the stimulation of preexisting, cross-reactive B cells. Both synchronous and classical antibody isotype responses have been reported following SARS-CoV-2 infection (18–21). The sensitivity of our assay improved overall when convalescent-phase samples ($\geq$10 DPSO) compared to acute-phase samples ($<$10 DPSO) were used for all SARS-CoV-2-specific antibody isotypes. The assay performance for individual antigens reported here is consistent with previous studies that have reported sensitivities for various SARS-CoV-2 IgG tests ranging from 82% to 100% using convalescent-phase samples (22–24). While the serum IgA and IgM assays reached >96% and 90% sensitivities, respectively,

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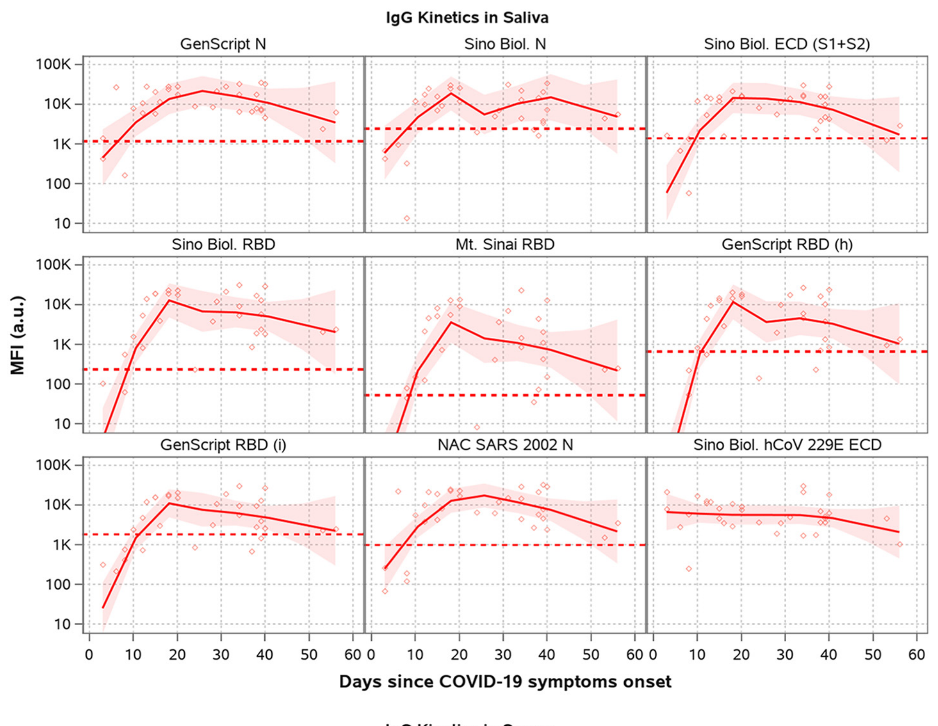

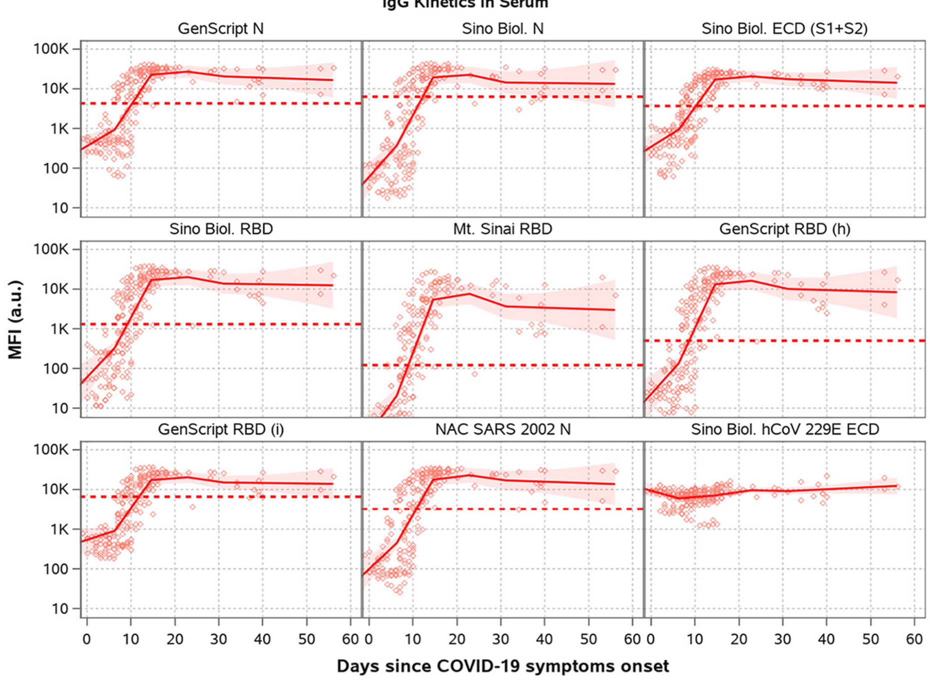

FIG 5 Comparison of saliva and serum SARS-CoV-2 antigen-specific IgG responses by days post-symptom onset (DPSO). The trajectories of IgG responses (red solid lines) and confidence intervals (semitransparent background) were estimated using a LOESS curve. Dashed red lines indicate cutoff values for each antigen. Sino Biol., Sino Biological; NAC, Native Antigen Company; N, nucleocapsid protein; ECD, ectodomain; S1/S2, S1 or S2 subunit of the spike protein; RBD, receptor binding domain; (h), produced in human cells; (i), produced in insect cells; MFI, median fluorescence intensity; a.u., arbitrary units.

at >14 DPSO, the sensitivity in saliva for these isotypes remained low. Both the mean fluorescence intensity and the MFI for most SARS-CoV-2 antigen-specific IgA responses in saliva increased with time since symptom onset and were significantly elevated at >14 DPSO compared to negative-control samples (see Table S2 in the supplemental material). This provides evidence that the salivary IgA assay performance could be

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improved, perhaps by optimizing sample dilutions or by optimizing other assay parameters (8, 9). Of note is the higher cutoff for IgA than for IgG, which is due to the high standard deviation in the antibody signal to the various antigens using pre-COVID-19-era specimens. A higher sample dilution when detecting salivary IgA might result in better discrimination (lower standard deviation among negative samples) between known positive and negative samples (8, 9). Nonspecific binding of IgA antibodies or cross-reactivity with other viruses could contribute to this background. Although we harvested GCF, which is enriched with oral mucosal transudate, IgM antibodies, which are significantly larger than IgG or IgA antibodies, are much less abundant in this type of salivary specimen due to their larger size (12).

Cross-reactive antibody responses elicited by related viruses are common and can compromise the performance of serological assays. We hypothesized that SARS-CoV-1 and SARS-CoV-2 may exhibit cross-reactivity to N, which is 90% conserved (20); however, RBD, which is much less conserved among betacoronaviruses, would exhibit greater specificity (15, 25). We found that all (24/24; 100%) saliva samples from COVID-19 cases collected at >14 DPSO reacted with the closely related SARS-CoV-1 2002 N in the IgG assay. Of course, this antigen could still be used for SARS-CoV-2 antibody testing, as the cross-reactivity would be relevant only if SARS-CoV-1 and SARS-CoV-2 were cocirculating in the same human population. We did not specifically evaluate whether common hCoVs elicit cross-reactive antibodies causing false-positive results in our SARS-CoV-2 assay; however, this is very unlikely given that prepandemic samples and COVID-19 samples collected at <10 DPSO consistently tested negative for antibodies to SARS-CoV-2 antigens. We included one hCoV antigen (hCoV-229E ECD) in the panel. Most sera from early and late COVID-19 cases and negative-control samples reacted to this antigen, consistent with a high prevalence of hCoV exposure in the general population (26, 27); however, there appeared to be a small but significant boost in antibodies to hCoV-229E ECD in COVID-19 convalescent-phase samples. It would be interesting for future work to investigate whether this is due to direct cross-reactivity at conserved regions of spike, nonspecific bystander activation, or other phenomena. Ultimately, it does not appear that cross-reactivity from other hCoVs will be a confounding issue in detecting SARS-CoV-2-specific antibodies in humans (15, 28).

We conducted a sensitivity analysis comparing the cutoff values and the resulting sensitivity and specificity, derived using 363 heat-inactivated versus the 90 non-heat-inactivated prepandemic negative saliva samples used in this study, for 6 out of the 11 antigens displayed in Fig. 4. The results of the sensitivity analysis showed that while the cutoff value changed after heat inactivation for all six antigens, this resulted in only minimal changes in the sensitivity and specificity of some antigens and did not alter the overall positive/negative classification based on the applied algorithm. Using cutoff values derived from heat-inactivated prepandemic negative saliva samples, the specificity of the N protein, for example, decreased from 99% to 98%, whereas the specificity for Sino Biological RBD increased from 98% to 99%, and the sensitivity at >14 DPSO decreased from 100% to 96%, whereas the sensitivity for Sino ECD increased from 96% to 100%. Because none of the negative pre-and postpandemic saliva samples showed IgG binding to both N and any of the RBD/spike antigens, and all of the >14 DPSO samples were reactive to N and at least one of the RBD/spike antigens, our algorithm maintained 100% sensitivity and 100% specificity at 14 DPSO after heat inactivation. Thus, the results of this sensitivity analysis suggest that our overall findings are not impacted by applying cutoff values derived from non-heat-inactivated prepandemic negative saliva samples to heat-inactivated pandemic samples.

This study has several limitations. Our sample set was not large, especially at early time points for saliva, and did not include longitudinal saliva specimens from the same donor, limiting the robustness of our findings in saliva. Such samples will be increasingly available and will increase statistical power for the analyses presented here. Future studies should improve on the robustness of these findings by including a larger sample size at all time points and longitudinal saliva specimens from the same donor. Additional investigation with late-convalescent-phase saliva and sera will be important

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to determine the durability of SARS-CoV-2-specific IgG responses. Well-characterized sera from other hCoV and zoonotic CoV infections would be useful to more fully address the potential cross-reactivity of antibodies following SARS-CoV-1, Middle East respiratory syndrome CoV (MERS-CoV), hCoV-OC43, hCoV-HKU1, hCoV-229E, and hCoV-NL63 infection with SARS-CoV-2 proteins. We did not have uniform information on the severity of disease for COVID-19 cases included in this study, which may impact the quality or kinetics of antibody responses (18). Previous studies suggest that antibody responses are elevated among individuals with severe infection (18, 19, 29). Similarly, comprehensive sociodemographic data were not available, precluding any investigation of potential effects of age, sex, and other factors on antibody responses. We did not determine receiver operating characteristic (ROC)-optimized MFI cutoffs in this analysis. However, the cutoffs used in this study (average for negative samples plus 3 standard deviations) are conservative. Future analyses should identify ROC-optimized cutoffs, which could refine the reported sensitivity and specificity of this saliva assay. Additional replicates should also be used to assess intra- and interassay variability and determine a lower limit of detection for each antigen. Finally, because of sample availability, the specimen sets used in the serum and saliva assays are not all paired specimens from the same subject, and a direct comparison of our serum versus salivary assays is not possible. However, our data from testing the available paired specimens show a good correlation of IgG responses in serum and saliva. Thus, the data shown here represent a proof of principle that antibody testing from saliva specimens performs promisingly and warrants further study and development. A logical next step would be to perform a head-to-head comparison of this novel saliva assay with other antibody tests approved for clinical use.

In summary, we demonstrate that SARS-CoV-2 antigen-specific antibody responses in saliva reflect those observed in serum and that salivary SARS-CoV-2 antigen-specific IgG can be used to accurately detect prior SARS-CoV-2 infection. We have developed and validated a saliva-based multiplex immunoassay and identified SARS-CoV-2-specific IgG responses that can detect prior SARS-CoV-2 infection with high sensitivity (anti-N IgG [100% sensitivity and 99% specificity]) and specificity (anti-RBD IgG [93% sensitivity and 100% specificity]) at ≥10 DPSO. An accurate saliva-based antibody test for prior SARS-CoV-2 infection would improve our ability to perform public health interventions in the current pandemic. This noninvasive method for comprehensive determination of prior SARS-CoV-2 infection will facilitate large-scale serosurveillance to evaluate population seropositivity. As SARS-CoV-2 vaccine candidates progress through clinical trials, such noninvasive tests will be critical to identify potential immunity gaps and susceptible populations to inform targeted vaccination efforts as well as companion diagnostics for vaccine trials (30). Furthermore, saliva assays can be used to monitor correlates of protection and the force of transmission in community-based settings, before and after the implementation of vaccination/prevention strategies, to determine the effectiveness of population-based interventions and direct future preventative strategies.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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input on antigen selection, assay design, and interpretation of results. Y.C.M. and D.L.T. provided input on study design and interpretation of results. B.D. provided input on interpretation of results. W.A.C., O.L., P.P.C., and H.B.L. shared samples and data for the analysis and provided input on interpretation of results. M.H.C. developed the project concept. M.H.C., N.R., J.K.F., A.C.S., and S.E. led and coordinated specimen collection efforts and reviewed and edited the manuscript. C.D.H. developed the project concept and guided the laboratory work.

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