Sensitive Serology Measurements in the Saliva of Individuals with COVID-19 Symptoms Using a Multiplexed Immunoassay

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Background: There are numerous benefits to performing salivary serology measurements for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative pathogen for coronavirus disease 2019 (COVID-19). Here, we used a sensitive multiplex serology assay to quantitate salivary IgG against 4 SARS-CoV-2 antigens: nucleocapsid, receptor-binding domain, spike, and N-terminal domain.

Methods: We used single samples from 90 individuals with COVID-19 diagnosis collected at 0 to 42 days postsymptom onset (PSO) and from 15 uninfected control subjects. The infected individuals were segmented in 4 groups (0–7 days, 8–14 days, 15–21 days, and >21 days) based on days PSO, and values were compared to controls.

Results: Compared to controls, infected individuals showed higher levels of antibodies against all antigens starting from 8 days PSO. When applying cut-offs with at least 93.3% specificity at every time interval segment, nucleocapsid protein serology had the best sensitivity at 0 to 7 days PSO (60% sensitivity [35.75% to 80.18%], ROC area under the curve [AUC] = 0.73, $P = 0.034$). Receptor-binding domain serology had the best sensitivity at 8 to 14 days PSO (83.33% sensitivity [66.44%–92.66%], ROC AUC = 0.90, $P < 0.0001$), and all assays except for N-terminal domain had 92% sensitivity (75.03%–98.58%) at >14 days PSO.

Conclusions: This study shows that our multiplexed immunoassay can distinguish infected from uninfected individuals and reliably (93.3% specificity) detect seroconversion (in 60% of infected individuals) as early as the first week PSO, using easy-to-collect saliva samples.

INTRODUCTION

Given the continued health and socioeconomic impact of the coronavirus disease 2019 (COVID-19) pandemic, determining its seroepidemiological characteristics is of paramount importance for understanding seropositivity rates against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and guiding appropriate resource management. In addition to tracking disease

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IMPACT STATEMENT

This study shows the sensitivity and specificity of salivary serology measurements for severe acute respiratory syndrome coronavirus 2 using a multiplexed immunoassay. Given the benefits of saliva as a biofluid for analyte quantitation (e.g., ease of sampling and processing) coupled with the automated fashion of our multiplexed technology, this assay can be used for population-wide seropositivity testing in severe acute respiratory syndrome coronavirus 2 infection.

incidence and prevalence at population scale, antibody testing can be used to model disease transmission, screen asymptomatic infections, assess vaccine responsiveness and durability of antibody production post-vaccination or -natural infection (1–8). When there is evident correlation between antibody levels and protection, serology can also be used to identify individuals at higher risk of reinfection and therefore inform vaccine prioritization strategies as needed (9).

The utility of serosurveillance is especially evident as infection with SARS-CoV-2 results in the development of serum antibodies in 90% of infected individuals within the first 2 weeks post-symptom onset (PSO) (10–13). Although serum is the most studied matrix for antibody detection, venous blood sampling presents several financial and logistical limitations to population-scale surveying. On the other hand, saliva is a noninvasive alternative with potential for self-collection that circumvents the majority of challenges present with serum testing. Since antibody concentration in saliva is several orders of magnitude lower than in serum, assays would require high analytical sensitivity (11, 14, 15). Nevertheless, the efficacy of saliva-based serology testing has been demonstrated for the surveillance and diagnosis of other pathogens (16–18).

Salivary antibodies are derived from the blood pool of IgG that can leak into the saliva via the gingival crevicular fluid or are produced locally by the salivary glands (19, 20). The potential for saliva as a biofluid for anti-SARS-CoV-2 antibody sampling is supported by the strong correlation observed in antibody responses measured in serum and saliva during and post- infection (7, 11, 21–23). Antibodies to SARS-CoV-2 have been detected in self-collected saliva specimens transported without refrigeration, viral inactivation, or preservatives (24). Despite the acknowledged need for saliva-based serology tests for SARS-CoV-2, none are yet commercially available.

In this study, we used a hypersensitive multiplex assay to quantitate anti-SARS-CoV-2 antibodies in saliva. Specifically, we measured IgG reactive to 4 SARS-CoV-2 antigens: nucleocapsid, receptor-binding domain (RBD), spike, and N-terminal domain (NTD). Samples from individuals with COVID-19 diagnosis collected at 0 to 42 days PSO were segmented in 4 groups (0–7 days, 8–14 days, 15–21 days, and >21 days) and compared to individuals without COVID-19. The diagnostic performance of every assay at selected time intervals PSO was also assessed. These observations represent natural immune responses as they were measured before the rollout of vaccines for COVID-19.

MATERIALS AND METHODS

Sample Collection

A total of 105 retrospectively collected saliva specimens (collected from March 2020–September

2020, prior to the broad availability of SARS-CoV-2 vaccines) were obtained under approval of the Sinai Health System Research Ethics Board (REB no. 02-0118 U). Of these, 15 samples were collected from non-COVID-19 patients (no prior COVID-19 diagnosis and negative SARS-CoV-2 PCR test) and 90 from patients who were diagnosed with COVID-19 by clinical nasopharyngeal swab or midturbinate nasal swab-based PCR at a network of hospitals including Sinai Health System, Sunnybrook Health Centre, North York General Hospital, and Michael Garron General Hospital (Toronto, Canada). Samples were randomly collected at different time points PSO (ranging from 0–42 days), or at other disease diagnosis for the non-COVID-19 samples.

Patients were asked under informed consent to spit into a sterile 50 mL-specimen container (2.5 mL of saliva), which was topped with 2.5 mL of phosphate-buffered saline. Samples were transported to the microbiology laboratory at Sinai Health System, where they were treated with 1% Triton X 100 at room temperature for 1 h before freezing at −80°C, within 8 h from time of collection, using a standardized protocol. Samples were stored for 2 to 8 months (median = 7 months, interquartile range = 2 months). Prior to processing, all samples were subsequently thermally inactivated by incubation at 65°C for 30 min.

Serology Measurements

Sample preparation has been previously described (25). Serology measurements were collected using kits and reagents that are commercially available from Meso Scale Discovery® (MSD). On the day of sample testing, saliva was thawed at room temperature and centrifuged briefly to pull down any food particles or mucus. To assess sample quality, samples were visually verified to be saliva and not predominantly phlegm or mucus.

Prior to analysis, saliva samples were further diluted 5-fold with sample diluent (MSD® Diluent 2). Samples were assayed in a 96-well plate format using MSD V-PLEX® COVID-19 Coronavirus Panel 2 kits for measuring IgG antibody responses (K15369U). Each well of the plates included an antigen array that enabled the multiplexed measurement of antibody responses against 9 different coronavirus antigens as well as BSA as a negative control. These included 4 SARS-CoV-2 antigens (nucleocapsid, spike, RBD, and NTD) and spike proteins from 5 other coronaviruses (SARS-CoV-1 and endemic coronaviruses 229E, HKU1, NL63, and OC43). Assay protocols were run according to the manufacturer's protocol except for the use of sample diluents and dilution factors (as described previously) that were optimized for saliva.

For quantitation of antibody responses, an 8-point calibration curve was run in duplicate on all plates and the signals for each antigen reactivity were fit to a 1/Y2-weighted 4-parameter logistic curve. Samples were run in duplicate, and the antibody concentration against each antigen was interpolated from the 4-parameter logistic curve and corrected for dilution. The concentrations were presented in arbitrary intensity units per mL (IU/mL) that were defined relative to the assigned values of the reference standard.

Measurement of Total Antibody Levels

Total levels of IgG, IgM, and IgA immunoglobulin in saliva were measured using MSD's Isotyping Panel 1 Human/NHP Kit (K15203D) according to the manufacturer's directions. Samples were diluted 1000-fold, and levels are reported in μg/mL. Calibration and quantitation were carried out as described in the serology measurements section.

Statistical Analysis

Data were processed in Excel. Further data transformation, statistical analyses, and data visualization were performed in Prism (GraphPad). For every assay, concentrations below the lower limit of detection were set to the lower limit of

detection, defined as the concentration resulting in a signal 2.5 DS above the assay background. Concentrations above the upper limit of quantitation were set to the values of the top calibrator. The infected subjects were segmented in 4 groups (0–7 days, 8–14 days, 15–21 days, and >21 days) based on the time interval PSO. Differences between time intervals PSO and controls were analyzed with a Kruskal–Wallis test, followed by pairwise multiple comparisons using Dunn test. Differences in the total levels of IgG between time intervals PSO and controls were assessed with a Brown–Forsythe and Welch ANOVA test followed by multiple comparisons using the Dunnett test. For all analyses, statistical significance was set to $P < 0.05$.

Diagnostic Performance and Determination of Cut-off Values

A ROC curve analysis was performed to evaluate percent sensitivity and specificity of anti-SARS-CoV-2 serology measurements at various cut-off values for every time interval PSO. Moreover, the ROC area under the curve (AUC) was used to assess the assay's overall accuracy in classifying seropositive subjects from controls. Finally, the ROC curve was used to select the optimum cut-off concentration for every assay at selected time-interval segments (0–7, 8–14, >14 days, all time points combined), based on the following criteria: (*a*) at least 93% specificity and the maximum identifiable likelihood ratio were achieved; (*b*) cut-off values were the same (or approximately the same, with less than 15% variation) across all time-interval segments for the same assay. By applying the first criterion (*a*), the candidate cut-off values were the same across all time points and resulted in the highest sensitivity value. Thus, for every assay we achieved the maximum Youden's index (Specificity + Sensitivity −1) for our given specificity, which is a common method for selecting cut-off points (26, 27). Spike was an exception, as 2 cut-off values were determined, although with less than 15% difference between time intervals (see results). Percent specificity and sensitivity values are reported with 95% CI values; IU/mL values were converted to standard WHO values for nucleocapsid, spike, and RBD proteins by multiplying the IU/mL values with their conversion factor (nucleocapsid = 0.00236, spike = 0.00901, RBD = 0.02720), as previously established (28). Statistical significance was set to $P < 0.05$.

RESULTS

Relevant Time Points of Detectable Immunity against SARS-CoV-2 Antigens

Salivary IgG levels reactive to four SARS-CoV-2 antigens (nucleocapsid, spike, RBD, and NTD) for noninfected and infected individuals are shown in Fig. 1. Overall, IgG levels rose significantly beyond 8 to 14 days PSO as compared to uninfected controls. The most significant difference between controls and 8 to 14 days PSO occurred with anti-RBD levels ($P = 0.008$) (Fig. 1, B), followed by anti-spike levels ($P = 0.0010$) (Fig. 1, C), anti-nucleocapsid levels ($P = 0.0022$) (Fig. 1, A), and, lastly, anti-NTD levels ($P = 0.0341$) (Fig. 1, D). The trend of increasing antibody levels continued throughout the time window of our study (up to 42 days PSO) for all antigens, and such levels were significantly higher compared to controls.

Diagnostic Performance

The diagnostic performance for every assay at every time interval was determined by the assay's efficacy to classify seropositive subjects from noninfected controls based on the ROC curve analysis and the AUC metric. Specifically, we constructed ROC curves and identified the AUC and associated *P*-value for every SARS-CoV-2 marker at every time interval (0–7 days, 8–14 days, 15–21 days, and >21 days) (Fig. 2). As early as 7 days PSO, all assays

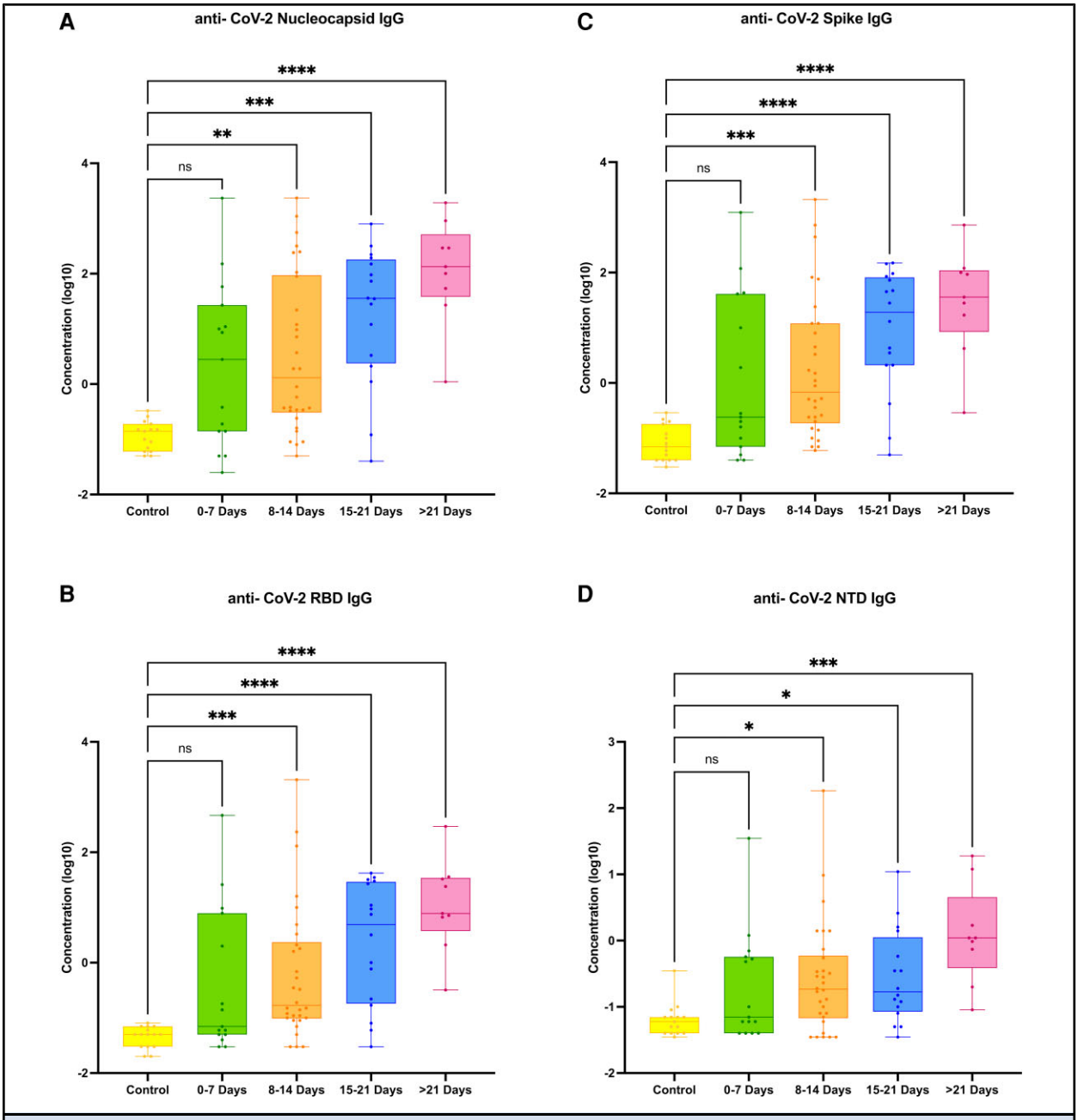

Fig. 1. Log-transformed concentration [intensity units/mL (IU/mL)] of IgG against SARS-CoV-2 (CoV-2): nucleocapsid (A), spike (B), RBD (C), and NTD (D) proteins as measured in uninfected individuals (control) and individuals infected with SARS-CoV-2 at various time points PSO (0–7, 8–14, 15–21, >21 days). *P-value < 0.05, **P-value < 0.01, ***P-value < 0.001, **** P-value < 0.0001.

except for NTD distinguished significantly (albeit moderately) infected from uninfected individuals ($AUC_{Nucleocapsid} = 0.73$, $P = 0.034$; $AUC_{Spike} = 0.76$, $P = 0.017$; $AUC_{RBD} = 0.74$, $P = 0.03$; $AUC_{NTD} = 0.66$, $P = 0.14$). From 8 days PSO onwards, all assays distinguished infected from uninfected controls, and

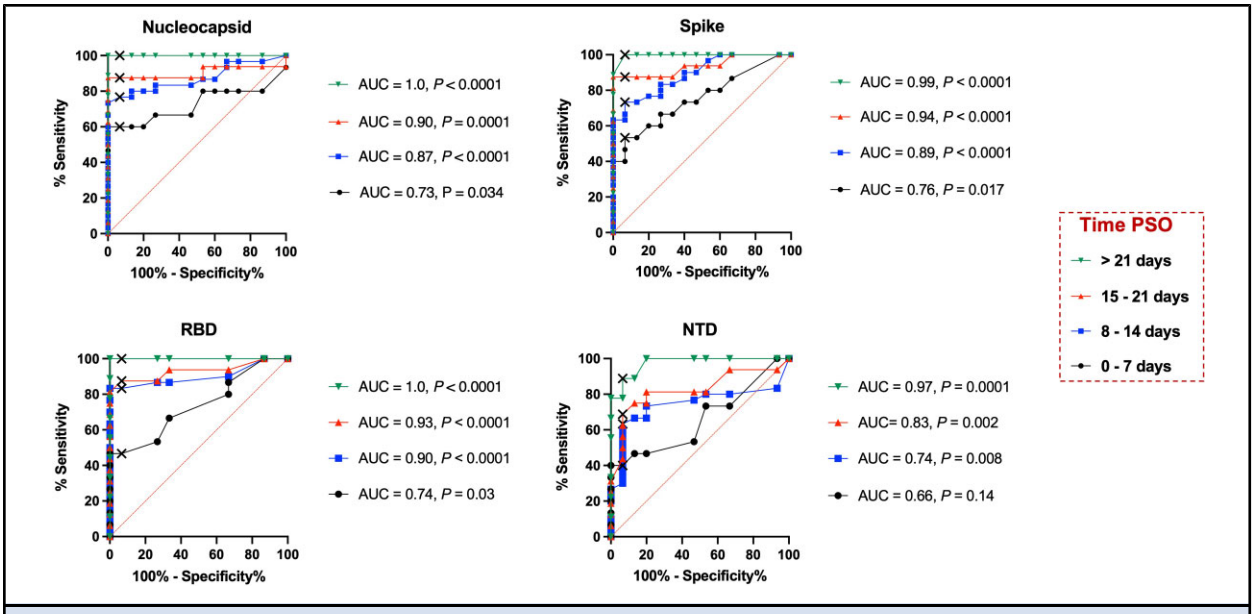

Fig. 2. Diagnostic performance of serology measurements against SARS-CoV-2 proteins at various time points PSO. ROC curves along with AUC and *P*-values indicate efficacy of serology measurements in distinguishing seropositive subjects from noninfected controls. Symbol *x* indicates cut-off thresholds as reported in Table 1.

this performance improved as time PSO advanced (Fig. 2; 8–14 days, 15–21 days, > 21 days PSO intervals).

Determination of Cut-off Points

ROC curves were also used to determine time point-specific percent sensitivity, specificity, and likelihood ratio profiles at various cut-offs. The same cut-off threshold was applied to all time intervals PSO except for spike, for which we derived 2 thresholds (see later discussion). Table 1 shows percent sensitivity (and related likelihood ratios) of serological measurements for each SARS-CoV-2 antigen at selected time intervals PSO (0–7 days, 8–14 days, >14 days, and all time points combined), with at least 93% specificity (see methods). More specifically, the cut-off for nucleocapsid was 0.295 IU/mL (6.962×10^{-4} WHO IU/mL [same across all time intervals]); for spike, 0.23 IU/mL (2.0723×10^{-3} WHO IU/mL [for time intervals 0–7, 8–14 days, and all time points combined]) and 0.26 IU/mL (5.388×10^{-3} WHO IU/mL [for time intervals >14 days]); for RBD, 0.075 IU/mL (2.04×10^{-3} WHO IU/mL [same across all time intervals]); and for NTD, 0.11 IU/mL (no WHO equivalent available [same across all time intervals]).

At the classification cut-points, IgG against SARS-CoV-2 nucleocapsid protein can identify 60% of the infected individuals by 7 days PSO, followed by IgG against spike protein (53.33%). By 14 days, IgG against RBD can identify 83.33% of infected individuals, followed by nucleocapsid (76.67%) and spike protein (73.33%). After 14 days PSO, IgG against nucleocapsid, spike, and RBD proteins have 92% sensitivity (75.03%–98.58%). All time-point sensitivity was highest for nucleocapsid and RBD (78.57% [67.61%–86.56%], likelihood ratio = 11.79), followed by spike (75.71% [64.50%–84.25%], likelihood ratio = 11.36) (Table 1). Sensitivity of NTD

Table 1. Percent (%) sensitivity (with 95% CI and likelihood ratio) for serology measurements against SARS-CoV-2 proteins at selected time intervals PSO.

SARS-CoV-2 protein target [cut-off value]	Diagnostic metric	0–7 days (n = 15)	8–14 days (n = 30)	>14 days (n = 25)	All time points (n = 70)
Nucleocapsid	% Sensitivity	60.00	76.67	92	78.57
[0.295 IU/mL]	95% CI	35.75–80.18	59.07–88.21	75.03–98.58	67.61–86.56
	Likelihood Ratio	9.00	11.50	13.80	11.79
Spike	% Sensitivity	53.33	73.33	92	75.71
[0.23 IU/mL]	95% CI	30.12–75.19	55.55–85.82	75.03–98.58	64.50–84.25
[0.26 IU/mL]	Likelihood Ratio	8.00	11.00	13.80	11.36
RBD	% Sensitivity	46.67	83.33	92	78.57
[0.075 IU/mL]	95% CI	24.81–69.88	66.44–92.66	75.03–98.58	67.61–86.56
	Likelihood Ratio	7.00	12.50	13.80	11.79
NTD	% Sensitivity	40.00	63.33	76	62.86
[0.11 IU/mL]	95% CI	19.82–64.25	45.51–78.13	56.57–88.50	51.15–73.23
	Likelihood Ratio	6.00	9.50	11.40	9.429

Specificity is at least 93.3% (70.2%–99.7%). N refers to number of samples in the SARS-CoV-2-infected groups. For all assays, the same cut-off value was applied to all time intervals PSO except for spike, for which 0.23 IU/mL was applied for time intervals <14 days as well as for all time points column, whereas 0.26 IU/mL was applied to time interval >14 days PSO. For WHO IU/mL equivalents see results section.

serology was overall 18.7% lower compared to the other 3 proteins combined.

Immunity against SARS-CoV-2 and Other Coronaviruses in Control Subjects

Control subjects (who were not diagnosed with COVID-19) had high levels of antibodies to the 4 pre-COVID-19 endemic coronaviruses 229E, HKU1, NL63, and OC43 compared to IgG against SARS-CoV-2, further confirming absence of IgG against SARS-CoV-2 (Fig. 3). As expected, in this group anti-SARS-CoV-2 and SARS-CoV-1 IgG levels were multiple log values lower than IgG against other circulating coronaviruses. Specifically, control samples showed significantly higher levels of antibodies to endemic viruses compared to all SARS-CoV-2 antigens (Fig. 3 shows statistical differences only against SARS-CoV-2 spike [229E: $P = 0.0001$; HKU1: $P < 0.0001$; NL63: $P = 0.0004$; OC43: $P < 0.0001$]), but there was no significant difference between levels of IgG against SARS-CoV-1 and SARS-CoV-2 spike ($P > 0.9999$).

Quality of Samples

As previously established (24), we found that saliva samples had expected levels of immunoglobulins to indicate sample integrity and to conclude that the detected salivary antibodies did not result from oral bleeding. Median levels of total salivary IgG were 3.4 µg/mL, 1.9 µg/mL, 1.8 µg/mL, 3.4 µg/mL, and 4.2 µg/mL for subjects in the control, 0 to 7 days, 8 to 14 days, 15 to 21 days, and >21 days groups, respectively. These concentrations were within the range of total salivary IgG published previously (0.4–93 µg/mL) (29) and similar to the median value reported for self-collected saliva (9.92 µg/mL). Also, total salivary IgG levels were statistically similar between all groups (Brown–Forsythe and Welch ANOVA $P = 0.1494$).

DISCUSSION

In this study, we quantified the levels of salivary IgG against 4 proteins (full length or domains) of

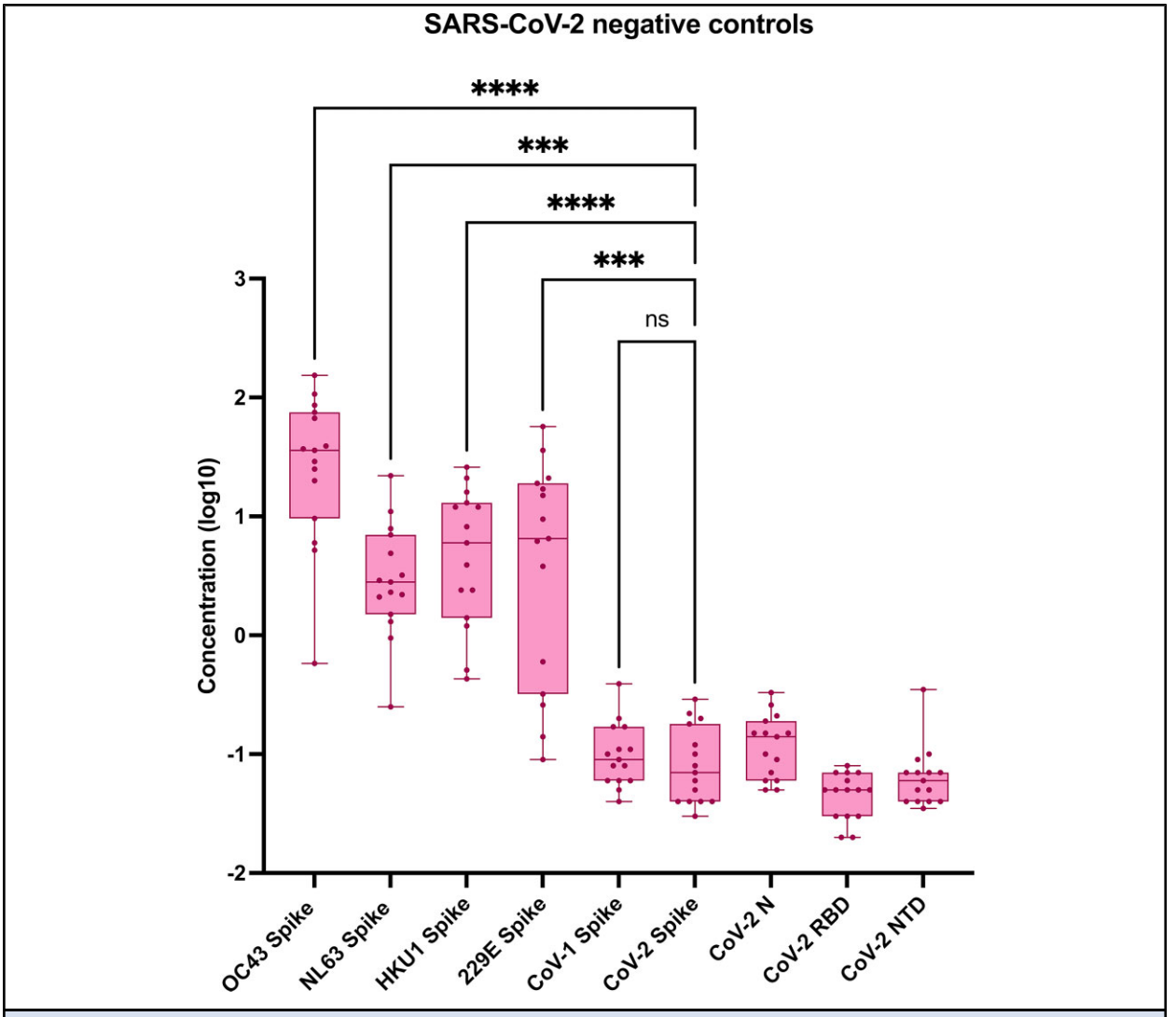

Fig. 3. Absence of antibodies against SARS-CoV-2 proteins in uninfected controls. Log- transformed concentrations of IgG against SARS CoV-2 antigens (nucleocapsid, RBD, spike, NTD, SARS-CoV-1, and NL63-, HKU1-, 229E-, and OC43- Spike protein collected from SARS-CoV-2 negative individuals). ******P*-value < 0.001, ** *P*-value < 0.0001.**

SARS-CoV-2 (nucleocapsid, spike, RBD, and NTD) in a cohort of infected individuals and uninfected controls using an ultrasensitive multiplexed immunoassay. This assay can detect antibodies against nucleocapsid, NTD, RBD, and spike as low as 0.046 IU/mL (1.09×10^{-4} WHO IU/mL), 0.003 IU/mL (no WHO equivalent), 0.035 IU/mL (9.52×10^{-4} WHO IU/mL), and 0.049 IU/mL (4.41×10^{-4} WHO IU/mL), respectively (28). There are multiple benefits to using saliva to establish seropositivity in SARS-CoV-2 infection, including noninvasive and straightforward sampling and processing, and specimen stability at physiological temperatures (24) and freezing-temperature (−20°/−80°C) conditions (30, 31). These, coupled with the use of a quantitative multiplexed

immunoassay that can be automated, provide unique benefits for population-wide seropositivity testing.

It has been previously shown that saliva and blood serology measurements for SARS-CoV-2 correlate (7, 11, 21, 22, 29, 32). Most recently, we showed improved sensitivity of salivary serological testing in a cohort of self-reported infected individuals using a multiplexed immunoassay (24). Here we extend these observations by establishing the clinical sensitivity and specificity of our assay at relevant time points of COVID-19. Using the same multiplexed assay, the classification cut-offs for nucleocapsid, spike, and RBD were established previously as 3.2 IU/mL, 0.96 IU/mL, and 0.24 IU/mL, respectively, to encompass 98% of control values (24). Here, the classification cut-off values for nucleocapsid, spike, and RBD at 0 to 7 and 8 to 14 days PSO were 0.295 IU/mL (6.962×10^{-4} WHO IU/mL), 0.23 IU/mL (2.0723×10^{-3} WHO IU/mL), and 0.075 IU/mL (2.04×10^{-3} WHO IU/mL), which were orders of magnitudes lower than previously established (24), and, while they encompass 93.33% of control samples, they exhibit over 77% of sensitivity (ranging from 73.33% [spike] to 83.33% [RBD]). The thresholds identified here may be lower than previously measured due to the differences in saliva collection and processing. Samples were diluted (2×) with PBS containing Triton and heat inactivated, which has been shown to lower detectable antibody levels (7, 11). Moreover, here the samples were stored in the freezer (−80°C) for a longer duration, which may have resulted in some IgG degradation (31). These findings emphasize the notion that classification thresholds should be established based on the collection method and study design, although such practice contributes to well-known challenges around comparing seropositivity findings across various studies (33).

When considering all time intervals PSO, we conclude that the best performing serology markers were for nucleocapsid and RBD proteins, whereas NTD serology had the lowest diagnostic value. Serology against nucleocapsid protein can identify 60% of symptomatic individuals as early as up to 7 days PSO. IgG against RBD can identify 83.33% of symptomatic patients within 2 weeks PSO. After 2 weeks, the diagnostic value of nucleocapsid, spike, and RBD serology is the same, identifying 92% of symptomatic individuals. Taken together, these observations suggest that salivary serology measurements against nucleocapsid, RBD, and spike proteins can provide the highest diagnostic value in the assessment of seropositivity for SARS-CoV-2, followed by anti-NTD IgG measurements.

The diagnostic performance of our assay in saliva vs serum remains to be established. However, based on previous studies using comparable experimental design but in serum samples (i.e., same multiplexed technology for the same SARS-CoV-2 proteins, assessed during similar disease time frames), showed overall higher sensitivity than our current salivary serology assay (34, 35). Notably, in the study by Nandakumar et al. at time points < 7 days post-PCR testing, the sensitivity of serum serology was similar to our saliva serology, with values ranging between 37.8% and 56.8% (at 99.5% specificity) (34). In the study by Johnson et al., the sensitivity of serum serology for spike, RBD, and nucleocapsid at 0 to 7 days PSO was higher than the present study, with values ranging from 60% to 75% (at 92%–97% specificity) (35). Taken together, these studies suggest that overall serum serology for SARS-CoV-2 proteins might have improved clinical sensitivity compared to saliva, but the assay's sensitivity depends on the timing of sample collection relative to disease onset. Furthermore, a direct comparison of the multiplexed assays in serum vs saliva using the same samples is needed to accurately establish the assay's diagnostic performance in both biofluids.

Some limitations are important to mention. The first limitation of our study is the relatively small sample size and incomplete annotation of the samples. Additionally, we cannot directly confirm

that the infected individuals are seropositive for anti-SARS-CoV-2 antibodies. Of note, cut-off thresholds were set based on controls who were noninfected (PCR result negative) and had no antibodies against SARS-CoV-2 (levels of saliva antibodies against SARS-CoV-2 proteins were multiple log values lower than antibodies against common cold coronaviruses; Fig. 3). As such, for determining the diagnostic value of our assays based on ROC-identified classification cut-offs, a fundamental assumption was that all SARS-CoV-2 individuals developed antibodies against the virus, which is an overestimation. Indeed, it has been shown that seroconversion occurs in 90% of infected persons within 2 weeks PSO (10–13). Therefore, assuming the observations in the current study are representative of the larger population, we can expect that the true sensitivity profile of our assay will improve in larger cohorts. Another limitation is that the relatively long duration of sample storage prior to quantitation may have influenced the detectable levels of antibodies. While saliva is relatively stable in freezing temperatures, analyzing samples after 3 months of storage for the detection of IgG antibodies is not recommended (31). Of note, at the time of sample collection (March–September 2020), the incidence of COVID-19 was relatively low and access to SARS-CoV-2-positive samples had logistical challenges, resulting in longer sample storage. However, given that the total IgG concentration was within the expected range, IgG degradation (if any) due to extended sample storage was minimal. Additionally, because the collection of control samples was concurrent to COVID-19-positive samples, it is expected that any storage effects were normalized across all study groups.

When interpreting the results of this study, the timing of sample collection relative to the COVID-19 pandemic is important. During that time (March–September 2020), the disease prevalence was low in most parts of the world, and vaccines were unavailable. For example, in Canada, cumulatively 4200/1 million of population cases were reported by the end of September 2020, compared to the current cumulative total of 105 000/1 million cases (June 2022) (36). Therefore, the uninfected controls in the present study were truly seronegative, and the responses in the infected individuals represent natural humoral responses, most likely primary. However, as of the writing of this article (June 2022), not only has the total cumulative incidence of COVID-19 increased, but also most of the world's population has received at least one COVID-19 vaccine dose (36). Relatedly, the current serologic profile against SARS-CoV-2 at a population-wide scale is more complex compared to the time of sample collection, consisting of antibodies that represent, in the simplest scenario (37, 38), (*a*) natural primary or secondary humoral responses following first or subsequent infection, (*b*) vaccine-induced humoral responses, (*c*) a combination of the previous, or (*d*) no detectable antibodies due to waning of immunity or absence of infection/vaccination. Thus the IgG concentrations reported in this study are relevant to the timing of sample collection and should not be used as an absolute benchmark to classify seropositivity in the post-vaccination phase of the pandemic.

Notwithstanding, the present study presents important strengths. Specifically, we show the successful application of a multiplexed technology for the quantitation of antibodies against 4 common SARS-CoV-2 proteins, using easy-to-collect saliva samples from symptomatic PCR-confirmed subjects. We further characterize the diagnostic performance of the salivary serology assays at clinically relevant time intervals from symptom onset, illustrating that clinical sensitivity and accuracy change during the disease course. Lastly, to establish accurate cut-off points, special care was taken to select negative controls that were not infected by SARS-CoV-2 and lacked antibodies against the 4 SARS-CoV-2 proteins.

In conclusion, we show the high sensitivity and specificity profile of salivary SARS-CoV-2 serology in symptomatic individuals, based on the detection of IgG against nucleocapsid, spike, RBD, and NTD proteins, using a multiplexed immunoassay. We conclude that IgG against nucleocapsid, RBD, and spike proteins have the highest diagnostic value by detecting as many as 83.33% (24.81%–92.66%) and 92% (75.03%–98.58%) of infected individuals within the first 2 weeks from symptom onset and thereafter, respectively.

Nonstandard Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; PSO, post-symptom onset; RBD, receptor-binding domain; NTD, N-terminal domain; MSD, Meso Scale Discovery®; IU/mL, intensity units per mL; AUC, area under the curve.

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REFERENCES

1. Assadiasl S, Fatahi Y, Zavvar M, Nicknam MH. COVID-19: significance of antibodies. Hum Antibodies 2020;28:287–97.
2. Dobaño C, Alonso S, Fernández de Sevilla M, Vidal M, Jiménez A, Pons Tomas G, et al. Antibody conversion rates to SARS-CoV-2 in saliva from children attending summer schools in Barcelona, Spain. BMC Med 2021;19:309.
3. Earle KA, Ambrosino DM, Fiore-Gartland A, Goldblatt D, Gilbert PB, Siber GR, et al. Evidence for antibody as a protective correlate for COVID-19 vaccines. Vaccine 2021;39:4423–8.
4. Li K, Huang B, Wu M, Zhong A, Li L, Cai Y, et al. Dynamic changes in anti-SARS-CoV-2 antibodies during SARS-CoV-2 infection and recovery from COVID-19. Nat Commun 2020;11:6044.
5. Peeling RW, Wedderburn CJ, Garcia PJ, Boeras D, Fongwen N, Nkengasong J, et al. Serology testing in the COVID-19 pandemic response. Lancet Infect Dis 2020;20:e245–9.
6. Petherick A. Developing antibody tests for SARS-CoV-2. Lancet 2020;395:1101–2.
7. Pisanic N, Randad PR, Kruczynski K, Manabe YC, Thomas DL, Pekosz A, et al. COVID-19 serology at population scale: SARS-CoV-2-specific antibody responses in saliva. J Clin Microbiol 2020;59:e02204-20.
8. West R, Kobokovich A, Connell N, Gronvall GK. COVID-19 Antibody tests: a valuable public health tool with limited relevance to individuals. Trends Microbiol 2021;29:214–23.
9. Bubar KM, Reinholt K, Kissler SM, Lipsitch M, Cobey S, Grad YH, et al. Model-informed COVID-19 vaccine prioritization strategies by age and serostatus. Science 2021;371:916–21.
10. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, et al. A serological assay to detect SARS-CoV-2 seroconversion in humans. Nat Med 2020;26:1033–6.
11. Isho B, Abe KT, Zuo M, Jamal AJ, Rathod B, Wang JH, et al. Persistence of serum and saliva antibody responses to SARS-CoV-2 spike antigens in COVID-19 patients. Sci Immunol 2020;5:eabe5511.
12. Long Q-X, Liu B-Z, Deng H-J, Wu G-C, Deng K, Chen Y-K, et al. Antibody responses to SARS-CoV-2 in patients with COVID-19. Nat Med 2020;26:845–8.

13. Premkumar L, Segovia-Chumbez B, Jadi R, Martinez DR, Raut R, Markmann A, et al. The receptor binding domain of the viral spike protein is an immunodominant and highly specific target of antibodies in SARS-CoV-2 patients. Sci Immunol 2020;5:eabc8413.
14. Li X, Pomares C, Peyron F, Press CJ, Ramirez R, Geraldine G, et al. Plasmonic gold chips for the diagnosis of toxoplasma gondii, CMV, and rubella infections using saliva with serum detection precision. Eur J Clin Microbiol Infect Dis 2019;38:883–90.
15. Liu T, Hsiung J, Zhao S, Kost J, Sreedhar D, Hanson CV, et al. Quantification of antibody avidities and accurate detection of SARS-CoV-2 antibodies in serum and saliva on plasmonic substrates. Nat Biomed Eng 2020;4: 1188–96.
16. Estévez PT, Satoguina J, Nwakanma DC, West S, Conway DJ, Drakeley CJ. Human saliva as a source of anti-malarial antibodies to examine population exposure to plasmodium falciparum. Malar J 2011;10:104.
17. Granade TC, Phillips SK, Parekh B, Gomez P, Kitson-Piggott W, Oleander H, et al. Detection of antibodies to human immunodeficiency virus type 1 in oral fluids: a large-scale evaluation of immunoassay performance. Clin Diagn Lab Immunol 1998;5: 171–5.
18. Vohra P, Belkhode V, Nimonkar S, Potdar S, Bhanot R, Izna, et al. Evaluation and diagnostic usefulness of saliva for detection of HIV antibodies: a cross-sectional study. J Fam Med Prim Care 2020;9:2437–41.
19. Brandtzaeg P. Do salivary antibodies reliably reflect both mucosal and systemic immunity? Ann N Y Acad Sci 2007; 1098:288–311.
20. Sheikh-Mohamed S, Isho B, Chao GYC, Zuo M, Cohen C, Lustig Y, et al. Systemic and mucosal IgA responses are variably induced in response to SARS-CoV-2 mRNA vaccination and are associated with protection against subsequent infection. Mucosal Immunol 2022;15: 799–808.
21. Heaney CD, Pisanic N, Randad PR, Kruczynski K, Howard T, Zhu X, et al. Comparative performance of multiplex salivary and commercially available serologic assays to detect SARS-CoV-2 IgG and neutralization titers. J Clin Virol 2021;145:104997.
22. MacMullan MA, Ibrayeva A, Trettner K, Deming L, Das S, Tran F, et al. ELISA detection of SARS-CoV-2 antibodies in saliva. Sci Rep 2020;10:20818.
23. Sullivan PS, Sailey C, Guest JL, Guarner J, Kelley C, Siegler AJ, et al. Detection of SARS-CoV-2 RNA and antibodies in diverse samples: protocol to validate the sufficiency of provider-observed, home-collected blood, saliva, and oropharyngeal samples. JMIR Public Health Surveill 2020; 6:e19054.
24. Campbell C, Padmanabhan N, Romero D, Joe J, Gebremeskel M, Manjula N, et al. Quantitative serology for SARS-CoV-2 using self-collected saliva and finger-stick blood. Sci Rep 2022;12:6560.
25. Campbell C, Roblin D, Padmanabhan N, Romero D, Joe J, Fathi L, et al. Saliva-based SARS-CoV-2 serology using at-home collection kits returned via mail. Sci Rep 2022;12: 14061.
26. Habibzadeh F, Habibzadeh P, Yadollahie M. On determining the most appropriate test cut-off value: the case of tests with continuous results. Biochem Medica 2016;26:297–307.
27. Hajian-Tilaki K. Receiver operating characteristic (ROC) curve analysis for medical diagnostic test evaluation. Casp J Intern Med 2013;4:627–35.
28. Meso Scale Discovery. V-PLEX COVID-19 serology kits. https://www.mesoscale.com/~/media/files/product%20inserts/v-plex%20covid-19%20serology%20assays%20insert.pdf (Accessed September 2022).
29. Riis JL, Bryce CI, Stebbins JL, Granger DA. Salivary total immunoglobulin G as a surrogate marker of oral immune activity in salivary bioscience research. Brain Behav Immun Health 2020;1:100014.
30. Chiappin S, Antonelli G, Gatti R, De Palo EF. Saliva specimen: a new laboratory tool for diagnostic and basic investigation. Clin Chim Acta 2007;383:30–40.
31. Schipper RG, Silletti E, Vingerhoeds MH. Saliva as research material: biochemical, physicochemical and practical aspects. Arch Oral Biol 2007;52:1114–35.
32. Faustini SE, Jossi SE, Perez-Toledo M, Shields AM, Allen JD, Watanabe Y, et al. Detection of antibodies to the SARS-CoV-2 spike glycoprotein in both serum and saliva enhances detection of infection. Preprint at https://www.medrxiv.org/content/10.1101/2020.06.16.20133025v1 (2020).
33. Chan Y, Fornace K, Wu L, Arnold BF, Priest JW, Martin DL, et al. Determining seropositivity—a review of approaches to define population seroprevalence when using multiplex bead assays to assess burden of tropical diseases. PLoS Negl Trop Dis 2021;15:e0009457.
34. Nandakumar V, Profaizer T, Lozier BK, Elgort MG, Rychert J, Slev PR, et al. Evaluation of a novel multiplex platform for simultaneous detection of IgG antibodies against the 4 main SARS-CoV-2 antigens. J Appl Lab Med 2022;7:698–710.
35. Johnson M, Wagstaffe HR, Gilmour KC, Mai AL, Lewis J, Hunt A, et al. Evaluation of a novel multiplexed assay for determining IgG levels and functional activity to SARS-CoV-2. J Clin Virol 2020;130:104572.
36. Ritchie H, Mathieu E, Rodés-Guirao L, Appel C, Giattino C, Ortiz-Ospina E, et al. Our World Data: Coronavirus pandemic (COVID-19). https://ourworldindata.org/coronavirus (Accessed September 2022).
37. Alejo JL, Mitchell J, Chang A, Chiang TPY, Massie AB, Segev DL, et al. Prevalence and durability of SARS-CoV-2 antibodies among unvaccinated US adults by history of COVID-19. JAMA 2022;327:1085–7.
38. Damluji AA, Christenson RH, deFilippi C. Clinical application of serologic testing for coronavirus disease 2019 in contemporary cardiovascular practice. J Am Heart Assoc 2021;10:e019506.