Analysis of SARS-CoV-2 Antibodies in COVID-19 Convalescent Plasma using a Coronavirus Antigen Microarray

Authors

Rafael R. de Assis (1)
Aarti Jain (1)
Rie Nakajima (1)
Algis Jasinskas (1)
Jiin Felgner (1)
Joshua M. Obiero (1)
Oluwasanmi Adenaiye (2)
Sheldon Tai (2)
Filbert Hong (2)
Philip Norris (3)
Mars Stone (3)
Graham Simmons (3)
Anil Bagri (4)
Martin Schreiber (5)
Andreas Buser (6)
Andreas Holbro (6)
Manuel Battegay (6)
Donald K. Milton (2)
Prometheus Study Group
Huw Davies (1)
Laurence M. Corash (4)
Michael P. Busch (3)
Philip L. Felgner (1)
Saahir Khan (8)*
*Corresponding Author: saahirk@uci.edu

Affiliations

1. Department of Physiology and Biophysics, School of Medicine, University of California Irvine, Irvine, CA
2. Institute for Applied Environmental Health, School of Public Health, University of Maryland, College Park, MD
3. Vitalant Research Institute, San Francisco, CA
4. Cerus Corporation, Concord, CA
5. Department of Surgery, Oregon Health & Science University, Portland, OR
6. Regional Blood Transfusion Service, Swiss Red Cross, University Hospital Basel, University of Basel, Basel, Switzerland
7. Division of Infectious Diseases & Hospital Epidemiology, University Hospital Basel, University of Basel, Basel, Switzerland
8. Division of Infectious Diseases, Department of Medicine, University of California Irvine Health, Orange, CA
Abstract

The current practice for diagnosis of COVID-19, based on SARS-CoV-2 PCR testing of pharyngeal or respiratory specimens in a symptomatic patient at high epidemiologic risk, likely underestimates the true prevalence of infection. Serologic methods can more accurately estimate the disease burden by detecting infections missed by the limited testing performed to date. Here, we describe the validation of a coronavirus antigen microarray containing immunologically significant antigens from SARS-CoV-2, in addition to SARS-CoV, MERS-CoV, common human coronavirus strains, and other common respiratory viruses. A comparison of antibody profiles detected on the array from control sera collected prior to the SARS-CoV-2 pandemic versus convalescent blood specimens from virologically confirmed COVID-19 cases demonstrates complete discrimination of these two groups. This array can be used as a diagnostic tool, as an epidemiologic tool to more accurately estimate the disease burden of COVID-19, and as a research tool to correlate antibody responses with clinical outcomes.
Background

COVID-19 caused by the SARS-CoV-2 virus is a worldwide pandemic with significant morbidity and mortality estimates from 1-4% of confirmed cases\(^1\). The current case definition for confirmed SARS-CoV-2 infection relies on PCR-positive pharyngeal or respiratory specimens, with testing largely determined by presence of fever or respiratory symptoms in an individual at high epidemiologic risk. However, this case definition likely underestimates true prevalence, as individuals who develop subclinical infection that does not produce fever or respiratory symptoms are unlikely to be tested, and testing by PCR of pharyngeal or respiratory specimens is only around 60-80% sensitive depending on sampling location and technique and the patient’s viral load\(^2\). Widespread testing within the United States is also severely limited by the lack of available testing kits and testing capacity limitations of available public and private laboratories. Therefore, the true prevalence of SARS-CoV-2 infection is likely much higher than currently reported case numbers would indicate.

Serology can play an important role in defining the true prevalence of COVID-19, particularly for subclinical infection\(^2\). Early studies of serology demonstrate high sensitivity to detect confirmed SARS-CoV-2 infection, with antibodies to virus detected approximately 1 to 2 weeks after symptom onset\(^3\). Unlike PCR positivity, SARS-CoV-2 antibodies are detectable throughout the disease course and persist indefinitely\(^4\). Multiple serologic tests have been developed for COVID-19\(^5\) including a recently FDA-approved lateral flow assay. However, these tests are limited to detection of antibodies against one or two antigens, and cross-reactivity with antibodies to other human coronaviruses that are present in all adults\(^6\) is currently unknown. Prior use of serology for detection of emerging coronaviruses focused on antibodies against the spike (S) protein, particularly the S1 domain, and the nucleocapsid (N) protein\(^7\). However, the optimal set of antigens to detect strain-specific coronavirus antibodies remains unknown.

Protein microarray technology can be used to detect antibodies of multiple isotypes against hundreds of antigens in a high throughput manner\(^8,9\), so is well suited to serologic surveillance studies. This technology, which has previously been applied to other emerging coronaviruses\(^10\), is based on detection of binding antibodies, which are well-correlated with neutralizing antibodies\(^11\) but do not require viral culture in biosafety level 3 facilities. Recently, our group developed a coronavirus antigen microarray (CoVAM) that includes antigens from SARS-CoV-2 and tested it on human sera collected prior to the pandemic to demonstrate low cross-reactivity with antibodies from human coronaviruses that cause the common cold, particularly for the S1 domain\(^2\). Here, we further validate this methodology using convalescent blood specimens from COVID-19 cases confirmed by positive SARS-CoV-2 PCR.

Methodology

Specimen Collection

The SARS-CoV-2 convalescent blood specimens from nasopharyngeal SARS-CoV-2 PCR-positive individuals were collected from different sources to increase the number of positive specimens available for analysis. Two sera and plasma samples were obtained from acute COVID-19 patients from the Oregon Health Sciences University Hospital.
(OHSU), Portland, OR. These were sourced from discarded clinical laboratory specimens exempted from informed consent and IRB approval under condition of patient anonymity. Four plasma samples were obtained from outpatients of the University Hospital Basel, University of Basel, Basel, Switzerland. These patients were screened in accordance with Swiss regulations on blood donation and approved as plasma donors according to the Blood Transfusion Service of the Swiss Red Cross with informed consent. These donors were diagnosed with COVID-19 based on SARS-CoV-2 positive nasopharyngeal swab PCR tests. At time of plasma donation, each had two negative nasopharyngeal swab SARS-CoV-2 PCR- tests and negative SARS-CoV-2 PCR tests in blood, and they were qualified as plasma donors. Plasma was collected from these convalescent donors at the Regional Blood Transfusion Service of the Swiss Red Cross in accordance with national regulations. One convalescent plasma was isolated from a large-volume apheresis collection following standard protocol from a documented recovered COVID-19 blood donor who was more than 28 days post symptomatic.

The negative control sera used in this study were collected between November 2018 and May 2019 for a larger study where residents of a college resident community in the Eastern United States were monitored prospectively to identify acute respiratory infection (ARI) cases using questionnaires and RT-qPCR, so as to characterize contagious phenotypes including social connections, built environment, and immunologic phenotypes. A total of 144 de-identified blood specimens were tested on the CoV antigen microarray. Electronic informed consents including future research use authorization was obtained under protocols approved by the Institutional Review Boards (IRBs) of the University of Maryland and the Department of Navy Human Research Protections Office.

**Coronavirus Antigen Microarray**

The coronavirus antigen microarray used in this investigation includes 67 antigens across subtypes expressed in either baculovirus or HEK-293 cells (Table 1). These antigens were provided by Sino Inc. (Wayne, PA) as either catalog products or custom synthesis service products. The antigens were printed onto microarrays, probed with human sera, and analyzed as previously described. Briefly, lyophilized antigens were reconstituted to a concentration of 0.1 mg/mL in phosphate-buffered saline (PBS) with 0.001% Tween-20 (T-PBS) and then printed onto nitrocellulose-coated slides from Grace Bio Labs (GBL, Bend, OR) using an OmniGrid 100 microarray printer (GeneMachines). The microarray slides were probed with human sera diluted 1:100 in 1x GVS Fast Blocking Buffer (Fischer Scientific) overnight at 4°C, washed with T-TBS buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20 in ddH2O adjusted to pH 7.5 and filtered) 3 times for 5 minutes each, labeled with secondary antibodies to human IgA and IgG conjugated to quantum dot fluorophores for 2 hours at room temperature, and then washed with T-TBS 3 times for 5 minutes each and dried. The slides were imaged using ArrayCam imager (Grace Bio Labs, Bend, OR) to measure background-subtracted median spot fluorescence. Non-specific binding of secondary antibodies was subtracted using saline control. Mean fluorescence of the 4 replicate spots for each antigen was used for analysis.
Statistical Analyses

The mean fluorescence intensity (MFI) of each antigen was determined by the average of the median fluorescence signal of four replicate spots. The fluorescence signal for each spot was determined by its signal intensity subtracted by the background fluorescence. Antigens containing a human Fc tag were removed from the analysis, as the secondary antibodies used for quantification are known to bind to human Fc; non-human Fc tag did not interfere with the assay. All statistical analyses were conducted using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). MFI was normalized using the `normalize.quantiles.use.target` function from the `proprocessCore` package (version 1.48.0). As a target for normalization, a vector containing the median MFI for IgG or IgA was constructed. Descriptive statistics were used to summarize the IgA and IgG reactivity measured as MFI. Wilcoxon Rank Sum tests with p < 0.05 corrected for multiple comparisons were used to compare the mean differences between groups.

Receiver Operating Characteristic Area Under the Curve (ROC AUC) values for each antigen were calculated by comparing positive and negative specimens using the `pROC` package (version 1.16.2). Antigens were ranked based on their ROC AUC values, and high performing antigens with ROC AUC > 0.95 were identified. Data visualization was performed using the `ggplot2` package (version 3.3.0).

Results

Discrimination of SARS-CoV-2 Convalescent Plasma using Coronavirus Antigen Microarray

The coronavirus antigen microarray was used to detect IgG and IgA antibodies against a panel of antigens, including coronavirus spike protein (S) as separated receptor-binding (RBD), S1, and S2 domains or whole protein (S1+S2) and nucleocapsid protein (NP), from multiple coronaviruses including SARS-CoV-2, SARS-CoV, MERS-CoV, and the four common cold coronaviruses (HKU1, OC43, NL63, 229E) as listed in Table 1. To determine the antibody profile of SARS-CoV-2 infection, the differential reactivity to these antigens was evaluated for SARS-CoV-2 convalescent plasma from PCR-positive individuals (positive group) and sera collected prior to the COVID-19 pandemic from naïve individuals (negative group) as shown in Figure 1.

The positive group demonstrates high IgG reactivity to SARS-CoV-2 NP, S2, and S1+S2 antigens and moderate IgG reactivity to SARS-CoV-2 S1 and RBD antigens, while the negative group demonstrates low IgG cross-reactivity to SARS-CoV-2 S1+S2 and no cross-reactivity to other SARS-CoV-2 antigens (Figure 2). The positive group also demonstrates high IgG cross-reactivity with SARS-CoV S2 and S1+S2 antigens, while the negative group demonstrates low cross-reactivity with MERS-CoV S1+S2 and no cross-reactivity with other SARS-CoV and MERS-CoV antigens. The two groups do not differ significantly in reactivity to common cold coronaviruses and other seasonally circulating respiratory viruses. Similar trends are observed for IgA but with lower reactivity overall (Figure 3). The positive group again demonstrates high IgA reactivity to SARS-CoV-2 NP, S2, and
S1+S2 and moderate IgA reactivity to SARS-CoV-2 S1 with high IgA cross-reactivity to SARS-CoV NP, while the negative group demonstrates low IgA cross-reactivity to all SARS-CoV-2, SARS-CoV, and MERS-CoV antigens.

Selection of High-Performing Antigens to Detect SARS-CoV-2 Infection

Each coronavirus antigen was evaluated for performance in discriminating the positive group from the negative group across a full range of assay cutoff values to generate Receiver Operating Characteristic (ROC) curves for which Area Under Curve (ROC AUC) was measured (Figure 4). High-performing antigens for detection of IgG or IgA defined by ROC AUC > 0.95 included all SARS-CoV-2 antigens and MERS-CoV S2 for IgG and SARS-CoV-2 S2 and S1+S2 for IgA (Table 2). Each of these antigens discriminated between the positive group and the negative group with high significance (Figure 5).

Discussion

This study reveals several insights into the antibody response to SARS-CoV-2 infection. The antibody profiles of naïve individuals include high IgG reactivity to common cold coronaviruses with low-level cross-reactivity with S2 domains from SARS-CoV-2 and other epidemic coronaviruses, which is not surprising given the high degree of sequence homology and previously observed serologic cross-reactivity between S2 domains of betacoronaviruses, a group that includes SARS-CoV-2, SARS-CoV, MERS, and common cold coronaviruses HKU1 and OC43. However, naïve individuals do not show cross-reactivity to other SARS-CoV-2 antigens. Even for the nucleocapsid protein, which also has high sequence homology between betacoronaviruses, cross-reactivity is only seen between SARS-CoV-2 and SARS-CoV and not with MERS-CoV or common cold coronaviruses. In addition, the quantitative difference between high antibody reactivity to SARS-CoV-2 S2 in the positive group and low-level antibody cross-reactivity in the negative group is large enough that these antigens still discriminate these groups with high significance.

This study also informs antigen selection and design for population surveillance and clinical diagnostic assays and vaccine development. The observation that naïve individuals with antibodies to common cold coronaviruses do not show cross-reactivity to SARS-CoV-2 nucleocapsid protein dispels concerns that the high sequence homology of this protein across betacoronaviruses would impair its performance as a diagnostic or vaccine antigen. The low-level antibody cross-reactivity of naïve individuals for SARS-CoV-2 spike protein containing S2 domain may not preclude its use as a diagnostic antigen given large quantitative difference in antibody reactivity between positive and negative groups, but this cross-reactivity may influence response to vaccination with spike protein antigens containing the S2 domain.

The coronavirus antigen microarray can be useful both as an epidemiologic tool and as a research tool. The high throughput detection of SARS-CoV-2-specific antibody profiles that reliably distinguish COVID-19 cases from negative controls can be applied to large-scale population surveillance studies for a more accurate estimation of the true prevalence of disease than can be achieved with symptom-based PCR testing. In
addition, detection of these antibodies in SARS-CoV-2 convalescent plasma donations can provide validation prior to clinical use for passive immunization. The variation in the SARS-CoV-2 antibody profiles among acute and convalescent donors suggests that epitope characterization of convalescent donor plasma will be informative for evaluation of passive immune therapy efficacy in COVID-19 patients. The central role of inflammation in the pathogenesis of severe COVID-19\textsuperscript{16} can be more closely studied by analyzing both strain-specific and cross-reactive antibody responses, particularly to test hypotheses regarding antibody-dependent enhancement with critical implications for vaccine development\textsuperscript{17}.

Conclusions

A coronavirus antigen microarray containing a panel of antigens from SARS-CoV-2 in addition to other human coronaviruses was able to reliably distinguish convalescent plasma of PCR-positive COVID-19 cases from negative control sera collected prior to the pandemic by detecting both strain-specific and cross-reactive antibodies. Further studies are needed to apply this methodology to large-scale serologic surveillance studies and to correlate specific antibody responses with clinical outcomes.

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Author Disclosures

The coronavirus antigen microarray is intellectual property of the Regents of the University of California that is licensed for commercialization to Nanommune Inc. (Irvine, CA), a private company for which Philip L. Felgner is the largest shareholder and several co-authors (de Assis, Jain, Nakajima, Jasinskas, Obiero, Davies, and Khan) also own shares. Nanommune Inc. has a business partnership with Sino Biological Inc. (Beijing, China) which expressed and purified the antigens used in this study. The convalescent plasma used in this study was collected for clinical use by independent blood centers using licensed plasma or platelet processing systems manufactured by Cerus Corporation, for which multiple authors (Corash, Bagri) are shareholders and employees. Manuel Battegay, Andreas Buser and Andreas Holbro are employees of the University of Basel and have no conflicts of interest.
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Figure 1. Heatmap for coronavirus antigen microarray. The heatmap shows IgG (A) and IgA (B) reactivity measured as mean fluorescence intensity across four replicates, against each antigen organized into rows color coded by virus, for sera organized into columns classified as positive (convalescent from PCR-positive individuals) or negative (prior to pandemic from naïve individuals). Reactivity is represented by color (white = low, black = mid, red = high).
Figure 2. Normalized IgG reactivity of positive and negative sera on coronavirus antigen microarray. The plot show IgG reactivity against each antigen measured as mean fluorescence intensity (MFI) with full range (bars) and interquartile range (boxes) for convalescent sera from PCR-positive individuals (positive, red) and sera from naïve individuals prior to pandemic (negative, blue). Below the plot, the heatmap shows average reactivity for each group (white = low, black = mid, red = high). The antigen labels are color coded for respiratory virus group.
**Figure 3.** Normalized IgA reactivity of positive and negative sera on coronavirus antigen microarray. The plot shows IgG reactivity against each antigen measured as mean fluorescence intensity (MFI) with full range (bars) and interquartile range (boxes) for convalescent sera from PCR-positive individuals (positive, red) and sera from naïve individuals prior to pandemic (negative, blue). Below the plot, the heatmap shows average reactivity for each group (white = low, black = mid, red = high). The antigen labels are color coded for respiratory virus group.
Figure 4. ROC curves for high-performing antigens. ROC curves showing sensitivity versus specificity for discrimination of positive and negative sera were derived for each individual high performing antigen (ROC AUC ≥ 0.95) for both IgG and IgA (solid blue line) and compared to no discrimination (ROC AUC = 0.5, dashed black line).
Figure 5. Normalized antibody reactivity of positive and negative sera for high-performing antigens. IgG and IgA reactivity against each high-performing antigens (ROC AUC > 0.95) measured as mean fluorescence intensity (MFI) for convalescent sera from PCR-positive individuals (positive, red) and sera from naïve individuals prior to pandemic (negative, blue) are shown as box plots, including full range (bars), interquartile range (boxes), median (black line), and individual sera (dots) with p-values for each antigen calculated by Wilcoxon Rank Sum test.
Table 1. Content of coronavirus antigen microarray. The virus group, subtype, and strain, protein, GenBank identification where available, expression system, gene construct, and vendor source and catalog number are shown for each antigen.
Table 2. Receiver Operating Characteristic Area Under Curve (ROC AUC) for SARS-CoV-2, SARS-CoV, and MERS-CoV antigens. ROC AUC values for discrimination of positive and negative sera were derived for each individual antigen for both IgG and IgA and ranked, and high-performing antigens with ROC AUC > 0.95 are indicated above the lines.
