Novel Serological Test for Immune Response to SARS-CoV-2 Virus

INVENTOR
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SHORT DESCRIPTION
Scientists at Rush University Medical Center have developed and validated a new serological assay to identify individuals that have had an immune response to SARS-CoV-2. Built on the Luminex immunobead platform, this multiplexed assay simultaneously evaluates four antigens (Spike (S), Nucleocapsid (N), Membrane (M), and Envelope (E) glycoproteins) from the SARS-CoV-2 virus in a fully-automated workflow that provides fast and accurate diagnostic results.

This test was specifically engineered to provide greater confidence in diagnostic results through the use of an algorithm integrating results from four independent SARS-CoV-2 antigen assays and requires no less than two analytical findings to deliver a diagnostic result. This approach provides superior test performance than most FDA-approved tests (that evaluate only a single antigen) and a significant reduction in the rate of ‘False Negative’ misclassifications - which has been the primary issue plaguing serological assays, including those with Emergency Use Authorization under the FDA.

ASSAY PERFORMANCE OVERVIEW
Validation studies against 1042 patients with either a PCR-confirmed Covid-19 status (n=44) or collected prior to the Covid-19 pandemic (n=998) showed excellent performance characteristics, including an overall accuracy of 99.62% and sensitivity/specificity figures of 100% and 99.6%, respectively. Eligible matrices include venous serum/plasma specimens or capillary blood (delivered via dried blood spot cards).

APPLICATIONS
- Screening of non-symptomatic individuals for seropositivity, indicating potential immunity for returning to work or other public functions
- Quantitative monitoring of an immune response to a SARS-CoV-2 vaccine, as part of a clinical trial.
- Stratification of convalescent plasma as a therapeutic approach for COVID-19 based on four independent antibody titers.

ADVANTAGES
- Multiplex format provides greater coverage of SARS-CoV2 epitopes and greater confidence in a diagnostic result than any ‘single-antigen’ serological test.
- Lower incidence of false positive/negative results due to assessment of multianalyte testing
- Rapid turnaround time and cost-effective.
- Amenable to “at home” sampling via finger-prick/dried blood spot card devices with no loss in test performance characteristics.
Novel Serological Test for Immune Response to SARS-CoV-2 Virus

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Abstract
Coronavirus disease 2019 (Covid-19) is a global pandemic that has impacted the lives of the entire world’s population. Accurate and effective testing mechanisms to identify those with active infections or have convalesced is necessary to properly monitor and control this infectious agent. To this end, we have recently developed a serological method for assessment of an immune response to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pathogen based on simultaneous evaluation of four viral antigens, including the Spike, Nucleocapsid, Membrane, and Envelope glycoproteins, and built on the Luminex immunobead platform. Validation studies against 1042 patients have provided test performance characteristics of 100% Sensitivity, 99.6% Specificity, and 99.62% overall accuracy, relative to PCR-based assignments. This quantitative method is highly effective in applications that include diagnosis of those with past infections (symptomatic or asymptomatic), epidemiological studies/ guidance for policy decisions, monitoring titers during vaccine trials, and rationalized donor selection for therapeutic ‘convalescent plasmapheresis’ strategies.

Introduction
The recent pandemic of Coronavirus disease 2019 (Covid-19) has greatly impacted the United States with nearly 2 million cases reported and 112,133 deaths according to the US Center for Disease Control and Prevention (as of 6/10/2020)1. While PCR-based testing for the SARS-CoV-2 virus has been the clinical standard for point of care testing, limitations to this approach in both extent of testing and technical performance make it only one solution to accurately identifying cases in the population2,3. Serological testing approaches that evaluate an immune response to pathogen infection and may provide a more comprehensive appreciation of infection rates within the population and help direct policy decisions regarding social distancing, ‘return to work’ guidance, help guide vaccine development, and provide a means to identify donor candidates for ‘convalescent plasma’ therapeutic strategies4-6.

The goal of the present study was to develop a robust method for evaluating serological specimens for evidence of an immune response to SARS-CoV-2 that is rapid, scalable to population-based studies, and meets performance expectations outlined by the US Food and Drug Administration.

Methods
Specimens were acquired from the clinics at Rush University Medical Center, Rush Oak Park, and Riverside Healthcare. Sera and EDTA-plasma were obtained via conventional phlebotomy and processed using standard laboratory protocols. Aliquots were stored at -80 °C until the time of evaluation and were not subjected to more than 3 freeze/thaw cycles. All cases were obtained with Rush Institutional Review Board (IRB) approval. Covid-19 positive cases (n=44) were obtained from the Department of Pathology’s clinical labs based on a positive result from polymerase chain reaction (PCR)-based point of care testing for SARS-CoV-2; subsequent serological specimens were obtained upon follow-up exam at least 12 days from symptom onset/6-days from PCR-based diagnostics. The Covid-19 negative population (n=998) was obtained prior to the global pandemic (i.e. before 6/2019) by the Rush Biorepository as control specimens, with inclusion limited to those accrued during peak annual flu-season (i.e. December-March).

All assays are performed using the Luminex immunobead platform in either 96- or 384-well microtiter plate formats. Bead sets for SARS-CoV-2 Spike (S), Nucleocapsid (N), Membrane (M), and Envelope (E) glycoproteins were developed and analytically validated ‘in house’ using commercially-available immunoreagents and standard protocols, as previously described7-10. Each assay uses a serial dilution series of commercial anti-antigen polyclonal antibodies for each bead set (in multiplex) to generate a standard curve for quantitation of all assay results.
with a 5-parametric fit algorithm. All patient specimens (sera or plasma) were diluted 500-fold using assay buffer immediately before evaluation and processed with duplicate sampling. Read precision thresholds were set to a percent coefficient of variation (%CV) of 10%. Briefly, all assay steps were separated by exhaustive bead washes with assay buffer using a BioTek 405 plate washer. Primary specimen incubations with the 4-plex bead set range 0.5-2h, with 1h being the most common time used. This was followed by application of the biotin-conjugated, anti-human IgG detection antibody for 30’ and application of the streptavidin Phycoerythrin conjugated (SAPE) reporting moiety. Reads were conducted on a FlexMAP 3D multiplate reader (Luminex Corporation) using xPONENT® v4.0 software. Resultant concentration levels for each specimen were evaluated with a decision tree algorithm to classify each case as Covid seropositive seronegative. Experimentally determined values were then contrasted to the accepted patient status (based on PCR or time of accrual) and statistically evaluated using a ‘confusion matrix’ calculator (http://onlineconfusionmatrix.com/).

Results

Patients enrolled in the current study were selected based on the following characteristics: Covid-19 Positive - all Covid-19 patients presented at RUMC with flu-like symptoms and met diagnostic criteria for PCR-based testing, as specified by the Center of Disease Control. Serological specimens from these cases were obtained during a follow-up evaluation at least 12 days from symptom onset. Clinical characteristics of this cohort were unavailable given these were obtained as remnant specimens from the clinical labs. Covid-19 Negative: this control cohort was accrued by the Rush Biorepository prior to June, 2019 and selected to only contain those collected during peak flu season (December – March) to enhance potential confounding respiratory pathogen infection prevalence. Patient characteristics for the Covid-19 negative cohort had the following demographic characteristics: median age was 64.2 years with 44.8% males and a racial breakdown of White – 66.2%, Black – 24.2%, Hispanic – 5.1%, Asian – 2.3%, other – 2.9%. Approximately 74% of this cohort had a comorbidity of chronic obstructive pulmonary disorder (COPD).

Specimens were evaluated manually with the ‘Rush SARS-CoV-2 immune response panel’ by experienced laboratory personnel and generated median overall %CV values that was 8.9% for all evaluations. A total of 44 cases of Covid-19 positive (by PCR) and 998 cases of Covid-19 negative were evaluated for their IgG fractions by this method and classified by the ‘decision tree’ algorithm presented in Figure 1 (threshold values for classification withheld). This analysis resulted in 44 cases classified positive (TP), no cases of false negative (FN), 994 cases classified negative (TN), and 4 cases of false positive (FP). Figure 2 shows the results of confusion matrix calculations:

<table>
<thead>
<tr>
<th>Measure</th>
<th>Value</th>
<th>Derivations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>1.0000</td>
<td>TPR = TP / (TP + FN)</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.9960</td>
<td>SPC = TN / (FP + TN)</td>
</tr>
<tr>
<td>Precision</td>
<td>0.9167</td>
<td>PPV = TP / (TP + FP)</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>1.0000</td>
<td>NPV = TN / (TN + FN)</td>
</tr>
<tr>
<td>False Positive Rate</td>
<td>0.0040</td>
<td>FPR = FP / (FP + TN)</td>
</tr>
<tr>
<td>False Discovery Rate</td>
<td>0.0033</td>
<td>FDR = FP / (FP + FN)</td>
</tr>
<tr>
<td>False Negative Rate</td>
<td>0.0000</td>
<td>FNR = FN / (FN + TP)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>0.9962</td>
<td>ACC = (TP + TN) / (P + N)</td>
</tr>
<tr>
<td>F1 Score</td>
<td>0.9965</td>
<td>F1 = 2TP / (2TP + FP + FN)</td>
</tr>
<tr>
<td>Matthews Correlation Coefficient</td>
<td>0.9555</td>
<td>TP<em>TN + FP</em>FN / sqrt((TP+FP)<em>(TP+FN)</em>(TN+FP)*(TN+FN))</td>
</tr>
</tbody>
</table>

Figure 2. Calculated values of performance characteristics based on ‘Confusion Matrix’
This analysis provided a 100% Sensitivity and 99.6% specificity, with a 99.62% overall classification accuracy, relative to PCR-based assessments. These performance characteristics greatly exceed specified limits by the FDA under the Emergency Use Authorization and is eligible for review to be an approved diagnostic method upon application. Evaluation of total Ig (IgA+IgM+IgG) fractions in earlier patients has previously shown improved sensitivity in specimens collected within 6 days of symptom onset (data not shown).

Conclusions
We have successfully developed a method that quantitatively evaluates in a single assay an individual’s immune response to four distinct SARS-CoV-2 antigens that has excellent performance characteristics for diagnostic applications, epidemiological studies, or policy decisions for ‘return to work’ guidance. The method is highly robust and amenable to automation to accommodate scaling to population-based testing protocols. Protocols for dried blood spot sampling/specimen delivery have been developed and will be submitted to the FDA for approval under the Emergency Use Authorization (EUA) along with sampling from conventional venipuncture.

References