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SARS-CoV-2–Specific Antibody Detection for Seroepidemiology: A Multiplex Analysis Approach Accounting for Accurate Seroprevalence

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Background. The COVID-19 pandemic necessitates better understanding of the kinetics of antibody production induced by infection with SARS-CoV-2. We aimed to develop a high-throughput multiplex assay to detect antibodies to SARS-CoV-2 to assess immunity to the virus in the general population.

Methods. Spike protein subunits S1 and receptor binding domain, and nucleoprotein were coupled to microspheres. Sera collected before emergence of SARS-CoV-2 (n = 224) and of non-SARS-CoV-2 influenza-like illness (n = 184), and laboratory-confirmed cases of SARS-CoV-2 infection (n = 115) with various severities of COVID-19 were tested for SARS-CoV-2–specific IgG concentrations.

Results. Our assay discriminated SARS-CoV-2–induced antibodies and those induced by other viruses. The assay specificity was 95.1%–99.0% with sensitivity 83.6%–95.7%. By merging the test results for all 3 antigens a specificity of 100% was achieved with a sensitivity of at least 90%. Hospitalized COVID-19 patients developed higher IgG concentrations and the rate of IgG production increased faster compared to nonhospitalized cases.

Conclusions. The bead-based serological assay for quantitation of SARS-CoV-2–specific antibodies proved to be robust and can be conducted in many laboratories. We demonstrated that testing of antibodies against multiple antigens increases sensitivity and specificity compared to single-antigen–specific IgG determination.

Keywords. COVID-19; IgG; spike S1; RBD; nucleoprotein; endemic coronavirus; multiplex bead-based immune assay; specificity; sensitivity; influenza-like Illness (ILI).

Coronavirus disease 2019 (COVID-19) caused by the newly emerged severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a pandemic in a largely immune-naive population. The presence of specific antibodies is currently being investigated to assess the induction of an immune response in patients and to assess the degree of exposure and immunity in the general population [1–3]. As it is a recently emerged coronavirus variant, the kinetics and degree of immunity induced following contact with the virus and COVID-19 disease are largely unknown.

SARS-CoV-2 expresses a spike protein, highly similar to spike of SARS-CoV, which binds to angiotensin converting enzyme 2 (ACE2) [4, 5]. Binding of antibodies to the receptor binding domain (RBD) of spike neutralizes the ability of the virus to infect cells [6]. In addition, antibodies are detected against other viral proteins, including nucleoprotein (N) [7]. N is shielded within the virion and therefore N-specific antibodies are probably unable to neutralize the virus. Although N may not be involved in neutralization of the virus, antibodies to N could provide an indicator of exposure to the virus. Antibodies to N induced by SARS-CoV reportedly recognize N of SARS-CoV-2 but not of seasonal coronaviruses [8].

Estimates of the prevalence of seroconversion as proxy for protection of the general population may support health decision making, including the decision to lift lockdown measures. To appropriately apply an assay for serosurveys we need to know the precision of the assay, that is the sensitivity and specificity, which are variable between currently available tests [9, 10]. Performing and sustaining large studies to assess changing population immunity requires high-throughput screening assays that are robust and accurate [11]. Many countries now aim to assess the protective status of the general population for COVID-19 using antibody assays. To guarantee high specificity, the assay should be validated with a representative number of sera from patients infected with other coronaviruses and other pathogens causing influenza-like illness (ILI), but this is often

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lacking [11–13]. To date, COVID-19 prevalence of seroconverted individuals is relatively low and there is a risk of significant overestimation if an assay has insufficient specificity (Supplementary Table 1). Thus, high specificity is important at this stage [11, 12].

Our laboratory has extensive experience in developing multiplex assays to quantify antibodies to many bacterial and viral pathogens in the general population, of which most are part of the national immunization program [1, 14–17]. We developed a high-throughput and highly quantitative bead-based multiplex immunoassay to assess the prevalence of seropositivity in the general population, and also anticipating the introduction of future SARS-CoV-2 vaccines. By multiplexing a broader range of SARS-CoV-2 antigens in a single assay we may generate a better understanding of the proportion of persons that have seroconverted. Moreover, in a multiplex assay positivity can be compared among antigens to provide a more detailed evaluation of the antibody levels and to enhance assay performance [17]. The developed assay was tested on samples from COVID-19 patients with various severities of disease collected at multiple timepoints to determine the kinetics of seroconversion.

METHODS

Serum Samples

Serum samples were obtained from the following cohorts: (1) a random selection of individuals (n = 224) from a national (Dutch) cohort representing all age groups and obtained 3 years prior to SARS-CoV-2 emergence (Pienter3 study, Netherlands trial register number NL5467); (2) individuals (Supplementary Table 2) with proven non-SARS-CoV-2 ILI caused by human coronaviruses (n = 110, HCoV ILI) or other viruses (n = 74, non-HCoV ILI) obtained from the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands (trial register number NL4666) [18], and from Erasmus Medical Center, Rotterdam, collected prior to the SARS-CoV-2 outbreak and at least 2 weeks after polymerase chain reaction (PCR) detection of the virus; and (3) sera of 115 laboratory-(PCR) confirmed COVID-19 patients that were either hospitalized (n = 50) or outpatient (n = 65) (Erasmus Medical Center and RIVM, Medical Ethical Committee number METC 06/282). Of the nonhospitalized cases, 34% were male and the median age was 42.6 years (95% confidence interval [CI], 40.0–45.2). Of the hospitalized patients 51% were admitted to the intensive care unit. Of the ward and ICU patients, 78% and 75% were male and the mean age was 62.1 years (95% CI, 55.5–68.8) and 61.8 years (95% CI, 55.7–97.8), respectively. Paired samples, collected between days 3 and 40 after disease onset, were available from 73 COVID-19 patients. Ethical approval was obtained from the Erasmus Medical Center Medical Ethical Committee (MEC-2015–306) to anonymously analyze the ILI and COVID-19 samples. Informed consent and voluntary informed consent were provided where applicable.

Assay Procedure

The steps in assay validation were similar to recently developed bead-based multiplex immunoassays for CMV, EBV, and RSV, with minor modifications as described below [16, 17]. For the multiplex bead-based immune assay the following antigens obtained from Sino Biological were used: SARS-CoV-2 monomeric spike S1 (40591-V08H), RBD (40592-V08B), and nucleoprotein (N) (40588-V08B). Microplex fluorescent beads were activated in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.5. The proteins were diluted to a concentration of 0.2 mg/mL in phosphate-buffered saline (PBS) pH 7.4 and added at 5 µg per 75 µL of activated beads.

An internal reference sample was created by pooling 13 sera of COVID-19 patients with varying immunoglobulin G (IgG) concentrations. An arbitrary antibody concentration unit of 100 was assigned on the basis of the mean fluorescence intensity (MFI) signal in the upper limit of linearity of a 3-fold serial dilution of the reference sample.

Sera (25 µL) diluted 1:400 and 1:8000 in SM01 buffer (Surmodics) plus 2% fetal calf serum were incubated with antigen-coated beads for 45 minutes at room temperature at 750 rpm in the dark. Following incubation, samples were washed 3 times with PBS, incubated with phycoerythrin-conjugated goat anti-human IgG for 30 minutes and washed. Samples were acquired on an LX200 or FM3D (Luminex). MFI was converted to arbitrary units (AU/mL) by interpolation from a 5-parameter logistic standard curve, using Bioplex Manager 6.2 (Bio-Rad Laboratories) software and exported to Microsoft Excel.

Assay Validation

Different batches of antigen-conjugated beads were incubated with serially diluted sera to test linearity and parallelism between bead conjugations, reference, and serum samples. Assay robustness was tested by analyzing a serum panel by 3 different operators on independent days using 2 different bead and 2 reference batches. The ability to discriminate IgG concentrations between COVID-19 patients and controls was evaluated by receiver operator characteristic (ROC) analyses. To select the optimal assay defaults, both the Youden J statistic, which balances between sensitivity and specificity, and a specificity-optimized cutoff (specificity of at least 98.5% for low-prevalence settings of 5%–10%) were selected.

Data Analyses

Data were entered into GraphPad Prism 8.4.1 to generate graphs and perform statistical analyses. Reproducibility of the assay was evaluated using R^2 and coefficient of variation (% CV) calculated by standard deviation divided by average × 100. For the ROC analyses antibody concentrations of cross-sectional Pienter3 participants (n = 224), ILI patients with coronavirus (n = 74), or other viral infection (n = 110) were used as the negative control group and PCR-confirmed COVID-19 samples (n = 115) with various clinical severities were used in

the positive group. We selected for serum samples that were obtained more than 10 days post onset of disease symptoms to meet a reasonable degree of seroconversion, as shown in recent reports [8, 19]. Both the Youden J statistic-determined cutoff and the specificity-optimized cutoff (specificity of at least 98.5%) were determined.

To compare differences in concentrations, data were log-transformed and tested with either a *t* test between 2 groups, or 1-way ANOVA and Tukey's multiple comparison test to compare multiple groups and adjusted *P* values reported. Antibody kinetics was fitted using a nonlinear 4-parameter least square fit in Graphpad Prism 8.4.1.

RESULTS

Performance of the Assay

We prepared a reference serum by pooling 13 PCR-confirmed COVID-19 sera and tested serial dilutions in the multiplex assay consisting of distinct fluorescent beads coupled to SARS-CoV-2 nucleoprotein (N), S1, and the S1 subunit RBD (Figure 1A). This was repeated for varying batches of beads to assess consistency of performance. The assay was able to quantify concentrations in a 1000 to 10 000-fold concentration range, using a single dilution of the serum. To reliably quantify antibody concentrations between the reference serum and test samples, we confirmed that the reference and a selection of samples display the same rate of decline of fluorescence signal with increasing dilutions, which is referred to as parallelism (Figure 1B). These data show that the triplex assay is a highly quantitative assay to detect antibodies to SARS-CoV-2.

Applying an assay in large population and longitudinal studies requires reproducibility of assay results. Therefore, antibody concentrations were determined in independent experiments performed on 6 different days, using a selection of 214 samples for RBD and 268 samples for N and S1 with different concentrations of SARS-CoV-2 antibodies (Figure 1C). In addition, the reproducibility test was performed by 3 different technicians using different bead batches and references to reflect the expected maximum variability of the assay over time. Comparison of sample data determined on 2 independent assays runs resulted in an R^2 of 0.982, 0.985, and 0.988 for N, S1, and RBD, respectively (Figure 1C). The obtained % CVs were 19.1, 25.5, and 14.6 for N, S1, and RBD, respectively, showing that the assay results were reproducible.

Sensitivity and Specificity

Sera of 115 PCR-confirmed COVID-19 patients after 10 days of symptoms were tested in the assay and the results compared to a control panel of 408 sera collected prior to the outbreak of SARS-CoV-2. In COVID-19 patients, high concentrations of IgG were observed to all 3 antigens (Figure 2A). Despite clear discrimination of IgG concentrations between groups of control and COVID-19 patients, some samples overlapped between the 2 groups. Therefore, the specificity and sensitivity of the assay to discriminate between COVID-19 patients and controls using IgG concentrations was evaluated by an established statistical standard to analyze assay performance, the ROC analyses. For the ROC analyses, concentration data of hospitalized and nonhospitalized COVID-19 disease cases were included to provide a realistic evaluation of the performance of the assay (Figure 2A). The area under the curves ranged from 0.9839 to 0.9859 (Figure 2B). The ROC generated cutoff concentrations of 14.8, 0.85, and 8.21 AU/mL using the ROC Youden J statistic. To gain a higher specificity of the assay optimized for a low population seroprevalence, the cutoff concentrations were 19.7, 2.37, and 19.1 for N, S1, and RBD, respectively (Figure 2C). The latter cutoffs resulted in a specificity of 98.5%, 99.0%, and 98.5% at a sensitivity of 89.4%, 84.4%, and 83.6% for N, S1, and RBD, respectively.

IgG in Non-SARS-CoV-2 Infections and SARS-CoV-2 Infections of Various Severities

To study how our assay discriminates between antibodies of individuals with different laboratory-confirmed viral infections, antibodies were measured in a cross-sectional population panel (n = 224), a panel of noncorona ILI patients (non-HCoV ILI, n = 74), and non-SARS-CoV-2 corona ILI patients (HCoV ILI, n = 110) and compared to PCR-confirmed COVID-19 patients' samples. Some of the COVID-19 patients were admitted to hospital (n = 50) because of severe COVID-19 and these were compared to nonhospitalized COVID-19 cases (n = 65). For each of the 3 negative control groups the majority of the samples had concentrations below the cutoff for all 3 antigens (Figure 3A). The number of false-positive samples ranged from 5 to 6 out of 404 or 408 samples tested for the different antigens. The non-HCoV and HCoV ILI panels were from persons infected with multiple different non-SARS viruses including 4 different endemic coronavirus (Supplementary Table 2). The proportion of false positives did not increase by testing the convalescent sera from patients with a laboratory (PCR)-confirmed infection with either of the 4 seasonal coronaviruses (Figure 3B, and data not shown), indicating that the antigens used in the assay are selective for SARS-CoV-2–induced antibodies. Comparison of PCR-confirmed SARS-CoV-2 patients samples shows that all hospitalized patients induced antibodies to N and the majority of hospitalized patients induced antibodies to S1 and RBD. The majority of the nonhospitalized cases showed antibody concentrations above the cutoff for N, whereas around 10% of the nonhospitalized patients did not produce antibodies above the cutoffs for S1 and RBD. Overall, the concentrations of antibodies in serum samples from patients that were hospitalized were significantly higher compared to patients that were not hospitalized.

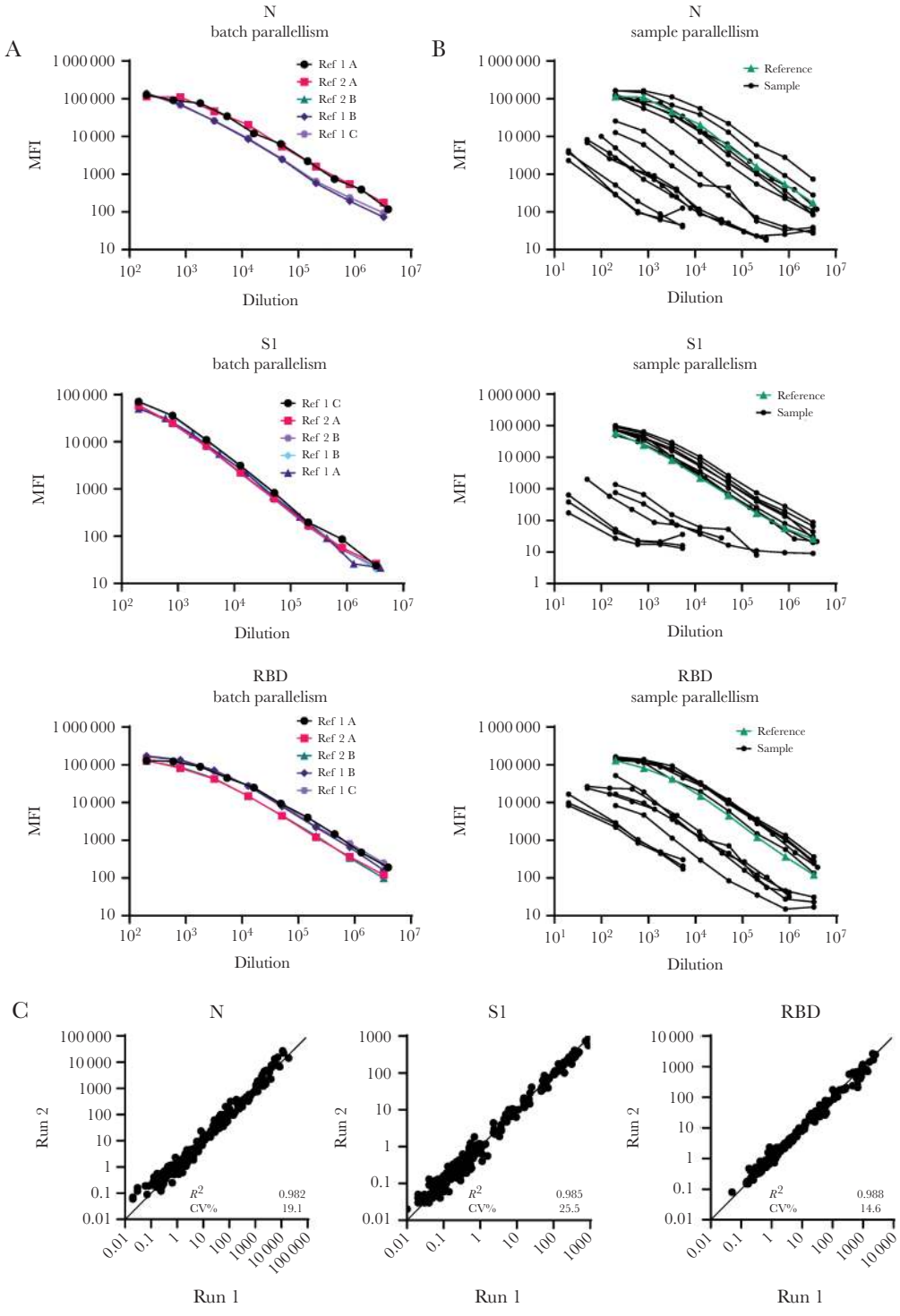


Figure 1. Assay development and validation *A*, Different lots of antigen-bead conjugations were used to determine the consistency of the mean fluorescence signal of a titrated pooled reference serum. Two different references consisting of pooled sera of COVID-19 patients (Ref 1 and Ref 2) were tested in independent runs designated A, B or C. *B*, The reference and serum samples were serially diluted to test parallelism for reliable quantification of antibody concentrations. *C*, Samples were tested in 2 independent runs by different technicians using different bead and reference batches to test robustness of the triplex assay. Concentrations in AU/mL are shown. Abbreviations: AU, arbitrary units; MFI, mean fluorescence intensity; N, nucleoprotein; RBD, receptor binding domain; S1, spike protein subunit 1.

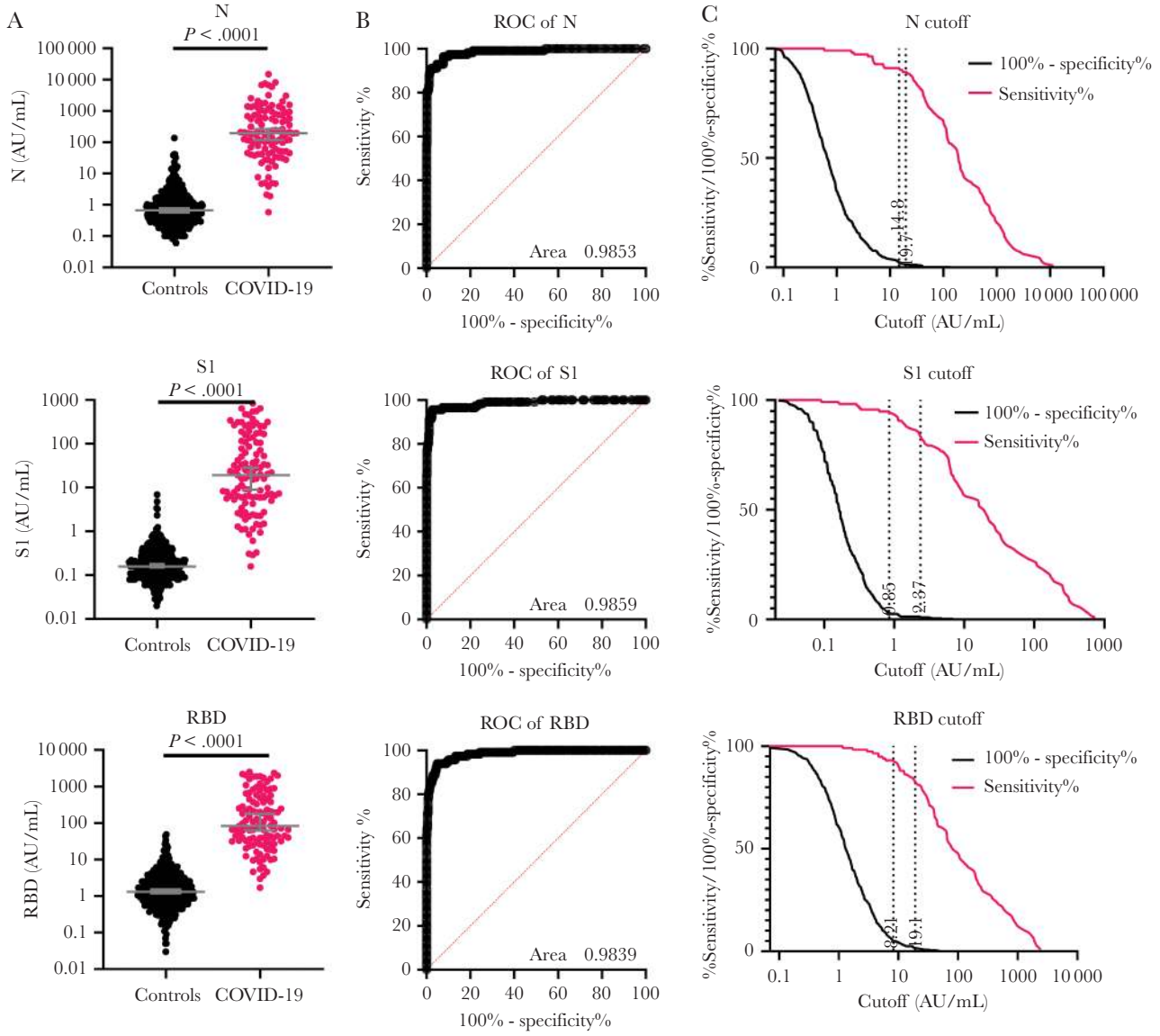


Figure 2. Ability of the assay to identify COVID-19 patients. *A*, Control sera (n = 408) and COVID-19 sera (n = 115) collected after day 10 of symptoms were tested and compared for concentrations of IgG. Median concentration and 95% confidence intervals are shown. *B*, The sera tested in (*A*) were analyzed by ROC. *C*, The ROC data were used to determine Youden J statistic cutoff (lower cutoff) and a specificity-optimized cutoff of at least 98.5% specificity (higher cutoff). Abbreviations: AU, arbitrary unit; IgG, immunoglobulin G; N, nucleoprotein; RBD, receptor binding domain; ROC, receiver operator characteristic; S1, spike protein subunit 1.

Kinetics of Seroconversion

Following infection, an immune response is initiated, resulting in the production of serum antibodies. To study the time between onset of disease symptoms and the development of antibodies, paired serum samples were collected from the majority of patients. Data were separated for patients that were either admitted to the hospital or not (Figure 4A and 4B). Apart from the paired samples from 2 patients that were obtained before 7 days after onset of disease, all other hospitalized cases showed seroconversion for all 3 antigens tested (Figure 4A). In line with other reports, hospitalized COVID-19 patients seroconverted around day 10 of disease onset. Of 53 nonhospitalized cases, 48 seroconverted, whereas 5 showed slight increases in concentrations but failed to formally cross the cutoff value for any of the 3 analytes to be regarded a specific seroconversion. Hospitalized patients reached a plateau of antibody production shortly after 2 weeks from onset of symptoms, which took at least 25 days for the nonhospitalized cases (4–10 fold lower slope; Figure 4C and 4D). As a consequence of the slower increase of antibody concentrations the time to detectable antibodies was delayed, especially with respect to antibodies reacting to S1 and RBD. The variance in the nonhospitalized cases was high compared to the hospitalized cases, which is illustrated by the lower R^2 of the nonlinear least square fit of the 2 patient groups.

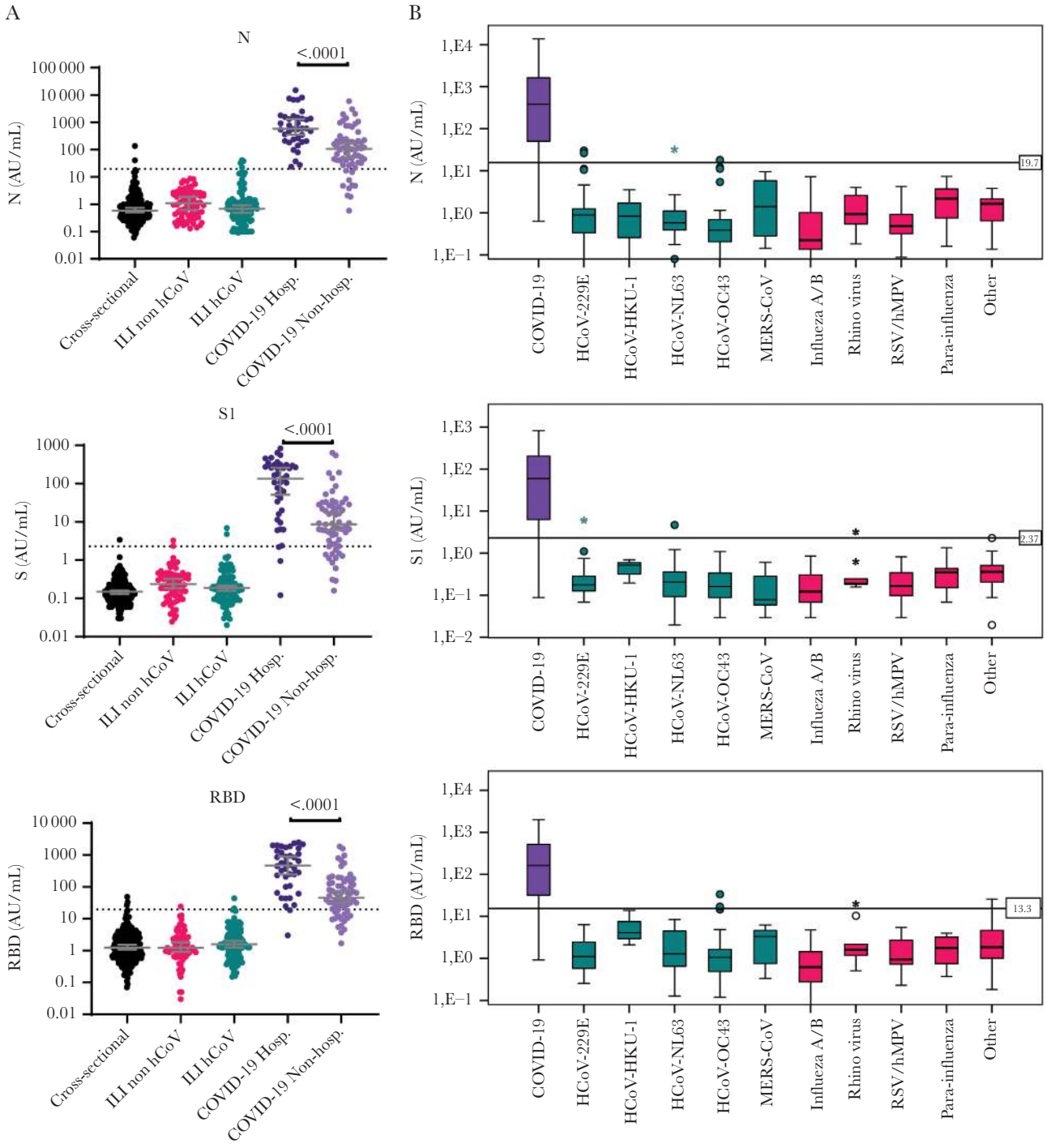
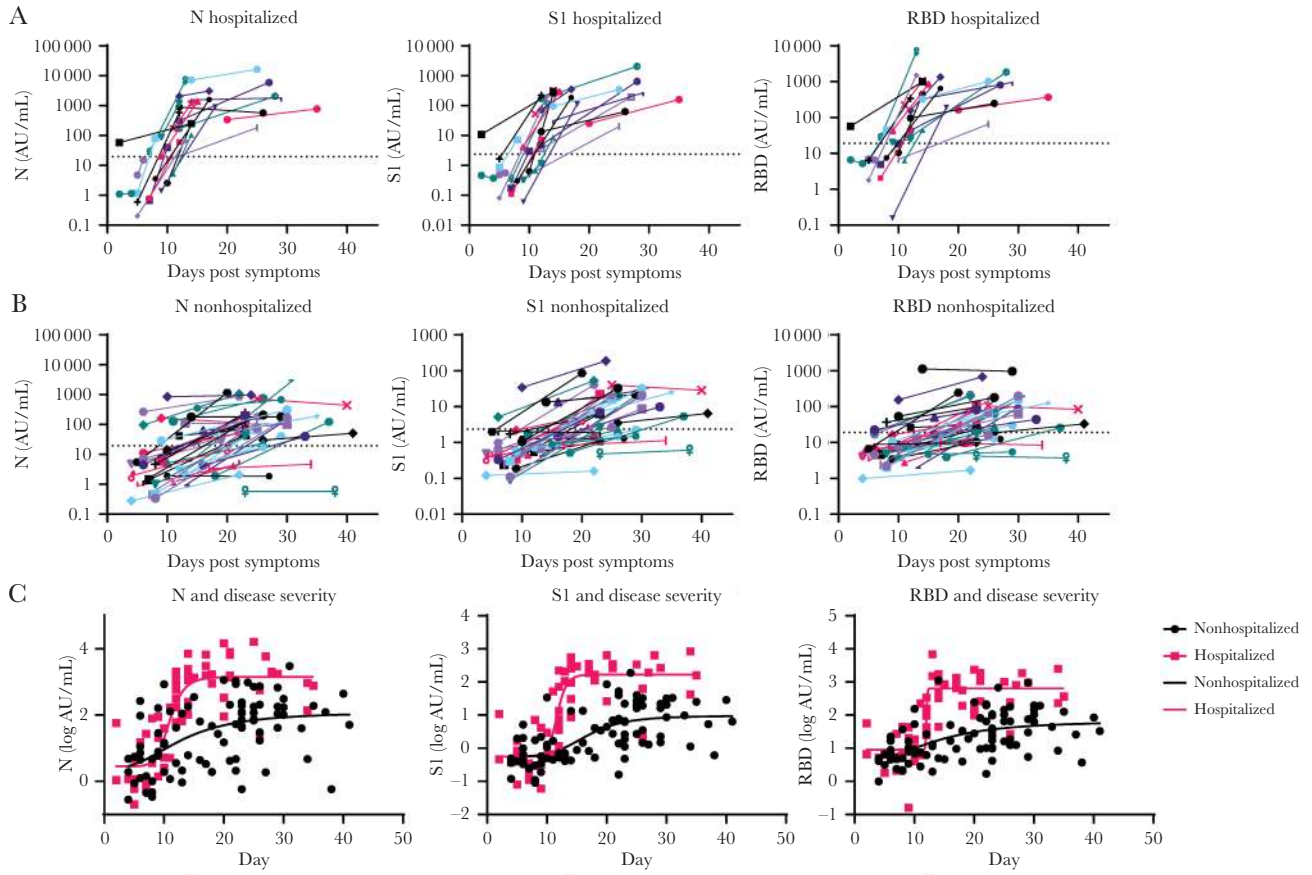


Figure 3. Discrimination of COVID-19 patients with varying severity from a cross-sectional population panel and ILI patients. *A*, Individuals from the cross-sectional panel aged 3–90 years (n = 224), ILI patients with noncoronavirus (n = 75), and non-SARS-CoV-2 seasonal coronavirus-infected ILI patients (n = 109) were compared to hospitalized and nonhospitalized COVID-19 patients. Median concentration and 95% confidence intervals and statistical results (adjusted *P* values of Tukey multiple comparison) between the groups are shown. *B*, Laboratory-confirmed viral infections (see Supplementary Table 2) and concentration data of ILI patients are shown to confirm that the assay discriminates SARS-CoV-2–specific antibodies from antibodies induced by various laboratory-confirmed viral infections. Abbreviations: AU, arbitrary unit; COVID-19, coronavirus disease 2019; HCoV, human coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; N, nucleoprotein; non-HCoV, noncoronavirus; RBD, receptor binding domain; RSV, respiratory syncytial virus; S1, spike protein subunit 1.



D

N	Hospitalized	
	No	Yes
Slope	2.66	8.24
R^2	0.296	0.696
P value	<.0001	

S1	Hospitalized	
	No	Yes
Slope	4.71	10.6
R^2	0.311	0.714
P value	<.0001	

RBD	Hospitalized	
	No	Yes
Slope	1.76	11.1
R^2	0.188	0.668
P value	<.0001	

Figure 4. Kinetics of antibody production after disease onset in hospitalized and nonhospitalized COVID-19 patients. Paired samples were analyzed to identify changes in IgG concentrations in hospitalized (*A*) and nonhospitalized (*B*) COVID-19 patients. *C*, The log-transformed concentration data of the samples shown in (*A*) and (*B*) were fitted with a 4-parameter nonlinear least squared fit. *D*, Of each patient with paired samples available, 1 sample was selected randomly and data were fitted to estimate the slope, R^2 of the fits, and the difference between the fitted lines determined. Abbreviations: AU, arbitrary unit; COVID-19, coronavirus disease 2019; IgG, immunoglobulin G; N, nucleoprotein; RBD, receptor binding domain; S1, spike protein subunit 1.

Multiplexed Evaluation of Seroconversion of Different Severities of COVID-19

The engagement of different structural SARS-CoV-2 proteins in 1 serological determination (multiplex testing) instead of 1 protein could improve the sensitivity and the specificity. If only 1 analyte is analyzed, the sensitivities for hospitalized cases were 94.1%, 94.3%, and 100% for RBD, S1, and N, respectively, using the specificity-optimized cutoff (Table 1). Using the ROC Youden J statistic cutoff the sensitivities were 97.1% for both S1 and RBD and 100% for N. Nonhospitalized cases typically had lower concentrations of IgG, which reduced the sensitivity: 76.3% for S1 and up to 82.7 % for N using the specificity-optimized cutoff. Using the Youden J statistic cutoff, the sensitivity increased to 91.3% for S1.

In this multiplex approach an increased sensitivity can be obtained by evaluating a sample as positive when either 1 of the antibody concentrations determined is higher than the set cutoff (logical OR analysis in Table 1). Any combination of antigen reached a sensitivity of 100% when N was used in hospitalized cases and ranged from 90.4% (S1 or RBD) up to 95.1% (N or S1 or RBD) using the specificity-optimized cutoff. Applying the Youden J statistic cutoff resulted in a sensitivity for nonhospitalized cases of at least 92.8% (N or S1) up to 98.8% (N or S1 or RBD). The specificity of the Youden J analyses using N or S1 or RBD dropped to 90.9%. This specificity is far too low for serosurveillance purposes in areas of low prevalence. The specificity-optimized cutoff (95.8%–97.8%) is clearly better, which may be considered adequate if the true prevalence in the

Table 1. Specificity and Sensitivity of Single and Multiplex Analyses of Seroconversion

		Specificity optimized		Youdens J statistic	
Antigen	Hospitalized	Sensitivity, %	Specificity, %[a]	Sensitivity, %	Specificity, %[a]
Single					
N	Yes	100	98.7	100	98.2
N	No	82.7		85.2	
S1	Yes	94.3	99.1	97.1	98.1
S1	No	76.3		91.3	
RBD	Yes	94.1	98.0	97.1	94.5
RBD	No	78.3		91.6	
OR					
N or S1	Yes	100	97.8	100	96.3
N or S1	No	88.0		92.8	
N or RBD	Yes	100	96.7	100	92.8
N or RBD	No	91.7		95.2	
S1 or RBD	Yes	97.1	97.2	97.1	92.7
S1 or RBD	No	90.4		96.4	
N or S1 or RBD	Yes	100	95.8	100	90.9
N or S1 or RBD	No	95.1		98.8	
AND					
N and S1	Yes	93.5	100	96.8	100
N and S1	No	70.5		83.3	
N and RBD	Yes	93.5	100	96.8	100
N and RBD	No	69.6		82.3	
S1 and RBD	Yes	91.2	100	97.1	100
S1 and RBD	No	64.6		87.3	
N and S1 and RBD	Yes	90.3	100	96.8	100
N and S1 and RBD	No	59.7		80.5	

Abbreviations: N, nucleoprotein; RBD, receptor binding domain; S1, spike protein subunit 1.

[a]Specificities per antigen apply to both rows, hospitalized yes/no.

population is above 20%. Because in most countries the overall COVID-19 seroprevalence is currently under 20%, high specificity is required to provide reliable seroprevalence estimates (illustrated in Supplementary Table 1). This could be achieved by defining a sample positive when at least 2 antibody test results in multiplex are above the cutoff. This resulted in a specificity of 100% for any of the combinations and both the specificity-optimized and the Youden J statistic-determined cutoffs (logical AND; Table 1). As expected, this increased specificity comes at the expense of the sensitivity. Here, if only S1 and RBD are taken into consideration, this combination resulted in the highest possible sensitivity of 87.3% and 97.1% for nonhospitalized and hospitalized patients, respectively.

DISCUSSION

We aimed to develop a high-throughput quantitative assay to measure true concentrations of antibodies to spike S1, spike RBD, and N of SARS-CoV-2. The assay presented here uses a very small sample volume, which can be obtained from, for example, fingerstick blood, while retaining highly quantitative output. This bead-based multiplex immunoassay generates robust results and is able to discriminate COVID-19 with different degrees of disease severity, especially from day 10 of disease onward. The results of the assay presented here provide detailed insight into the performance of the assay in terms of parallelism between the references and sera containing different concentrations of antibodies. In addition, we show consistency of assay results when the same samples are measured on independent days, by different investigators using different batches of reagents, basically incorporating all potential variability.

Large population studies are in high demand to provide insight into the spread of the virus and the protective status of the population, which can be used for policy makers to manage the pandemic or lift the lockdown measures [2, 3, 11, 12]. Assays results have to be accurate to generate reliable seroprevalence data of the general population. In addition to knowing the performance of an assay, we need to understand how the majority of infections in the general population relate to the induction of detectable antibodies. Our data comparing hospitalized and nonhospitalized cases revealed that milder disease results in both lower levels of antibodies and later seroconversion, which is in line with previous reports [19, 20]. Also, comorbidities may play a role in the production of specific serum antibodies following infection, which warrants further study. Approximately 10% of the nonhospitalized cases in our selection did not show any seroconversion at all, indicating that such mild infections

may not be detected by serological assays. However, assay performance could be improved by adding other SARS-Cov-2 proteins or subunits of these to further improve the sensitivity of the assay to detect low seroconversion in some cases.

Essential performance characteristics of assays aiming to identify seroprevalence in the population are the specificity and sensitivity. The specificity and sensitivity determine the positive and negative predictive value (PPV and NPV) of the assay given the prevalence of seropositivity in the population [21]. In current low-prevalence settings insufficient specificity will generate a low PPV, resulting in a significant overestimation of the proportion of seropositive individuals (illustrated in Supplementary Table 1). However, the accuracy of the reported sensitivity and specificity of an assay also highly depends on the patient selection used for this evaluation, for example, using sera of severe COVID-19 patients will result in beneficial statistics of an assay because of the acknowledged higher antibody concentration and seroconversion rate [22]. These statistics will not apply in a population serosurvey where the majority of persons will not develop severe COVID-19. For this reason, we included a heterogeneous group of COVID-19 patients' samples, consequently reducing sensitivity. Scoring samples positive if at least 2 of the analytes generated positive results improved the specificity of the assay to 100% at a sensitivity > 90%. At a true seroprevalence of 5%, this would provide a seroprevalence estimate of 4.5% and therefore would be much more accurate than using a single analyte. We recommend transparent reporting of underlying assay performance using heterogeneous panels of controls and COVID-19 patients. Furthermore, implementation of international reference materials as being distributed by, for example, the National Institute for Biological Standards and Control, to facilitate comparison of seroepidemiological data between studies and countries is greatly recommended [1, 23].

From an immunological point of view, it needs to be established which SARS-CoV-2–specific antibodies correlate with protection. Antibodies to RBD of S1 have been shown to associate with neutralization of the virus in vitro, and preliminary data indicate that the antibodies reported in our assay correlate quantitatively with virus neutralization in vitro as well [6]. The data presented here show detection of total IgG. Another study has shown that IgG subclasses are not equally induced by SARS-CoV-2 infection, with a bias towards the production of IgG3, at least in the first weeks after infection [24]. Infection with SARS-CoV-2 also induces the production of IgA and IgM, which can contribute to protection and in vitro neutralization of the virus, but these isotypes are currently not captured by our assay [7, 8, 25]. Follow-up studies are needed to establish the longevity of the production of antibodies, the degree of protection antibodies confer through various Fc receptor-mediated and other mechanisms, and how B-cell memory is induced. Such studies should also consider different viral loads detected in a patient and degree of severity of COVID-19.

In conclusion, we developed a robust multiplex assay to detect antibodies to SARS-CoV-2 in small blood volumes. Our study is unique in validating the assay against HCoV and non-HCoV ILI panels. Because of the differences in seroconversion rates and quantitative antibody concentrations among nonhospitalized COVID-19 cases, which represents the majority of patients in the general population, further investigation is required to improve assay performance for serosurveys in general. We show the advantages of multiplexed analysis in determining seroconversion and provide a framework for reliable seroprevalence estimates in different settings.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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