Multiplexed Serum Antibody Screening Platform Using Virus Extracts from Endemic *Coronaviridae* and SARS-CoV-2

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ABSTRACT: The presence of antibodies against endemic corona-viruses has been linked to disease severity after SARS-CoV-2 infection. Assays capable of concomitantly detecting antibodies against endemic coronaviridae such as OC43, 229E, NL63, and SARS-CoV-2 may help to elucidate this question. We developed a serum screening platform using a bead-based Western blot system called DigiWest, capable of running hundreds of assays using microgram amounts of protein prepared directly from different viruses. Characterization of the immunoassay for detection of SARS-CoV-2 specific antibodies revealed a sensitivity of 90.3% and a diagnostic specificity of 98.1%. Concordance analysis with the SARS-CoV-2 immunoassays available by Roche, Siemens, and Euroimmun indicates comparable assay performances (Cohen's κ ranging from 0.8874 to 0.9508). Analogous assays for OC43, 229E, and NL63 were established and combined into one multiplex with the SARS-CoV-2 assay. Seroreactivity for different coronaviruses was detected with high incidence, and the multiplex assay was adapted for serum screening.

KEYWORDS: SARS-CoV-2, COVID-19, endemic coronavirus, serology, Luminex, Western blot
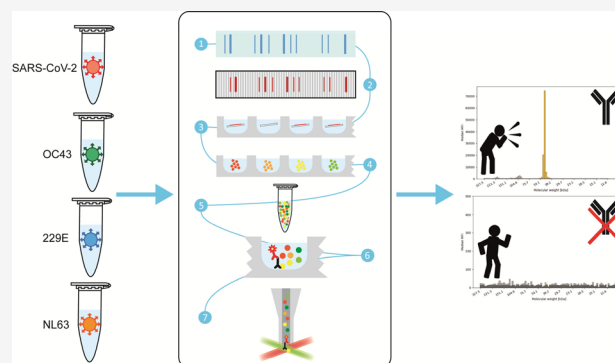
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a newly identified beta coronavirus that crossed the species barrier and found its way into the human population in 2019. It causes the coronavirus disease 2019 (COVID-19), and the ongoing pandemic has a devastating effect on wide parts of the human population.[1] The virus is highly contagious causing the disease to spread very rapidly, yet symptoms of infected individuals vary widely. A fraction of COVID-19 patients develop a fatal course of the disease, while mild COVID-19 cases are frequently observed.[2] Different comorbidity factors were recently identified, whereas the prediction of the course of the disease is not yet possible.[3] Protective antibodies formed after infection are associated with viral clearance, but the occurrence of high antibody titers has also been linked to more serious forms of the disease.[4] A role of pre-existing and cross-reacting antibodies, from endemic coronaviruses, that recognize proteins from SARS-CoV-2 is discussed,[5,6] and a phenomenon termed antibody-dependent enhancement (ADE), which is linked to existing antibodies, might be one of the reasons for life-threatening symptoms occurring during later stages of COVID-19.[7−9] In contrast, a pre-existing cross-reactive T cell memory for SARS-CoV-2 does exist in a significant proportion of the population,[10,11] and

it is likely to be caused by previous infections with endemic coronaviruses. This observation might explain some of the heterogeneity observed in COVID-19, yet the role of the antibody response against these viruses remains elusive. Assays capable of detecting antibodies against endemic coronaviridae, such as OC43, 229E, and NL63, will help us to understand a possible role of existing antibodies against these human coronaviruses during COVID-19. Available systems are using recombinant antigens to detect viral protein-directed antibodies in serum or plasma samples. This approach is not only economical but also makes the generation of large reagent batches feasible, allowing for the generation of vast numbers of assays required for systematic sample screening.[12,13] Here, we employ a novel way of building a serologic assay system to detect and characterize anti-SARS-CoV-2 antibodies. The

utilized approach is based on the classical Western blot procedure, which has been modified to be run as a high-throughput assay system. The use of antigens reflecting the complete pathogen proteome for antibody detection has been employed since the late 1970s[14] and was subsequently proven to be useful for identifying proteins recognized during the humoral immune response. In lysates prepared from infectious virus particles, not only are all possible viral proteins present and can be probed in one assay, but also the use of native-like antigens should enable the detection of antibodies recognizing relevant protein modifications present in the naturally occurring pathogen.

The DigiWest procedure, which is employed here, is a variant of the classical Western blot. It addresses the most obvious disadvantages of Western blotting, namely, its low throughput, high antigen consumption, and poor reproducibility.[15] In the DigiWest, the assay signal is generated on microspheres rather than on a membrane, thus allowing the use of fast and standardized assay protocols on the Luminex platform. Due to the possibility of multiplexing, multiple antigens from different viruses can be probed at the same time, enabling the setup of semiquantitative seroreactivity screens.

■ RESULTS

DigiWest for Detecting Serum Antibodies against SARS-CoV-2. Here, we used the DigiWest for size dependent separation of virus proteins representing the entire viral proteome and for their subsequent immobilization on microspheres in order to adapt this technology to serum analysis (Figure 1). As a first step for detecting serum antibodies recognizing viral proteins, lysates from infectious SARS-CoV-2 virus particles were prepared in SDS-PAGE loading buffer. DigiWest was performed as described using 0.5 μg of virus protein, and Luminex microspheres sufficient to run hundreds of assays were generated. Detection of total protein on the loaded DigiWest beads (Figure 2a) showed characteristic protein bands for the lysate. Using an antibody generated against the SARS-CoV-2 nucleocapsid, a prominent peak at 47.2 kDa (Figure 2b) was detected, which is consistent with the expected size of the protein. Another antibody generated against the SARS-CoV-2 spike protein detects a prominent peak at 141 kDa, the expected molecular weight (Figure 2c). In the next step, human sera were diluted 1:200 in an optimized and modified serum assay buffer and incubated with the DigiWest microspheres. High signals were detected from COVID-19 convalescent sera. Most sera showed their main peak of reactivity at 47 kDa, i.e., the size corresponding to the SARS-CoV-2 nucleocapsid protein (Figure 2e). For SARS-CoV-2 negative samples, no or very low signals were obtained (Figure 2d). Assay background was found to be variable, but since the determined signal intensities only consist of the peak area, reliable values were calculated using the DigiWest evaluation tool.[15] Since reactivity against the nucleocapsid protein was consistently found in COVID-19 convalescent sera, these values were used for describing SARS-CoV-2 seroreactivity.

Multiplexed DigiWest for Detecting Serum Antibodies Recognizing Different Human *Coronaviridae*. In order to expand the assay to cover human endemic coronaviruses, virus lysates from the two alpha coronaviruses 229E and NL63 and from the beta coronavirus OC43 were processed as described, and equivalent DigiWest assays were established and combined into one assay system. When using
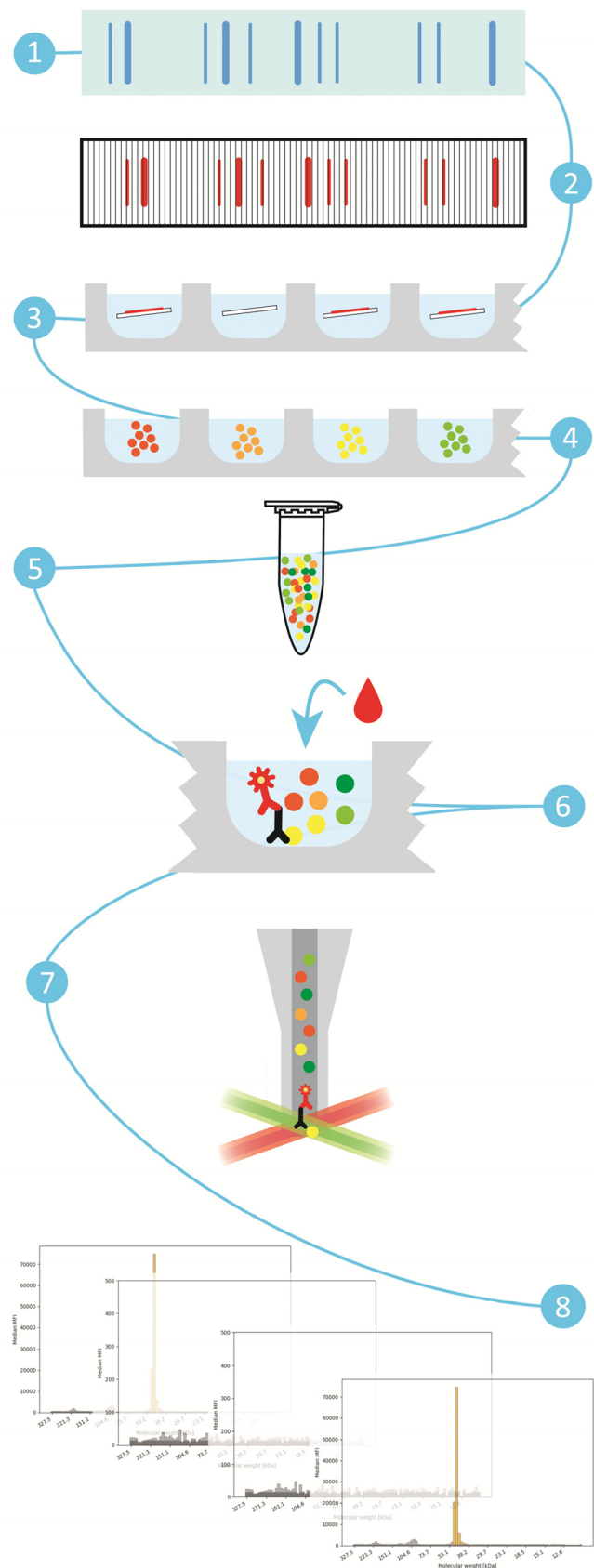
Figure 1. Schematic overview of the DigiWest workflow (modified from Treindl et al.[15] CC BY 4.0): (1) Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). (2) Blotting of proteins to membrane and biotinylation of immobilized proteins directly on the membrane. Cutting of sample lanes into 96

Figure 1. continued

stripes to generate 96 molecular weight fractions immobilized on the membrane. (3) Elution of the proteins in 96-well plates. (4) Loading of biotinylated proteins onto 96 distinct Neutravidin-coated magnetic Luminex bead populations. (5) Pooling into bead pools and reconstitution of the initial sample lane. (6) Immunoassay: aliquots of the generated bead pool (<0.5%) are incubated with specimen before PE-labeled secondary antibodies are added for signal generation. (7) Readout using a Luminex instrument, (8) reconstitution of the initial lane and data analysis.

sera from SARS-CoV-2 negative individuals, seroreactivity against endemic viral proteins was found for a large fraction of tested samples. As for the SARS-CoV-2 DigiWest assay, the main serological activity for the different viruses was detected at a molecular weight corresponding to nucleocapsid proteins. To prove that the detected proteins are indeed the nucleocapsids of the different coronaviruses, we produced recombinant versions of the nucleocapsid proteins of all tested viruses. We used the purified proteins in a different DigiWest experiment and compared the obtained signals with the signals obtained from the whole virus lysate DigiWest (Figure 3a). In the whole virus lysate, the observed molecular weight of SARS-CoV-2 nucleocapsid protein was 47.2 kDa with a calculated molecular weight of 45.6 kDa. For OC43 nucleocapsid protein, 229E nucleocapsid protein, and NL63 nucleocapsid protein, the values were 53.1 kDa (calculated 49.3 kDa), 45.4 kDa (calculated 43.5 kDa), and 42.1 kDa (calculated 42.3 kDa),

respectively. Thus, in all cases obtained, molecular weights are in good agreement with the expected values. The molecular weights were confirmed via DigiWest using recombinant proteins (Figure 3b). A small set of 12 sera was used to detect seroreactivity on virus lysates and on the recombinant nucleocapsid proteins. The correlating signal was detected, and this confirmed that the detected reactivity is directed against the nucleocapsid proteins.

Evaluation of the Characteristics of the SARS-CoV-2 Serological Assay. To characterize the performance of the DigiWest, we used the final multiplexed assay now comprising virus lysates of SARS-CoV-2, 229E, OC43 and NL63 to screen a set of characterized samples.[11] Among the analyzed sera, there were 195 SARS-CoV-2 PCR positive specimens, 49 prepandemic samples and 19 self-reported negative samples. The complete data set is available online in Supplementary Data 1. To define the assay cutoff for SARS-CoV-2 seropositivity, 49 prepandemic and noninfected control samples were employed. The highest signal value detected in this group was 1295 average fluorescence intensity (AFI). In a second step, the lowest value of all SARS-CoV-2 PCR-positive specimens still above this intensity (1968 AFI) was defined as a seropositive for SARS-CoV-2. The mean of these two measurements was calculated and defined to be the cutoff for seroconversion (1632 AFI). After the definition of the cutoff, a test set of 53 negative and 31 positive samples was used to determine specificity and sensitivity. The complete data set is available in Supplementary Data 3. Using the defined cutoff, an assay specificity of 98.1% (CI 94.5−100%)
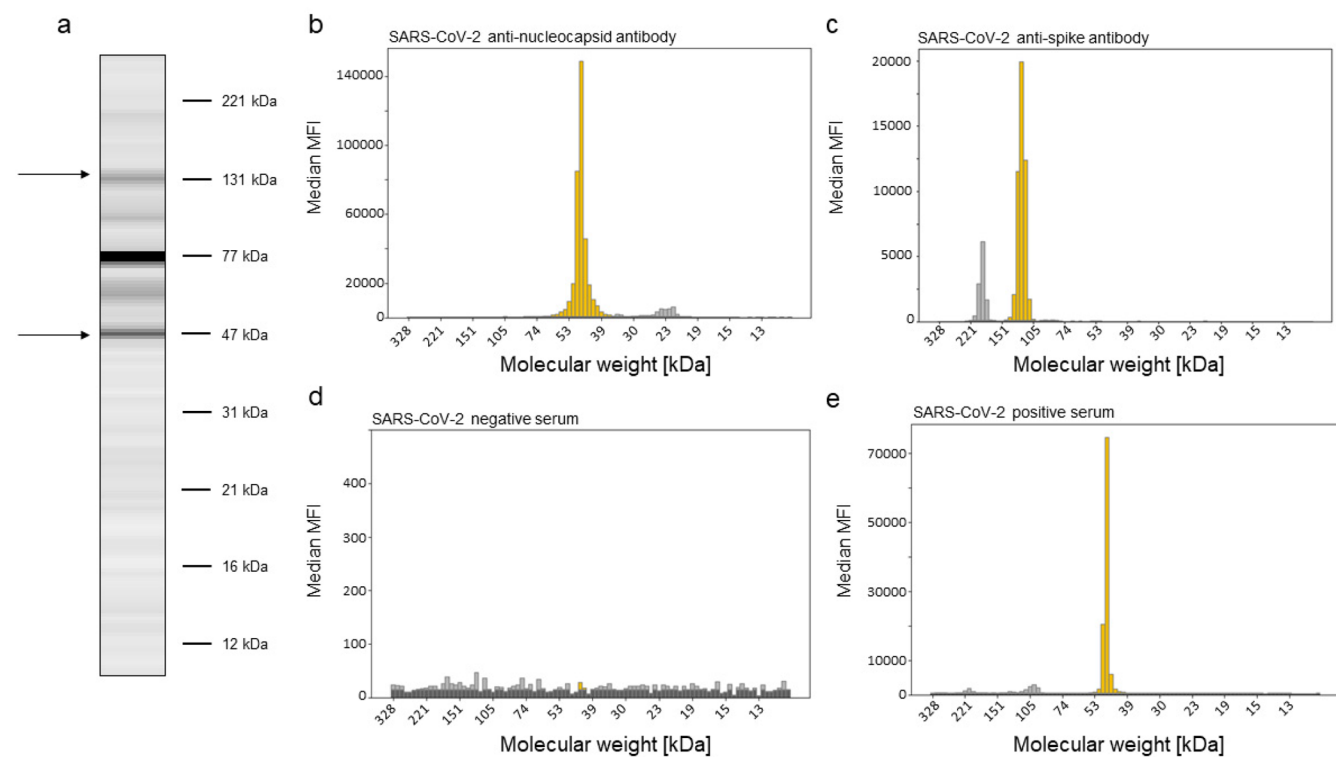
Figure 2. Protein detection on SARS-CoV-2 virus lysate loaded DigiWest beads. Virus proteins were size separated by the DigiWest procedure and transferred to microspheres. In (a), a total protein stain of the separated proteins is shown; data are represented as a Western blot mimic,[15] thereby resembling a SDS-PAGE lane. The marked protein bands corresponds to the viral spike and nucleocapsid protein. An anti-SARS-CoV-2 nucleocapsid antibody detects this protein at the expected molecular weight (47.2 kDa) (b). A different antibody detects the spike protein at the expected molecular weight (141 kDa) (c). Serum from a SARS-CoV-2 PCR positive patient reacts with the nucleocapsid protein (47.2 kDa), giving high fluorescent intensity (e), whereas in a negative serum no peaks are detected (d).
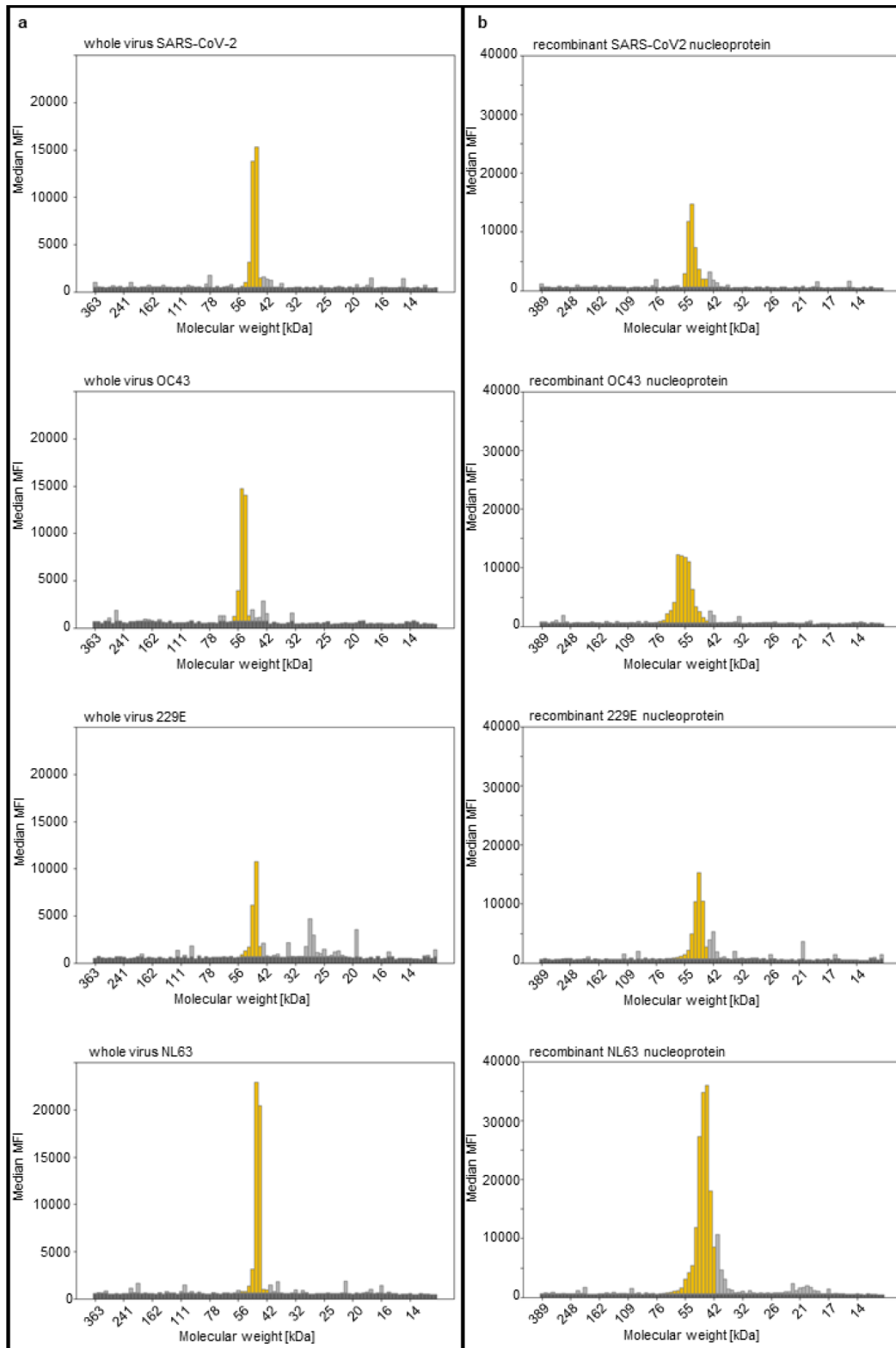
Figure 3. Multiplexed detection of nucleocapsid protein from SARS-CoV-2, OC43, 229E, and NL63. Reactivity of a patient serum was tested on whole virus lysates of the different coronavirus types (a) and on recombinant nucleocapsid proteins of the different viruses (b) using multiplexed DigiWest assays. The used SARS-CoV-2 positive serum shows antibody reactivity on whole virus lysates for (i) SARS-CoV-2, (ii) OC43, (iii) 229E, and (iv) NL63 (a). In (b), the same serum is incubated with a DigiWest bead set loaded with recombinant nucleocapsid from (i) SARS-CoV-2, (ii) OC43, (iii) 229E, and (iv) NL63. As for the whole virus lysates, antibody reactivity is observed; for SARS-CoV-2, a peak at 47.2 is kDa detected; for the endemic *Coronaviridae* OC43, 229E, and NL63, peaks at the molecular weights at the respective sizes of 53.1, 45.4, and 42.1 kDa, respectively, are found.

was found. Furthermore, 3/28 (10.7%) samples from SARS-CoV-2 PCR positive specimens showed no seroconversion, yielding a sensitivity of 90.3% (CI 79.9−100%). The positive predictive value is 0.966 (CI 0.899−1.0), and the negative predictive value is 0.945 (CI 0.855−1.0). These fundamental characteristics of the newly established assay system are comparable to published values for different commercially available SARS-CoV-2 immunoassays.[16,17]

To demonstrate the dynamic range of the serologic DigiWest assay, three SARS-CoV-2 positive sera with different AFIs were serially diluted with a negative serum (Figure 4). Good signal linearity was seen in the dilution curve and seropositivity was detected for higher AFIs down to a serum dilution of 1:5000.
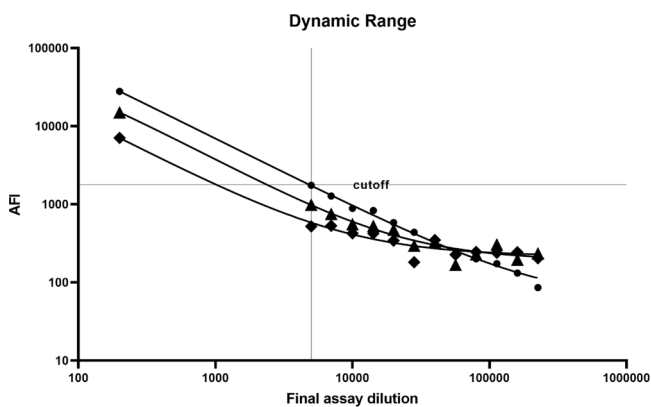
Figure 4. Dynamic range of the DigiWest serological assay. Sera of three SARS-CoV-2 positive patients were diluted in serum of a SARS-CoV-2 negative donor (serial dilution, 13 steps ranging from 1:25 to 1:1131). The mixtures were further diluted 1:200 in serum assay buffer, and the immunoassay was performed. Shown are the final dilutions of positive sera (X-axis) and the resulting average fluorescence intensities (AFI). Logistic regression was performed using a sigmoidal fit and 4-parameter logistics: (●) positive sample 1 (bottom, 54.50; top, 1052419; IC_{50}, 3.317; Hill slope, −0.8797; log IC_{50} 0.5208); (▲) positive sample 2 (bottom, 192.5; top, 106 711; IC_{50}, 29.65; Hill slope, −0.9556; log IC_{50}, 1.472); (◆) positive sample 3 (bottom, 217.7; top, 938327; IC_{50}, 0.9145; Hill slope, −0.9118; log IC_{50}, −0.03881)

For closer evaluation of the assay performance, we reanalyzed the complete sample set using the (i) Elecsys anti-SARS-CoV-2 assay (Roche Diagnostics), (ii) ADVIA Centaur SARS-CoV-2 (Siemens Healthcare Diagnostics),[18] (iii) EUROIMMUN SARS-CoV-2 IgG ELISA, and (iv) EUROIMMUN SARS-CoV-2 IgA ELISA test systems. Further information on the assay procedures is provided in the Methods section. Concordance (Cohen's κ) and correlation (Spearman's r) analyses were performed, and the different assay characteristics were compared and visualized (Figure 5). Concordance of DigiWest vs Roche was found to be 0.9508 (95% CI; 0.91−0.99 Figure 5a), and for DigiWest vs Siemens Cohen's κ was 0.9100 (95% CI; 0.86−0.96 Figure 5b). Concordance of DigiWest vs Euroimmun IgG was calculated in two ways: if the borderline results were considered positive, Cohen's κ was found to be 0.9180 (95% CI; 0.87−0.97), and if considered negative the concordance was 0.8874 (95% CI; 0.83−0.94 Figure 5c). When comparing the DigiWest based IgG detection to the Euroimmun based IgA test and borderline results were considered positive, a value of 0.7493 (95% CI;

0.67−0.83) was found. If the borderline results were considered negative, Cohen's κ was found to be 0.7512 (95% CI; 0.67−0.83).

Correlation analysis utilizing Spearman's r revealed a positive correlation of all investigated assays (Figure 5d). The highest correlation for DigiWest was found with the Roche system (Spearman's r = 0.91; 95% CI; 0.89−0.93). Spearman's r for DigiWest and Siemens was found to be 0.87 (95% CI; 0.83−0.90). Spearman's r for DigiWest and Euroimmun IgG and IgA was calculated at 0.87 (95% CI; 0.84−0.90) and 0.78 (95% CI; 0.72−0.82), respectively. The highest overall correlation was found between Siemens and Euroimmun IgG (Spearman's r = 0.94; 95% CI; 0.93−0.96), and the lowest overall correlation was found between Euroimmun IgA and Roche (Spearman's r = 0.73; 95% CI; 0.66−0.78).

Multiplexed Detection of Antibodies against SARS-CoV-2, OC43, 229E, and NL63. By integrating DigiWest assays for 229E, OC43, and NL63 into the detection system for SARS-CoV-2, concomitant detection of the presence of antibodies binding to antigens derived from the different *Coronaviridae* becomes possible. In the analyzed sample set, reactive antibodies against all endemic coronaviruses were detected with high frequency (Supplementary Data 1). To estimate the reactivity against the other human endemic coronaviruses, a provisional cutoff for OC43, 229E, and NL63 was defined at the same value as was determined for SARS-CoV-2 (1632 AFI). For SARS-CoV-2 negative sera, 82.4% showed reactivity against OC43 nucleocapsid, 95.6% against 229E, and 100% against NL63. For SARS-CoV-2 positive samples, the numbers were 79.5% against OC43, 99% against 229E, and 98.5% against NL63. The overall reactivity was 80.2% against OC43, 98.1% against 229E, and 98.9% against NL63. Despite the high frequency of antibodies directed against the endemic coronaviruses OC43, 229E, and NL63 in SARS-CoV-2 negative sera, no recognition of SARS-CoV-2 proteins was observed in these samples. This directly translates into the high specificity of the SARS-CoV-2 assay system and reveals only minor or no cross-reactivity of existing antibodies with the existing SARS-CoV-2 nucleocapsid protein. The correlation analysis between all coronaviruses (including SARS-CoV-2) showed values ranging from 0.03 to 0.75 (Figure 6). The highest correlation was observed for antibodies recognizing the nucleocapsid protein of 229E and NL63 with a Spearman's r of 0.75 indicating a possible cross-reactivity.

■ DISCUSSION

The use of proteins from clinically relevant pathogens as antigens for antibody detection is a classical method for identifying an individual immune response.[19] While this classical approach has distinct drawbacks, e.g., the need for isolation of large amounts of pathogen and poor assay reproducibility when using different protein batches, it also provides substantial advantages. With this assay, using denatured and reduced virus lysates, linear epitopes with and without post-translational modifications could be detected. Modifications only found in the authentic proteins are present in antigen preparations, and therefore, the identification of reactive antibodies against these possible pathogen-derived antigens should be feasible. In addition, the generation of protein extract from pathogens of different strains is often technically uncomplicated and fast. This may turn out to be especially useful when a comparative analysis of antigen
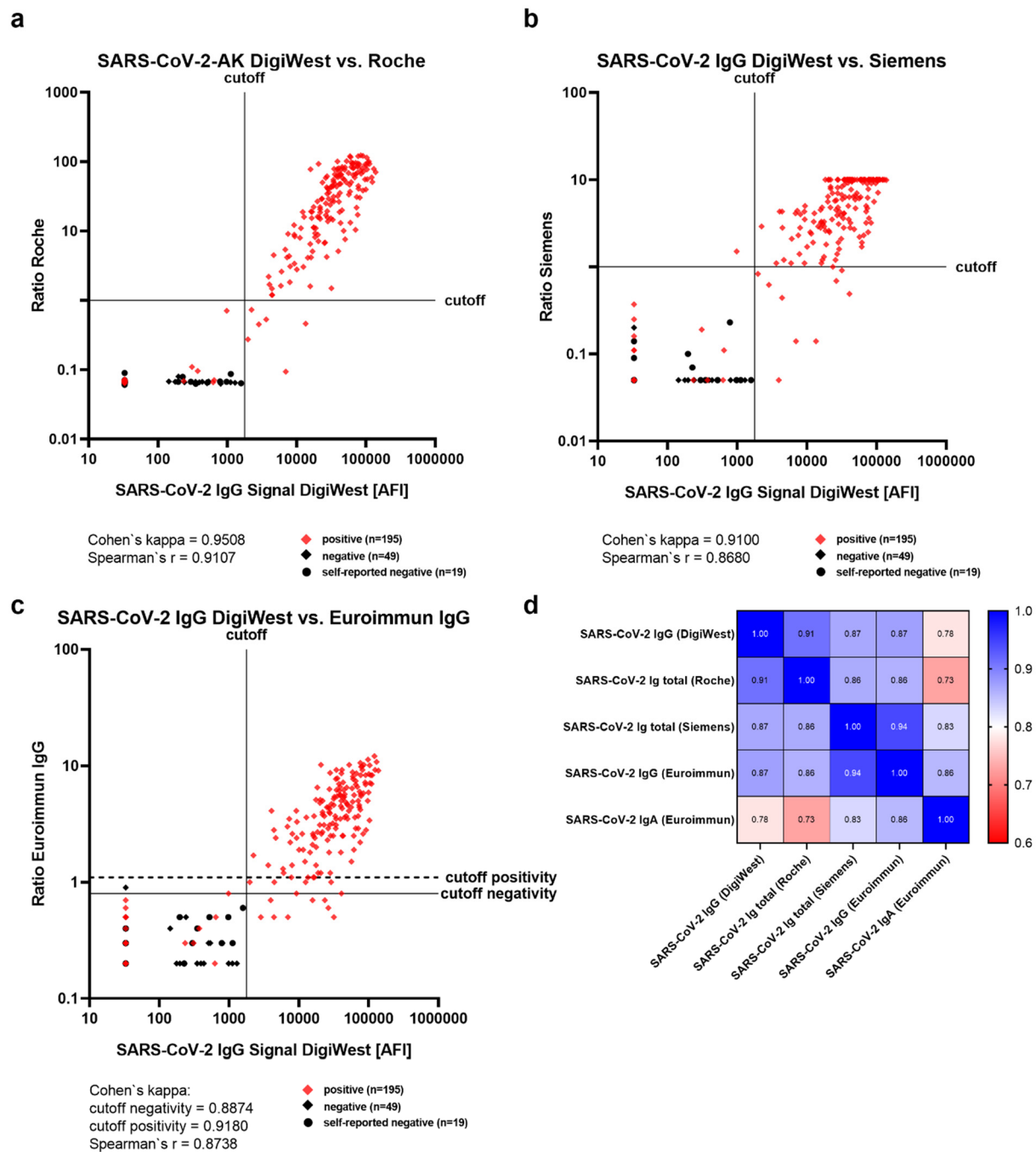
Figure 5. Comparison of the DigiWest seroconversion assay with commercially available SARS-CoV-2 assays. Concordance (Cohen's κ) and correlation coefficients (Spearman's r) of DigiWest data and the commercially assays from Roche (a), Siemens (b), and Euroimmun IgG (c) were calculated and are shown below the plotted data; cutoff values are depicted as a black line in the scatter plot. For the Euroimmun IgG, two different κ values were calculated; when borderline results (as defined by the manufacturer) were considered positive, κ was 0.9180. If the borderline results were considered negative, the concordance for Euroimmun IgG was 0.8874. In (d), the correlation coefficients (Spearman's r) between all used assays are shown in a heat map. The highest value (Spearman's r = 0.91) for DigiWest was found for the Roche system, and the lowest value (Spearman's r = 0.78) for DigiWest vs Euroimmun IgA is shown of Spearman's r values.

preparations from closely related pathogenic agents is of interest. Such an analysis may facilitate the identification of relevant cross-reacting antibodies directly on a wide variety of antigenic structures. These advantages may help set up systems that take an unbiased approach to characterize the humoral immune response.

Here we describe the setup of such an assay system using protein extracts prepared directly from infectious SARS-CoV-2 virus particles. The employed DigiWest procedure is an

immunoblot system that closely resembles the classical Western blot procedure. After SDS-PAGE based protein size separation, proteins are immobilized on polystyrene microspheres and assay readout is performed on the Luminex assay platform. An amount of 10 μg of protein is sufficient to generate batches of assay material sufficient for thousands of serum analyses; this directly translates into good assay reproducibility. In addition, the use of the Luminex platform for readout enables a high assay throughput without the need
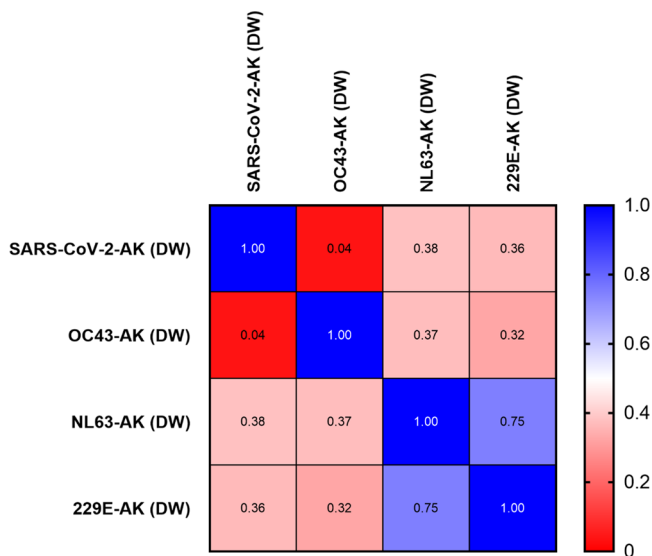
Figure 6. Spearman's rank correlation of SARS-CoV2 and endemic coronavirus types in the serological DigiWest (DW) assay. Data generated for SARS-CoV-2, OC43, 229E, and NL63 were used for correlation analysis, and Spearman's rank coefficients were calculated for assay pairing. Results are displayed as heat map of Spearman's *r* values. A high correlation (Spearman's *r* 0.75) was found between NL63 and 229E indicating cross-reactivity.

for producing recombinant proteins. As in Western blotting, the assay gives direct information on the size of the recognized proteins, and antigenic proteins can often be directly identified. When using COVID-19 convalescent sera, a specific antibody response to a protein of 47 kDa corresponding to the nucleocapsid protein of SARS-CoV-2 was recurrently seen. Reactivity against other viral proteins was present in individual serum samples, yet the nucleocapsid protein was identified as the major antigen in this assay. The observed low seroreactivity against the spike protein could be due to the fact that reduced and denatured proteins are present in the DigiWest and that these are not recognized by most of the anti-spike antibodies in the serum. This indicates that a strong antibody response can be detected on denatured N protein, while other viral proteins are not detected.

For detailed evaluation of the performance of the newly developed assay for detecting anti-SARS-CoV-2 antibodies, a set of more than 250 well-characterized sera was employed, in which sera were mainly taken from a clinical study on T-cell response after SARS-CoV-2 infection.[11] By using four different serological assays that are in use in clinical routine laboratories, we showed high concordance (Cohen's κ 0.86−0.94) between all systems. This demonstrates high standards for all tested assays. Interestingly, the highest concordance (0.94) was found between the Siemens assay system and the Euroimmun IgG assay, with both assays mainly detecting the spike protein. Nearly the same κ value (0.91) was calculated for the Roche and the DigiWest system, both of which use the nucleocapsid protein as the detected antigen. The Euroimmun IgA showed slightly different assay characteristics, which is most likely due to the fact that it is the only assay that exclusively detects IgA immunoglobulins. Yet, no principal differences in assay characteristics were observed and all assays showed reliable detection of seroprevalence after SARS-CoV-2 infection.

Antibodies against endemic coronaviruses are frequently found in human individuals.[20] These viruses cause mild diseases and are associated with approximately 20% of the common colds.[21,22] However, when comparing the sequences of the virus genome, the degree of similarity between the SARS-CoV-2 and these viruses is astonishingly high.[23] This similarity has led to speculations that antibodies against these endemic viruses may also possess protective properties against SARS-CoV-2.[24] The presence of these antibodies might explain the vastly diverse courses of disease. Therefore, the DigiWest assay system was expanded by using lysates from alpha coronaviruses 229E and NL63 as well as from the beta coronaviruses OC43, thus enabling the detection of serum antibodies recognizing antigens from four coronaviruses in one assay. The implementation of these assays directly succeeded the method used for SARS-CoV-2 and seroreactivity toward the nucleocapsid protein was frequently found for these coronaviruses.

As expected, a very high rate of infection for all of the coronaviruses was found, yet no obvious indication of cross-reactivity to the SARS-CoV-2 proteins was seen in negative SARS-CoV-2 samples. This is in contrast to the described T-cell response that can be triggered by peptides derived from the SARS-CoV-2 nucleocapsid.[11,25] In a first, smaller scale screening approach the described assay system was used in combination with an antibody neutralization assay that detects the presence of serum antibodies capable of neutralizing SARS-CoV-2.[26] In this work, we show evidence that antibodies against the endemic coronavirus 229E contribute to SARS-CoV-2 neutralization. This result proves the advantage of a multiplexed system for detecting serum antibodies directed against different closely related viral pathogens, and this may help to understand the highly variable immune responses observed in different individuals.

As a serological assay system, the DigiWest is not only a novelty, but it allows the setup of a highly specific assay within a very short time frame. Its flexibility enables the integration of antigen extracts from all kinds of sources, and it is capable of detecting a wide variety of serum antibodies since complex mixtures of different pathogen-derived proteins can be probed in one reaction. Yet, the setup of the system is complex and requires (i) propagation and handling of the pathogens and (ii) generation of the antigen loaded microspheres, (iii) before the actual serum assay is performed. Only specialized research laboratories do have the capability to perform all of these steps. The fact that the three steps of the DigiWest procedure can easily be separated changes the situation. It opens the possibility to bring the technology to the large number of clinical and research laboratories that use the widely distributed Luminex platform for serum analysis. Large batches of antigen loaded beads, sufficient to run tens of thousands of assays can be produced by specialized laboratories using the established DigiWest workflow. These bead sets are stable, and the actual serum screening is easily performed in such clinical laboratories.

While this will broaden the applicability of the approach substantially, the setup of such an assay is mainly interesting when approaching specific questions that cannot be answered easily when using standard serological assay systems. Here we show that the fast setup of an assay for detecting antibodies against novel pathogens is possible by using crude protein extracts. Other questions may include the identification of antigenic structures in complex protein mixtures from bacteria and viruses. Running multiplexed serological assays that combine antigens from similar pathogens to identify the

binding characteristics of existing antibodies may even be important to identify changes in immunogenicity during the development of new pathogenic strains.

METHODS

Experimental Design. Assays capable of detecting antibodies against endemic *Coronaviridae* such as OC43, 229E, and NL63 will provide a better understanding of pre-existing antibodies against these closely related *Coronaviridae* during COVID-19. The use of antigens prepared directly from isolated virus particles and their use in the bead-based Western blot system DigiWest provide a fast and simple way of generating a multiplexed assay capable of detecting seroreactivity against these viruses. Starting with clinically characterized serum samples with a documented presence of anti SARS-CoV-2 antibodies and protein lysates prepared from SARS-CoV-2, a specific assay is built and the characteristics of the system are determined. In a second step, protein extracts from OC43, 229E, and NL63 are used to setup analogous assays and are later integrated into one multiplexed assay system.

Patients and Blood Samples. A total of 263 pre-existing and deidentified serum samples were used for assay development. Ethical approval was granted from the Ethics Committee of University Hospital Tübingen; samples from 193 SARS-CoV-2 polymerase chain reaction (PCR) positive individuals (179/2020/BO2) and of 18 self-reported negative samples were collected (179/2020/BO2). A self-reported healthy serum sample ($n = 1$) and self-reported convalescent serum after SARS-CoV-2 infection ($n = 2$) were obtained at the NMI under the guidelines of the local ethics committees (495/2018/BO2). Sample collection for each donor was performed approximately 3−8 weeks after the end of symptoms and/or negative virus smear. In addition, samples from healthy donors obtained from Central BioHub before 8/2019 were used as negative controls ($n = 49$). All available information can be found in Supplementary Data 1 and 2. For the determination of sensitivity and specificity, a test set of 31 SARS-CoV-2 PCR positive individuals and 53 self-reported negative individuals was used (9122/BO/K/2020). All information on these samples can be found in Supplementary Data 3.

SARS-CoV-2 Virus Lysate. To prepare SARS-CoV-2 virus lysate, the supernatant of infected human Caco-2 cells was purified. Briefly, Caco-2 cells were infected 1:10−1:500 with clinical isolate 200325_Tü1. At 48 h postinfection, the supernatant was collected, centrifuged, and frozen. A volume of 900 μL of supernatant was added to 200 μL of 20% sucrose and centrifuged for 90 min at 4 °C and 14 000 rpm. The supernatant was discarded, and a PBS washing step was done, followed by another centrifugation step. The supernatant was discarded, and the viral pellet was resuspended in 25 μL of lithium dodecyl sulfate (LDS) sample buffer (Life Technologies) and heated for 5 min at 95 °C.

Multiplex Serum Reactivity Test via DigiWest. Whole viral protein lysates from 229E, OC43, and NL63 (ZeptoMetrix Corp) and SARS-CoV-2 were used for DigiWest as described. First, viral protein lysates were subjected to gel electrophoresis and Western blotting using the NuPAGE system. Membranes were washed with PBST (0.1% Tween-20, PBS), and membrane-bound proteins were biotinylated by adding 50 μM NHS-PEG12-Biotin (Thermo Fisher Scientific) in PBST for 1 h. After washing in PBST, membranes were dried overnight. Subsequently, the Western blot lanes were cut

into 96 strips of 0.5 mm width and were transferred to a 96-well plate (Greiner Bio-One). For protein elution, 10 μL of elution buffer was added to each well (8 M urea, 1% Triton-X100 in 100 mM Tris-HCl pH 9.5). The protein eluates were diluted with 90 μL of dilution buffer (5% BSA in PBST, 0.02% sodium azide). Neutravidin-coated MagPlex beads (Luminex) of a distinct color ID were added to the protein eluates, and binding was allowed overnight; 500 μM PEG12-biotin in PBST was added to block remaining Neutravidin binding sites. The bead containing fractions were pooled, and thereby the original Western blot lanes were reconstituted. Beads were washed in PBST and resuspended in storage buffer (1% BSA, 0.05% azide, PBS). The generated bead set represents the proteomes of the four coronaviruses (SARS-CoV-2, OC43, 229E, NL63), and reactivity against all proteins can be tested in one assay.

For serum incubation, 5 μL of the bead mix was equilibrated in 50 μL of serum assay buffer (Blocking Reagent for ELISA (Roche) supplemented with 0.2% milk powder, 0.05% Tween-20 and 0.02% sodium azide, 25% Low Cross buffer (Candor Bioscience), 25% IgM-reducing agent buffer (ImmunoChemistry)). Serum assay buffer was discarded, and 30 μL of diluted patient serum (1:200 in serum assay buffer) was added and incubated for 2 h at room temperature on a shaker. After washing in PBST, 30 μL of phycoerythrin labeled anti-human IgG secondary antibody (diluted 1:200 in serum assay buffer; Dianova) was added and the plate was incubated for 45 min at 23 °C. The beads were washed twice with PBST, and readout was performed on a Luminex FlexMAP 3D platform.

The DigiWest analysis tool was used to assess serum reactivity against the viral proteins.[15] Virus protein-specific peaks were identified, and average fluorescence intensity (AFI) values were calculated by integration of peak areas. To detect the nucleocapsid and the spike protein of SARS-CoV-2, commercially available antibodies were used (Sino Biologicals; nucleocapsid 40143-R019; spike protein 40591-MM42). Incubation was performed as described previously.[27]

Generation of Expression Constructs for Production of Viral Antigens. The cDNAs encoding the nucleocapsid proteins of SARS-CoV-2, OC43, NL63, and 229E (GenBank accession numbers QHD43423.2; YP_009555245.1; YP_003771.1; NP_073556.1) were produced by gene synthesis (Thermo Fisher Scientific) and cloned including N-terminal hexahistidine (His6)-tag by standard techniques into NdeI/HindIII sites of the bacterial expression vector pRSET2b (Thermo Fisher Scientific).

Protein Expression and Purification. For production of the viral nucleocapsid proteins, the respective expression constructs were used to transform *E. coli* BL21(DE3) cells. Protein expression was induced in 1 L of TB medium at an optical density (OD600) of 2.5−3 by addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 20 °C. Cells were harvested by centrifugation (10 min 6000g), and the pellets were suspended in binding buffer (1× PBS, 0.5 M NaCl, 50 mM imidazole, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM MgCl2, 150 μg/mL lysozyme (Merck), and 625 μg/mL DNaseI (Applichem)). The cell suspensions were sonified for 15 min (Bandelin Sonopuls HD70, power MS72/D, cycle 50%) on ice, incubated for 1 h at 4 °C in a rotary shaker, and sonified again. After centrifugation (30 min at 20 000g), urea was added to a final concentration of 6 M to the soluble protein extract. The extract was filtered through a a 0.45 μm filter and loaded on a pre-equilibrated 1 mL

HisTrapFF column (GE Healthcare). The bound His-tagged nucleocapsid proteins were eluted by a linear gradient (30 mL) ranging from 50 to 500 mM imidazole in elution buffer (1× PBS, pH 7.4, 0.5 M NaCl, 6 M urea). Elution fractions (0.5 mL) containing the His-tagged purified proteins were analyzed via standard SDS-PAGE followed by staining with InstantBlue Coomassie stain (Expedeon). Immunoblotting using an anti-His antibody (Penta-His antibody, #34660, Qiagen) in combination with a donkey anti-mouse antibody labeled with AlexaFluor647 (Invitrogen) on a Typhoon Trio analyzer (GE-Healthcare, excitation 633 nm, emission filter settings 670 nm BP 30) was performed to confirm protein integrity.

Commercial Serological Assays. *SARS-CoV-2 IgG and IgA ELISA (EUROIMMUN AG).* The 96-well SARS-CoV-2 IgG ELISA and the 96-well SARS-CoV-2 IgA ELISA assay (EUROIMMUN) were performed on an automated BEP 2000 Advance system (Siemens Healthcare Diagnostics GmbH) according to the manufacturer's instructions. The ELISA assay detects anti-SARS-CoV-2 IgG and IgA, directed against the S1 domain of the viral spike protein, and relies on an assay-specific calibrator to report a ratio of specimen absorbance to calibrator absorbance. The final interpretation of positivity is determined by a ratio above a threshold value given by the manufacturer: positive (ratio ≥ 1.1), borderline (ratio = 0.8–1.0), or negative (ratio < 0.8). Quality control was performed following the manufacturer's instructions on each day of testing.

Elecsys Anti-SARS-CoV-2 Immunoassay (Roche Diagnostics GmbH). The Elecsys anti-SARS-CoV-2 assay is an ECLIA (electrogenerated chemoluminescence immunoassay) assay designed by Roche Diagnostics GmbH and was used according to the manufacturer's instructions. It is intended for the detection of high affinity antibodies (including IgG) directed against the nucleocapsid protein of SARS-CoV-2 in human serum. Readout was performed on the Cobas ae411 analyzer. Negative results were defined by a cutoff index (COI) of <1.0. Quality control was performed following the manufacturer's instructions on each day of testing.

SARS-CoV-2 Total (COV2T) Immunoassay (Siemens Healthcare Diagnostics Inc.). The ADVIA Centaur SARS-CoV-2 Total (COV2T) assay is a chemiluminescent immuno-assay intended for qualitative detection of total antibodies (including IgG and IgM) against SARS-CoV-2 in human serum and was used according to the manufacturer's instructions. The system reports ADVIA Centaur COV2T assay results in index values and as nonreactive < 1.0 or reactive ≥ 1.0. Nonreactive samples are considered negative for SARS-CoV-2 antibodies; reactive samples are considered positive for SARS-CoV-2 antibodies.

Statistical Analysis. Sensitivity and specificity for each assay were calculated using the results of the PCR-testing as the gold standard. Concordance was calculated using Cohen's κ with 95% confidence intervals (CI).[28] Correlation was calculated using Spearman's r with 95% CI. For determining the dynamic range, a sigmoidal, 4-parameter logistic regression was used to fit the data and interpolate the dilution factor at the cutoff signal. All statistical analyses were performed using GraphPad Prism 8 or R studio (ver. 1.3.959).

Data Availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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△S.F. and F.R. contributed equally to this work. S.F. and F.R. planned, designed and performed experiments and data analysis; A.S., M.B., and D.J. planned and performed experiments; P.K., B.T., F.W., and U.R designed, cloned, expressed, and purified the nucleocapsid proteins; N.R. and M.S. prepared SARS-CoV-2 virus lysate; S.H. and A.P. performed sample analysis; A.N., J.W., A. B., M.T., and T.J. arranged sample and data collection; G.K., N.S.M., T.J., and K.S.L. supported the study planning; S.F., F.R., and M.T. wrote the manuscript. All authors reviewed the manuscript.

Notes

The authors declare the following competing financial interest(s): M.F.T. has filed patent PCT/EP2012/062403 that covers parts of the method. The remaining authors declare no competing financial interests.

■ REFERENCES

(1) Wu, F., Zhao, S., Yu, B., Chen, Y. M., Wang, W., Song, Z. G., Hu, Y., Tao, Z. W., Tian, J. H., Pei, Y. Y., Yuan, M. L., Zhang, Y. L., Dai, F. H., Liu, Y., Wang, Q. M., Zheng, J. J., Xu, L., Holmes, E. C., and Zhang, Y. Z. (2020) A New Coronavirus Associated with Human Respiratory Disease in China. *Nature* 579 (7798), 265−269.

(2) Yang, X., Yu, Y., Xu, J., Shu, H., Xia, J., Liu, H., Wu, Y., Zhang, L., Yu, Z., Fang, M., Yu, T., Wang, Y., Pan, S., Zou, X., Yuan, S., and Shang, Y. (2020) Clinical Course and Outcomes of Critically Ill Patients with SARS-CoV-2 Pneumonia in Wuhan, China: A Single-Centered, Retrospective, Observational Study. *Lancet Respir. Med.* 8 (5), 475−481.

(3) Rebello, C. J., Kirwan, J. P., and Greenway, F. L. (2020) Obesity, the Most Common Comorbidity in SARS-CoV-2: Is Leptin the Link? *Int. J. Obes.* 44, 1810.

(4) Long, Q.-X., Tang, X.-J., Shi, Q.-L., Li, Q., Deng, H.-J., Yuan, J., Hu, J.-L., Xu, W., Zhang, Y., Lv, F.-J., Su, K., Zhang, F., Gong, J., Wu, B., Liu, X.-M., Li, J.-J., Qiu, J.-F., Chen, J., and Huang, A.-L. (2020) Clinical and Immunological Assessment of Asymptomatic SARS-CoV-2 Infections. *Nat. Med.* 26, 1200.

(5) Huang, A. T., Garcia-Carreras, B., Hitchings, M. D. T., Yang, B., Katzelnick, L. C., Rattigan, S. M., Borgert, B. A., Moreno, C. A., Solomon, B. D., Trimmer-Smith, L., Etienne, V., Rodriguez-Barraquer, I., Lessler, J., Salje, H., Burke, D. S., Wesolowski, A., and Cummings, D. A. T. (2020) A Systematic Review of Antibody Mediated Immunity to Coronaviruses: Kinetics, Correlates of Protection, and Association with Severity. *Nat. Commun.* 11 (1), 1−16.

(6) Ng, K. W., Faulkner, N., Cornish, G. H., Rosa, A., Harvey, R., Hussain, S., Ulferts, R., Earl, C., Wrobel, A. G., Benton, D. J., Roustan, C., Bolland, W., Thompson, R., Agua-Doce, A., Hobson, P., Heaney, J., Rickman, H., Paraskevopoulou, S., Houlihan, C. F., Thomson, K., Sanchez, E., Shin, G. Y., Spyer, M. J., Joshi, D., O'Reilly, N., Walker, P. A., Kjaer, S., Riddell, A., Moore, C., Jebson, B. R., Wilkinson, M., Marshall, L. R., Rosser, E. C., Radziszewska, A., Peckham, H., Ciurtin, C., Wedderburn, L. R., Beale, R., Swanton, C., Gandhi, S., Stockinger, B., McCauley, J., Gamblin, S. J., McCoy, L. E., Cherepanov, P., Nastouli, E., and Kassiotis, G. (2020) Preexisting and de Novo Humoral Immunity to SARS-CoV-2 in Humans. *Science (Washington, DC, U. S.)* 370, 1339.

(7) Arvin, A. M., Fink, K., Schmid, M. A., Cathcart, A., Spreafico, R., Havenar-Daughton, C., Lanzavecchia, A., Corti, D., and Virgin, H. W. (2020) A Perspective on Potential Antibody-Dependent Enhancement of SARS-CoV-2. *Nature* 584, 353.

(8) Tetro, J. A. (2020) Is COVID-19 Receiving ADE from Other Coronaviruses? *Microbes Infect.* 22 (2), 72−73.

(9) Shrock, E., Fujimura, E., Kula, T., Timms, R. T., Lee, I.-H., Leng, Y., Robinson, M. L., Sie, B. M., Li, M. Z., Chen, Y., Logue, J., Zuiani, A., McCulloch, D., Lelis, F. J. N., Henson, S., Monaco, D. R., Travers, M., Habibi, S., Clarke, W. A., Caturegli, P., Laeyendecker, O., Piechocka-Trocha, A., Li, J. Z., Khatri, A., Chu, H. Y., Villani, A.-C., Kays, K., Goldberg, M. B., Hacohen, N., Filbin, M. R., Yu, X. G., Walker, B. D., Wesemann, D. R., Larman, H. B., Lederer, J. A., and Elledge, S. J. (2020) Viral Epitope Profiling of COVID-19 Patients Reveals Cross-Reactivity and Correlates of Severity. *Science (Washington, DC, U. S.)* 370 (6520), No. eabd4250.

(10) Mateus, J., Grifoni, A., Tarke, A., Sidney, J., Ramirez, S. I., Dan, J. M., Burger, Z. C., Rawlings, S. A., Smith, D. M., Phillips, E., Mallal, S., Lammers, M., Rubiro, P., Quiambao, L., Sutherland, A., Yu, E. D., da Silva Antunes, R., Greenbaum, J., Frazier, A., Markmann, A. J., Premkumar, L., de Silva, A., Peters, B., Crotty, S., Sette, A., and Weiskopf, D. (2020) Selective and Cross-Reactive SARS-CoV-2 T Cell Epitopes in Unexposed Humans. *Science (Washington, DC, U. S.)* 370, 89−94.

(11) Nelde, A., Bilich, T., Heitmann, J. S., Maringer, Y., Salih, H. R., Roerden, M., Lubke, M., Bauer, J., Rieth, J., Wacker, M., Peter, A., Horber, S., Traenkle, B., Kaiser, P. D., Rothbauer, U., Becker, M., Junker, D., Krause, G., Strengert, M., Schneiderhan-Marra, N., Templin, M. F., Joos, T. O., Kowalewski, D. J., Stos-Zweifel, V., Fehr, M., Rabsteyn, A., Mirakaj, V., Karbach, J., Jager, E., Graf, M., Gruber, L.-C., Rachfalski, D., Preuß, B., Hagelstein, I., Marklin, M., Bakchoul, T., Gouttefangeas, C., Kohlbacher, O., Klein, R., Stevanovic, S., Rammensee, H.-G., and Walz, J. S. (2021) SARS-CoV-2 T-Cell Epitopes Define Heterologous and COVID-19-Induced T-Cell Recognition. *Nat. Immunol. 22*, 74−85.

(12) Amanat, F., Stadlbauer, D., Strohmeier, S., Nguyen, T. H. O., Chromikova, V., McMahon, M., Jiang, K., Arunkumar, G. A., Jurczyszak, D., Polanco, J., Bermudez-Gonzalez, M., Kleiner, G., Aydillo, T., Miorin, L., Fierer, D. S., Lugo, L. A., Kojic, E. M., Stoever, J., Liu, S. T. H., Cunningham-Rundles, C., Felgner, P. L., Moran, T., García-Sastre, A., Caplivski, D., Cheng, A. C., Kedzierska, K., Vapalahti, O., Hepojoki, J. M., Simon, V., and Krammer, F. (2020) A Serological Assay to Detect SARS-CoV-2 Seroconversion in Humans. *Nat. Med. 26* (7), 1033−1036.

(13) Becker, M., Strengert, M., Junker, D., Kerrinnes, T., Kaiser, P. D., Traenkle, B., Dinter, H., Haering, J., Zeck, A., Weise, F., Peter, A., Hoerber, S., Fink, S., Ruoff, F., Bakchoul, T., Baillot, A., Lohse, S., Cornberg, M., Illig, T., Gottlieb, J., Smola, S., Karch, A., Berger, K., Rammensee, H.-G., Schenke-Layland, K., Nelde, A., Maerklin, M., Heitmann, J. S., Walz, J. S., Templin, M. F., Joos, T. O., Rothbauer, U., Krause, G. G., and Schneiderhan-Marra, N. (2021) Exploring beyond clinical routine SARS-CoV-2 serology using MultiCoV-Ab to evaluate endemic coronavirus cross-reactivity. *Nat. Commun. 12*, 1152.

(14) Burnette, W. N. (1981) Western Blotting": Electrophoretic Transfer of Proteins from Sodium Dodecyl Sulfate-Polyacrylamide Gels to Unmodified Nitrocellulose and Radiographic Detection with Antibody and Radioiodinated Protein A. *Anal. Biochem. 112* (2), 195−203.

(15) Treindl, F., Ruprecht, B., Beiter, Y., Schultz, S., Döttinger, A., Staebler, A., Joos, T. O., Kling, S., Poetz, O., Fehm, T., Neubauer, H., Kuster, B., and Templin, M. F. (2016) A Bead-Based Western for High-Throughput Cellular Signal Transduction Analyses. *Nat. Commun. 7* (1), 12852.

(16) Tang, M. S., Hock, K. G., Logsdon, N. M., Hayes, J. E., Gronowski, A. M., Anderson, N. W., and Farnsworth, C. W. (2020) Clinical Performance of Two SARS-CoV-2 Serologic Assays. *Clin. Chem. 66*, 1055.

(17) Tang, M. S., Hock, K. G., Logsdon, N. M., Hayes, J. E., Gronowski, A. M., Anderson, N. W., and Farnsworth, C. W. (2020) Clinical Performance of the Roche SARS-CoV-2 Serologic Assay. *Clin. Chem. 66*, 1107.

(18) Hörber, S., Soldo, J., Relker, L., Jürgens, S., Guther, J., Peter, S., Lehmann, R., and Peter, A. (2020) Evaluation of Three Fully-Automated SARS-CoV-2 Antibody Assays. *Clin. Chem. Lab. Med. 58*, 2113.

(19) Leung, D. T. M., Tam, F. C. H., Ma, C. H., Chan, P. K. S., Cheung, J. L. K., Niu, H., Tam, J. S. L., and Lim, P. L. (2004) Antibody Response of Patients with Severe Acute Respiratory Syndrome (SARS) Targets the Viral Nucleocapsid. *J. Infect. Dis. 190* (2), 379−386.

(20) Sariol, A., and Perlman, S. (2020) Lessons for COVID-19 Immunity from Other Coronavirus Infections. *Immunity 53*, 248.

(21) Masse, S., Capai, L., Villechenaud, N., Blanchon, T., Charrel, R., and Falchi, A. (2020) Epidemiology and Clinical Symptoms Related to Seasonal Coronavirus Identified in Patients With. *Viruses 12* (6), 630.

(22) Gorse, G. J., Patel, G. B., Vitale, J. N., and O'Connor, T. Z. (2010) Prevalence of Antibodies to Four Human Coronaviruses Is Lower in Nasal Secretions than in Serum. *Clin. Vaccine Immunol. 17* (12), 1875−1880.

(23) Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J., Sheng, J., Quan, L., Xia, Z., Tan, W., Cheng, G., and Jiang, T. (2020) Genome Composition and Divergence of the Novel Coronavirus (2019-NCoV) Originating in China. *Cell Host Microbe 27* (3), 325−328.

(24) Nickbakhsh, S., Ho, A., Marques, D. F. P., McMenamin, J., Gunson, R. N., and Murcia, P. R. (2020) Epidemiology of Seasonal Coronaviruses: Establishing the Context for the Emergence of Coronavirus Disease 2019. *J. Infect. Dis. 222* (1), 17−25.

(25) Grifoni, A., Weiskopf, D., Ramirez, S. I., Mateus, J., Dan, J. M., Moderbacher, C. R., Rawlings, S. A., Sutherland, A., Premkumar, L., Jadi, R. S., Marrama, D., de Silva, A. M., Frazier, A., Carlin, A. F., Greenbaum, J. A., Peters, B., Krammer, F., Smith, D. M., Crotty, S., and Sette, A. (2020) Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals. *Cell 181* (7), 1489−1501.

(26) Ruetalo, N., Businger, R., Althaus, K., Fink, S., Ruoff, F., Hamprecht, K., Flehmig, B., Bakchould, T., Templin, M. F., and Schindler, M. Neutralizing Antibody Response in Non-Hospitalized SARS-CoV-2 Patients. *medRxiv*, September 22, 2020, ver. 1. DOI: 10.1101/2020.08.07.20169961.

(27) Treindl, F., Zabinsky, E., Kling, S., Schwarz, M., Braeuning, A., and Templin, M. F. (2020) Array-Based Western-Blotting Reveals Spatial Differences in Hepatic Signaling and Metabolism Following CAR Activation. *Arch. Toxicol. 94* (4), 1265−1278.

(28) McHugh, M. L. (2012) Lessons in Biostatistics Interrater Reliability: The Kappa Statistic. *Biochem. Medica 22* (3), 276−282.