Evaluation of neutralizing antibodies against SARS-CoV-2 variants after infection and vaccination using a multiplexed surrogate virus neutralization test

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Nonstandard Abbreviations: sVNT, surrogate virus neutralization test; plex-sVNT, multiplex surrogate virus neutralization test; ACE2, angiotensin converting enzyme-2 receptor protein; PRNT, plaque reduction neutralization test; VOC, variant of concern; VOI, variant of interest; ICU, intensive care unit; RFI, relative fluorescence intensity; RBD, receptor binding domain

Abstract

BACKGROUND: The SARS-CoV-2 virus has mutated and evolved since the inception of the COVID-19 pandemic bringing into question the future effectiveness of current vaccines and antibody therapeutics. With evolution of the virus updated methods for the evaluation of the immune response in infected and vaccinated individuals are required to determine the durability of the immune response to SARS-CoV-2 variants.

METHODS: We developed a multiplexed surrogate virus neutralization test (plex-sVNT) that simultaneously measures the ability of antibodies in serum to inhibit binding between angiotensin converting enzyme-2 (ACE2) and 7 SARS-CoV-2 trimeric spike protein variants, including wild type, B.1.1.7(α), B.1.351(β), P.1(γ), B.1.617.2(δ), B.1.617.1(κ), and B.1.429(ε). The assay was validated against a plaque reduction neutralization test (PRNT).

We evaluated 170 samples from 97 COVID-19 patients and 281 samples from 188 individuals that received the Pfizer-BioNTech or Moderna mRNA vaccines.

RESULTS: The plex-sVNT demonstrated >96% concordance with PRNT. Antibody neutralization activity was significantly reduced for all SARS-CoV-2 variants compared to wild type in both the infected and vaccinated cohorts. There was a decline in overall antibody neutralization activity, within both cohorts, out to 5 months post infection or vaccination, with the rate of decline being more significant for the vaccinated.

CONCLUSIONS: The plex-sVNT provides a correlative measure to PRNT and a convenient approach for evaluating antibody neutralization against SARS-CoV-2 variants. Neutralization of SARS-CoV-2 variants is reduced compared to wild type and declines over the ensuing months after exposure or vaccination within each cohort, however it is still unknown what degree of neutralizing capacity is protective.

INTRODUCTION

SARS-CoV-2 has infected nearly 200 million people with 4 million deaths worldwide in the first year and a half of the COVID-19 pandemic. The release of highly effective coronavirus vaccines has greatly reduced the rate of severe disease and death in areas where vaccination rates are high. Correlates of immunity in an individual are still not well defined. SARS-CoV-2 continues to mutate which raises uncertainty regarding the effectiveness and duration of the immune response following natural infection and vaccination. Gain-of-function viral variants can result in improved replication capacity, viral infectivity, transmissibility, and neutralizing antibody escape. SARS-CoV-2 Variants of Concern (VOCs) have been identified and currently include; B.1.1.7 (UK, 201/501Y.V1, Alpha, or α), B.1.351 (South Africa, 20H/501Y.V2, Beta, or β), P.1 (Brazil, Gamma, or γ), and B.1.617.2 (India, Delta, or δ). Additional Variants of Interest (VOIs) currently include; B.1.525 (Eta, or η), B.1.526 (Iota, or ι), B.1.617.1 (India, Kappa, or κ), C.37 (Peru, Lambda, or λ), and B.1.621 (Columbia, Mu, or μ). Other variants such as B.1.429 (California, Cal20, Epsilon, or ε), were once listed as VOCs or VOIs and have since been removed from the list as others have emerged and become more dominant. Emerging data suggests that these VOCs/VOIs are susceptible to neutralization by SARS-CoV-2 antibodies in convalescent and vaccinee serum, however, to a lesser degree than wild-type virus (1-4). Most of these studies evaluated one time-point soon after vaccination with one specific vaccine for a limited number of variants, limiting the ability to compare: 1) neutralizing capacity against multiple variants within a specific group (e.g. α, β, γ, and δ after infection) 2) neutralizing activity in different groups (e.g. natural infection vs. vaccination), and 3) the rate of decline between time-points (e.g. 1 vs. 5 months after onset of symptoms/first vaccination) for all variants in different groups (e.g. natural infection vs. vaccination).

Various methods used to measure the SARS-CoV-2 humoral response have been described including qualitative and quantitative methods for total antibody or antibody subclasses (IgG, IgM, IgA) (1, 4), IgG avidity (5), and antibody neutralization activity. Plaque reduction neutralization tests (PRNT) measure SARS-CoV-2 neutralizing antibody titer and involve the use of live pathogens and target cells (6, 7). These laborious tests require a high degree of expertise and expose laboratory personnel to infection risks, limiting the widespread availability of testing. Surrogate virus neutralization tests (sVNTs) based on antibody-mediated blockage of molecular interactions have been described (8-10). An sVNT measures the competitive inhibition of the interaction between a viral structural protein and angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2 on host cells. Like PRNT, sVNTs detect neutralizing antibodies in an isotype-independent manner, offering a key advantage over antibody concentration assays. Here we present a multiplexed sVNT (plex-sVNT) method for the simultaneous evaluation of the ability of antibodies produced after infection or vaccination to inhibit the interaction between ACE2 and SARS-CoV-2 trimeric spike protein containing current VOCs. No studies to date have evaluated neutralizing antibodies from infected and vaccinated individuals for all major variants of concern using the same analytical method for comparison.

MATERIALS AND METHODS

Sample Collection

Ethical review. Two separate protocols, one for remnant specimens from patients who had COVID-19 natural infection (IRB #20-30387) and the other for COVID-19 vaccinated healthy individuals (IRB #20-33062), were approved by the Institutional Review Board of the University of California, San Francisco. The committee judged that written consent was not required for use

of remnant specimens. Written informed consent was obtained for blood collections from COVID-19 vaccinated individuals.

Subjects and specimens. The COVID-19 infection cohort utilized remnant serum or plasma samples (n=170) from routine clinical hospital laboratory testing. All samples were collected at least 12 days from symptom onset with an attempt to collect samples across the range of days out to 6 months. When two or more samples were collected for a patient, they were separated by at least 7 days. The median number of specimens per subject was 1 for the non-ICU patients and 2 for ICU patients. All patients (n=97) had positive results by SARS-CoV-2 real-time polymerase chain reaction (RT-PCR) in nasopharyngeal swabs between March and July 2020. Clinical data were extracted from electronic health records and included demographic information, patient-reported symptom onset date and indicators of disease severity. Patients were categorized based on their level of care; patients admitted to an intensive care unit at any time were classified as ICU patients, whereas those admitted to a hospital or managed as outpatients were considered non-ICU patients. The criteria for ICU admission at the hospital remained the same throughout the course of the study. No patients received convalescent plasma or monoclonal antibody drugs.

The COVID-19 vaccination cohort (n=188) utilized serum samples (n=281) collected via phlebotomy from healthcare workers. All individuals received either the Pfizer-BioNTech (n=150) or Moderna (n=38) vaccine between December 2020 and February 2021. No one had a previous COVID-19 infection as determined by self-report and all had a negative test for antibodies to the SARS-CoV-2 nucleocapsid protein. All samples were collected at least 7 days after second vaccination and 28 days (Pfizer-BioNTech) or 35 days (Moderna) from first vaccination with an attempt to collect samples across the range of days out to 6 months. When

two or more samples were collected from a subject, they were separated by at least 7 days. The median number of specimens per subject was 1 for the Pfizer-BioNTech and 2 for Moderna. Demographic data and dates of vaccination were collected following informed consent.

All samples were maintained at -20 ˚C for the duration of the study. After thawing at room temperature, samples were briefly vortexed before testing in singlicate.

Recombinant trimeric spike proteins and ACE-2-FC fusion proteins

Recombinant trimeric spike protein variants comprising wild-type Wuhan strain, UK B.1.1.7 (α), South African B.1.351 (β), Brazilian P.1 (γ), two Indian B.1.617.1 and B.1.617.2 (κ and δ) and California B.1.429 (ε, receptor binding domain only) were purchased from Icosagen, Estonia. Recombinant trimeric spike protein for the WA1 strain was not commercially available. Besides variant specific mutations, each trimeric protein contains aa 14-1211, plus two extra amino acids (AS) in the N-terminus, trimerization domain and His-6 tag at its C-terminus. Also added is a GS linker between the protein and trimerization domain as well as a GSG linker between the trimerization domain and His-tag. The furin cleavage site RRAR between Spike S1 and S2 is mutated to GSAS.

Variant specific mutations to trimeric proteins include HV 69-70 del, Y144 del, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H for B.1.1.7 (α), L18F, D80A, D215G, LAL 242-244 del, R246I, K417N, E484K, N501Y, D614G, A701V for B.1.351 (β), L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F for P.1 (γ), T19R, G142D, E156G, del_F157-R158, L452R, T478K, D614G, P681R for B.1.617.2 (δ) and G142D, E154K, L452R, E484Q, D614G, P681R for B.1.617.1 (κ). All recombinant proteins

were expressed in Chinese hamster ovary-based cell line and cell free supernatant used to purify proteins by Ni-affinity chromatography and filtration.

The gene encoding the recombinant human ACE2-Fc fusion protein comprising extracellular 18-741 amino acid domain of human ACE-2 receptor protein (NP_068576.1) fused to 225 amino acid human Fc fragment at the carboxyl terminus via a 9-residue glycine spacer was transfected into HEK-293_sus cells. The expressed soluble fusion ACE2-Fc protein was purified over Protein A resin column. The eluted protein was buffer exchanged into PBS, PH 7.0 and biotinylated using EZ-link sulfo NHS-biotin at 50 molar excess of biotin reagent to ACE2-Fc fusion protein (Thermo Scientific). Unbound biotin was removed via desalting over Thermo Fisher Zebra spin column.

Multiplexed SARS-CoV-2 surrogate virus neutralization test (plex-sVNT)

Trimeric spike protein variants were coupled separately to spectrally distinct paramagnetic beads. Briefly, SARS-CoV2 neutralizing antibodies present in serum/plasma compete with biotinylated-human ACE2-Fc protein for binding to trimeric proteins that are coupled to the beads (**Fig. 1A and 1B**). After washing, the beads are incubated with streptavidin-phycoerythrin (SA-PE; Agilent) conjugate followed by additional washes. Washed beads are suspended in buffer followed by passage through the detector that measures the relative fluorescent signal (RFI) associated with beads on the BioPlex 2200 platform (Bio-Rad Laboratories). The identity of the dyed beads is determined by the fluorescence of the dyes, and the amount of antibody captured by the antigen is determined by the fluorescence of the attached PE. Raw data are calculated in relative fluorescence intensity (RFI). The RFI signal is inversely proportional to the concentration of SARS-CoV-2 neutralizing antibodies in the sample. An

optimal signal was observed using 1 ug/mL biotinylated ACE2-Fc protein and 6 ug/mL SA-PE conjugate.

Direct binding of the recombinant ACE2 receptor protein to trimeric proteins was demonstrated using fluorescence of the attached PE to the biotinylated ACE2 receptor protein in the presence of normal healthy pre-pandemic serum. The observed RFI signal was normalized to calculate average fluorescent intensity/µg variant specific protein. Dose dependent inhibition of ACE2 binding to trimeric protein by neutralizing antibodies present in the infected and vaccinated cohort samples was calculated by subtracting the fluorescence of the test sample from the mean fluorescence of the normal healthy samples while % inhibition was calculated as follows: 1 – (RFI of the test sample/RFI of the normal sample).

The assay cutoff of 25% inhibition for the ACE2 - trimeric protein binding was established based on 99th percentile cutoff using commercially available healthy normal, pregnancy and potential cross reactant samples (N = 866) collected prior to November 2019. Precision was determined for low (34%), medium (65%) and high (91%) percent inhibition. Within-run, between-run, and total imprecision ranged from 0.5% to 3.9%, 1.1% to 3.4%, and 1.2% - 7.1%, respectively. Linearity was assessed using the WHO international standard for SARS-CoV-2 antibody (NIBSC code 20/136). The assay was log-log linear (r=0.99) from 80% (corresponding to 1000 IU/mL) to 21% (corresponding to 31.5 IU/mL). A high sample corresponding to 100% inhibition was used to demonstrate extended linearity from 80%-100%.

PRNT SARS-CoV2 neutralization assay

The PRNT assay was conducted in a biosafety level 3 (BSL-3) laboratory at the Colorado State University Infectious Disease Research Center (Fort Collins, CO). Samples were heat

inactivated for 30 min at 56 °C, and serial twofold dilutions were prepared in a 96-well plate (Greiner Bio One). Viral stock (strain hCoV-19/USA/WA1/2020, BEI Resources) containing approximately 200 plaque-forming units (pfu) per 0·1 ml was added to each well containing plasma dilutions. Following an incubation period at 37°C in a 5% CO_2 incubator, 6-well plates (Greiner Bio One) containing recently confluent Vero cells (ATCC) were inoculated with the virus–plasma mixtures. After a second incubation period at 37°C, 2 ml of overlay (2× MEM with 4% FBS [Peak Serum] and agarose) was added to each well. After 24 h incubation at 37°C, a second overlay containing neutral red (Millipore Sigma) was dispensed into each well and the number of plaques was counted 48–72 h after initial inoculation. The highest dilution of plasma that inhibited plaque formation by 50% ($PRNT_{50}$) and 90% ($PRNT_{90}$) was determined based upon the titer of the viral stock and the number of plaques present at each dilution. Samples with $PRNT_{50}$ titers of <1:20 are considered negative for neutralizing antibodies.

Statistical analysis

All data were entered into the Research Electronic Data Capture (REDCap) database. Comparisons for parameters passing the Shapiro–Wilk test for normality were performed using a paired, two-tailed *t*-test where statistical significance was defined as $P<0.05$. Correlation between the plex-sVNT and PRNT was determined using Spearman coefficients. Positive and negative percent agreement (PPA, NPA) were performed using multiple cutoffs for neutralizing titers since the true protective titer has not been established. Ideal cutoffs for the plex-sVNT method that maximized PPA and NPA were calculated using the Youden Index. Neutralizing antibody (% inhibition or %I) measurements for the COVID-19 variants compared to wild type were analyzed with-in each group (ICU, non-ICU, Pfizer-BioNTech, Moderna) using one-way

repeated measures ANOVA, followed by the Fisher Least Significant Difference test. The percent inhibition for SARS-CoV-2 variants was compared to wild-type using Deming regression and Bland-Altman analysis with determination of the Spearman correlation. The rate of decline of percent inhibition versus days since symptom onset or first vaccination was determined using simple linear regression analysis and calculation of the 95% confidence interval (95% CI). Statistical analysis was performed using Analyse-it software (method validation edition, version 4.95) and GraphPad Prism (version 9.2.0).

RESULTS

The COVID-19 infection cohort included 97 SARS-CoV-2 RT-PCR positive patients, 41 (42%) admitted to the hospital and 56 outpatients (**Table 1**). Of the hospitalized patients, 28 (68%) were admitted to the ICU and 22 (54%) required mechanical ventilation. The ICU patients were older (median age 54.7 y) and a higher percentage were male (64.3%) compared to non-ICU patients (43.5 y and 43.5%). The median number of days from symptom onset to sample collection was significantly shorter for ICU patients (29) compared to non-ICU patients (55).

The COVID-19 vaccination cohort included 188 individuals that received two doses of an mRNA vaccine, 150 Pfizer-BioNTech and 38 Moderna (**Table 1**). The median age for those receiving the Pfizer (41.5 y) and Moderna (39.5 y) vaccination were similar. Both groups were predominately female, with a higher percentage of females in the Pfizer (78.7%) compared to Moderna (60.5%) group. The median time between vaccine doses differed by 7 days for Pfizer (21) and Moderna (28), while the median number of days from first vaccine dose to sample collection was not statistically different.

The performance of the plex-sVNT using wild-type trimeric spike protein was compared against the PRNT virus neutralization assay for 76 samples from the COVID-19 infection cohort and 102 commercially available healthy normal, pregnancy and potential cross reactant samples. The Spearman correlation between the plex-sVNT assay (cut-off, 25% inhibition) and $PRNT_{50}$ (cut-off, 1:10) and $PRNT_{90}$ (cut-off, 1:20) was 0.80 (95%CI: 0.69-0.88, P <0.001), and 0.88 (95%CI: 0.81-0.93, P<0.0001), respectively (**Fig. 2, A and B**).

The positive agreement and negative agreement for 178 samples was 96% (95%CI: 88.9-98.6%) and 99% (95%CI: 97.4%-99.8%) , respectively, for both $PRNT_{50}$ and $PRNT_{90}$ using the plex-sVNT upper 99th percentile cut-off of 25%. PPA and NPA using optimized sVNT cut-offs compared to increasing $PRNT_{50}$ titers are shown in **Figure 2C**. The PPA ranged from 100% ($PRNT_{50}$ 1:20 and plex-sVNT 19%) to 93.9% ($PRNT_{50}$ 1:640 and plex-sVNT 54%). The NPA ranged from 99.0% ($PRNT_{50}$ 1:20 and plex-sVNT 19%) to 93.0% ($PRNT_{50}$ 1:640 and plex-sVNT 54%).

Antibodies capable of neutralizing the interaction between SARS-CoV-2 variants and ACE2 were present in all individuals tested from all groups, however, there was a high degree of variability in neutralizing capacity between subjects likely given the diverse timing of sample collection relative to infection or vaccination (**Figure 3**). Overall, the median percent inhibition was lower for all variants compared to wild type in all four groups. The difference reached statistical significance for all variants in all groups compared to wild type (*P<0.0001*), except epsilon for the COVID-19 non-ICU group (*P=0.31*). The decrease in percent inhibition towards the different variants followed the same trend for all 4 groups. Neutralizing antibodies ranking from lowest percent inhibition to highest compared to wild type were beta, gamma, alpha, delta/kappa, and then epsilon. The decrease in neutralizing capacity towards the variants

compared to wild type was less pronounced for the COVID-19 ICU group. The heterogeneity between the four groups (**Table 1**) does not allow for further comparison of results between groups. For the entire dataset (all samples in all groups), Deming regression for SARS-CoV-2 variants compared to wild-type demonstrated slopes ranging from 1.21 (delta) to 1.33 (beta) and Spearman correlations ranging from r_S=.960 (kappa) to r_S=.993 (alpha) (Figure 4). Bland-Altman biases for percent inhibition were -16 (alpha), -21 (beta), -19 (gamma), -8 (delta), and -8 (kappa) (**Figure 4**) and -3 (epsilon – data not shown).

Figure 5 shows the percent inhibition of neutralizing antibodies for wild type SARS-CoV-2 versus days since symptom onset or first vaccination for the COVID-19 infection and vaccination cohorts. There was an inverse correlation between percent inhibition and days since symptom onset/vaccination out to 6 months in all groups except the non-ICU infected group. This may be due to the limited number of data points for the non-ICU group between 120 and 160 days or the large inter-individual variation in % inhibition prior to 120 days. For wild type, the decline was more pronounced (P<0.0001) in the Pfizer-BioNTech group (slope -0.27, 95% CI: -0.32 - -0.23)) compared to the Moderna group (slope -0.15, 95% CI: -0.20 - -0.11) and both vaccine groups (P<0.0001) compared to the COVID-19 ICU group (slope -0.04, 95% CI: -0.07 - -0.01). The results were similar for the variants compared to wild type in the vaccination groups with slightly lower percent inhibition overall (**Figure 5 E and F**).

DISCUSSION

This paper describes a bead-based multiplexed method for the analysis of neutralizing antibodies against SARS-CoV-2 variants. More specifically it measures the ability of infection- or vaccine-induced antibodies to inhibit binding between the SARS-CoV-2 trimeric spike protein

(wild-type and variant) and ACE2, serving as an in-vitro surrogate for in-vivo viral-host invasion. The use of trimeric recombinant spike protein variants offers advantages over isolated protein subunits or domains, such as the receptor binding domain (RBD) since it mimics the natural conformation of the native spike protein. Emerging SARS-CoV-2 variants exhibit mutations or deletions in the spike structural domain other than the RBD or S1 subunit; therefore, use of the trimeric spike protein has significant advantages for evaluating antibody activity against variants.

To date, only one sVNT method has received emergency use authorization from the US FDA (cPASS, GeneScript) for the analysis of neutralizing antibodies to wild-type virus, however, this method is an enzyme-linked immunoassay designed for batch testing in a high complexity laboratory. The plex-sVNT method is a high throughput assay that can measure neutralizing antibodies to wild type and emerging variants in one test simultaneously on an automated analyzer with all results obtained in 52 minutes. The high concordance and correlation with a gold-standard PRNT supports its utility as a viable alternative to time-consuming conventional cell-based assays which are limited in availability, and only capable of testing one variant at a time. The PRNT and plex-sVNT methods were compared using the respective assay cut-offs (titer and % inhibition). A more clinically relevant comparison would use the titer and % inhibition that signify viral protection, which are likely higher than the cut-offs, however, these are not yet defined. Assay agreement remained high (>93%) between $PRNT_{50}$, at increasing titers from 1:20 to 1:640, compared to plex-sVNT using optimized cutoffs, suggesting that the plex-sVNT would be a viable surrogate to PRNT regardless of the cut-off used. Limitations in sample volume prohibited comparisons between PRNT and plex-sVNT for the variants. As variants continue to emerge, such as the most recent omicron variant, the plex-sVNT described

here can be adapted to monitor neutralizing antibody responses after infection and vaccination, however the process required for regulatory approval would likely hamper timely widespread clinical implementation.

This study identified neutralizing activity of infection- and vaccine- elicited antibodies against 6 SARS-CoV-2 variants, suggesting that some immunity is retained against the current variants. The ability of antibodies to neutralize binding of variant trimeric spike protein to ACE2 was reduced compared to wild type, for both the COVID-19 infection and vaccination cohorts. Neutralizing antibodies ranking from lowest to highest bias in percent inhibition compared to wild type were beta, gamma, alpha, delta/kappa, and then epsilon. The decrease in neutralizing capacity towards the variants was less pronounced for the COVID-19 ICU group. There was an inverse correlation between percent inhibition and days since symptom onset/vaccination out to 6 months in all groups except the non-ICU infected group. Within the vaccine cohort, the rate of decline was significantly faster for Pfizer-BioNTech compared to Moderna for wild type and all variants. The lack of timed longitudinal samples in the study, resulting from the use of remnant samples, prohibited further analysis of the durability of the immune response.

The significance of these finding with regards to an individual's immune response is currently unknown. Data on the rate of re-infection or vaccine breakthrough for these variants, will be the subject of future epidemiologic research. Other mechanisms within the human immune system, such as the existence of T-cell function and memory B-cells may be sufficient to protect the vaccinated and those previously infected with SARS-CoV-2. A recent study examined the breakthrough infection rate for individuals who received the Pfizer vaccine for SARS-COV-2 variants and reported a higher incidence for the α strain after the first dose, and a higher incidence for the β strain after the second dose (12). In another study, Bergwerk et al.

characterized 39 healthcare workers who developed a COVID-19 infection after receiving a SARS-CoV-2 vaccine. During the peri-infection period, they found lower neutralizing antibody titers in blood compared with non-infected matched controls (13). They also showed that higher antibody neutralizing capacity was associated with lower viral loads. The plex-sVNT would be an appropriate test should evaluation of a larger population prove to be warranted for research and clinical investigations.

It is still unknown what degree of neutralizing capacity of SARS-CoV-2 antibodies correlates with significant immunity or complete protection from COVID-19 infection. The heterogeneity in methodologies used to evaluate the humoral immune response complicates interpretation across studies. Estimates of neutralization capacity against variants compared to wild-type vary substantially between studies likely due to variations in assay design and populations studied (1-4). To our knowledge this is the first study to evaluate neutralizing antibodies in both infected and vaccinated individuals for multiple variants using the same analytical method for comparison. More studies are needed to determine the true utility of measuring the SARS-CoV-2 antibody response over-time to inform clinical and population health outcomes.

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References

1. Pegu A, O'Connell S, Schmidt SD, O'Dell S, Talana CA, Lai L, et al. Durability of mRNA-1273 vaccine-induced antibodies against SARS-CoV-2 variants. Science 2021;373:1372-7.
2. Xie X, Lui Y, Liu J, Zhang X, Zou J, Fontes-Garfias CR, et al. Neutralization of SARS-CoV-2 spike 69/70 deletion, E484K and N501Y variants by BNT162b2 vaccine-elicited sera. Nat Med 2021;27:620-1.
3. Edara VV, Norwood C, Floyd K, Lai L, Davis-Gardner ME, Hudson WH, et al. Infection- and vaccine-induced antibody binding and neutralization of the B.1.351 SARS-CoV-2 variant. Cell Host Microbe 2021;29:516-21.
4. Trombetta CM, Marchi S, Viviani S, Manenti A, Benincasa L, Ruello A, et al. Serum Neutralizing Activity against B.1.1.7, B.1.351, and P.1 SARS-CoV-2 Variants of Concern in Hospitalized COVID-19 Patients. Viruses 2021;13:1347.
5. Luo YR, Chakraborty I, Yun C, Wu AHB, Lynch KL. Kinetics of SARS-CoV-2 Antibody Avidity Maturation and Association with Disease Severity. Clin Infect Dis 2020;72:301-8.
6. Payne S. Methods to Study Viruses. In Viruses: from understanding to investigation. 1st Ed. New York (NY): Elsevier; 2017. p. 37–52.
7. Rogers TF, Zhao F, Huang D, Beutler N, Burns A, He WT, et al. Isolation of potent SARS-CoV-2 neutralizing antibodies and protection from disease in a small animal model. Science 2020;369:956-63.
8. Bošnjak B, Stein SC, Willenzon S, Cordes AK, Puppe W, Bernhardt G, et al. Low serum neutralizing anti-SARS-CoV-2 S antibody levels in mildly affected COVID-19

convalescent patients revealed by two different detection methods. Cell Mol Immunol 2021;18:936-44.

9. Tan CW, Chia WN, Qin X, Liu P, Chen MIC, Tiu C, et al. 2020. A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2–spike protein–protein interaction. Nat Biotechnol 2020;38:1073-8.
10. Meyer B, Reimerink J, Torriani G, Brouwer F, Godeke GJ, Yerly S, et al. Validation and clinical evaluation of a SARS-CoV-2 surrogate virus neutralisation test (sVNT). Emerg Microbes Infect 2020;9:2394–403.
11. Luo YR, Yun C, Chakraborty I, Wu AHB, Lynch KL. A SARS-CoV-2 label-free surrogate virus neutralization test and a longitudinal study of antibody characteristics in COVID-19 patients. J Clin Microbiol 2021;59:e0019321.
12. Kustin T, Harel N, Finkel U, Perchik S, Harari S, Tahor M, et al. Evidence for increased breakthrough rates of SARS-CoV-2 variants of concern in BNT162b2-mRNA-vaccinated individuals. Nat Med 2021;27:1379-84.
13. Bergwerk M, Gonen T, Lustig Y, Amit S, Lipsitch M, Cohen C, et al. Covid-19 breakthrough infections in vaccinated health care workers. N Engl J Med 2021;385:1474-84.

Table 1. Demographics, clinical characteristics and sample information for the COVID-19 infection and SARS-CoV-2 vaccinated cohorts.

Characteristic[1]	COVID-19 Non-ICU patients	COVID-19 ICU patients	Pfizer-BioNTech Vaccination	Moderna Vaccination
Number of subjects	69	28	150	38
Age				
Median [IQR]	48 [32 - 60]	57.5 [43 - 63]	41.5 [34 - 50]	39.5 [32 - 56]
Mean (SD)	47.0 (17.4)	54.7 (12.0)	43.1 (10.6)	43.7 (14.6)
Male sex	30 (43.5)	18 (64.3)	32 (21.3)	15 (39.5)
Admitted to hospital	13 (18.8)	28 (100)	N/A	N/A
Mechanical ventilation	0 (0)	22 (78.6)	N/A	N/A
Days between doses Median [min – max]	N/A	N/A	21 [18 – 35]	28 [21 – 33]
Median specimens per subject	1 [1 – 1]	2 [2 – 4]	1 [1 – 2]	2 [2 – 2]
Total number of specimens	77	93	208	73
Days from:				
Onset of symptoms*	55 [12 - 174]	29 [12 - 225]	N/A	N/A
First vaccine dose*	N/A	N/A	91.5 [28 - 160]	108 [36 - 157]

[1]Medians [interquartile range], mean (standard deviation), N (%), or *median [minimum – maximum] are reported. N/A – not applicable.

Figure captions

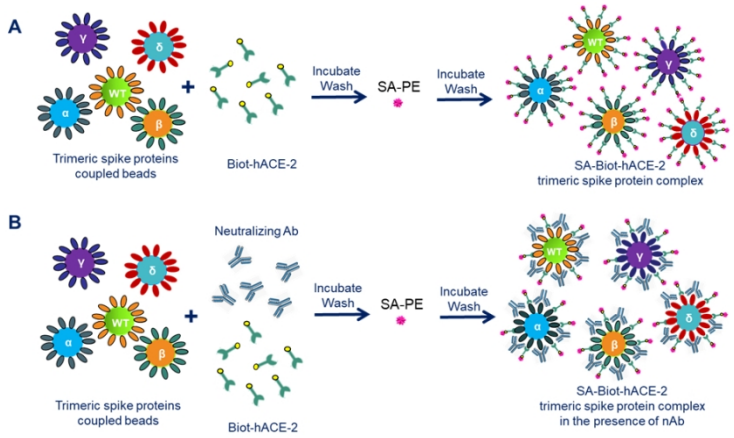
Fig. 1. Multiplex SARS-CoV-2 surrogate virus neutralization test (plex-sVNT). (A) Binding of biotinylated ACE2 to spectrally distinct paramagnetic beads coupled with trimeric spike protein (wild type or variant) in the absence of neutralizing antibodies. The amount of biotinylated ACE2 bound to the beads is measured by the fluorescence of the attached Phycoerythrin (PE). (B) Competition between biotinylated ACE2 and neutralizing antibodies in the sample for binding to trimeric spike proteins. The presence of neutralizing antibody in patient sample blocks ACE2 binding thereby reducing the signal.

Fig. 2. Correlation between the plex-sVNT and $PRNT_{50}$ (A) and $PRNT_{90}$ (B). Dashed lines represent the discriminative line between positive and negative for each assay. (C) Concordance between PRNT and the multiplex surrogate virus neutralization test.

Fig. 3. Percent inhibition of neutralizing antibodies against SARS-CoV-2 variants. (A) COVID-19 non-ICU patients (n=77). (B) COVID-19 ICU patients (n=93). (C) Pfizer/BioNTech vaccinated individuals (n=208). (D) Moderna vaccinated individuals (n=73). Box and whisker plots represent the median and upper and lower quartile (box) with min and max (whiskers) and mean (+). All variants were statistically different ($P<0.0001$) from wild-type for all groups (A-D) except epsilon compared to wild-type for the COVID-19 non-ICU group ($P=.31$)

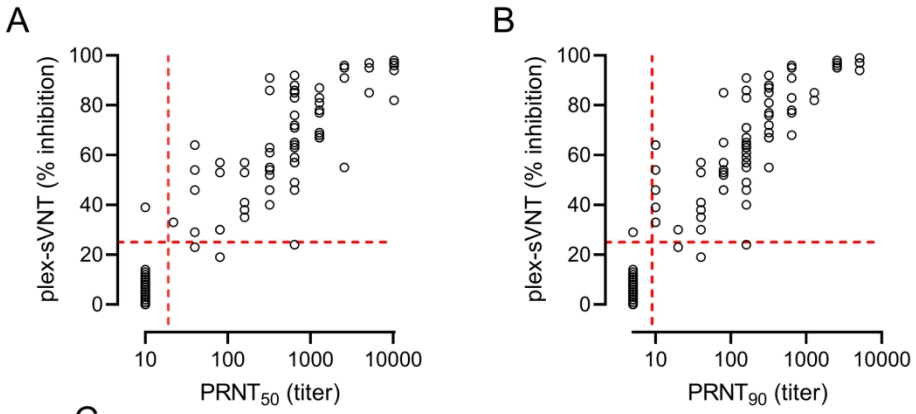
Fig. 4. Deming regression (A-E) and Bland-Altman (F-J) analysis for SARS-CoV-2 variants compared to wild-type. Slope and spearman correlation is shown for each SARS-CoV-2 variant compared to wild-type.

Fig. 5. Plot of percent inhibition by neutralizing antibodies vs. days since symptoms onset or first vaccination. Percent inhibition of ACE2 binding to wild type trimeric spike protein for (A) COVID-19 ICU (n=93, slope -0.04, 95% CI: -0.07 - -0.01), (B) COVID-19 non-ICU (n=77, slope – undetermined), (C) Pfizer-BioNTech (n=208, slope -0.27, 95% CI: -0.32- -0.23), and (D) Moderna (n=73, slope -0.15, 95% CI: -0.20 - -0.11). Decline in percent inhibition of ACE2 binding to variant trimeric spike protein for (E) Pfizer/BioNTech (slopes range from -0.25 - -0.33) and (F) Moderna (slopes range from -0.15 - -0.30).



338x190mm (96 x 96 DPI)

Figure 2



C

PRNT 50 Titer	plex-sVNT Cutoff	% Inhibition	PPA (%) (95% CI)	NPA (%) (95% CI)
1:20	99th percentile	25%	96.0 (88.9 - 98.6)	99.0 (94.7 -99.8)
	Optimized	19%	100 (95.1 -100)	99.0 (94.7 -99.8)
1:80	99th percentile	25%	97.1 (90.0 - 99.2)	94.5 (88.5 - 97.5)
	Optimized	29%	97.1 (90.0 - 99.2)	94.5 (88.5 - 97.5)
1:160	99th percentile	25%	98.4 (91.7 - 99.7)	91.2 (84.6 - 95.1)
	Optimized	38%	96.9 (89.3 - 99.1)	94.7 (90.0 - 97.6)
1:320	99th percentile	25%	98.3 (91.0 - 99.7)	87.4 (80.2 - 92.2)
	Optimized	46%	94.9 (86.1 - 98.3)	94.1 (88.4 - 97.1)
1:640	99th percentile	25%	98.0 (89.3 - 99.6)	80.6 (73.0 - 99.6)
	Optimized	54%	93.9 (83.5 - 97.9)	93.0 (87.3 - 96.3)

182x167mm (300 x 300 DPI)

Figure 3

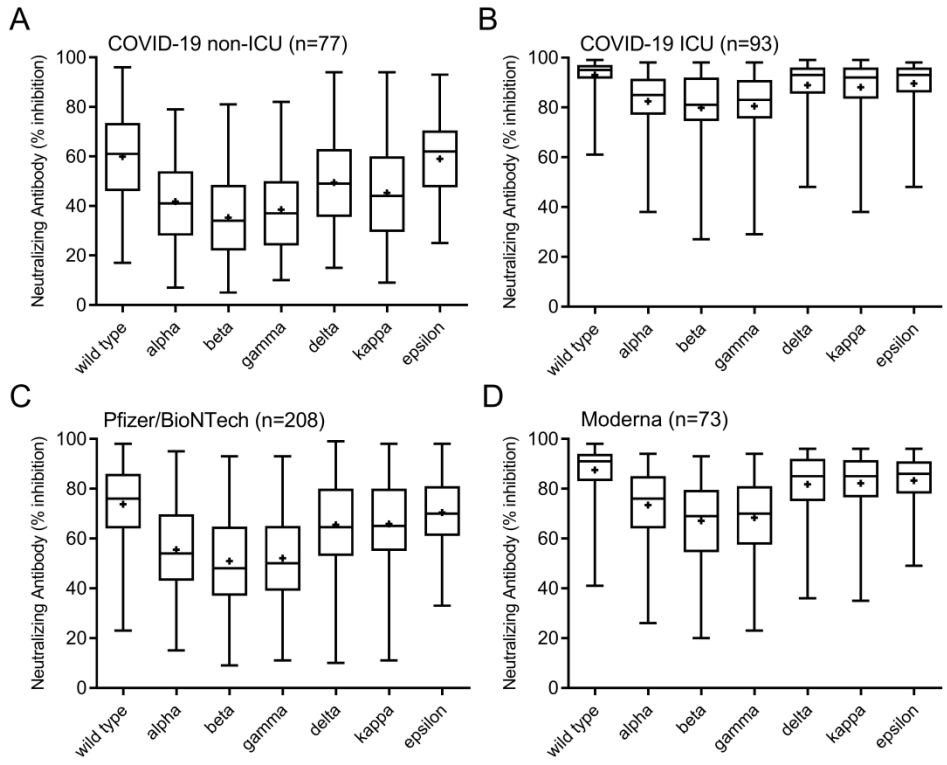


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Figure 4

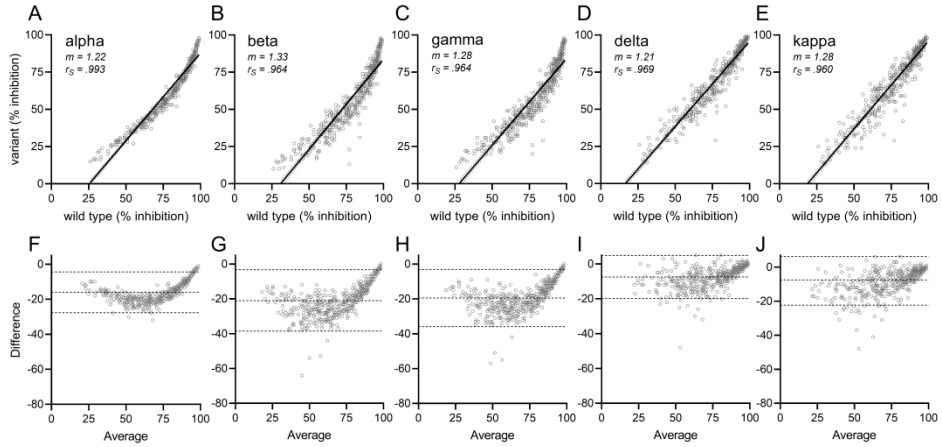

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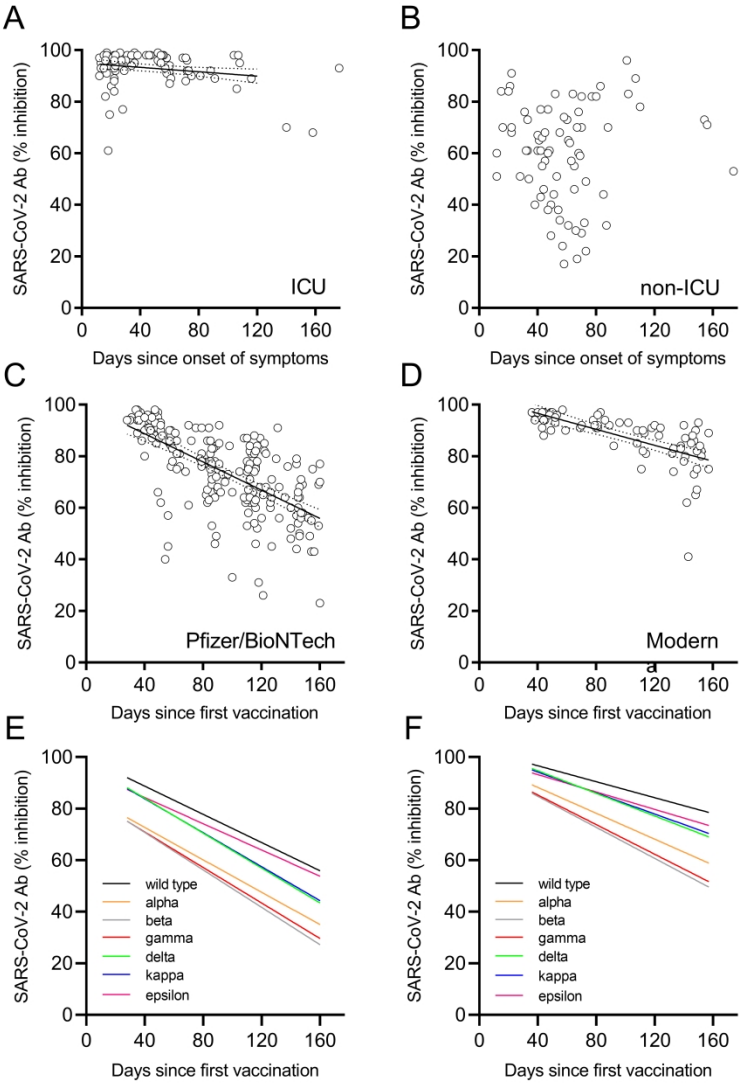
Figure 5



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