# A SARS-CoV-2 surrogate virus neutralization test (sVNT) based on antibody-mediated blockage of ACE2-spike (RBD) protein-protein interaction

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# Abstract

At this critical moment of the international response to the COVID-19 outbreak, there is an urgent need for a robust serological test to detect neutralizing antibodies to SARS-CoV-2. Such a test is not only important for contact tracing, but for determining infection rate, herd immunity and predicted humoral protection. The current gold standard is a virus neuralization test (VNT) requiring live virus and a biosafety level 3 (BSL3) laboratory. On the other hand, the ELISA- or lateral flow-based assays are for the detection of binding antibodies, which does not directly correlate with their neutralizing ability. Here we report a SARS-CoV-2 surrogate virus neutralization test (sVNT) that is designed to detect total neutralizing antibodies in an isotype- and species-independent manner. Our simple and rapid test is based on antibody-mediated blockage of virus-host interaction between the ACE2 receptor protein and the receptor binding domain (RBD) of the viral spike protein. The test has been validated with two COVID-19 patient cohorts in two different countries, achieving 100% specificity and 95-100% sensitivity and is capable of differentiating antibody responses from other known human coronaviruses. Importantly, the sVNT does not require BSL3 containment, thereby making the test immediately accessible to the global community.


# Introduction

The COVID-19 outbreak was first recognized in December 2019 in Wuhan, China[1], which has since spread to all parts of the world resulting in a total 2,160,207 infections with 146,088 deaths as of 18 April, 2020[2]. The causative agent was identified as 2019-nCoV, subsequently designated SARS-CoV-2[3,4], which belongs to the species *SARS-related coronavirus* (SARSr-CoV), same as for SARS-CoV, the causative agent of the SARS outbreak 17 years ago[5].

While molecular detection, such as polymerase chain reaction (PCR) and next generation sequencing (NGS), played and continue to play an important role in acute diagnosis and monitoring of genetic changes of the virus, there is now an urgent need for a reliable and versatile serological or antibody test. Such a test is needed for retrospective contact tracing, investigation of asymptomatic infection rate, accurate determination of case fatality rate, assessment of herd immunity and humoral protective immunity in recovered patients and recipients of vaccine candidates, and in the search for the natural reservoir host and intermediate host(s)[6]. Research laboratories and pharmaceutical companies are racing to produce antibody tests that can detect COVID-19 infection with sufficient specificity and sensitivity[6]. There are two types of antibody tests one can aim for. The first type is the virus neutralization test (VNT) which detects neutralizing antibodies (NAbs) in a patient's blood. VNT requires handling live SARS-CoV-2 in a specialized biosafety level 3 (BSL3) containment facility which is tedious and time consuming, taking 2-4 days to complete. Pseudovirus-based virus neutralization test (pVNT) is similar, but still requires the use of live viruses and cells although handled in a BSL2 laboratory[7,8]. All other assays, such as ELISA and lateral flow rapid tests, represent the second assay type which detect only binding antibodies, and not NAbs[6,9-11].

In this study, we established a surrogate virus neutralization test (sVNT) which detects NAbs, but without the need to use any live virus or cells and can be completed in 1-2 hours in a BSL2 lab. Using purified receptor binding domain (RBD) protein from the viral spike (S) protein and the host cell receptor ACE2, our test is designed to mimic the virus-host interaction by direct protein-protein interaction in a test tube or an ELISA plate well. This highly specific interaction can then be neutralized, i.e., blocked by highly specific NAbs in patient or animal sera in the same manner as in a conventional VNT.

# Results

### Biochemical simulation of virus-receptor interaction and antibody-mediated neutralization

Immediately after SARS-CoV-2 was identified as the causative agent of the COVID-19 outbreak, it was shown that the human angiotensin converting enzyme-2 (hACE2) is the main functional receptor for viral entry[3]. We hypothesized that the virus-receptor binding can be mimicked *in vitro* via a protein-protein interaction using purified recombinant hACE2 and the RBD of the SARS-CoV-2 S protein. This interaction can be blocked by virus NAbs present in the test serum, using the same principle as a conventional VNT conducted using live virus inside a BSL3 facility (Fig. 1a and b).

In our study, the direct binding was demonstrated using different SARS-CoV-2 proteins conjugated with horseradish peroxidase (HRP). There is a dose-dependent specific binding between hACE2 and RBD or S1, but not with the nucleocapsid (N) protein, with the RBD producing the best binding characteristics (Fig. 1c). The HRP-RBD protein was chosen for subsequent studies. We then demonstrated that the specific RBD-hACE2 binding can be blocked or neutralized by COVID-19 sera in a dose-dependent manner, but not by sera from healthy controls (Fig. 1d). To prove that the same principle works with the closely related SARS-CoV, which also uses hACE2 as the entry receptor[12], we repeated the similar experiments and proved that the SARS-CoV RBD performed in an almost identical manner in this new test format (Fig. 1e, f), termed surrogate virus neutralization test (sVNT).

### Isotype- and species-independent neutralization

One of the advantages of sVNT is its ability to detect total antibodies in patient sera, in contrast to most SARS-CoV-2 antibody tests published or marketed, which are almost all isotype-specific, mostly for IgM or IgG, with some for IgA[9-11]. From a panel of 77 COVID-19 positive sera from patients in Singapore, we have designated four groups based on IgM or IgG ELISA levels, determined by our in-house capture ELISA assays (see Methods), present in the patient convalescent sera: a) high IgM/low IgG; b) low IgM/high IgG; c) low IgM/ low IgG; and d) high IgM/high IgG. All groups showed strong neutralization activity in the sVNT (Fig. 2), demonstrating the isotype-independent performance of the assay. It is worth to note that for panel c with low IgM/IgG, the % inhibition in sVNT is still significant at 70-75%, demonstrating its superior sensitivity as this group of sera were deemed negative or weakly positive with isotype-specific capture ELISA based on IgM or IgG alone.

We then tested different animal sera in the sVNT assays to demonstrate species-independent performance. Results from three independent rabbits immunized with the SARS-CoV-2 RBD protein, demonstrate very potent neutralizing activity in the SARS-CoV-2 sVNT (Fig. 3a). Similarly, sera from ferrets infected with SARS-CoV (Fig. 3b) and rabbits immunized with inactivated SARS-CoV (Fig. 3c) also demonstrate an efficient dose-dependent inhibition of the hACE2-SARS-CoV RBD interaction in the SARS-CoV sVNT.

### Specificity against other hCoVs and comparison of SARS sera collected in 2003 vs 2020

To demonstrate specificity, we tested different panels of sera against other known human coronaviruses (hCoVs) and confirmed that the SARS-CoV-2 sVNT can differentiate antibody responses between COVID-19 and other coronavirus infections (Fig. 3d). For SARS sera, there is some level of cross reactivity as expected from their close genetical relatedness and previous published studies[3,7]. But the difference in neutralization is statistically significant, and hence the sVNT can be used to differentiate COVID-19 infection from past SARS infection. For human sera from patients with 229/NL63 or OC43 infection and alpaca sera from experimental MERS-CoV infection, there is no detectable cross neutralization.

During the investigation of potential cross reactivity between SARS sera and SARS-CoV-2 virus, we made several important observations. Firstly, despite the lack of cross neutralization by SARS sera against the live SARS-CoV-2 virus in VNT observed by us and other groups[13], we detected some level of cross neutralization in sVNT (Fig. 3d), indicating sVNT is more sensitive than VNT; secondly, SARS NAbs are detectable for at least 17 years in recovered patients (Fig. 3f); thirdly, the cross neutralization level is higher in the 2020 SARS sera than the 2003 samples (Fig. 3d) although the homologous neutralizing level of the 2020 SARS sera (Fig. 3f) is lower than the 2003 SARS sera (Fig. 3e); lastly, we have found that the N-specific antibody level is much lower in the 2020 SARS sera than the 2003 samples (Fig. 3g).

### Correlation between live virus VNT and biochemical sVNT

A panel of COVID-19 sera with different levels of SARS-CoV-2 NAbs as shown by sVNT (Suppl Fig. 1) were chosen for a comparative and correlation study between the live virus based VNT and the RBD-hACE2 based sVNT. The results demonstrate good overall the correlation between the two assays (Fig. 4a and Suppl Table 1). The SARS-CoV-2 sVNT is more sensitive than VNT. At the 50% inhibition cutoff, which is considered a stringent cutoff as evident from the titration curves in Suppl. Fig. 1, all COVID-19 patients sera showed neutralization at 1:20 or greater with the COVID-19 Patient 13 serum reaching a neutralization titer equal to or greater than 640 (Suppl. Fig. 1).

### Validation with two cohorts of positive and negative sera from two countries

To validate the performance of the SARS-CoV-2 sVNT, we tested two different cohorts of positive and negative sera. The assay was performed in two different countries by two independent groups to further assure reliability and reproducibility. For the first cohort, we tested 77 sera from PCR-confirmed COVID-19 patients in Singapore collected on days 14-33 after symptom onset and 75 healthy control sera. All

control sera were negative, resulting in a 100% specificity. Using a cutoff at 20% inhibition, the assay sensitivity is at 100%. The sensitivity decreases to 95.6% when a 40% cutoff is used (Fig. 4b). For the second cohort, we tested 50 sera each of healthy controls and PCR-confirmed COVID-19 patients in Nanjing, China, sampled on days 27-61 after symptom onset. The specificity is 100%. The sensitivity is 98% and 96% using a 20% and 40% cutoff, respectively (Fig. 4c).

# Discussion

We are more than 100 days into the COVID-19 outbreak and attention worldwide, both in the scientific community and for policy makers, has shifted focus from acute diagnostic strategy and capacity to the use of serology for the “exit strategy”, relying on accurate assessment of infection prevalence at the individual and population (herd) level. Discussion and debate on the role of serology has intensified greatly in this context[6].

While there are many COVID-19 lab-based or point-of-care (POC) antibody test kits commercially available, none are capable of measuring NAbs. VNT or pVNT remain the only platform for detection of NAbs. Both require live virus and cells, highly skilled operators, are less sensitive in general, and take days to obtain results. VNT and pVNT are thus not suitable for mass production and testing, even in the most developed nations.

The World Health Organization (WHO) has recently cautioned that positive results from antibody tests do not equal to protective immunity[14] due to two aspects or obstacles. Firstly, most, if not all, current testing done at large scale is for detection of binding antibodies only and does not measure NAbs; secondly, the presence of NAbs may or may not correlate with protection. While the second aspect will take much more in-depth scientific and clinical research to resolve in the specific context of COVID-19 infection, past experiences with viral infection in general argue that in most recovered patients NAb level is a good indicator of protective immunity, despite the fact that some patients may not obey this “rule of thumb”[15,16]. In this study, we have developed a novel sVNT platform to tackle the first obstacle.

The data presented here demonstrated that sVNT is as specific as, and more sensitive than VNT. The results obtained from sVNT correlates well with VNT. The major advantage of sVNT is that it can be rapidly conducted in most research or clinical labs without the need to use live biological materials and biosafety containment. The sVNT is also amenable to high throughput testing and/or fully automated testing after minimal adaptation.

Another advantage of sVNT is its ability to detect SARS-CoV-2 antibodies in a species-independent manner. As the origin of SARS-CoV-2 and early transmission event remain elusive, the sVNT assay will be ideally suited for “virus hunting” as past studies have amply demonstrated that serological surveys are more superior than molecular detection as the virus-specific antibodies last much longer in animals than the viral genetic material[17-19]. Sampling serum for antibody detection is also more reliable than other sampling approaches used for molecular detection as the target tissues can vary from virus to virus[20-22].

In addition, sVNT offers a key advantage over most ELISA or POC tests in its ability to detect total NAbs in an isotype-independent manner. This will not only simplify the testing strategy, but also further increase test sensitivity. As shown in Fig. 2c for the serum panel of COVID-19 patients showing low IgM and IgG in the isotype-specific ELISAs, the sVNT assay still detected significant level of NAbs. Although the mechanism needs further investigation, there are at least two possibilities: the presence of other Ig isotypes or neutralization synergy or cooperativity from the combination of different isotype antibodies targeting different neutralization critical epitopes, as previously observed for HIV and other viruses[23-25].

Results obtained for the two SARS serum panels are very interesting. The long lasting NAbs 17 years after initial infection is encouraging news for recovered COVID-19 patients considering the close relationship of the two viruses. The mechanism and biological significance of the increased cross neutralization towards SARS-COV-2 coupled with the decrease/disappearance of N-specific antibodies 17 years after infection warrants further investigation in the context of better understanding SARSr-CoV immune response dynamics.

In summary, we have addressed the challenge of COVID-19 serology with a new approach that enables the detection of NAbs in an easy, safe, rapid and inexpensive manner with enhanced specificity and sensitivity. While the sVNT assay may never be able to completely replace the conventional VNT, our data indicate that their performance is generally well correlated. Its application can cover many aspects of COVID-19 investigation from contact tracing, sero-prevalence survey, reservoir/intermediate animal tracking to assessment of herd immunity, longevity of protective immunity and efficacy of different vaccine candidates.

# Methods

**Cells and virus.** Human embryonic kidney (HEK293T) cells (ATCC# CRL-3216) and African green monkey kidney, clone E6 (Vero-E6) cells (ATCC# CRL-1586) were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. SARS-CoV-2, isolate BetaCoV/Singapore/2/2020 (Accession ID EPI_ISL_406973), was used for virus neutralization test on Vero-E6 cells as described previously[26].

**Panels of human and animal sera used in this study.** In Singapore, COVID-19 patient sera used in this study was from the Singapore PROTECT study as described [13]. Sera from recovered SARS patients from 2003 were as previously described [15]. For SARS recall sampling in 2020, we contacted and then obtained blood from consenting individuals previously admitted for SARS (ethics approval number: NHG DSRB E 2020/00091). The hCoV serum panel included post-infection samples from subjects confirmed CoV 229/NL63 and CoV OC43 positive using the SeeGene RV12 respiratory multiplex kit in a previous study (ethics approval number: NUS-IRB 11-3640)[27]. Negative control sera were obtained from residual serum samples from previous unrelated studies. In Nanjing, China, COVID-19 convalescent sera were collected with written informed consent and approved by the ethics committee of the Second Hospital of Nanjing (ethics approval number: 2020-LS-ky003). Rabbit anti-SARS-CoV-2 RBD sera were purchased

from GenScript. Rabbit and ferret anti-SARS-CoV sera, and alpaca anti-MERS-CoV sera were as described in previous studies[28,29].

**Direct binding and sVNT assay**. For direct binding, hACE2 protein (GenScript) was coated at 100 ng/well in 100 mM carbonate-bicarbonate coating buffer (pH 9.6). HRP-conjugated SARS-CoV-2 N, S1, RBD or HRP-conjugated SARS-CoV-RBD (all purchased from GenScript) was added to the hACE2 coated plate at different concentration in OptEIA assay diluent (BD) for 1h at room temperature. Unbound HRP-conjugated antigens were removed by five phosphate buffered saline, 0.05% tween-20 (PBST) washes. Colorimetric signal was developed on the enzymatic reaction of HRP with chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB) (Invitrogen). Equal volume of TMB stop solution (KPL) was added to stop the reaction, and the absorbance reading at 450 nm and 570 nm were acquired using Cytation 5 microplate reader (BioTek). For the surrogate neutralization test (sVNT), 6 ng of HRP-RBD (from either virus) was pre-incubated with test serum at the final dilution of 1:20 for 1 h at 37°C, followed by hACE2 incubation for 1 h at room temperature. Inhibition (%) = (1 - Sample OD value/Negative Control OD value) x100.

**Indirect ELISA**. SARS-CoV-2 and SARS-CoV N proteins were expressed from the pcDNA3.1 SARS-CoV-2 N and pDualGC SARS-CoV N transfected HEK293T cells and purified using Ni Sepharose (GE healthcare). For indirect ELISA, 100 ng of each protein was coated onto MaxiSORP ELISA plate (Nunc) using 100 mM carbonate buffer and blocked with BD OptEIA (BD). COVID-19, SARS patient sera were tested at a dilution of 1:50 and detected by Goat-anti-human IgG-HRP (Santa Cruz) at 1:10,000 dilution. The chromogenic signal was developed using TMB substrate (Invitrogen) and the reaction was stop with TMB stop solution (KPL). Absorbance readings at 450 and 570 nm were obtained using Cytation 5 microplate reader (Bio-Tek).

**Capture ELISA**. 96-well Maxisorp plates (Nunc) were coated with 10 µg/ml of anti-human IgM (SeraCare) or anti-human IgG (Jackson labs) in bicarbonate buffer overnight at 4$^{o}$C. Wells were blocked using BD OptEIA assay diluent (BD) for 1 h at 37$^{o}$C and heat-inactivated sera diluted 1:50 were next added and incubated for 1 h at 37$^{o}$C. Following extensive washing, SARS-CoV-2-HRP (GenScript) diluted 4 µg/ml was added and incubated for 30 min at 37$^{o}$C. Chromogenic reaction was quantified following the addition of TMB substrate (Invitrogen) and stop solution (KPL SeraCare). The absorbance of the samples was measured at 450 nm and the background at 570 nm. Negative controls consisted of 37 naïve human sera. Results are presented as fold-change over average reading of negative controls.

**Statistical analysis.** Statistical analysis was perform using GraphPad Prism software with the Kruskal-Wallis test to compare multiple groups, followed by Dunn's multiple comparisons test. Data were considered significant if * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

# Declarations

**Online content.** Any methods, additional references, Nature Research reporting summaries, supplementary information, acknowledgements; details of author contributions and competing interests are available at [Article DOI].


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### Author contributions

L-FW conceived and guided the study. CWT, WNC and DEA performed laboratory work including data analysis. MI-CC, ZH, BEY, Y-JT, YY and DCL provided necessary samples and coordination for the study. L-FW initiated the manuscript writing with input from all authors.

### Conflict of interest

A patent application has been filed for the content disclosed in this study and a SARS-CoV-2 sVNT Kit is under development with an industrial partner for commercialization.

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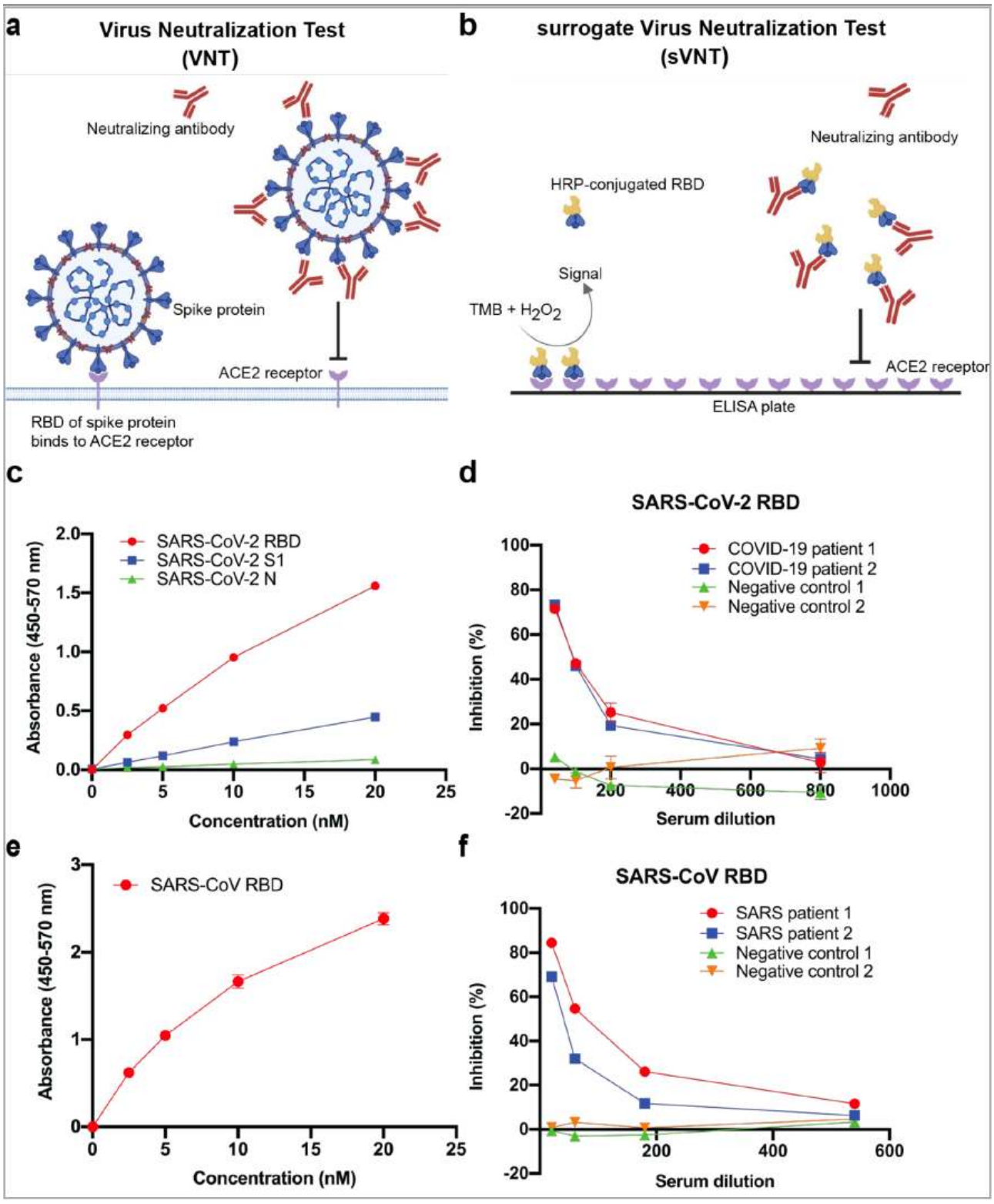
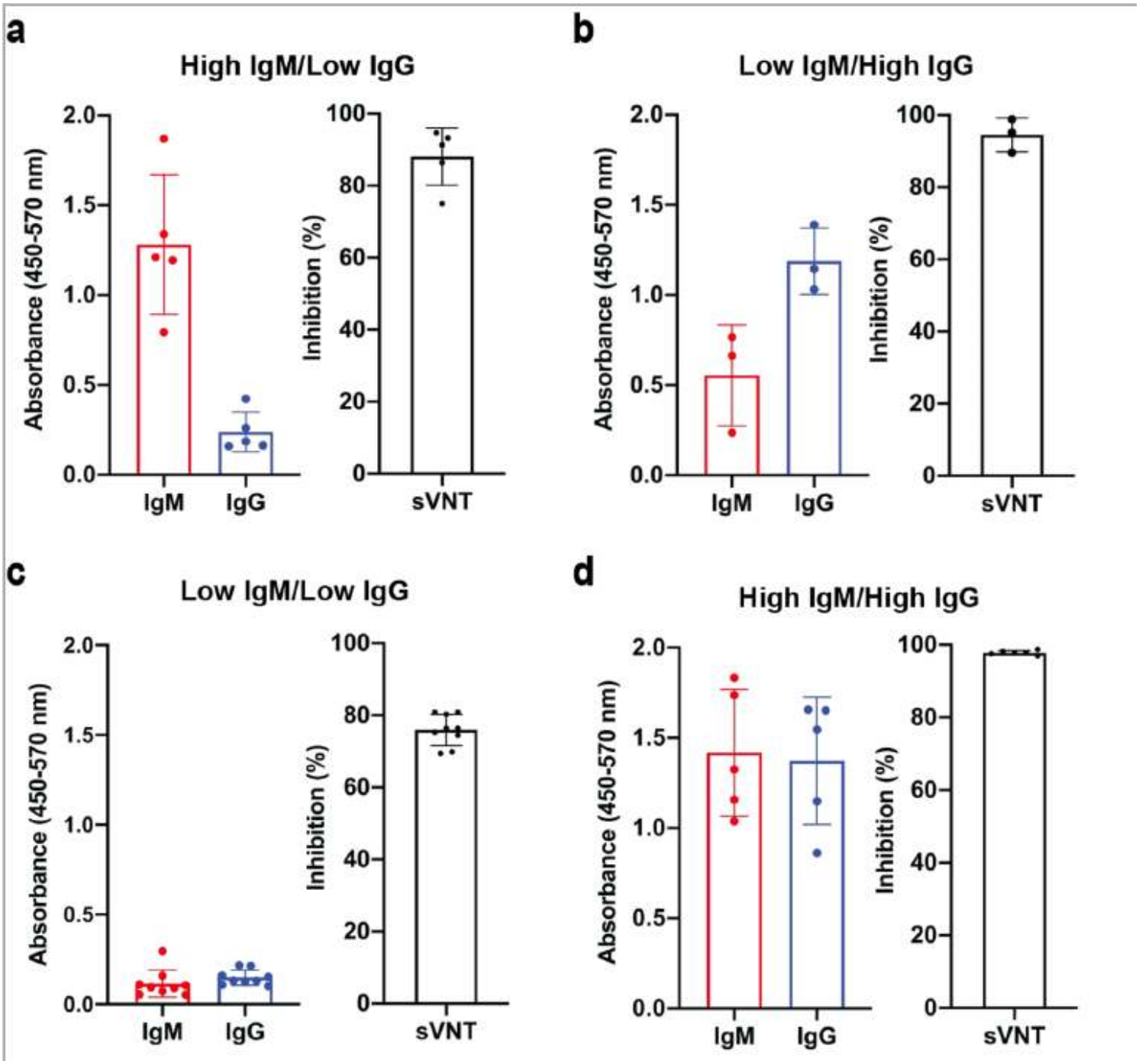
# Figures




## Figure 1

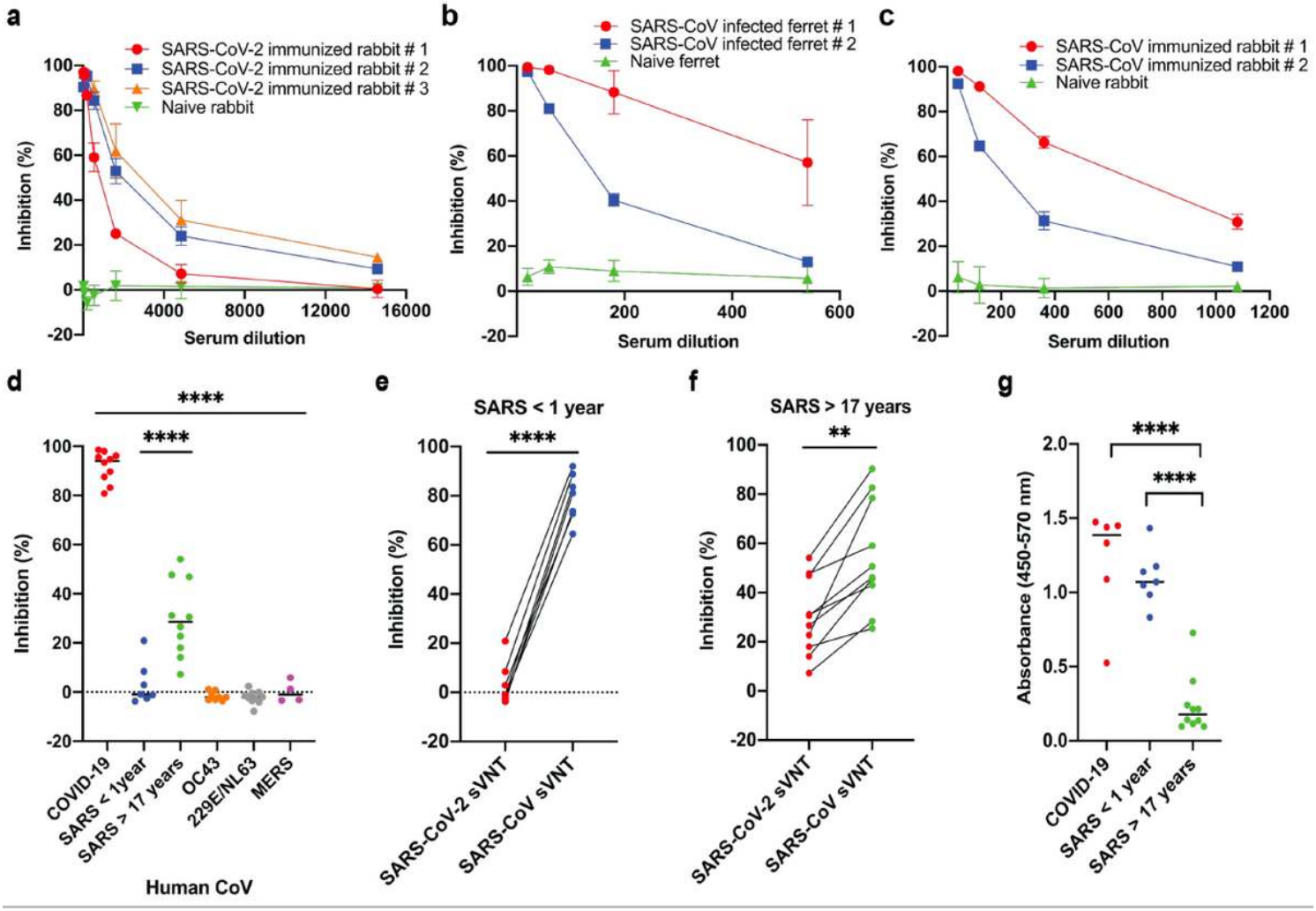
Principle and initial validation of the SARS-CoV-2 surrogate virus neutralization test (sVNT). (a) Mechanism of conventional virus neutralization test (VNT). Anti-SARS-CoV-2 neutralizing antibodies block SARS-CoV-2 Spike protein from binding to hACE2 receptor proteins on the host cell surface. (b) In the sVNT assay, anti-SARS-CoV-2 neutralizing antibodies block HRP-conjugated RBD protein from binding to the hACE2 protein pre-coated on an ELISA plate. (c) Binding of HRP-conjugated SARS-CoV-2 N, S1 and

RBD proteins to hACE2. (d) Inhibition of SARS-CoV-2 RBD-hACE2 interaction by COVID-19 patient sera. (e) Binding of HRP-conjugated SARS-CoV RBD to hACE2. (f) Inhibition of SARS-CoV RBD-hACE2 interaction by SARS patient sera




## Figure 2

Isotype-independent neutralization by human sera with different levels of IgM and IgG antibodies. (a) High IgM/Low IgG (n = 5); (b) Low IgM/High IgG (n = 3); (c) Low IgM/Low IgG (n = 9); (d) High IgM/High IgG (n = 5). The IgM and IgG levels were determined by isotype-specific capture ELISA detailed in Methods.




## Figure 3

Species-independent and virus-specific neutralization. (a) Rabbit anti-SARS-CoV-2 RBD sera from immunization (n =3). (b) Ferret anti-SARS-CoV sera from infection (n = 2); (c) Rabbit anti-SARS-CoV sera from immunization (n = 2). (d) SARS-CoV-2 sVNT using different coronavirus sera: human COVID-19 sera (n = 10), human SARS sera sampled in 2003 (n = 7, <1 year), human SARS-CoV sera sampled in 2020 (n = 10, >17 years), human OC43 sera (n = 8), human 229E/NL63 sera (n = 10), MERS-CoV sera from experimentally infected alpaca (n = 4). (e) Comparative analysis of homologous and heterologous NAb levels for the 2003 SARS serum panel. (f) Comparative analysis of homologous and heterologous NAb levels for the 2020 SARS serum panel. (g) Comparative analysis of homologous N-specific antibodies in the three serum cohorts. SARS-CoV-2 N protein indirect ELISA for COVID-19 sera and SARS-CoV N protein indirect ELISA for the two SARS serum panels.

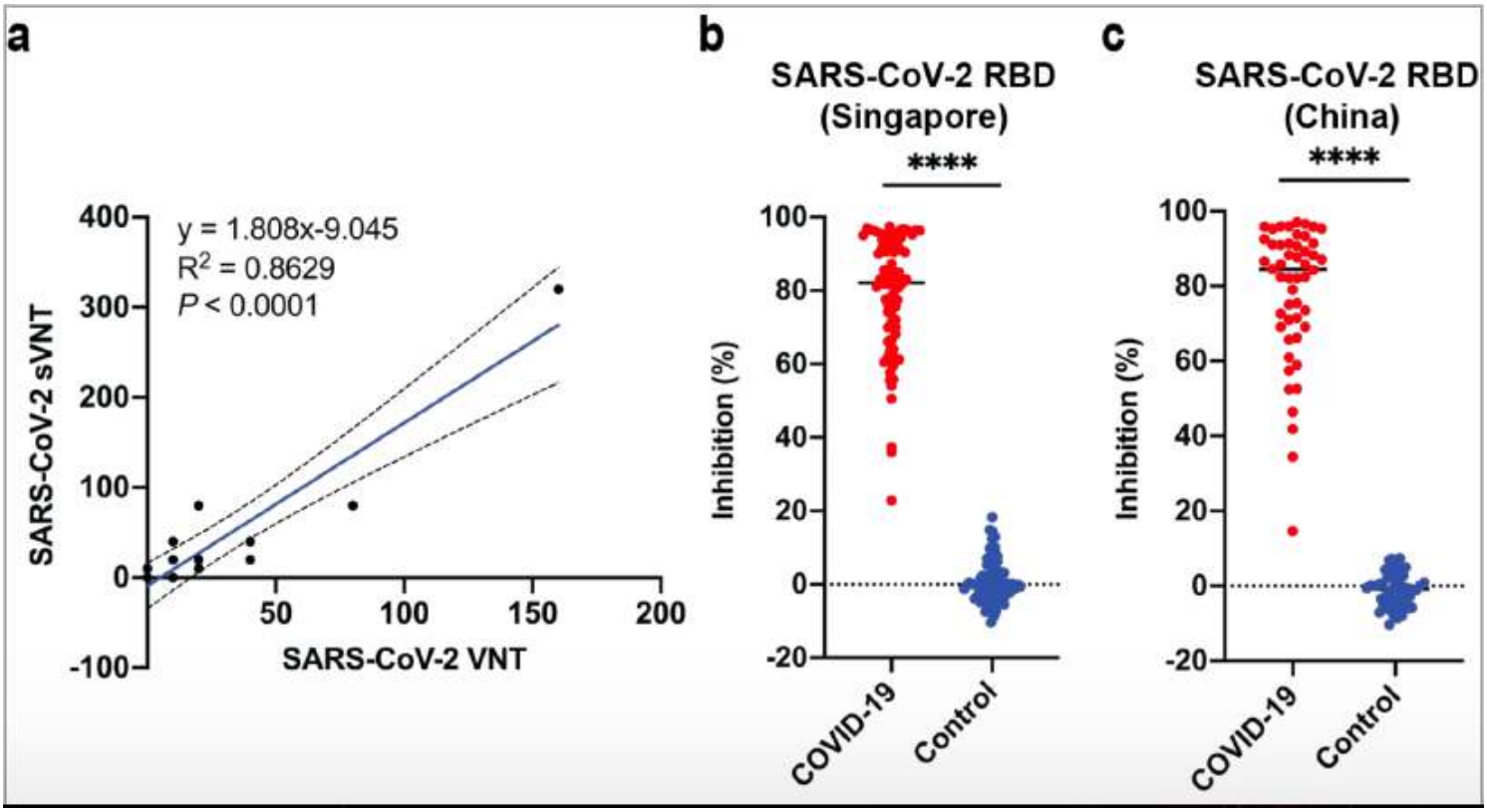



**Figure 4**

Correlation between sVNT and VNT and sVNT testing with two COVID-19 patient cohorts from two different nations. (a) Correlation analysis for 13 COVID-19 sera with different levels of SARS-CoV-2 antibodies by VNT and sVNT at 70% inhibition. Testing of healthy control and COVID-19 serum cohorts in Singapore (b) (COVID-19 n = 77, control n = 75) and Nanjing (c), China (COVID-19 n = 50, control n = 50).

# Supplementary Files

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