






Evaluation of a SARS-CoV-2 Surrogate Virus Neutralization Test for Detection of Antibody in Human, Canine, Cat, and Hamster Sera

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ABSTRACT Surrogate neutralization assays for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that can be done without biosafety level 3 containment and in multiple species are desirable. We evaluate a recently developed surrogate virus neutralization test (sVNT) in comparison to 90% plaque reduction neutralization tests (PRNT₉₀) in human, canine, cat, and hamster sera. With PRNT₉₀ as the reference, sVNT had sensitivity of 98.9% and specificity of 98.8%. Using a panel of immune sera corresponding to other coronaviruses, we confirm the lack of cross-reactivity to other coronaviruses in SARS-CoV-2 sVNT and PRNT₉₀, except for cross-reactivity to SARS-CoV-1 in sVNT.

KEYWORDS SARS coronavirus 2, COVID-19, serology, neutralization, seroepidemiology, human, animal, SARS-CoV-2, antibody, canine, cat, hamster, surrogate virus neutralization

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019 to cause a pandemic. For seroepidemiology studies and in outbreak investigations, it is important to detect antibody responses in humans and animals to ascertain evidence of past infection with SARS-CoV-2. Antibody assays that are transferable across species are desirable because SARS-CoV-2 infects pets and other farmed animals (e.g., mink) (1–3), for monitoring antibody responses in experimental animal models (4), and in studies to identify the natural animal reservoir of SARS-CoV-2. Enzyme-linked immunosorbent assays (ELISAs) of the virus spike receptor binding domain (RBD), which has the fewest cross-reactive epitopes in common with other coronaviruses, or of the whole spike protein or nucleoprotein are widely used for detection of antibody in humans (5–7). Virus neutralization assays are used for confirming positive results but require handling live virus in biosafety level 3 (BSL-3) containment facilities or the use of a pseudotyped virus (6).

ELISAs used for detection of antibody in other animal species require assays to be reoptimized with the relevant species-specific anti-Ig conjugate for detecting immunoglobulins of each species. For some species, relevant anti-Ig reagents may not be

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TABLE 1 Performance characteristics of sVNT in comparison with PRNT₉₀^a

Study group	No. sVNT pos/PRNT ₉₀ pos (PRNT titer and sVN% inhibition)	No. sVNT neg/ PRNT ₉₀ neg	No. sVNT pos/ PRNT ₉₀ neg	sVNT neg/ PRNT ₉₀ pos	Total
SARS-CoV-2 infected and control human sera					
<10 days after illness onset	6 ^b	22	1	1	30
≥10 days after illness onset	166 ^b	5	3	1	175
Control humans collected in 2017	0	196	0	0	196
SARS-CoV-2 infected and control canine sera					
SARS-CoV-2 infected dogs	3 (PRNT titers 1:40, 1:80, and 1:160; sVNT inhibition 33, 66, and 78, respectively)	1 (acute serum, later seroconverted)	0	0	4
Control dogs	0	40	0	0	40
SARS-CoV-2 infected and control cats					
SARS-CoV-2 infected cats	2 (PRNT titers 1:320 and 1:80; sVNT inhibition 83 and 75, respectively)	0	0	0	2
Control cats	0	59	0	0	59
SARS-CoV-2 infected and control hamsters					
SARS-CoV-2 infected hamsters	3 (PRNT titers 1:320, 1:640, and 1:640; sVNT inhibition 83, 83, and 83, respectively)	0	0	0	3
Control hamsters	0	2	0	0	2
Total	180	325	4	2	511

^aPRNT₉₀, 90% plaque reduction neutralization test; sVNT, surrogate virus neutralization test.

^bCorrelations between PRNT₉₀ titers and percent surrogate virus neutralization (sVNT%) inhibition levels shown in Fig. 1.

available. The currently available generic alternative has been the use of virus neutralization test (VNTs), which usually involve handling live virus in BSL-3 containment. A “surrogate” VNT (sVNT) that can be done in BSL-2 containment has recently been reported (8). It is an assay that relies on competitive inhibition of the interaction of ACE-2 receptor coated on an ELISA plate with horseradish peroxidase (HRP)-labeled virus spike receptor binding domain. We used a panel of sera from patients or animals with real-time PCR (RT-PCR)-confirmed SARS-CoV-2 infection and corresponding controls to evaluate this sVNT in comparison to the “gold standard” 90% plaque reduction neutralization tests (PRNT₉₀).

MATERIALS AND METHODS

Sera. Sera from patients with RT-PCR-confirmed SARS-CoV-2 infection ($n = 205$) and negative controls ($n = 196$) collected from blood donors in 2017 were used (6). Among the 205 SARS-CoV-2-infected humans, 38 were <20 years of age, 68 were 21 to 40 years of age, 55 were 41 to 60 years of age, and 44 were older than 60 years, with 119 of the overall cohort being male. Disease severity ranged from asymptomatic ($n = 18$) to mild ($n = 166$) to severe, with the latter defined as disease severe enough to require >3 liters of supplemental oxygen per min ($n = 19$); disease severity data were unavailable for two individuals. Dogs ($n = 4$) and two cats with RT-PCR-confirmed SARS-CoV-2 infection were included in the study (1, 9). Dog sera ($n = 40$) obtained from a study of canine influenza in 2017 (10) and sera from cats ($n = 59$) submitted for routine veterinary diagnostic testing in 2020 were used as controls. Hamster sera from experimentally infected hamsters and controls were available from a previous study (4).

Coronavirus immune sera. Immune sera specific for alphacoronaviruses (porcine respiratory coronavirus, feline infectious peritonitis virus, canine coronavirus, and porcine transmissible gastroenteritis virus), betacoronaviruses (SARS-coronavirus), and gammacoronaviruses (infectious bronchitis virus) were obtained from BEI Resources (animal CoV reagents were supplied to BEI Resources by Linda Saif (<http://www.beiresources.org/About/BEIResources.aspx>) or generated by Linda Saif (see Table 2). Stanley Perlman provided mouse serum to mouse hepatitis virus (strains A59 and JHM) (Table 1). The homologous antibody titers to the immunizing virus were also provided by Linda Saif or Stanley Perlman (Table 2).

Serological tests. SARS-CoV-2 surrogate virus neutralization test kits were obtained from GenScript, Inc., NJ, USA, and the tests were carried out according to the manufacturer’s instructions. The test sera (10 μ l) and positive and negative controls were diluted 1:10 and mixed with an equal volume of horseradish peroxidase (HRP) conjugated to SARS-CoV-2 spike receptor binding domain (RBD) (6 ng) and incubated for 30 min at 37°C. A 100- μ l volume of each mixture was added to each well on the microtiter plate coated with ACE-2 receptor. The plate was sealed and incubated at room temperature for 15 min at 37°C. Plates were then washed with wash solution and tapped dry, and 100 μ l of 3,3',5,5'-

TABLE 2 SARS-CoV-2 sVNT and PRNT₉₀ cross-reactivity with immune sera corresponding to other coronaviruses^a

Genus	Antiserum	Homologous Ab titer by ELISA or virus neutralization (VN) as indicated	PRNT ₉₀ titer	sVN % inhibition	sVN result
<i>Alphacoronavirus</i>	Gnotobiotic pig antiserum to porcine respiratory coronavirus—NR-460	ELISA 1:1,200 ^b	<1:10	8.39	Negative
	Guinea pig antiserum to feline infectious peritonitis virus—NR-2518	ELISA 1:2,000 ^b	<1:10	13.17	Negative
	Guinea pig antiserum to canine coronavirus—NR-2727	ELISA 1:4,094 ^c	<1:10	17.20	Negative
	Gnotobiotic pig antiserum to porcine transmissible gastroenteritis virus—NR-458	ELISA 1:1,400 ^b	<1:10	12.76	Negative
<i>Betacoronavirus</i>	Guinea pig anti-SARS-CoV—NR-10361	VN 1:2,560 ^c	<1:10	81.14	Positive
	Rabbit antiserum for SARS-CoV S protein (control)—NRC-769	VN <1:10	<1:10	13.68	Negative
	Rabbit antiserum for SARS-CoV S protein (low titer)—NRC-770	VN 1:80	<1:10	78.44	Positive
	Rabbit antiserum for SARS-CoV S protein (medium titer)—NRC-771	VN 1:160	<1:10	75.30	Positive
	Rabbit antiserum for SARS-CoV S protein (high titer)—NRC-772	VN 1:640	1:10	79.59	Positive
	Human SARS convalescent plasma (E222P)	VN 1:160 ^e	<1:10	28.39	Positive
	Human SARS convalescent plasma (E229P)	VN 1:320 ^e	<1:10	36.39	Positive
	Mouse hepatitis virus (A59 strain) infected mouse	VN 1:1000 ^d	<1:10	14.76	Negative
	Hyperimmunized mouse serum to mouse hepatitis virus (JHM strain)	VN 1,1778 ^d	<1:10	9.73	Negative
<i>Gammacoronavirus</i>	Guinea pig antiserum to infectious bronchitis virus—NR-2515	ELISA 1:50,000 ^b	<1:10	15.16	Negative

^aAbbreviations: Ab, antibody; ELISA, enzyme-linked immunosorbent assay; PRNT₉₀, 90% plaque reduction neutralization test; SARS-CoV, severe acute respiratory syndrome coronavirus; sVN, surrogate virus neutralization. sVN inhibition of $\geq 20\%$ is regarded as positive (pos) and $< 20\%$ as negative (neg). All homologous antibody titers represent ELISA titers except for antibody titers to SARS-CoV and mouse hepatitis virus, which represent neutralizing antibody titers.

^bHomologous antibody titer data obtained from BEI Resources.

^cHomologous antibody titer data obtained from Linda Saif.

^dHomologous antibody titer data obtained from Stanley Perlman.

^eNeutralizing antibody titers from the Malik Peiris laboratory.

tetramethylbenzidine (TMB) solution was added to each well and incubated in the dark at room temperature for 15 min. The reaction was stopped by addition of 50 μ l of Stop Solution to each well and the absorbance read at 450 nm in an ELISA microplate reader. The assay validity was based on values representing optical density at 450 nm (OD₄₅₀) for positive and negative results falling within the range of recommended values. On the basis of the assumption that the positive and negative controls gave the recommended OD₄₅₀ values, percent inhibition of each serum was calculated as follows: percent inhibition = $(1 - \text{sample OD value/negative-control OD value}) \times 100$. Percent inhibition values of $\geq 20\%$ are regarded as positive results, while percent inhibition values of $< 20\%$ are regarded as negative results (8).

SARS-CoV-2 90% plaque reduction neutralization titer was determined as previously described using the virus isolate BetaCoV/Hong Kong/VM20001061/2020 (6). In brief, the assay was performed in duplicate using 24-well tissue culture plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) in a biosafety level 3 facility. Serial dilutions of each serum sample were incubated with 30 to 40 plaque-forming units of virus for 1 h at 37°C. The virus-serum mixtures were added onto preformed Vero E6 cell (ATCC CRL-1586) monolayers and incubated for 1 h at 37°C in an incubator in a 5% CO₂ atmosphere. The cell monolayer was then overlaid with 1% agarose in cell culture medium and incubated for 3 days, at which time the plates were fixed and stained. The antibody titer was defined as the highest serum dilution that resulted in $\geq 90\%$ reduction in the number of virus plaques (PRNT₉₀).

SARS-CoV-2 spike receptor binding domain ELISA was carried out as previously described (6). ELISA 96-well plates (Nunc MaxiSorp; Thermo Fisher Scientific) were coated overnight with 100 ng per well of the purified recombinant RBD protein in phosphate-buffered saline (PBS) buffer. The plates were then blocked with 100 μ l of ChonBlock blocking/sample dilution ELISA buffer (Chondrex Inc., Redmond, WA, USA) and incubated at room temperature for 2 h. Each serum or plasma sample was tested at a dilution of 1:100 in ChonBlock blocking/sample dilution ELISA buffer and added to the ELISA wells of each plate for 2 h of incubation at 37°C. After extensive washing with PBS containing 0.1% Tween 20, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (GE Healthcare) (1:5,000) was added for 1 h at 37°C. The ELISA plates were then washed five times with PBS containing 0.1% Tween 20. Subsequently, 100 μ l of HRP substrate (Ncm TMB One; New Cell and Molecular Biotech Co. Ltd., Suzhou, China) was added into each well. After 15 min of incubation, the reaction was stopped by adding 50 μ l of 2 M H₂SO₄ solution

and analyzed on a Sunrise (Tecan, Männedorf, Switzerland) absorbance microplate reader at 450-nm wavelength. The validation and optical density cutoff for a positive result were as described in the previous publication (6).

Statistical analysis and modeling. We used Pearson's correlation coefficient to compare correlations between assays.

Ethical statement. Collection of serum from patients with coronavirus disease 2019 (COVID-19) was approved by the institutional review boards (IRBs) of the respective hospitals, *viz.* Kowloon West Cluster [KW/EX-20-039 (144-27)], Kowloon Central/Kowloon East cluster (KC/KE-20-0154/ER2), and HKU/HA Hong Kong West Cluster (UW 20-273). The collection of healthy blood donor sera performed in 2017 was approved by the IRBs of The Hong Kong University and the Hong Kong Island West Cluster of Hospitals (IRB reference number UW16-254). The canine sera used were residual sera from samples collected as part of routine clinical care, and the Committee on the Use of Live Animals in Teaching and Research (CULATR) has waived animal ethics approval. Cat sera were residual sera collected as part of routine diagnosis. The experimental study on hamsters was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong (CULATR/5323-20).

RESULTS AND DISCUSSION

Sera from SARS-CoV-2-infected or control humans ($n = 401$), dogs ($n = 44$), cats ($n = 61$), or hamsters ($n = 5$) with known plaque reduction neutralization titer results were tested in the sVNT (Table 1). The overall concordance between the sVNT and PRNT₉₀ was 98.9%, with 505 of 511 giving agreement between the PRNT₉₀ and sVNT results. Of the six discrepant samples, all from SARS-CoV-2-infected humans, two sera were positive in PRNT (1:10 and 1:20) but negative in sVNT and four sera were positive in sVNT (inhibition of 23%, 32%, 54%, and 72%) but negative in PRNT₉₀; however, three of the latter were positive at the lower-stringency PRNT cutoff value of >50% reduction in plaque counts (Table 1). Of 175 sera from SARS-CoV-2-infected humans obtained 10 or more days after onset of illness, 166 (94.9%) were positive in both assays, with 167 (95.4%) being positive by PRNT and 169 (96.6%) positive by sVNT. It is known that many patients do not seroconvert in the first few days of illness; accordingly, 22 of 30 sera collected prior to day 10 after illness onset were seronegative in both assays. Six sera collected less than 10 days after onset of illness were positive by both sVNT and PRNT₉₀, whereas one serum sample each was positive in one but not the other assay. All 196 control human sera collected in 2017 (prior to the emergence of COVID-19) were negative in both assays. Thus, the sVNT was marginally more sensitive than PRNT₉₀ in detecting antibody responses in persons with confirmed SARS-CoV-2 infection.

Three of 4 sera collected from two RT-PCR-positive dogs were positive in both sVNT and PRNT₉₀. The antibody-negative serum was negative in both assays and was an early specimen collected from a dog (5 days after the dog, which had never developed symptoms, was taken to quarantine) which subsequently seroconverted as shown by results from both assays. The sera from both cats with RT-PCR-confirmed SARS-CoV-2 infections were positive in both assays, while sera from 59 control cats were negative in both assays.

Using a PRNT₉₀ antibody titer of $\geq 1:10$ as the gold standard for a positive antibody result, testing 511 sera from multiple species, the sVNT had 98.9% sensitivity and 98.8% specificity.

The sVNT is not meant to be quantitative as a result of testing a single serum dilution. However, there was a semiquantitative relationship between percent inhibition in the sVNT and the PRNT₉₀ titers (Fig. 1A). The Pearson's correlation coefficient between log-transformed 90% plaque reduction neutralization titer, calculated as $\ln\left[\log_2\left(\frac{\text{PRNT}_{90}}{10}\right) + 2\right]$, and percent inhibition in the surrogate virus neutralization test (sVNT_%) is 0.84 (P value < 0.01).

For reference, we also show the correlation between sVNT and the optical density on the spike RBD IgG antibody ELISA (Fig. 1B). The Pearson's correlation coefficient between spike RBD ELISA IgG optical density (ELISA_{OD}) and percent inhibition in the surrogate virus neutralization test (sVNT_%) is 0.74 (P value < 0.01).

We investigated cross-reactivity of immune sera to a range of alphacoronaviruses, betacoronaviruses, and gammacoronaviruses in the sVNT and PRNT₉₀, including antisera to feline infectious peritonitis virus, canine coronavirus, mouse hepatitis virus, and

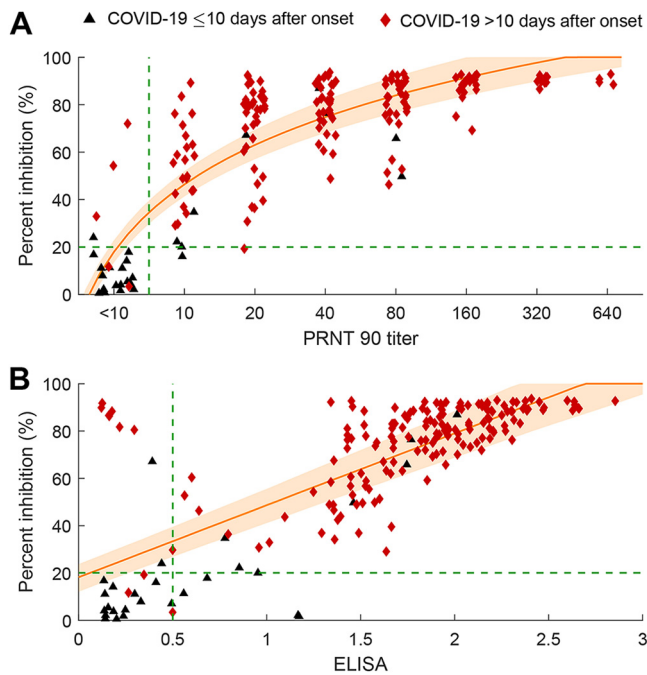


FIG 1 Correlation between percent inhibition in the surrogate virus neutralization test (sVNT) and (A) 90% plaque reduction neutralization test (PRNT₉₀) titer or (B) spike RBD ELISA IgG optical density of 205 sera from humans with results confirmed by RT-PCR. (A) Correlation between PRNT₉₀ and percent inhibition in the sVNT. We fitted a linear log regression model between percent inhibition in the sVNT (sVNT_%) and log-transformed PRNT₉₀ titers as follows: $sVNT_{\%} = k_1 \times \ln \left[\log_2 \left(\frac{PRNT_{90}}{10} \right) + 2 \right] + b_1$. The orange lines indicate estimated percent inhibition, and the orange shading indicates the 95% confidence interval. Blue dots represent the control samples. Black triangles represent samples from confirmed COVID-19 cases obtained less than 10 days since symptom onset, and red diamonds represent samples from confirmed cases obtained 10 days or more after symptom onset. For clarity, the control human sera are not included in the figure as they were negative in all three assays. (B) Correlation between percent inhibition in the sVNT and the spike RBD IgG ELISA optical density. We fitted a linear regression model between percent inhibition in the sVNT and the ELISA OD as follows: $sVNT_{\%} = k_2 \times ELISA_{OD} + b_2$.

SARS-CoV (Table 2). Two human SARS-CoV convalescent plasma samples were also included in this assessment. Cross-reactivity in the sVNT was detected with both SARS convalescent human plasma (homologous PRNT₉₀ titers of 1:160 and 1:320) and four of five hyperimmune sera to SARS-CoV. Cross-reactivity in the SARS-CoV-2 PRNT₉₀ was observed only with the high-titer hyperimmune rabbit sera to SARS-CoV (homologous neutralizing antibody titer of 1:640). SARS-CoV-2 and SARS-CoV-1 are closely related sarbecoviruses, and cross-reactivity of antibody binding to the RBD of these two viruses has been previously reported (11). This is unlikely to be a practical problem in testing human sera, as very few humans were previously exposed to SARS-CoV-1, which is known not to have been circulating in the human population after 2004. It is noted that the cross-reactivity of sVNT to SARS-CoV-2 appears greater than that seen with PRNT. Thus, when sVNT is used for testing animal sera, especially bat sera, the possibility of cross-reactivity between closely related viruses within the sarbecovirus group must be kept in mind. In contrast, neither assay had cross-reactivity with immune sera raised to other betacoronaviruses, alphacoronaviruses, or gammacoronaviruses.

In summary, we found excellent concordance between the sVNT and the gold standard PRNT₉₀ for SARS-CoV-2 antibody detection in human, dog, cat, and hamster sera. This assay would be of great utility as a species-independent and specific assay for primary testing for antibodies to sarbecoviruses (SARS-CoV-2 and SARS-CoV-1 and closely related viruses) in humans or animals. For example, in large-scale human seroepidemiology studies where ELISA is used as a screening assay, sVNT may be used to confirm the viral status of sera found to be ELISA positive. Alternatively, when investigating the natural animal reservoirs of sarbecoviruses, it may be used as a

primary screening assay of animal sera, irrespective of species. There was a subquantitative relationship between PRNT₉₀ titers and percent inhibition in the sVNT in a single serum dilution. Serial serum dilutions assayed on sVNT may improve quantitation, but this was not attempted in our study. We did not compare its performance with pseudoparticle neutralization assays for antibody, which is an alternative though technically more demanding method for testing for SARS-CoV-2 neutralization. The sVNT has the advantages of technical simplicity, speed (requiring only a few hours for completion), and the fact that it does not require cell culture facilities or BSL-3 containment.

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We declare that we have no conflict of interest.

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