Performance comparison of micro-neutralization assays based on surrogate SARS-CoV-2 and WT SARS-CoV-2 in assessing virus-neutralizing capacity of anti-SARS-CoV-2 antibodies

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Abstract
We compared neutralization assays using either the wild-type severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus or surrogate neutralization markers, using characterized sera. We found the results of the neutralization assays 75% concordant overall and 80% concordant for samples with high antibody levels. This demonstrates that commercial surrogate SARS-CoV-2 assays offer the potential to assess anti-SARS-CoV-2 antibodies’ neutralizing capacity outside CL-3 laboratory containment.
Table 1. Comparison of PRNT<sub>50</sub>/PRNT<sub>90</sub>, cVNT and sVNT results for a well-characterized panel of samples

| PRNT<sub>50</sub> | PRNT<sub>90</sub> | cVNT |
|------------------|------------------|------|
| POS | NEG | POS | NEG | POS | NEG | POS | NEG |
| True POS (n=21) | sVNT POS | 21 | 0 | 20 | 1 | 20 | 1 |
| sVNT NEG | 0 | 0 | 0 | 0 | 0 | 0 |
| True NEG (n=19) | sVNT POS | 0 | 1† | 0 | 1† | 0 | 1† |
| sVNT NEG | 0 | 18 | 0 | 18 | 0 | 18 |

*Characterized samples were obtained from the National Microbiology Laboratory, Winnipeg, Manitoba, Canada. PRNT, plaque reduction neutralization assay; PRNT<sub>50</sub>, 50% reduction of plaque compared to control; PRNT<sub>90</sub>, 90% reduction of plaque compared to control; cVNT, conventional viral neutralization test; sVNT, surrogate viral neutralization test. †SARS-CoV-1-positive/SARS-CoV-2-negative sample.

before the onset of the coronavirus disease 2019 (COVID-19) pandemic. All sera from COVID-19 PCR-confirmed patients had been shown to be reactive for anti-SARS-CoV-2 antibodies by at least two commercial assays that detected anti-spike antibodies: Siemens ADVIA Centaur SARS-CoV-2 Total (S1 RBD antigen) and Ortho Vitros SARS-CoV-2 total antibody (RBD antigen). Further, 39/42 samples were also reactive by a third commercial assay that detected anti-nucleocapsid antibodies (Abbott SARS-CoV-2 IgG assay). Samples that were reactive on commercial platforms were selected to represent a range of signal to cut-off (S/CO) ratios.

Conventional virus microneutralization test (cVNT)
The challenge virus used in these assays was obtained from the NML, and was designated as hCoV-19/Canada/ON-VIDO-01–2020. The cVNT was carried out as follows: twofold dilutions of sera were heated at 56 °C for 30 min to inactivate complement. To each dilution, 100 TCID<sub>50</sub> of SARS-CoV-2 was added, incubated for 2 h at 37 °C, and then transferred to microtitre plate wells containing monolayers of Vero E6 cells. Each sample was tested in duplicate. The development of cytopathic effect (CPE) was monitored over 3 days; the neutralizing titre was the lowest dilution without CPE. If only one well at the 1:8 dilution showed CPE, the CVNT result was recorded as equivocal.

Plaque reduction neutralization test (PRNT)
The SARS-CoV-2 PRNT was performed as described elsewhere [6]. The highest serum dilutions resulting in 50 and 90% reduction in plaques compared with controls were defined as the PRNT<sub>50</sub> and PRNT<sub>90</sub> endpoint titres, respectively. PRNT<sub>50</sub> titres and PRNT<sub>90</sub> titres ≥1:20 were considered positive for SARS-CoV-2 neutralizing antibodies.

Surrogate virus microneutralization test (sVNT)
sVNT tests (GenScript USA, Inc., NJ, USA) were performed following the manufacturer’s instructions. Diluted sera (1:9 dilution) were mixed with HRP-conjugated SARS-CoV-2 RBD at 1:1 ratio and incubated at 37°C for 30 min. One hundred microlitres of the mixture was added to a microtitre plate and incubated at 37°C for 15 min. The plate was then washed four times with wash buffer, before the addition of 100 µl 3,3′,5,5′-tetra-methylbenzidine solution per well, followed by incubation in the dark at 20–25°C for 15 min. Stop solution was added to each well and the plate was read immediately at 450 nm. Results were considered to be positive for neutralizing antibodies if the percentage inhibition of the sample was 20% or greater.

RESULTS
For the NML panel, PRNT<sub>50</sub>/PRNT<sub>90</sub>, cVNT and sVNT assays yielded comparable results for all 40 samples (Table 1). Of 19 SARS-CoV-2-negative samples, 1 sample had detectable neutralizing capacity by sVNT but not PRNT<sub>50</sub>/PRNT<sub>90</sub> and cVNT assays. This sample was obtained from a recovered SARS-CoV-1 patient and the cross-reaction is not entirely surprising, considering the ~73% similarity of the RBD motif of the two viruses. The only other SARS-CoV-1-positive sample on the panel was negative by all three tested assays. Of 21 samples from SARS-CoV-2-positive patients, 20 had fully concordant positive results by all assays and 1 had a positive sVNT and PRNT<sub>90</sub> result but negative cVNT and PRNT<sub>50</sub> results, potentially indicative of the ability of sVNT assay to detect low(er) levels of neutralizing antibodies (Table 1).

All BCCDC PHL samples negative for SARS-CoV-2 antibodies by high-volume platforms (Siemens, Ortho and Abbott) were also negative on the sVNT assay and had titres of <1:8 on cVNT (Table 2). All negative samples showed inhibition of binding of less than 11% in the sVNT. All samples that tested positive by commercial serology assays (n=42) were positive for inhibition by sVNT, but cVNT results were variable (Table 2). For samples testing positive in both cVNT and sVNT assays, the extent of antibody-mediated inhibition of binding to ACE-2 did not correlate with time of sample collection from disease onset, with variable levels of inhibition observed at both periods. The level of inhibition in sVNT showed a higher correlation with indices generated in the Siemens assay (Fig. 1a) than with the S/CO ratios of either the Abbott and Ortho assays (Fig. 1b, c). Samples from known positive patients with negative cVNT titres (<1:8) showed lower inhibition by sVNT compared to samples positive for neutralizing antibodies by both assays (44 vs 92%). For the BCCDC panel, there was an overall positive agreement of 62% between the sVNT and cVNT assays for COVID-19-positive sera and 100% negative agreement on pre-pandemic sera; positive agreement increased to 71% when equivocal results by cVNT were counted as positive.

DISCUSSION
We compared the GenScript sVNT assay to cVNT, PRNT and commercial CLIA assays. There was a high concordance of results for PRNT, cVNT and sVNT assays on the panel received from the
NML, while the BCCDC panel yielded more discrepant results by sVNT and cVNT. Overall, sVNT–cVNT agreement on sera from patients diagnosed by PCR for the combined NML–BCCDC panels was 75% (or 81% with equivocal cVNT results included as positive). Detectable inhibition of virus/viral epitopes binding to ACE2 receptor by sVNT but not cVNT assay could be indicative of the higher sensitivity of the sVNT method. For a single positive NML sample sVNT demonstrated inhibition concordant with PRNT\textsubscript{50} results, while PRNT\textsubscript{90} and cVNT results were negative. sVNT–cVNT concordance was greater for samples with higher S/CO ratios in commercial assays, but test outcome was unaffected by time of collection from disease onset. Similar findings were reported recently using two sVNT assays [7]. This is in contrast to recently reported results for GenScript sVNT, wherein samples

Table 2. Comparison of sVNT and cVNT results for a BCCDC PHL panel of serum samples from SARS-CoV-2-infected and uninfected patients

| Sample                      | Siemens S/CO | sVNT result | cVNT result |
|-----------------------------|--------------|-------------|-------------|
| Pre-pandemic                | 49           | NEG         | 49          |
| 0–13 days from disease onset| 11           | 1–4.9       | POS 2 POS   |
|                             |              | 5->10       | POS 9 POS   |
|                             |              | 1–4.9       | POS 9 NEG   |
|                             |              | ≥14 days    | POS 1 EQ    |
|                             |              | from disease onset | 31 | 5->10 | POS 22 NEG 2 |

sVNT, surrogate viral neutralization test; cVNT, conventional viral neutralization test.

![Graphs](image-url)

**Fig. 1.** Percentage binding inhibition by sVNT assay relative to S/CO ratios obtained for positive samples on Siemens (a), Ortho (b) and Abbott (c) high volume CLIA serology platforms. S/CO, signal-to-cutoff ratio; sVNT, surrogate viral neutralization test.
collected >14 days post-disease onset were more likely to be positive by sVNT than samples collected <14 days after onset [8]. Moreover, while Meyer et al. [8] found a pseudovirus-based VNT assay to be more sensitive than the GenScript sVNT assay, we found that the GenScript sVNT assay, at least in our hands, was more sensitive than a SARS-CoV-2-based cVNT assay. This could be due to the different in vitro infectivity of SARS-CoV-2 vs the VSV-based pseudovirus for host cells, as well as the variability of epitopes present for neutralization on SARS-CoV-2 virus, vs those utilized in the production of pseudovirus constructs, vs those utilized in the production of competitive inhibition surrogate neutralization assays. Given that during the natural infection, the host immune system is expected to generate antibodies against an array of exposed epitopes, partial availability of epitopes for neutralization/inhibition may contribute to differences in results between cVNT, sVNT and pseudovirus VNT assays. This, similarly, can also play into the discrepancies with regard to the presence/absence of observed differences in neutralizing capacity of sera collected early/later post-infection. Depending on whether the epitope of choice for the surrogate or pseudovirus system is the main target of generated antibodies both early and later in infection, the expected results would differ. Such considerations might become particularly important when assessing cross-neutralizing capacity post-vaccination vs post-infection with different variant strains of SARS-CoV-2.

If sVNT is indeed able to detect lower titres of neutralizing antibodies, it is yet to be determined whether these lower levels translate into in vivo virus-neutralizing capacity. Similarly, the potential requirement to modify ‘positive’ cut-offs on sVNT assay(s) for observed inhibitory effect needs to be evaluated. Interpretations of all these assays are further complicated by the fact that there are currently no clear in vivo correlates established for either the cVNT or the PRNT_{sub} assays.

A limited number of studies have been conducted on SARS CoV-2 neutralization tests [9, 10]. It has been shown that neutralizing antibody titres may vary among recovered COVID-19 patients [11], which suggests that other immune markers such as T-cells and cytokines are likely to contribute to viral clearance. Recently, Prévost et al. [12] found the neutralizing capacity of serum to substantially decrease in hospitalized COVID-19 patients over time, which is somewhat concordant with our cVNT findings. Whether and over what period neutralizing antibody titres correlate with protection in vivo still needs to be determined. Laboratory-developed [13] and commercially available sVNT assays allow for accessible high-volume assessments of antibody neutralizing capacity [14]. However, where clinical applications are concerned, suitable positive and negative cut-offs, ideally against in vivo correlates of protection, need to be evaluated and established.

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Author contributions

These authors contributed equally to this work.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The study was authorized by the Provincial Health Officer and approved by the Clinical Research Ethics Board of the University of British Columbia (H20-01089).

References

1. Mathuria JP, Yadav R R. Laboratory diagnosis of SARS-CoV-2 - A review of current methods. J Infect Public Health 2020;13:901–905.
2. Caeseele PV, Network for the CPHL, Bailey D, Chemists for the CS of C, Forge SE, Canada for the A of MM and ID. SARS-CoV-2 (COVID-19) serology: implications for clinical practice, laboratory medicine and public health. CMAJ 2020;192:E793–9.
3. Maeda A, Maeda J. Review of diagnostic plaque reduction neutralization tests for flavivirus infection. Vet J 2013;95:33–40.
4. Zakhartchouk AN, Liu Q, Petric M, Babiuk LA. Augmentation of immune responses to SARS coronavirus by a combination of DNA and whole killed virus vaccines. Vaccine 2005;23:4385–4391.
5. Skowronski DM, Hottes TS, Janjua NZ, Puruch D, Sabaiduc S, et al. Prevalence of seroprotection against the pandemic (H1N1) virus after the 2009 pandemic. CMAJ 2010;182:1851–1856.
6. Valcourt EJ, Kathy Manguiat K, Robinson A. Evaluation of a commercially-available surrogate virus neutralization test for severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). Diag Microbiol Infect Dis 2021;99.
7. Luchsingler LL, Ransegnola BP, Jin DK, Muecksk, F, Weisblumy, E T. Serological assays estimate highly variable SARS-CoV-2 neutralizing antibody activity in recovered COVID-19 patients. J Clin Microbiol 2020;58.
8. Meyer B, Reimerink J, Torriani G, Brouwer F, Godeke G-J, et al. Validation and clinical evaluation of a SARS-CoV-2 surrogate virus neutralisation test (sVNT). Emerg Microbes Infect 2020;9:2394–2403.
9. Jiang S, Hillyer C, Du L. Neutralizing antibodies against SARS-CoV-2 and other human coronaviruses. Trends Immunol 2020;41:355–359.
10. Poh CM, Carissimo G, Wang B, Amrnon SN. Lee CY-P, et al. Two linear epitopes on the SARS-COV-2 spike protein that elicit neutralising antibodies in COVID-19 patients. Nat Commun 2020;11:2806.
11. Ni L, Ye F, Cheng M-L, Cheng Y, Deng Y-Q, et al. Detection of SARS-CoV-2-specific humoral and cellular immunity in COVID-19 convalescent individuals. Immunity 2020;52:971–977.
12. Prévost J, Gasser R, Beaudoin-Bussières G, Richard J, Duerr R, et al. Cross-sectional evaluation of humoral responses against SARS-CoV-2 spike. Cell Rep Med 2020;1:100126.
13. Abe KT, Li Z, Samson R, Samavarchi-Tehrani P, Valcourt EJ, et al. A simple protein-based surrogate neutralization assay for SARS-CoV-2. JCI Insight 2020;5.
14. Bewley KR, Coombes NS, Gagnon L, McInroy L, Baker N, et al. Quantification of SARS-CoV-2 neutralizing antibody by wild-type plaque reduction neutralization, microneutralization and plaque-typed virus neutralization assays. Nature Protocols 2021;1:1–33.