Original Research

Performance evaluation of four surrogate Virus Neutralization Tests (sVNTs) in comparison to the *in vivo* gold standard test

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Abstract

Background: Several commercial surrogate Virus Neutralization Tests (sVNTs) have been developed in the last year. Neutralizing anti-SARS-CoV-2 antibodies through interaction with Spike protein Receptor Binding Domain (S-RBD) can block the virus from entering and infecting host cells. However, there is a lack of information about the functional activity of SARS-CoV-2 antibodies that may be associated with protective responses. For these reasons, to counteract viral infection, the conventional virus neutralization test (VNT) is still considered the gold standard. The aim of this study was to contribute more and detailed information about sVNTs' performance, by determining *in vitro* the anti-SARS-CoV-2 neutralizing antibody concentration using four different commercial assays and then comparing the obtained data to VNT. **Methods**: Eighty-eight samples were tested using two chemiluminescence assays (Snibe and Mindray) and two ELISA assays (Euroimmun and Diesse). The antibody titers were subsequently detected and quantified by VNT. **Results**: The overall agreement between each sVNT and VNT was 95.45% for Euroimmun and 98.86% for Diesse, Mindray and Snibe. Additionally, we investigated whether the sVNTs were closer to the gold standard than traditional anti-SARS-CoV-2 antibody assays S-RBD or S1 based, finding a higher agreement mean value for sVNTs ($98.01 \pm 1.705\%$ vs $95.45 \pm 1.921\%$; $p < 0.05$). Furthermore, Spearman's statistical analysis for the correlation of sVNT versus VNT showed r = 0.666 for Mindray; r = 0.696 for Diesse; r = 0.779 for Mindray and r = 0.810 for Euroimmun. **Conclusions**: Our data revealed a good agreement between VNT and sVNTs. Despite the VNT still remains the gold standard, the sVNT might be a valuable tool for screening wider populations.

Keywords: surrogate Virus Neutralization Tests; live virus neutralization test; anti-SARS-CoV-2 antibodies; immunoassays

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and resulting COVID-19 disease have presented a relevant challenge in laboratory diagnostics [1,2]. During the current pandemic, several SARS-CoV-2 diagnostic platforms have been developed and tested for clinical use. Serological assays are essential in the detection of anti-SARS-CoV-2 antibodies and in monitoring convalescent and vaccine-induced immunity [3]. Mainly, they play a supplementary but indispensable role in diagnosing suspected cases with negative swab, in asymptomatic or past SARS-CoV-2 infection patients, for epidemiological assessment, assuring the contact-tracing of positive cases

and identifying new foci, and in evaluating vaccine efficacy and therapeutic antibody development [4–8].

During the last 18 months, different serologic tests detecting specific SARS-CoV-2 antibodies were developed, some of which give quantitative results; however, these tests may detect all binding antibodies to a specific target, but they do not provide correct information on the potency of functional antibodies that may be associated with protective responses. In general, when analysing the correlation between antibody titer and neutralizing capacity it is necessary to distinguish between humoral immunity induced by natural infection and humoral immunity induced by vaccination. In fact, only in the latter do the amount of anti-

bodies and the neutralizing capacity correlate very well [9–12]. Therefore, in parallel to the development of reliable quantitative serological tests, there is an urgent need for fast and sensible functional assays to improve the understanding of SARS-CoV-2 immune responses, their kinetics over time and the knowledge of antibody persistence [13]. It is unclear how long SARS-CoV-2 neutralizing antibodies last and, as a result, whether they can provide long-term protection [14]. Although, some studies have shown a correlation between binding and neutralizing antibodies, neutralizing antibody assays are still a gold standard to judge the immunity tone of a patient [15,16].

Notably, the Virus Neutralization Test (VNT), and more specifically the Plaque Reduction Neutralization Test (PRNT) [17], is considered the gold standard for serological detection measuring neutralizing antibodies. It detects functional antibodies and its results demonstrate blocking virus infection, thus representing a strong correlative indicator of protection from disease. As PRNT variants, some Neutralization Tests (NTs) were developed by observing the Focus Reduction (FRNT) or the CytoPathic Effect (CPE) [18]. The principles are basically identical to those of PRNT, and are based on the single microscopy observation of infected foci or CPE reduction, instead of counting plaques.

All these assays use SARS-CoV-2 replicating virus. VNTs are inconvenient in that they require BioSafety Level (BSL)-3 laboratories and often require intense and time consuming (3–7 days) work as well as not being readily amenable to automatization [19].

Alternatively, pseudotype-based neutralization assays represent an improvement towards VNT with the advantage of being able to work at a lower containment level (BSL-2), in which case infectivity can be measured by marker gene expression using ELISA plate readers. However, they still require 1–2 days, skilled operators, and are affected by variations between laboratories and different preparations of viral pseudotypes.

Many efforts have been made to develop other methods to test sera for the presence of neutralizing antibodies that are more practical, simple, and at the same time, reliable.

These are commercial assays that detect SARS-CoV-2-specific antibodies directed against the Spike Receptor Binding Domain (S-RBD) protein and are capable of detecting antibodies showing neutralizing activity [20,21]. This year, several surrogate Virus Neutralization Tests (sVNTs) have appeared on the market that, based on the manufacturers' description, claim excellent correlation with the neutralizing titer.

Neutralizing antibodies, in most cases act by interacting with the binding between the S-RBD protein on the viral surface and its receptor, the angiotensin-2 converting enzyme (ACE-2) receptor, expressed on the target cell. Neutralizing antibodies can block virus entry and infection in the host cell through interaction with S-RBD [22,23]. This mechanism of virus entry inhibition into the target cell reduces viral replication and consequently viral load. Indeed, the appearance of neutralizing antibodies correlates with host immunity and protects, at least temporarily, against future reinfection [24,25]. Recent studies have shown that the level of neutralizing antibodies can vary markedly between patients with severe and mild-to-moderate symptoms [26,27].

However, there are no current data available regarding the titer of neutralizing antibodies providing patients protection from reinfection or from disease. Whilst the mechanism of protection certainly involves cellular immunity, there are supporting data which suggest neutralizing antibodies may be a correlate of protection [24,27,28].

To increase comparability of the results of serologic tests detecting neutralizing antibodies to support the identification of a possible protective correlate, it is crucial that all tests are calibrated and developed using a well-characterized and globally traceable reference standard. In this regard, an International Standard (IS) containing a known amount of anti-SARS-CoV-2 immunoglobulins with well-characterized neutralizing activity was made available by the WHO in December 2020 with the aim of allowing the accurate calibration of assays to the International Unit (IU) for neutralising antibodies or Binding Antibody Units (BAU) for binding antibodies, thereby reducing interlaboratory variation and creating a common language for reporting data [29]. In light of these premises, comparative performance data and proper validation are essential to guide the appropriate use of serology in COVID-19 diagnostics [13].

Our principal aim was to compare four different assay systems to determine *in vitro* SARS-CoV-2 neutralizing antibody concentrations and correlating the antibody levels with the gold standard VNT. Finally, we investigated whether the sVNTs performances were closer to the gold standard, comparing them with the traditional anti-SARS-CoV-2 S-RBD or S1 based antibody assays.

2. Materials and methods

Eighty-eight consecutive serum samples (F:M ratio 1.4:1, mean age = 57.0 ± 14.0 years) screened for anti-S protein antibodies at the Clinical Chemistry Laboratory of "Tor Vergata" University Hospital (Rome, Italy), were tested using four different commercial sVNTs for anti-SARS-CoV-2 IgG/IgA/IgM neutralizing antibodies and with the current gold standard live VNT. Our study cohort reasonably consisted of a mixed population (vaccinated subjects, recovered patients from COVID-19, and healthy individuals) and no longitudinal samples were included. Clinical data were obtained by retrospective medical records reviewed only for patients with discordant results between VNT and sVNTs assays. Table 1 illustrates the main features of each serological assay related to its

Table 1. Characteristics of sVNTs.

Manufacturer	Kit assay	Method	Cut-off	Conversion factor	Dynamic range	Assay principle
Mindray	*SARS-CoV-2 Neutralizing Antibodies*	CLIA	10 AU/mL	1 AU = 3.6547 IU/mL	2 AU/mL to 400 AU/mL	Neutralizing antibody in the sample competes with ACE2-ALP conjugate for binding sites of SARS-CoV-2 S-RBD immobilized in the para-magnetic microparticles
Snibe	*Maglumi SARS-CoV-2 Neutralizing Antibody*	CLIA	0.05 µg/mL	1 µg/mL = 405 IU/mL	0.050–30 µg/mL	Neutralizing antibody in the sample competes with recombinant SARS-CoV-2 S-RBD antigen labeled with ABEI for binding ACE2 antigen immobilized on magnetic microbeads
Euroimmun	*SARS-CoV-2 NeutraLISA*	ELISA	% IH <20: negative ≥20% IH <35: borderline % IH ≥35: positive	n.a.	% IH 0–100	Neutralizing antibody in the sample competes with the receptor biotinylated ACE2 (sample buffer) for the binding sites of the SARS-CoV-2 S1/RBD proteins immobilized on the plate
Diesse	*Chorus SARS-CoV-2 "Neutralizing" Ab*	ELISA	<20 BAU/mL	n.a.	20–1500 BAU/mL	Neutralizing antibody in the sample competes with the peroxidase-conjugated SARS-CoV-2 anti-S1 therapeutic monoclonal antibodies to bind S1 subunit fixed on the solid phase support

ABEI, N-(aminobutyl)-N-(ethylisoluminol); ALP, alkaline phosphatase; CLIA, chemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; IH, percentage of inhibition; n.a., not available.

method, declared cut-off, conversion factor, dynamic range and assay principle, according to the manufacturer's instructions. The linear dynamic ranges have been extended, conforming to the manufacturers' instructions. Additionally, the sera were also tested using eight different commercial kits for anti-SARS-CoV-2 IgG or total antibodies (tAb) detection, S-RBD or S1 based (**Supplementary File S1**).

2.1 Maglumi SARS-CoV-2 Neutralizing Antibody

The "Maglumi SARS-CoV-2 Neutralizing antibody" assay (Snibe Diagnostic, Shenzhen, China) for the detection of anti-SARS-CoV-2 IgG/IgA/IgM neutralizing antibodies was performed on the fully automated Maglumi analytical system (Snibe Diagnostic, Shenzhen, China). This assay is based on the competition between neutralizing antibodies and S-RBD antigens for the binding to ACE2. The linearity range is 0.050–30 μg/mL, cut-off value is 0.05 μg/mL. As declared by the manufacturer, the conversion factor to transform μg/mL in IU/mL is 405 and analytical sensitivity and specificity are both 100%.

2.2 SARS-CoV-2 Neutralizing Antibody

The "SARS-CoV-2 Neutralizing Antibody" chemiluminescent assay (Mindray Medical, Shenzhen, China) for the detection of anti-SARS-CoV-2 neutralizing antibodies was performed on the fully automated Mindray CL-1200i analytical system (Mindray Medical, Shenzhen, China). Neutralizing antibody in the sample competes with ACE2-alkaline phosphatase conjugate for binding sites of SARS-CoV-2 antigens. The linearity range is 2 AU/mL to 400 AU/mL, cut-off value is 10 AU/mL. As declared by the manufacturer, the conversion factor to transform AU/mL in IU/mL is 0.2734; sensitivity and specificity are 95.7% and 99.9%, respectively.

2.3 SARS-CoV-2 NeutraLISA

The "SARS-CoV-2 NeutraLISA" enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Lübeck, Germany) provides semiquantitative *in vitro* determination of neutralizing anti-SARS-CoV-2 antibodies. The assay is based on the competition between neutralizing antibodies and S-RBD antigens for the binding to ACE2. Briefly, during the first incubation, samples and controls are diluted with a sample buffer containing biotinylated ACE2. If neutralizing antibodies are present in the sample, they compete with the ACE2 receptor for the binding sites of the SARS-CoV-2 S1/RBD proteins. A second incubation step is performed to detect the bound ACE2 by the addition of peroxidase-labelled streptavidin, which catalyses a colour reaction. The intensity of the products absorbance is inversely proportional to the concentration of neutralizing antibodies in the sample.

The percentage of inhibition (% IH) is calculated with the following formula: % IH = 100% – (Extinction of patient sample $\times$ 100%/Extinction of blank).

Results below 20% IH are considered negative; $\geq$20% IH to <35% IH as borderline and $\geq$35% IH as positive. Sensitivity and specificity are 95.9% and 99.7%, respectively, as declared by the manufacturer.

2.4 Chorus SARS-CoV-2 "Neutralizing" Ab

The "Chorus SARS-CoV-2 "Neutralizing" Ab" (Diesse Diagnostica Senese, Siena, Italy) for the quantitative determination of total anti-S1 SARS-CoV-2 neutralizing antibodies was performed on the automated Chorus TRIO instrument (Diesse Diagnostica Senese, Siena, Italy).

The detection method is based on the principle of competitive testing using SARS-CoV-2 anti-S1 therapeutic monoclonal antibodies.

The SARS-CoV-2 anti-S1 antibodies present in the sample compete with a peroxidase-conjugated anti-S1 monoclonal antibody to bind the Spike protein S1 subunit Recombinant Binding Domain (RBD) site fixed on the solid phase support and available in limited numbers. The higher the concentration of antibodies present in the patient's serum, the lower the possibility of binding peroxidase-conjugated SARS-CoV-2 anti-S1 monoclonal antibody to the fixed antigen.

The results are expressed in BAU/mL, as declared by the manufacturer.

The samples are considered positive for values >50 BAU/mL; negative for values <20 BAU/mL and equivocal for all the values between 20–50 BAU/mL. The linearity range is 20–1500 BAU/mL. Samples >1500 BAU/mL can be diluted as declared by the manufacturer.

2.5 Live virus neutralization test

The live virus neutralization test (VNT) is a specialized type of immunoassay to detect antibodies able to inhibit virus replication *in vitro*. The live VNT was performed to establish the lower serum cut-off value to protect against SARS-CoV-2 infection.

The neutralizing antibodies titer was determined using the following 4 step-protocol: epithelial cell line VERO E6 culture, viral growth in cell culture and SARS-CoV-2 virus titration and micro-neutralization assay with subsequent cytopathic effect (CPE)-read out.

2.5.1 Cell culture

Epithelial cell line VERO E6, derived from the kidney of African green monkey *Cercopithecus aethiops*, was acquired from the American Type Culture Collection (ATCC-CRL 1586). Adherent sub-confluent VERO E6 cells monolayers were prepared in DMEM medium containing 10% FBS.

2.5.2 Viral growth in cell culture and SARS-CoV-2 virus titration

SARS-CoV-2 virus 2019-nCov/Italy-INMI1-strain (GISAID accession ID: EPI_ISL_412974) was purchased from Lazzaro Spallanzani Institute (Rome, Italy) via the European Virus Archive Global (EVAg).

The virus was propagated on VERO E6 using DMEM, supplemented with 2% FBS, cells were daily observed, and the virus was harvested until an 80–90% CPE was observed. This viral preparation was titrated in serial 1 Log dilutions to obtain a 50% tissue culture infective dose (TCID50) on 96-well culture plates of VERO E6 cells. The endpoint titer was calculated according to the Reed & Muench method based on eight replicates for each titration.

2.5.3 Micro-neutralization assay and CPE-read out

Sera were heat-inactivated for 30 minutes at 56 °C and two-fold serial dilutions, from 1:10 to 1:1280 were performed in duplicate in DMEM on 96-well culture plates. Then sera dilutions were mixed with an equal volume of viral solution containing 100 TCID50 of SARS-CoV-2 virus each well. The serum-virus mixture was incubated for 1 hour at 37 °C in a humidified atmosphere with 5% CO_2. After incubation, 100 μL of each dilution mixture was added in duplicate to a 24-hour hold cell plate containing a semi-confluent VERO E6 monolayer. The plates were then incubated for 3 days at 37 °C in a humidified atmosphere with 5% CO_2 and analyzed with an inverted optical microscope. The highest serum dilution able to protect more than 90% of the cells from CPE was considered the neutralization titer.

2.6 Statistical analysis

Results were expressed as the mean value with Standard Deviation (SD) when data were normally distributed. Otherwise, results were expressed as the median value with range. The Student t-test and the correlation coefficient (in the case of normally distributed data), the Wilcoxon's matched pairs t-test and the Spearman's coefficient of rank correlation (both in the case of non-normally distributed data) were used when required. p values less than 0.05 were considered statistically significant. Statistical analysis was performed using MedCalc Statistical Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014).

3. Results

In this study, all samples have been tested with four different commercial sVNTs for anti-SARS-CoV-2 neutralizing antibodies detection and compared with the VNT titer.

In addition, a qualitative test was used to evaluate the overall agreement rate between each sVNT and VNT. As shown in Table 2, a high agreement rate was demonstrated for all tests, ranging from 95.45% (Euroimmun) to 98.86% (Diesse, Mindray and Snibe). For each sVNT overall agreement, it has been associated a 2 × 2 contin-

gency table (**Supplementary File S2**) presenting the positive/negative number of samples for each serological assay compared to the True Positive (TP) and True Negative (TN) resulted by the gold standard (VNT). Furthermore, we assessed the overall agreement between eight traditional anti-SARS-CoV-2 antibody assays (S-RBD or S1 based) and VNT titers (Table 3). To this purpose, the VNT-agreement rates between anti-SARS-CoV-2 antibody assays and sVNTs (Fig. 1) were compared. The scatter plot graph shows that the sVNT mean value (98.01 $\pm$ 1.705%) was significantly higher than traditional anti-SARS-CoV-2 antibody assays (95.45 $\pm$ 1.921%) with a $p < 0.05$ (independent T test).
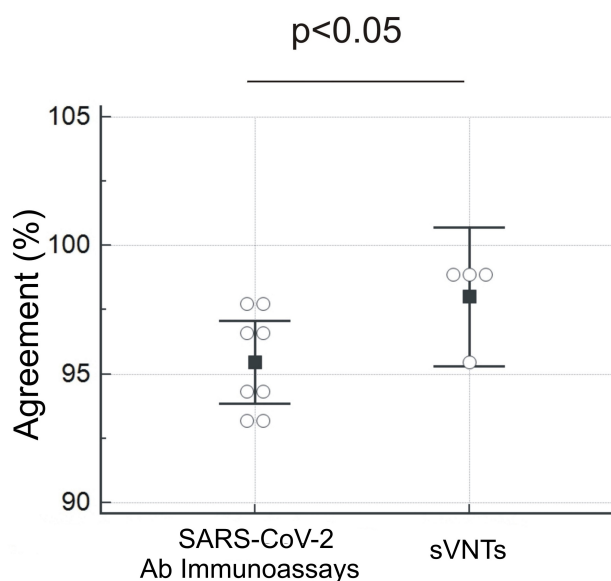

Fig. 1. Overall agreement of eight traditional anti-SARS-CoV-2 antibody assays (S-RBD or S1 based) vs sVNTs. The sVNTs mean value (98.01 $\pm$ 1.705%) was significantly higher than anti-SARS-CoV-2 antibody assays (95.45 $\pm$ 1.921%) with a $p < 0.05$ (Independent T test).

Table 2. Overall agreement (%) between sVNTs and VNT.

sVNT	%
Diesse sVNT	98.86
Mindray sVNT	98.86
Snibe sVNT	98.86
Euroimmun sVNT	95.45

In Table 4, the discordant results for each sVNT and VNT assay were compared to the patients' history: Euroimmun displayed four discordant cases; Diesse, Mindray and Snibe only one case. The discordances were possible false positive sVNT results except one possible false negative VNT case.

Table 3. Overall agreement (%) between anti-SARS-CoV-2 antibody assays and VNT.

Anti-SARS-CoV-2	%
Mindray S RBD IgG	94.32
Roche S RBD tAb	97.73
Snibe S RBD IgG	96.59
DiaSorin Trimeric S IgG	94.32
Thermo Fisher S1 IgG	93.18
Beckman S1 IgG	94.32
Euroimmun S1 IgG	96.59
Vircell VIRCLIA S1 IgG	93.18

All quantified sVNTs results were graphically correlated to VNT in Fig. 2. The dot plot graph illustrates on the vertical axis the sVNT antibody values and on the horizontal axis the neutralization titers, expressed as the reciprocal of the serum dilution, showing an increase in VNT titers correlated to the antibody concentrations. Spearman's statistical analysis was used in order to find the correlation of neutralizing antibodies levels versus VNT. The highest coefficient (r = 0.810) was obtained with Euroimmun sVNT (%) (Fig. 2D) and the lowest (r = 0.666) with Mindray sVNT (IU/mL) (Fig. 2B). Diesse sVNT (BAU/mL) achieved an r = 0.696 (Fig. 2A), while Maglumi sVNT (IU/mL) achieved an r = 0.779 (Fig. 2C). The statistical significance level estimated was $p < 0.001$ in all data. Finally we calculated the median value for all commercial assays at 1/160 VNT titer. Mindray, Maglumi and Diesse had similar median values and not significantly different; 715.6 IU/mL, 726.6 IU/mL and 967.0 IU/mL respectively. Euroimmun had a median value of 90.53%.

4. Discussion

After the first year of the COVID-19 outbreak, the role of serology shifted its focus from the acute diagnostic strategy of seroprevalence and protective herd immunity to (i) controlling the pandemic, to (ii) the critical measurements of individuals' neutralizing antibody levels for vaccine clinical trials, and finally (iii) research studies and disease prevention.

Traditionally SARS-CoV-2 antibody assays detect the isotypes of different antibodies and use different antigenic proteins. However, since competition with ACE2 may reveal the presence of potential neutralizing antibodies for RBD binding, recently various assays mainly based on the ACE2-RBD competition have spread widely and are known as sVNT.

Despite WHO efforts, only some of the commercial kits available today use the IU suggested for the neutralization assays. In fact, in our study only two out of the four sVNTs tested express their results as IU (Mindray and Maglumi), one as a percentage of HI (Euroimmun) and one as BAU/mL (Diesse). Also, VNT uses NTs instead of IU. Conversely, the harmonization process for anti-SARS-CoV-2 antibody assays has been achieved since most commercial assays use BAU/mL. Since the minimal neutralizing antibody threshold of protection from infection is still unknown, as is how long this protection might last after natural infection or vaccination, there is a great need for assays profiling patient responses to infection, and to establish acquired immunity against SARS-CoV-2 [30–32].

The sVNTs, given their high throughput nature and no need for biosafety level 3 containment, make them accessible to the wider community, enabling greater availability for effective mass testing.

Our data confirm its potential routine use as a screening test of neutralizing antibodies in a diagnostic laboratory scenario, given the high percentage of agreement with the gold standard test (95.45%–98.86%) [10]. In addition, agreement between sVNT values and VNT was higher than SARS-CoV-2 immunoassays agreement (98.01% vs 95.02%). Regarding the association between NTs and quantitative results, we observed a higher but not significant Spearman coefficient (sVNTs: r = 0.738 vs. SARS-CoV-2 immunoassays: r = 0.711).

However, the moderate correlation observed suggests that VNT cannot be replaced by the two examined assays groups, with the purpose of assessing functional antibody activity. Interestingly, the Euroimmun sVNT is the test with the highest number of qualitative discrepancies but with the strongest quantitative correlation, meaning the different aspects of the test. Our results are partially in agreement with a recent study [10] which showed an average correlation of 0.473, assessed by Spearman's test, between VNT and three different SARS-CoV-2 immunoassays. In detail, the correlation assessed by Matusali *et al*. [10] was lower compared to our results (0.473 vs. 0.724), possibly due to the different population studied (COVID-19 patients' sera vs routine sera) and to the assays examined. In fact, the tests used in our study were all anti-SARS-CoV-2 antibody assays S-RBD or S1 based, excluding nucleocapsid based assays with a lower neutralizing capacity activity [10]. Despite great efforts in recent months to develop reliable assays, the VNT still remains the gold standard, given its intrinsic characteristics. Indeed, the sVNTs may reveal only a portion of neutralizing antibodies and not measure the total neutralizing activity directed against epitopes outside the RBD, such as the N-terminal domain of the S protein [33–36].

Our correlation data between sVNT vs VNT show significant Spearman coefficients, with a statistical significance level $p < 0.001$ in all data, to confirm that high antibody concentrations correspond to high *in vitro* titer values, showing the applicable correlation for use in practice. From our data, it was shown that the best sVNT was Euroimmun assays, nevertheless, the new family of serological "ACE2-RBD competitive assays" are produced for fast and large-scale rapid testing, where a medium-good agreement is suf-

Table 4. Discordances between VNT and sVNTs.

VNT (titer)	sVNT	Patient's history	Comment
		Euroimmun sVNT	
1/160	Negative	HCW, history of COVID-19 in 2020, unvaccinated	false negative?
1/40	Negative	HCW, history of COVID-19 in 2020, unvaccinated	false negative?
1/40	Negative	Patient in treatment with Certolizumab for reumatoid arthritis, never had COVID19, unvaccinated, negative swab	asymptomatic COVID-19?
1/20	Negative	HCW, history of COVID-19 in 2020, unvaccinated	false negative?
		Diesse sVNT	
1/40	Negative	HCW, history of COVID-19 in 2020, unvaccinated	false negative?
		Mindray sVNT	
<1/10	Positive	HCW, history of COVID-19 in 2020, 1 vaccine dose given 15 days prior to the present blood test	true positive? higher sensitivity of the test compared to the VNT?
		Snibe sVNT	
1/160	Negative	HCW, history of COVID-19 in 2020, two months since 2nd dose of the vaccine to the present blood test	false negative?

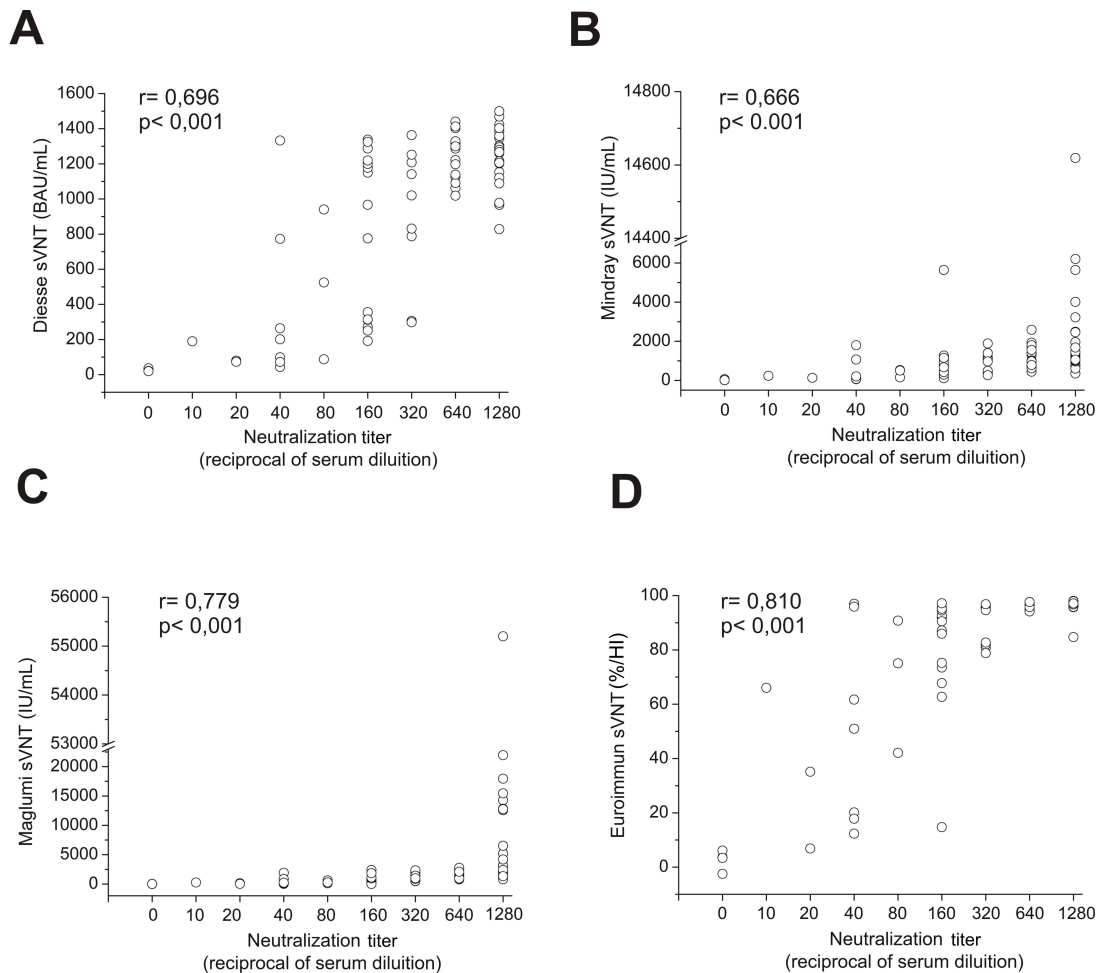**Fig. 2. Four graphs of correlation between sVNTs and VNT: (A) Diesse sVNT (BAU/mL) with r = 0.696. (B) Mindray sVNT (IU/mL) with r = 0.666. (C) Maglumi sVNT (IU/mL) with r = 0.779. (D) Euroimmun sVNT (% IH) with r = 0.819.** Spearman's test was used for correlation analysis.

ficient. Completely, they might help to provide a screening program to reduce the high number of samples undergoing VNT and thus, might be used as monitoring tools for a proper administration of vaccination or for an individual booster dose timing schedule.

Most evaluation studies have mainly compared serological assays with VNT results [37–43]. but, to the best of our knowledge, there are no studies that have considered the two different assay families separately, anti-SARS-CoV-2 antibody assays (S-RBD or S1 based) and sVNTs, compared with each other and against the gold standard.

The main limitation of the study is the small number of negative patients included due to the fact that we studied a routine population during the pandemic and the vaccination campaign, consequently scarce of negative samples. Therefore the bias resulting by a higher number of negative samples might alter the overall agreement. A second limitation of this study was that we did not use the Genscript (cPass) [44] as the sVNT reference method. Indeed, cPass is widely accepted as a reference in the literature and it is fully approved by the FDA. Unfortunately, at this stage it was not possible to perform further comparisons.

Finally, we calculated the median value for all commercial assays at 1/160 VNT titer. Mindray, Maglumi and Diesse had similar median values while Euroimmun could not be compared because it has a percentage unit of measurement. However, all tests show that as the concentration of serum antibodies increases, so does the VNT titer.

However, the strength of our work is that it is a so-called "real-life" research study typically designed to better reflect aspects of routine care.

Starting from these premises, our comparison study aims to contribute to the refinement of anti-SARS-CoV-2 antibody testing strategies for public health use.

Further studies on larger sample numbers are required to better understand the strengths and limitations of commercially available serological assays and for the flow chart optimization of COVID-19 serological tests, including both families of tests at different levels.

5. Conclusions

Serological studies are an aid to questions about the duration of immune protection and vaccine effectiveness. Although VNT still remains the gold standard, given the good agreement between VNT and sVNT, sVNT has proved to be a potential valuable tool for screening larger populations, due to shorter response times, lower costs, and good performance. In the next future sVNT could be used to personalize decision on vaccination shedule or disease therapy.

Author contributions

These should be presented as follows: MP, MI, MM, MN designed the research study. MI, MP, MN, VG, FT, SS performed the research. CC, AA, MM, PC, MB and SB provided critical revision of the manuscript and valuable comments. MI, MP, MN, LS, ER, FT, SS, BL analyzed the data. MI, MP, MM, MN, ER wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Conflict of interest

The authors declare that they have no conflict of interest. AA, ER are serving as the Editorial Board members of this journal. We declare that AA and ER had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to AP.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/2/10.31083/j.fbl2702074.

References

[1] Infantino M, Damiani A, Gobbi FL, Grossi V, Lari B, Macchia D, et al. Serological Assays for SARS-CoV-2 Infectious Disease: Benefits, Limitations and Perspectives. The Israel Medical Association Journal. 2020; 22: 203–210.

[2] Nuccetelli M, Pieri M, Gisone F, Sarubbi S, Ciotti M, Andreoni M, et al. Evaluation of a new simultaneous anti-SARS-CoV-2 IgA, IgM and IgG screening automated assay based on native inactivated virus. International Immunopharmacology. 2021; 92: 107330.

[3] Werner M, Pervan P, Gluck V, Zeman F, Koller M, Burkhardt R, et al. Evaluation of a Broad Panel of SARS-CoV-2 Serological Tests for Diagnostic Use. Journal of Clinical Medicine. 2021; 10: 1580.

[4] Pieri M, Nuccetelli M, Nicolai E, Sarubbi S, Grelli S, Bernardini S. Clinical validation of a second generation anti-SARS-CoV-2

IgG and IgM automated chemiluminescent immunoassay. Journal of Medical Virology. 2021; 93: 2523–2528.

[5] Theel ES, Slev P, Wheeler S, Couturier MR, Wong SJ, Kadkhoda K. The Role of Antibody Testing for SARS-CoV-2: is there one? Journal of Clinical Microbiology. 2020; 58: e00797-20.

[6] Pollán M, Pérez-Gómez B, Pastor-Barriuso R, Oteo J, Pérez-Olmeda M, Yotti R. SARS-CoV-2 seroprevalence in Spain - Authors' reply. The Lancet. 2020; 396: 1484–1485.

[7] Havers FP, Reed C, Lim T, Montgomery JM, Klena JD, Hall AJ, et al. Seroprevalence of Antibodies to SARS-CoV-2 in 10 Sites in the United States, March 23–May 12, 2020. JAMA Internal Medicine. 2020; 180: 1576.

[8] Martin C, Montesinos I, Dauby N, Gilles C, Dahma H, Van Den Wijngaert S, et al. Dynamics of SARS-CoV-2 RT-PCR positivity and seroprevalence among high-risk healthcare workers and hospital staff. Journal of Hospital Infection. 2020; 106: 102–106.

[9] Meschi S, Matusali G, Colavita F, Lapa D, Bordi L, Puro V, et al. Predicting the protective humoral response to a SARS-CoV-2 mRNA vaccine. Clinical Chemistry and Laboratory Medicine. 2021; 59: 2010–2018.

[10] Matusali G, Colavita F, Lapa D, Meschi S, Bordi L, Piselli P, et al. SARS-CoV-2 Serum Neutralization Assay: A Traditional Tool for a Brand-New Virus. Viruses. 2021; 13: 655.

[11] Carrillo J, Izquierdo-Useros N, Ávila-Nieto C, Pradenas E, Clotet B, Blanco J. Humoral immune responses and neutralizing antibodies against SARS-CoV-2; implications in pathogenesis and protective immunity. Biochemical and Biophysical Research Communications. 2021; 538: 187–191.

[12] Zheng J, Deng Y, Zhao Z, Mao B, Lu M, Lin Y, et al. Characterization of SARS-CoV-2-specific humoral immunity and its potential applications and therapeutic prospects. Cellular & Molecular Immunology. 2021; 1–8.

[13] Bubonja-Šonje M, Batičić L, Abram M, Cekinović Grbeša Đ. Diagnostic accuracy of three SARS-CoV2 antibody detection assays, neutralizing effect and longevity of serum antibodies. Journal of Virological Methods. 2021; 293: 114173.

[14] Kellam P, Barclay W. The dynamics of humoral immune responses following SARS-CoV-2 infection and the potential for reinfection. Journal of General Virology. 2020; 101: 791–797.

[15] Grzelak L, Temmam S, Planchais C, Demeret C, Tondeur L, Huon C, et al. A comparison of four serological assays for detecting anti-SARS-CoV-2 antibodies in human serum samples from different populations. Science Translational Medicine. 2020; 12: eabc3103.

[16] Peterhoff D, Glück V, Vogel M, Schuster P, Schütz A, Neubert P, et al. A highly specific and sensitive serological assay detects SARS-CoV-2 antibody levels in COVID-19 patients that correlate with neutralization. Infection. 2021; 49: 75–82.

[17] Bewley KR, Coombes NS, Gagnon L, McInroy L, Baker N, Shaik I, et al. Quantification of SARS-CoV-2 neutralizing antibody by wild-type plaque reduction neutralization, microneutralization and pseudotyped virus neutralization assays. Nature Protocols. 2021; 16: 3114–3140.

[18] Caccuri F, Zani A, Messali S, Giovanetti M, Bugatti A, Campisi G, et al. A persistently replicating SARS-CoV-2 variant derived from an asymptomatic individual. Journal of Translational Medicine. 2020; 18: 362.

[19] Walker GJ, Naing Z, Ospina Stella A, Yeang M, Caguicla J, Ramachandran V, et al. SARS Coronavirus-2 Microneutralisation and Commercial Serological Assays Correlated Closely for Some but Not All Enzyme Immunoassays. Viruses. 2021; 13: 247.

[20] Meyer B, Reimerink J, Torriani G, Brouwer F, Godeke G, Yerly S, et al. Validation and clinical evaluation of a SARS-CoV-2 surrogate virus neutralisation test (sVNT). Emerging Microbes &

Infections. 2020; 9: 2394–2403.

[21] Tan CW, Chia WN, Qin X, Liu P, Chen MI-, Tiu C, et al. A SARS-CoV-2 surrogate virus neutralization test based on antibody-mediated blockage of ACE2-spike protein-protein interaction. Nature Biotechnology. 2020; 38: 1073–1078.

[22] Jeyanathan M, Afkhami S, Smaill F, Miller MS, Lichty BD, Xing Z. Immunological considerations for COVID-19 vaccine strategies. Nature Reviews Immunology. 2020; 20: 615–632.

[23] Ju B, Zhang Q, Ge J, Wang R, Sun J, Ge X, et al. Human neutralizing antibodies elicited by SARS-CoV-2 infection. Nature. 2020; 584: 115–119.

[24] Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nature Medicine. 2021; 27: 1205–1211.

[25] Oguntuyo KY, Stevens CS, Hung CT, Ikegame S, Acklin JA, Kowdle SS, et al. Quantifying absolute neutralization titers against SARS-CoV-2 by a standardized virus neutralization assay allows for cross-cohort comparisons of COVID-19 sera. mBio. 2021; 12: e0249–20.

[26] Tomassetti F, Nuccetelli M, Sarubbi S, Gisone F, Ciotti M, Spinazzola F, et al. Evaluation of S-RBD and high specificity ACE-2-binding antibodies on SARS-CoV-2 patients after six months from infection. International Immunopharmacology. 2021; 99: 108013.

[27] Zost SJ, Gilchuk P, Case JB, Binshtein E, Chen RE, Nkolola JP, et al. Potently neutralizing and protective human antibodies against SARS-CoV-2. Nature. 2020; 584: 443–449.

[28] Feng S, Phillips DJ, White T, Sayal H, Aley PK, Bibi S, et al. Correlates of protection against symptomatic and asymptomatic SARS-CoV-2 infection. Nature Medicine. 2021; 27: 2032–2040.

[29] Kristiansen PA, Page M, Bernasconi V, Mattiuzzo G, Dull P, Makar K, et al. Who International Standard for anti-SARS-CoV-2 immunoglobulin. The Lancet. 2021; 397: 1347–1348.

[30] Mulligan MJ, Lyke KE, Kitchin N, Absalon J, Gurtman A, Lockhart S, et al. Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults. Nature. 2020; 586: 589–593.

[31] Sahin U, Muik A, Derhovanessian E, Vogler I, Kranz LM, Vormehr M, et al. COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses. Nature. 2020; 586: 594–599.

[32] Walsh EE, Frenck RW, Falsey AR, Kitchin N, Absalon J, Gurtman A, et al. Safety and Immunogenicity of Two RNA-Based Covid-19 Vaccine Candidates. New England Journal of Medicine. 2020; 383: 2439–2450.

[33] Chi X, Yan R, Zhang J, Zhang G, Zhang Y, Hao M, et al. A neutralizing human antibody binds to the N-terminal domain of the Spike protein of SARS-CoV-2. Science. 2020; 369: 650–655.

[34] McCallum M, De Marco A, Lempp FA, Tortorici MA, Pinto D, Walls AC, et al. N-terminal domain antigenic mapping reveals a site of vulnerability for SARS-CoV-2. Cell. 2021; 184: 2332–2347.e16.

[35] Suryadevara N, Shrihari S, Gilchuk P, VanBlargan LA, Binshtein E, Zost SJ, et al. Neutralizing and protective human monoclonal antibodies recognizing the N-terminal domain of the SARS-CoV-2 spike protein. Cell. 2021; 184: 2316–2331.e15.

[36] Yoshida S, Ono C, Hayashi H, Fukumoto S, Shiraishi S, Tomono K, et al. SARS-CoV-2-induced humoral immunity through B cell epitope analysis in COVID-19 infected individuals. Scientific Reports. 2021; 11: 5934.

[37] Krüttgen A, Lauen M, Klingel H, Imöhl M, Kleines M. Two novel SARS-CoV-2 surrogate virus neutralization assays are suitable for assessing successful immunization with mRNA-1273. Journal of Virological Methods. 2022; 299: 114297.

[38] Sholukh AM, Fiore-Gartland A, Ford ES, Miner MD, Hou YJ,

Tse LV, *et al*. Evaluation of Cell-Based and Surrogate SARS-CoV-2 Neutralization Assays. Journal of Clinical Microbiology. 2021; 59: e0052721.

[39] Müller K, Girl P, von Buttlar H, Dobler G, Wölfel R. Comparison of two commercial surrogate ELISAs to detect a neutralising antibody response to SARS-CoV-2. Journal of Virological Methods. 2021; 292: 114122.

[40] Mahmoud SA, Ganesan S, Naik S, Bissar S, Zamel IA, Warren K, *et al*. Serological Assays for Assessing Postvaccination SARS-CoV-2 Antibody Response. Microbiology Spectrum. 2021; 9: e0073321.

[41] Olbrich L, Castelletti N, Schälte Y, Garí M, Pütz P, Bakuli A, *et al*. Head-to-head evaluation of seven different seroassays including direct viral neutralisation in a representative cohort for SARS-CoV-2. Journal of General Virology. 2021; 102: 001653.

[42] Lamikanra A, Nguyen D, Simmonds P, Williams S, Bentley EM, Rowe C, *et al*. Comparability of six different immunoassays measuring SARS-CoV-2 antibodies with neutralizing antibody levels in convalescent plasma: From utility to prediction. Transfusion. 2021; 61: 2837–2843.

[43] Villalta D, Moratto A, Salgarolo V, Re MD, Giacomello R, Malipiero G. New-Generation Quantitative Immunoassays for SARS-CoV-2 Antibody Detection: need for Harmonization. Annals of Laboratory Medicine. 2022; 42: 113–116.

[44] Jung J, Rajapakshe D, Julien C, Devaraj S. Analytical and clinical performance of cPass neutralizing antibodies assay. Clinical Biochemistry. 2021; 98: 70–73.