Comparison of two commercial surrogate ELISAs to detect a neutralizing antibody response to SARS-CoV-2

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Abstract

Introduction: Reliable methods for the detection of SARS-CoV-2 neutralizing antibodies (NAbs) are essential for the evaluation of vaccine candidates and for the selection of convalescent plasma donors. Virus neutralization tests (NTs) are the gold standard for the detection and quantification of NAbs, but they are complex and require BSL3 facilities. In contrast, surrogate enzyme-linked immunosorbent assays (sELISA) offer the possibility of high-throughput testing under standard laboratory safety conditions. In this study, we investigated two commercial sELISA kits (GenScript, AdipoGen) designed for the detection of SARS-CoV-2 NAbs.

Methods: 276 plasma samples were screened using commercial IgG-ELISA and NAbs titers were determined by micro-neutralization test (micro-NT). In addition, all samples were tested in both sELISA. Sensitivity and specificity for both sELISA were determined in comparison to the micro-NT results.

Results: 57% of the samples were positive for SARS-CoV-2 NAbs in micro-NT, while 43% tested negative. Comparison with micro-NT results showed a sensitivity of 98.2% and a specificity of 69.5% for the GenScript ELISA. The AdipoGen ELISA had a sensitivity of 83.5% and a specificity of 97.8%. False negative results were obtained mainly on samples with low NAbs titers.

Conclusion: Both sELISA were able to qualitatively detect NAbs in plasma samples. Sensitivity and specificity differed between sELISA with GenScript superior in sensitivity and AdipoGen

superior in specificity. Both sELISA were unable to quantify NAbs, thus neither of them can completely replace conventional NTs. However, in a two-step diagnostic algorithm, AdipoGen could potentially replace NT as a subsequent confirmatory test due to its high specificity.

Keywords: COVID-19, SARS-CoV-2, antibodies, neutralizing, ELISA

Conflict of interest: The authors declare that there is no conflict of interest.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) first appeared in China at the end of 2019 and was subsequently identified as the causative agent of a new respiratory disease later known as coronavirus disease 2019 (COVID-19). Symptoms can range from mild and flu-like symptoms to severe and fatal lung disease [1-3]. Despite the immediate introduction of infection control measures, SARS-CoV-2 spread worldwide and soon became not only an urgent medical challenge but also a serious socioeconomic burden [4]. Government interventions to slow down the spread of the virus were quickly implemented and disrupted the daily lives of billions of people. Almost nine months after the start of the SARS-CoV-2 pandemic, the scientific community and policy-makers around the world have shifted their focus from diagnosing acute COVID-19 infections to serology and how it can be used to ease the constraints of daily life [5]. Antibody detection tests such as enzyme-linked immunosorbent assays (ELISAs) are widely used to estimate the prevalence and incidence of SARS-CoV-2 and dozens of companies now offer a variety of such immunoassays [6]. They can also help to determine case fatality rates more accurately and facilitate the search for natural reservoirs and intermediate hosts [7]. However, they lack the ability to verify neutralization, which is why they cannot distinguish between non-neutralizing antibodies (Abs) and NAbs. However, since virus neutralization plays a key role in the development of a (long-term) protective immune response, the ability to detect NAbs in patient samples is crucial [8]. Therefore, serological tests to detect NAbs against SARS-CoV-2 are an important aid in determining herd immunity and humoral protection, assessing vaccine efficacy during long-awaited clinical trials, and selecting convalescent plasma for intensive care treatment.

The current gold standard for detection and also quantification of functional NAbs in blood samples are virus neutralization tests. Several variants of the neutralisation test have been developed in the past: In the Plaque Reduction Neutralisation Test (PRNT), the virus plaques are

counted and compared with the initial concentration of the virus to determine the percentage reduction in total viral infectivity. In this way, PRNT endpoint titers can be calculated for each serum sample at each selected percentage reduction of viral activity (typically 50% or 90%). A disadvantage of PRNT is that it is labour-intensive and not easily adaptable for high throughput, which makes it difficult to use for large-scale surveillance and vaccine trials. The micro-NT is another variation whose informative value corresponds to the results of the PRNT90, but which is more suitable for the processing of large sample quantities due to the reduced amount of work needed. In this assay, the individual plaques are not counted, but the absolute virus growth in any well is measured.

However, all virus neutralisation tests depend on work with infectious viruses and can therefore only be performed for SARS-CoV-2 in a BSL3 laboratory environment, which greatly limits the number of laboratories that can perform them [9, 10]. Previously published pseudovirus-based NTs can be performed under BSL2 conditions, but also require the cultivation of infectious virus particles in cell cultures. There is therefore no significant time advantage compared to classical NTs [11, 12]. Other published alternatives include the use of the genetically modified fluorescent SARS-CoV-2 virus, which slightly reduces the time required to perform neutralization tests, but still requires the use of BSL3 facilities [13].

Given the current scale and pace of the pandemic, diagnostic laboratories everywhere are already finding it difficult to provide timely test results. As the demand for NAbs titers is expected to increase in the future, it is important to find faster, more scalable and automated high-throughput alternatives to traditional NT.

In this study we investigated two commercially available surrogate enzyme immunosorbent assays (sELISA) for the specific detection of SARS-CoV-2 NAbs in human blood samples: The SARS-CoV-2 Neutralizing Antibodies Detection Kit (AdipoGen Life Sciences, Liestal, Switzerland) and the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, NJ, USA).

We tested both kits side by side and in direct comparison with a micro-NT to determine both sensitivity and specificity of the two sELISAs and to test their potential as an alternative to conventional NT.

Material and Methods

Samples

A total of 276 human plasma samples were tested for SARS-CoV-2 specific NAbs. Of these plasma samples, 230 had previously tested positive for SARS-CoV-2 Abs in a commercial IgG ELISA (Euroimmun, Lübeck, Germany). A further 46 SARS-CoV-2 Abs negative plasma samples collected before the occurrence of SARS-CoV-2 (mid to late 2018) were used as negative controls.

The study was carried out in-line with “The Code of Ethics of the World Medical Association (Declaration of Helsinki)”. The use of plasma samples complied with the guidelines of the Central Ethics Committee of the German Medical Association (Dtsch Arztebl 2003; 100(23): A-1632). In accordance with these guidelines, the anonymised use of residual material from the samples sent to our laboratory for diagnostic purposes is permissible, provided that the patients have not decided against this procedure. All patients who had decided against this procedure were excluded from the analyses.

Micro-neutralisation test

Micro-NT analysis of plasma samples was performed as described before [14]. In brief, SARS-CoV-2 (strain MUC IMB-1) was cultured in Vero E6 cells. Virus stocks (50 TCID/50 µl) were prepared and stored at -80° C until further use. All micro-NTs were performed in 96-well culture plates (Greiner bio-one, Frickenhausen, Germany) on confluent cell monolayers. Plasma samples were diluted in Minimal Essential Medium (MEM, plus Non-Essential Amino Acids Solution and Antibiotic-Antimycotic Solution; all Invitrogen, ThermoFisher Scientific, Darmstadt, Germany) beginning with a ratio of 1:5 to a maximum of 1:80. A known positive and known negative plasma sample was used as a control together with a mock control and a virus back-titration on each plate.

Virus was pre-incubated with diluted plasma samples in duplicate for one hour at 37°C before the plasma-virus suspension was added to the wells. After an incubation period of 72 hours at 37°C (5% CO_2), the supernatants were discarded and the 96-well plates were fixed in 13% formalin/PBS, stained with crystal violet (0.1%) and titers were determined. The NAbs titer corresponded to the reciprocal of the highest plasma dilution that showed complete inhibition of CPE. The samples were classified as either "NT negative" (titer < 1:5) or "NT positive" (titer ≥1:5), with the highest possible titer being ≥1:80.

Surrogate enzyme linked immunosorbent assays (sELISA)

Two commercially available sELISA kits were used to determine the presence or absence of SARS-CoV-2 specific NAbs: The SARS-CoV-2 Neutralization Antibody Detection Kit (AdipoGen Life Sciences, Liestal, Switzerland) and the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, NJ, USA). Both tests use the competitive inhibition of the protein-protein interaction between a recombinant SARS-CoV-2 RBD protein and recombinant human ACE2 receptor (hACE2) to measure the specific neutralizing effect of antibodies in the patient sample. However, GenScript uses hACE2 coated plates for capture and HRP-conjugated SARS-CoV-2 RBD for detection while AdipoGen provides RBD-coated plates and uses HRP-conjugated hACE2 for detection. Both sELISA were performed in strict accordance with the manufacturer's instructions. All samples were tested in duplicate and the mean value of both measurements was used to calculate the relative inhibition. The samples were classified as either "positive" (inhibition ≥20%) or "negative" (inhibition <20%) as suggested by the manufacturers.

Results

Detection of SARS-CoV-2 neutralizing antibodies by NT

First, all samples were examined in the micro-NT. During the first experiment, only those plasma samples that showed a NAbs titer equal to or higher than 1:10 were initially considered positive, while all samples with a lower titer (<1:10) were considered negative.

Those initial results showed that 140 samples (50.7%) were positive to SARS-CoV-2 specific NAbs, while 136 samples (49.3%) were negative, including all negative control research samples from 2018. When comparing these results with the results of both sELISA, we noticed a high number of apparently false-positive results for both the GenScript (n=53) and the AdipoGen (n=16), resulting in rather low specificities (GenScript: 61%; AdipoGen: 88%) (Fig. 1).

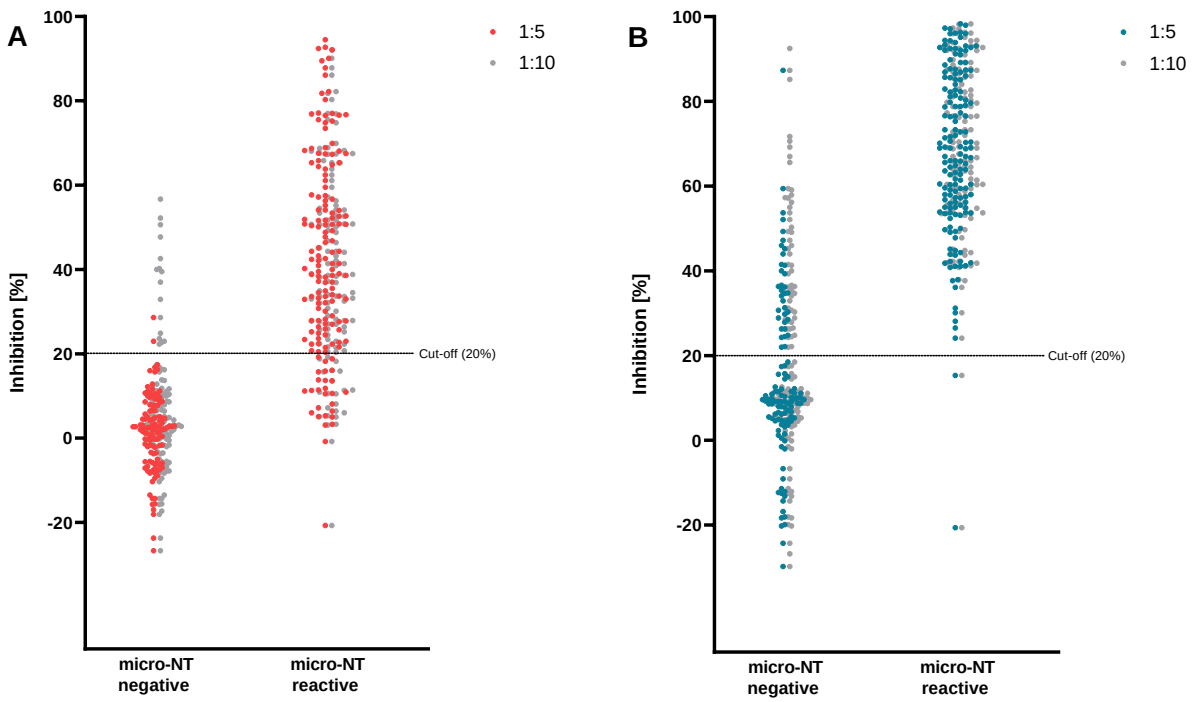

Fig. 1: Distribution of inhibition values determined by AdipoGen (A) and GenScript (B) within negative (≤1:5 (colored) and ≤1:10 (grey)) and positive NT results. (A) The AdipoGen ELISA is highly specific and gives almost no false positive results, while being less sensitive as indicated by a wide distribution of inhibition values of positive NT samples. **(B)** The GenScript ELISA is highly sensitive with very few false negative results, while it is much less specific with a wide range of inhibition values of negative NT samples. Specificities of both ELISAs benefited from the re-titration of initially negative NT samples as indicated by the original inhibition values (≤1:10) in grey.

After considering that enzymatic immunoassays are known to be generally more sensitive than NTs, we decided to re-titrate all 136 NT-negative samples from 1:5.

After re-titration, a total of 158 (57.2%) were positive, while 118 samples (42.8%) still showed no neutralizing effect in the NT even at a dilution of 1:5. Of the positive samples, 12% (n=19) had a titer of 1:5, 46.2% (n=73) had a titer of 1:10 and 21.5% (n=34) had a titer of 1:20. 10.1% each had a titer of 1:40 (n=16) and ≥1:80 (n=16) (Fig. 3).

This means that of the 230 plasma samples that initially tested positive in the IgG Euroimmun ELISA, only 68.7% (n=158) were positive (>1:5) in the NT. In contrast, all 46 plasma samples from 2018 had a titer of <1:5, confirming the specificity even at titers lower than 1:10.

Detection of NAbs with the SARS-CoV-2 Neutralizing Antibodies Detection Kit (AdipoGen)

The test was easy to perform and took about 2.5 hours from start to finish.

Of the 158 samples reactive in the micro-NT, 131 also tested positive for AdipoGen, resulting in a sensitivity of 82.9%. No false negative results were obtained on samples with a titer of 1:40 or a titer equal to or greater than 1:80. Of the 118 NT-negative samples, 116 were also negative in AdipoGen-sELISA, resulting in a specificity of 98.3% (Tab. 1), and the inhibition values of the negative NT results showed a low variance. In contrast, the scatter of inhibition values of positive NT results was much higher (Fig. 1A).

PLasma samples		AdiPoGen		GenScript	
		Positive	Negative	Positive	Negative
Total	276	133	143	158	84
VNT positive	158	131	27	156	2
VNT negative	118	2	116	36	82
Sensitivity [%].		82.9		98.7	
Specificity [%].		98.3		69.5	

Table 1: Comparison of sensitivity and specificity of the AdipoGen and GenScript

Detection of NAbs by cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript)

The test was easy to perform and only took about 1.5 hours from start to finish.

Of the 158 samples of NAbs confirmed with NT, 156 also tested positive with GenScript, resulting in a sensitivity of 98.7%. No false negative results were obtained on samples with a titer of 1:5, 1:20, 1:40 and equal to or greater than 1:80. The specificity was 69.5% with 36 false positive results (Tab. 1) and a high spread of inhibition values within the negative NT results (Fig. 1B).

Micro-NT vs. sELISA

While the micro-NT is able to quantify the amount of NAbs in a patient sample by titration, both sELISAs use relative inhibition [%] as readout. When comparing NT titers with relative inhibition, no clear correlation could be found in either sELISA. Simple linear regression confirmed this

observation (AdipoGen: R^2 0.515, Y=1.012x+12.19; GenScript: R^2 0.435, Y=1.109x+30.76) (Fig. 3). Thus, no conclusions can be drawn from inhibition values about titers.

In general, the higher the micro-NT titer, the less likely false negative sELISA results were obtained. No false negative sELISA results were observed in any of the examined plasma samples with a NT titer of > 1:40 (Fig. 2 and 3). Overall, the relative inhibitions determined by GenScript were generally slightly higher than those determined by AdipoGen (Figure 3).

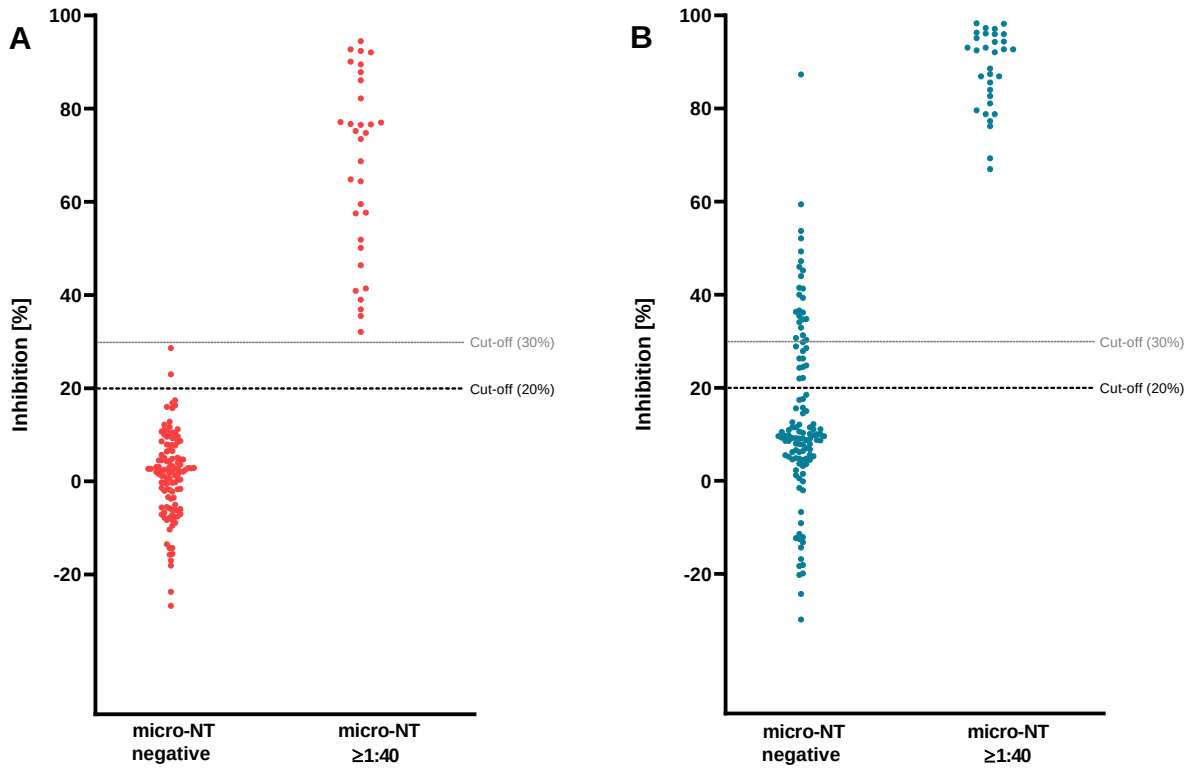

Fig. 2: Distribution of inhibition values determined by AdipoGen (A) and GenScript (B) within negative (≤1:5) and ≥1:40 micro-NT results. The AdipoGen ELISA is highly specific, but delivers two false-positive results at the manufacturer's recommended cut-off of 20% inhibition. By applying an increased cut-off of 30%, a 100% specificity is achieved without loss of sensitivity. The GenScript test remains less specific even with a 30% cut-off value with 24 false-positive results. For micro-NT results ≥1:40, the sensitivity of both sELISA is 100%.

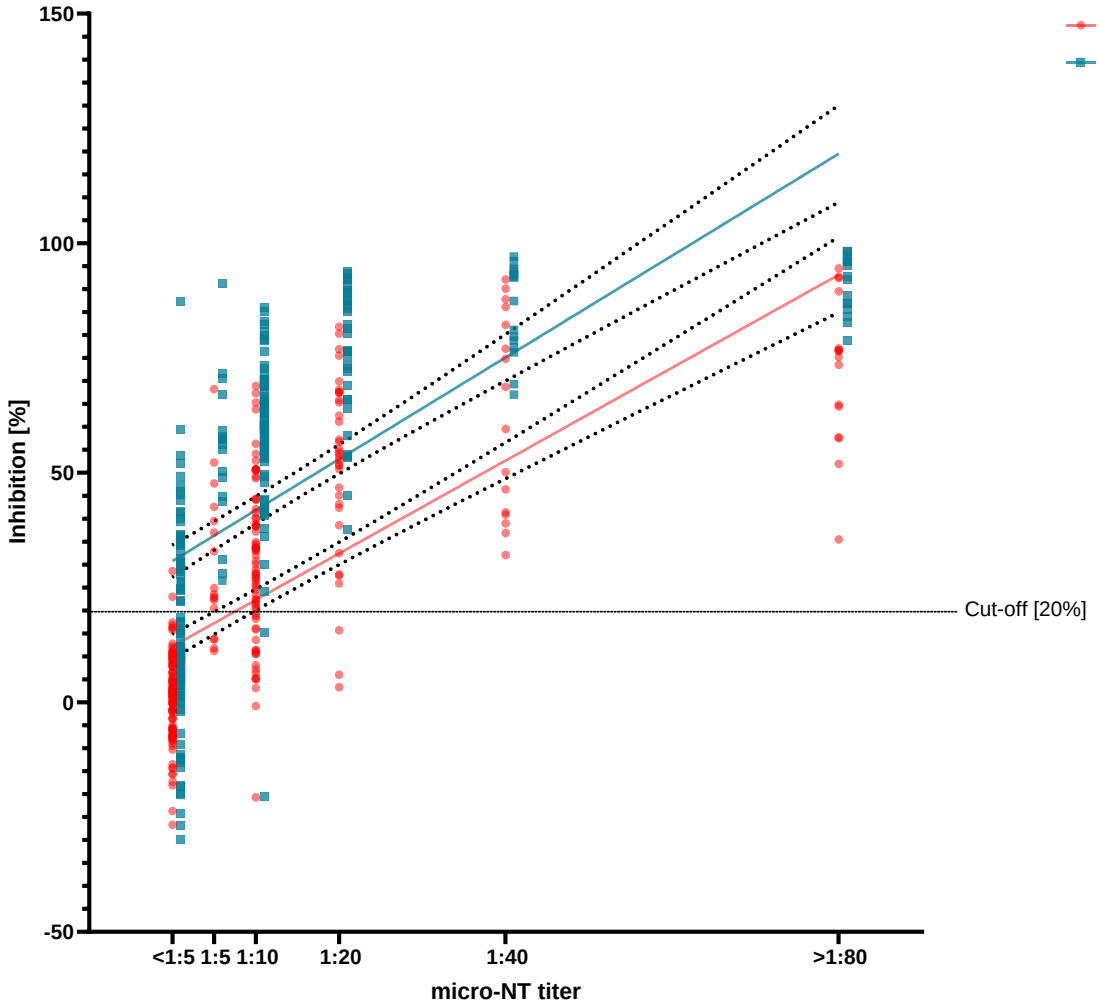

Fig. 3: Regression analysis of the 276 plasma samples tested shows no correlation between titer levels and sELISA inhibition values A Pearson regression analysis (95% confidence interval) was performed. The two-dimensional distribution shows no correlation with a wide spread of sELISA inhibition values, especially within low NT titers. Thus, no conclusion about exact titer levels can be drawn from inhibition values. No difference was observed between the two tested sELISAs: ^{R2} (AdipoGen): 0.515, ^{R2} (GenScript): 0.435)

Discussion

The importance of acquired immunity and neutralizing antibodies against SARS-CoV-2 has become a frequently discussed topic not only in the scientific community but also in the public debate among the general public and policy makers. Although the link between the detection of NAbs and the presence of protective immunity has not yet been finally established, NAbs are

already of great interest in confirming the efficacy of potential vaccines and in the selection of convalescent plasma donors. In this study we investigated two commercially available sELISA kits specifically designed to detect SARS-CoV-2 NAbs and compared their results with NAbs titers obtained by NT.

The data presented here initially confirmed that NAbs are not detectable in all patients who test positive for SARS-CoV-2 Abs in conventional antibody ELISA. We examined 230 samples that had previously tested positive for Abs in a commercial IgG ELISA (Euroimmun, Lübeck, Germany). However, only 68.7% (158/230) of these IgG positive samples were also positive in NT. This result differs from the results of a recently published study by GeurtsvanKessel et. al., in which the specificity of the Euroimmun IgG ELISA was reported to be 97-100% [10]. Assay performance was also determined by comparing the test result with NAbs titers. However, titers were measured by PRNT50 and were considered positive already at PRNT >20. However, the use of such low cut-off values leads to a considerable increase in the variability of the results and to a significant reduction in the specificity of the PRNT. Stricter PRNT90 or micro-neutralization test titers are much more specific by reducing background serum cross reactivities. They are therefore more suitable for evaluating test systems to be used in epidemiological studies, for diagnostic purposes or for selecting therapeutic convalescent plasma donors.

We were also able to show that both sELISA are easy to perform and have a significant time advantage over NT: GenScript took the least time from start to finish (1.5 hours), followed by AdipoGen (2.5 hours), while the micro-NT took 72 hours (plus the time for cell culture preparation). Furthermore, no BSL3 laboratory is required for both sELISA. In terms of assay performance (i.e. sensitivity and specificity) we found that the GenScript sELISA has a sensitivity of 98.7%. This was higher than the sensitivity determined for the AdipoGen sELISA (82.9%), which gave a higher number of false negative results (27/158). However, the high sensitivity of the GenScript sELISA correlated with a lower specificity of only 69.5%, which resulted in a large number of false positive results (36/118). In comparison, the AdipoGen sELISA had a much higher specificity of 98.3% with only two false positive results. Interestingly, both were just above the cut-off (23% and 28% respectively).

In general, false negative sELISA results might be attributed to the fact that the micro-NT is able to detect neutralization irrespective of specific epitopes while both sELISA are only able to detect NAbs specific for RBD. Thus, the differences in specificity and even more in sensitivity were somewhat surprising as both tests use the same principle to detect NAbs (i.e. the inhibition of

RBD-hACE2 interaction by NAbs). Nevertheless, the different orientation of the proteins during coating and detection might contribute to the differences in the results. The preincubation of plasma with soluble RBD (GenScript) instead of immobilized RBD (AdipoGen) seems to be more sensitive. At the same time, this interaction of soluble RBD and ACE-2 directly in plasma might be more prone to deliver false results due to different factors and components present in plasma possibly causing steric hindrance. In contrast, the AdipoGen sELISA performs a washing step eliminating these factors which might contribute to its higher specificity.

The fact that no conclusion about titer levels could be drawn from inhibition values is a clear disadvantage of both ELISAs. Especially for the selection of convalescent plasma for therapeutic purposes, an exact titer determination is crucial [17, 18]. However, sensitivity generally improved with higher titers and reached 100% for titers ≥1:40. At the same time, a slight increase of the cutoff value from 20% to 30% resulted in a specificity of 100% for the AdipoGen sELISA. Thus the AdipoGen achieved both a sensitivity and a specificity of 100% for samples with micro-NT titers ≥1:40 (Fig. 2). This could indeed be used in situations, where samples need to be evaluated for high titers (≥1:40), rather than exact titers such as screening strategies for the selection of convalescent plasma.

Our results show that due to their lower sensitivity (AdipoGen) or specificity (GenScript) and the lack of an absolute quantification possibility, neither of the two sELISA assays can currently fully replace the virus neutralization test. However, because NTs are such sophisticated tests, many laboratories use a two-step diagnostic algorithm to reduce the number of samples. Very often a fast and easy to perform IgG ELISA with high sensitivity is used as a screening test. Only IgG-positive samples are then analyzed using NT.

In this context, the AdipoGen sELISA could serve as a useful replacement. The slightly lower sensitivity (82.9%) could be compensated by the high sensitivity of the screening ELISA, while the high specificity (98.3%) would ensure very few false-positive results. Additionally, with an adjustment of the cut-off value to 30%, the specificity can be improved to 100%. However, such a test strategy is not applicable to questions where absolute quantification of virus neutralization is required, as is the case with large-scale screening studies on seroprevalence with emphasis on NAbs and humoral immunity. sELISA could potentially be a useful tool in the initial clinical evaluation studies of spike- or RBD-based vaccine candidates. However, the GenScript sELISA is not suitable in this context due to its low specificity. Instead, its high sensitivity may make it a useful alternative to classical antibody screening ELISAs.

In summary, our study shows that both SARS CoV-2 sELISA were able to qualitatively detect NAbs in human plasma samples. Sensitivity and specificity differed between the two sELISA, with GenScript superior in sensitivity and AdipoGen superior in specificity. Combined with the fact that both sELISA are not capable of quantifying NAbs, these results suggest that neither of them can completely replace conventional NT. However, in a two-step diagnostic algorithm where samples are pre-screened by conventional sensitive IgG ELISA, the AdipoGen sELISA could potentially replace the NT as a subsequent confirmatory test due to its high specificity for all questions that do not require quantification of NAbs.

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