SARS-COV-2 Neutralizing Activity in Serum Collected from Recovered and Vaccinated Health Care Workers

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Research Article

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Abstract

Vaccination against the ongoing COVID-19 is the key point in fight against the pandemic. The Spike (S) glycoprotein of SARS-CoV-2 is the major target of the neutralizing humoral response. We evaluated analytical and clinical performances of a surrogate virus neutralization test (sVNT) (iFlash-2019-nCoV Nab assay, Ylho, China) compared to the conventional neutralization tests (cVNT) and anti-S eCLIA assays (Roche Diagnostics, Switzerland) in recovered and/or vaccinated health care workers. Our results indicate that sVNT displayed high specificity and no cross reactivity. Both Roche and iFlash immunoassays were good in identifying cVNT serum dilution > 1:16. Optimal thresholds in identifying cVNT titers ≥ 1:16, were 74.5 U/ml and 49.4 IU/ml for anti-S eCLIA and sVNT, respectively. Our data show that Nab neutralizing antibodies titers depend on individuals and may abate over time. Specific assays such as sVNT may be a reliable complementary tool to routine anti-S serology assays.

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) epidemic, which was first reported in Wuhan, China, has rapidly spread worldwide and caused the COVID-19 pandemic [1]. As different vaccines become available, a better knowledge of their performances to induce a sufficient public immunity against COVID-19 is of major importance. The Spike (S) glycoprotein of SARS-CoV-2 is the major target of the neutralizing humoral response and is therefore used for the development of vaccines and monoclonal antibody (Ab) treatments [2–6]. Virus-specific neutralizing Abs (NAbs) play a key role in reducing viral replication and accelerating viral clearance of SARS-CoV-2 [7,8]. NAbs mainly act against the receptor-binding domain (RBD) of the SARS-CoV-2 S glycoprotein effectively blocking viral entry via preventing its binding to the angiotensin-converting enzyme 2 (ACE-2) receptor [9–12].

There is some evidence that immune protection against other human coronaviruses (HCoV) is not long-lasting, but there is also evidence that Ab against SARS-CoV-1 persists for years and displays potent neutralizing activity even in the absence of detectable IgG directed against SARS-CoV-1 nucleocapsid protein (N) [13,14]. However, recent descriptions of SARS-CoV-2 reinfection cases support recommendations to offer vaccination to highly exposed individuals (as healthcare workers (HCW)), who have recovered from a mild form of COVID-19 [15,16]. These authors also suggest that vaccination against SARS-CoV-2 will probably have a short-lasting protective effect, meaning that most people should get vaccinated periodically. Up to now, most serological studies have focused on hospitalized patients, but specific information on serologic response in individuals with mild infection is still scarce and mainly focused on seroconversion rates [17–24]. Recently, Marot et al. showed that neutralizing activity of Ab appears to be transient with a decline, or even a loss, of the NAb titers from 2 months after disease onset supporting vaccine recommendation of infected HCW [25]. However, in a context of vaccine shortage and/or mistrust in vaccines, it could be highly benefic to determine who really needs a boost and how many doses may be necessary depending on clinical history along with accurate serological information. Thus, serological

testing, especially to detect NAbs, is essential in identifying individuals who are potentially protected against re-infection.

In COVID-19 patients, NAbs can be detected within 2 weeks of symptom onset but in some cases, substantially longer [26–29]. However, the dynamics of NAbs and their correlation with anti-S Ab have not been explored in COVID-19 patients more than six months after symptom's onset. Moreover, it is still unclear whether current serological assays that detect anti-S Ab predict neutralizing activities or protection against viral re-infection [30].

NAbs can be detected with conventional neutralization tests (cVNT), and their presence is often correlated with protective immunity [31]. cVNT is considered as the gold standard [21] but it must be performed in biosafety level -3 laboratory (BSL-3), is time-consuming, labor-intensive, require several days of work, highly skilled operators, and is hardly standardized as compared to other serologic assays. Therefore, very few labs can run such tests, which are not suitable for mass testing. On the other hand, enzyme-linked immunosorbent assays (ELISA) and ELISA variants, such as lateral flow assay (LFA) and chemiluminescence immunoassays (CLIA) detect anti-S or anti-RBD Ab with high sensitivity but vary in their ability in predicting NAb activity [32–37], and therefor results may not directly correlate with protection [38,39].

In the present study, we evaluated analytical and clinical performances of a surrogate virus neutralization test (sVNT) (iFlash-2019-nCoV Nab assay, Ylho, China) that is designed to detect total NAbs in an isotype and species-independent manner, and can be completed in 1-2 hours in a BSL2 lab [40]. Anti-S eCLIA assay (Elecsys anti-SARS-CoV-2 S, Roche Diagnostics, Switzerland) can detect some NAb but not all Nab, and might also detect non-NAb. Indeed, although RBD might be the target for NAb, some RBD Ab cannot fully block the interaction between RBD and ACE2, which could fail in preventing re-infection. We compared cVNT, sVNT and anti-S eCLIA assays in immunocompetent subjects (mainly health care workers): 9-11 months after COVID-19, after 1st dose of vaccine, after 2nd dose of vaccine, and in patients with a history of COVID-19 after one dose of vaccine.

Material And Methods

This study was conducted in the Virology laboratory of Paul Brousse hospital (Paris, France). It was carried out in accordance with the Declaration of Helsinki as a retrospective non-interventional study with no addition to standard care procedures. Reclassifications of biological remnants into research material after completion of the ordered virological tests were obtained under number DC 2009-965 and received ethical approval of the CPP (Comité de Protection des personnes) Ile de France 7, N°CO-15-000, and following with French law. Informed consent was obtained from all subjects and/or their legal guardian(s).

Clinical samples

First, 54 serums sera were selected to determine thresholds of anti-S eCLIA and sVNT values predictive of neutralizing activity using cVNT as a gold standard (Fig. 1):

- 11 serum samples from vaccinated individuals either at least 14 days after 1st dose (N=7), or at least 7 days after 2nd dose (N=4);

- 41 serum samples from recovered COVID-19 patients either at least 3 months after onset of infection (N=36) and at least 6 months after onset of infection and 7 days after 1st dose of vaccine (N=5);

- As a control, 2 samples were collected from naïve and non-vaccinated individuals.

In a second step, 394 samples were collected to compare anti-S eCLIA and sVNT results (Fig. 2):

- 103 serum samples collected before the COVID-19 pandemic including 3 serum samples collected from patients with other HCoV infection;

- 27 serum samples collected at least 3 weeks after infection from patients with confirmed SARS-CoV-2 Variant (alpha, beta, or delta);

- 168 serum samples collected from naïve HCWs before vaccination (N=73), at least 14 days after 1st dose of vaccine (N=36), and at least 7 days after 2nd dose of vaccine (N=95);

- 96 serum samples collected from COVID-19 recovered HCWs 9-11 months after onset of infection (N=73) and at least 7 days after one dose of vaccine (=23).

Median age of HCWs was 53 (ranging from 22 to 86) and 64% were women. None was immunocompromised. All were vaccinated with Pfizer vaccine.

Conventional virus neutralization test (cVNT):

All procedures related to cVNT were performed in a BSL-3, in accordance with WHO recommendations. Vero E6 cells (African green monkeys, kidney) were grown at 37°C, 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM, 319966), supplemented with L-Glutamine, 10% Fetal Bovine Serum (FBS, FCS, Sigma-Aldrich, #F9665), and 1% Penicillin/Streptomycin/Neomycin (PSN, GIBCO 15640055). The SARS-CoV-2 virus was isolated from a RT-PCR confirmed COVID-19 patient hospitalized in Paul Brousse hospital (Villejuif, France) in December 2020, and amplified in Vero cells. We confirmed virus titer by TCID50 ($10^{7.5}$ PFU/ml) and RT PCR (CT: 9,5). Serum samples were decomplemented by heat inactivation (56°C for 30 min), subjected to serial two-fold dilution in PBS 1X (1:2 to 1:2048, each dilution 250µl). Different sera dilutions were incubated with 250µl of diluted virus (10^3 TCID50/ml) in DMEM with 2% FBS at 37°C, 5% CO2 for 1 hour, and then 100µl of each dilution was added to Vero cells seeded (3×104/well) in 96 well plates (4 technical replicates for each sera dilution, n:4). Plates were incubated at 37°C, 5% CO2 for 96 hours until microscopy examination on day 4 to assess the cytopathic effect (CPE). NAbs titer was

confirmed with the highest dilution of sera that inhibited 100% of the CPE (absence of cytopathic effect). cVNT titers ≥1:16 were considered positive for SARS-CoV-2 NAbs.

Surrogate virus neutralization test (sVNT):

The iFlash-2019-nCoV NAb assay is a paramagnetic particle chemiluminescent one-step immunoassay for quantitative determination of NAb in serum using an automated analyzer. This assay is based on Ab-mediated blockage of virus-host interaction between ACE2 receptor protein and RBD of the viral S protein. It is designed to mimic the virus-host interaction by direct protein-protein interaction in a test tube. In brief, NAb in the sample reacts with RBD antigen-coated microparticles to form a complex. In a second step, ACE2 conjugate is added to competitively bind to the RBD-coated particles which have not been neutralized by NAb from the sample. Bound particles are then detected thanks to a chemiluminescent reaction, and a calibration curve allows to quantify the amount of NAb. Results are expressed in IU/ml with a manufacturer providing a cut-off of 24 IU/ml.

Elecsys Anti-S eCLIA assay (Anti-S eCLIA)

Anti-S eCLIA was used for the quantitative determination of antibodies to the SARS-CoV-2 S protein receptor-binding domain (RBD). The quantification range of Anti-S eCLIA is 0.4 to 250 U/ml (results <0.4 U/ml are considered non-reactive, anti-S titers over the quantification range are expressed as >250 U/ml). Manufacturer positive cut-off is 0.4 U/ml.

Statistical analysis

Statistical analysis was performed with *GraphPad Prism* Software 9.1.2 (GraphPad, La Jolla, CA, USA). Wilson-brown statistic method with 95% confidence interval (p value <0.0001) was performed. Correlation test (Spearman) and Receiver operating characteristic curve (ROC) were conducted using *Analyse-it* software v5.65. Results with *p* values <0.05 were considered significant.

Results

Correlation of anti-S eCLIA, sVNT and cVNT results

Fifty-four samples from naïve, infected, and/or vaccinated patients were used to assess the correlation between anti-S eCLIA, sVNT and cVNT (Fig. 1). Overall 3/54 had no detectable **anti-S eCLIA** (titer <0.4 U), the remaining had titers ranging from 1.21 to > 250 U/ml. sVNT was performed on 45/54 samples with titers ranging from 0 to 12,377 IU/ml. Control samples had no neutralizing activity and no reactivity in any immunoassay. cVNT was performed on these 54 samples with neutralization results ranging from 0 to 1:1,024, and 20/54 (37%) had a significant neutralization in dilution ≥ 1:16.

Anti-S eCLIA and sVNT titers were correlated (Spearman's rs = 0.761, p<0.0001). Both Roche and iFlash immunoassays were good in identifying cVNT serum dilution > 1:16, with AUC (Area under the curve) of 0.950 (95% CI 0.889-1.011), and 0.870 (95% CI 0.752-0.988) (p<0.0001), for anti-S eCLIA and sVNT respectively. Immunoassays were equally discriminant. Optimal thresholds in identifying cVNT titers ≥

1:16, were 74.5 U/ml and 49.4 IU/ml for anti-S eCLIA and sVNT, respectively (so-called Nabs cut-offs). With these thresholds, sensitivity and specificity were 95% and 93.4% for anti-S eCLIA, and 82.4 and 85.7% for sVNT (Fig. 3).

sVNT performances vs cVNT

Specificity and sensitivity of sVNT were assessed on sera collected from patients before the COVID-19 pandemic (expected negative) and from patients who had recovered after confirmed COVID-19 infection (expected positive). All expected negative were indeed negative 103/103 (100% specificity) including samples collected from patients with other HCoV infection. Among 100 sera from recovered patients, 91% had detectable titers including 64/73 (87.7%) samples collected ≥ 9 months after infection, and 27/27 (100%) samples collected from patients with recent confirmed COVID-19 infection with a variant.

Anti-S eCLIA vs sVNT

Anti-S eCLIA Abs levels from hybrid immunized individuals (infected-vaccinated) and 2-doses vaccine immunized individuals are higher compared to other situations ($p<0.03$). NAbs level (sVNT) in sera from hybrid immunized individuals (infected-vaccinated), 2-doses vaccine immunized individuals and recovered patients from variants of SARS-CoV-2 infection are also higher compared to the other situations. ($p<0.0003$) (Fig. 4; Table 1).

Table 1
Comparison between sVNT and anti-S eCLIA titers in different HCW populations.

	sVNT			Anti-S Abs		
	Mean titer (Positive cut-off : >24 IU/ml)	N° and % (> 24 IU/ml)	N° and % (> 50 IU/ml)	Mean titer (Positive cut-off : >0.8 U/ml)	N° and % (> 0.8 U/ml)	N° and % (> 75 U/ml)
Naïve pre-vaccination	10.82 ±5	0 (0%)	0 (0%)	<0.4	0 (0%)	0 (0%)
14d post D1	86.26 ±198	21 (58%)	12 (33%)	64.78 ±90	28 (78%)	10 (28%)
7 d post D2	36611419,09 ±82643261	51 (86%)	50 (85%)	201.58 ±98	55 (93%)	47 (80%)
Infected 9 Mo	394 ±755	64 (88%)	42 (58%)	169 ±92	72 (99%)	56 (77%)
Infected 7d post D1	31306349,36 ±85874735	23 (100%)	22 (96%)	250± 41	23 (100%)	22 (96%)

Before vaccination, 73 serum samples were collected. All (73/73) were negative in Roche and sVNT assay with mean titers <0.4 U/ml and 10.82±5 IU/ml, respectively.

At least 14 days after 1st dose and before 2nd dose, 28/36 (78%) serum samples showed positive anti-S eCLIA titers (ranging from 2.88 to >250 U/ml (mean titers: 64.78± 90), and 21/36 (58%) were positive with sVNT (titres ranging from 24,792 to 1188,864 UI/ml (mean titers: 86.26±198)). Based on established NAb cut-offs, the number of individual with protective level of antibody dropped to 12/36 (33%) with sVNT and to 10/36 (28%) with anti-S eCLIA. At least 7 days after 2nd dose, 55/59 (93%) showed positive anti-S eCLIA titers (ranging from 1 to >250 U/ml (mean titers: 201.58 ±98)), and 51/59 (86%) were positive with sVNT (titres ranging from 26,88 to 240,000,000 IU/ml (mean titers: 36611419 ±82643261)). Based on established NAb cut-offs, 50/51 (85%) individuals, had sVNT titre >50 IU/ml and 47/59 (80%) had an anti-S eCLIA >75 U/ml.

Between 9 to 11 months after recovered COVID-19 infection, 73 samples were collected from COVID-19 healthcare workers. All were symptomatic with mild symptoms mainly cough, fever, breathless, body ache, headache, diarrhoea or anosmia. Overall, 72/73 (99%) showed presence of anti-S eCLIA (titres ranging from 10.39 to > 250 U/ml (mean titers: 169 ±92)), and 64/73 (88%) were positive with sVNT (titres ranging from 25 to 2697 UI/ml (mean titers: 394 ±755)). Based on established NAb cut-off, the number of individual with protective level of antibody dropped to 42/73 (58%) with sVNT and to 56/42 (77%) with anti-S eCLIA. In these recovered COVID-19 HCW, 23 had one dose of vaccine at least 6 months after onset on infection. All (23/23 (100%)) showed presence of anti-S eCLIA (titres ranging from 60.89 to > 250 UI/ml (mean titers: 31306349 ±85874735)) and positive sVNT (titres ranging from 35,712 to 240000002,4 IU/ml (mean titers: 250± 41)). Based on established NAb cut-off, 22/23 (96%) had sVNT titre >50 IU/ml and anti-S eCLIA >75 U/ml.

Discussion

We studied the neutralizing activity of the serum samples which is a biological marker often correlated to protective immunity [31], using a sVNT in comparison to classical cVNT. Both assays determine the functional ability of Ab to prevent virus binding to its receptor. These assays have also the advantage to study the overall serum neutralizing activity (both isotypes and epitope-specific Ab). However, cVNT can not be routinely performed on a large number of samples compared to the automated sVNT assay. Our results indicate that sVNT displayed high specificity and no cross reactivity. We also established that a threshold of 50 UI/ml with sVNT was predictive of a neutralizing activity of anti-SARS-CoV-2 Ab in cell culture (vs 24 IU/ml cutoff recommended by manufacturer) with sensitivity and specificity of 82.4% and 85.7% respectively.

It is well known that positive results from current large scale serological ELISA or CLIA assays, even those which specifically target S protein of SARS-CoV-2, do not necessarily indicate neutralizing immunity because they detect some NAb but not all NAb and might also detect non-NAb. Indeed, although RBD might be the target for NAb, some RBD Ab cannot fully block the interaction between RBD and ACE2,

which could fail in preventing re-infection. With the anti-S eCLIA assay, we have established a threshold of 75 UI/ml of anti-S, which was related to neutralizing activity of anti-SARS-CoV-2 antibodies in cVNT with sensitivity and specificity of 95% and 93.4% respectively. This threshold is quite far from the 0.8 UI/ml cutoff recommended by manufacturer and has to have an impact on clinical interpretation of results.

Our understanding of the duration and nature of protective immunity to SARS-CoV-2 is currently very limited. The kinetics of antibody-mediated immunity to SARS-CoV-2 infection, and how long this immunity lasts are unknown. Recently, in ferrets, authors showed that reinfected SARS-CoV-2 animals with high NAbs had attenuated viral replication and rapid viral clearance. Whereas direct-contact transmission was observed only from reinfected ferrets with low NAb titres, demonstrating a close correlation between NAb titers and protection in an animal model [41]. Although a recent study in a rhesus macaque model, did not find evidence of reinfection and therefor evoked a robust protective immune response when the animals were re-exposed to SARS-CoV-2 one month after the initial viral infection [42], only monkeys with high NAb titers were used for reinfection studies and few showed low levels of replication following reinfection. Overall, these studies indicate that NAb titers is a critical determinant in providing protection against reinfection; initial exposure to other human coronaviruses are known to fail to elicit a sufficient protective immune response with sometimes a higher viral load [43]. This result, consistent with our findings and those of other groups, indicates that SARS-CoV-2 infection elicits a neutralizing activity of sera, at least temporarily, which may be correlated with a protective humoral response.

Our data show that NAb titers in patients are variable, and may abate over time depending on individuals, which is in accordance with findings in patients infected with other human HCoV [44,45]. The short-term humoral immune response in COVID-19 patients is also highly consistent with that observed in patients infected with SARS-CoV-1 and MERS-CoV [46,47] who show a rapid decrease in virus-specific antibody titers within 3–4 months. Among the 73 recovered patients in our study, 64 (88%) patients showed low NAb titers (mean titers: 394 ±755) 9 to 11 months after infection, suggesting that the Ab titers may diminish with time, or that some recovered patients may not produce a high-titer neutralizing response during SARS-CoV-2 infection. Although it has been shown a positive correlation between NAb titers and IgG, in patients within 3 months after infection, our results indicates that more than 9 months after infection, anti-S serology may fail to find NAb and that specific assays such as SVNT may be a valuable complementary tool [48]. However, their response after one boost of vaccine (mean titers: 31306349,36 ±85874735) showed equivalent NAb titers compared to naïve patients who received two doses of vaccine (mean titers: 36611419,09 ±82643261). This information encourages performing serology in general population in order to identify individuals who might have had asymptomatic infection to optimize vaccination schedules, as now recommended in most countries.

As the COVID-19 pandemic continues to spread worldwide, most people infected with SARS-CoV-2 will recover from primary infection. To hamper the continuous spread of this virus, recovered individuals

would have sufficient NAb to protect themselves against reinfection. Recent studies have reported that asymptomatic COVID-19 patients exhibit lower Ab response than patients with severe disease do. Moreover, there is a rapid decline of Ab in asymptomatic patients compared to severe COVID-19 patients [49–51]. Hence, sVNT is reliable for assessment of herd immunity and humoral protective immunity in recovered/vaccinated patients. As sVNT will not be available in all labs, using it could be considered to revaluate routinely used serological assays to facilitate screening in general population and target individuals who should have one or two doses of vaccine. Also, in patients that are known to have poor immune response to vaccination (dialysed patients or those receiving rituximab for example), it would help to identify those who deserve a third dose of vaccine.

Although, cases of suspected SARS reinfection have continuously been rising among recovered COVID-19 patients, their immune response against the virus, especially the role of NAb was not fully characterized [52]. Our understanding of the duration and nature of protective immunity to SARS is currently very limited. Our data suggest that NAb may be absent in some patients who recovered from COVID-19 and in these specific individuals, a single boost of vaccine allows boost their pre-existing immunity and reach protective level. Given the current situation of significant part of population showing some mistrust in vaccines, or in case of vaccine shortage, sVNT assays or routine serological assays with newly established NAb cut-offs, would avoid vaccinate individuals that have titers indicating protective immunity. Studies are currently ongoing to evaluate usefulness of sVNT in immune patients at risk of severe COVID-19.

This present study had several limitations as systematic follow up, and sequential sampling was limited. More studies are required to assess the protection correlates after SARS-CoV-2 infection, such as the minimal NAb titer for protection. Further, it will be pertinent to study the protective role of Nab during reinfection in recovered COVID-19 patients and the role of cellular immune response. It will also be important to assess changes in the levels of antibodies directed against S protein and RBD over time, to improve our understanding of the potential protective immune response to SARS-CoV-2 infection.

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Figures

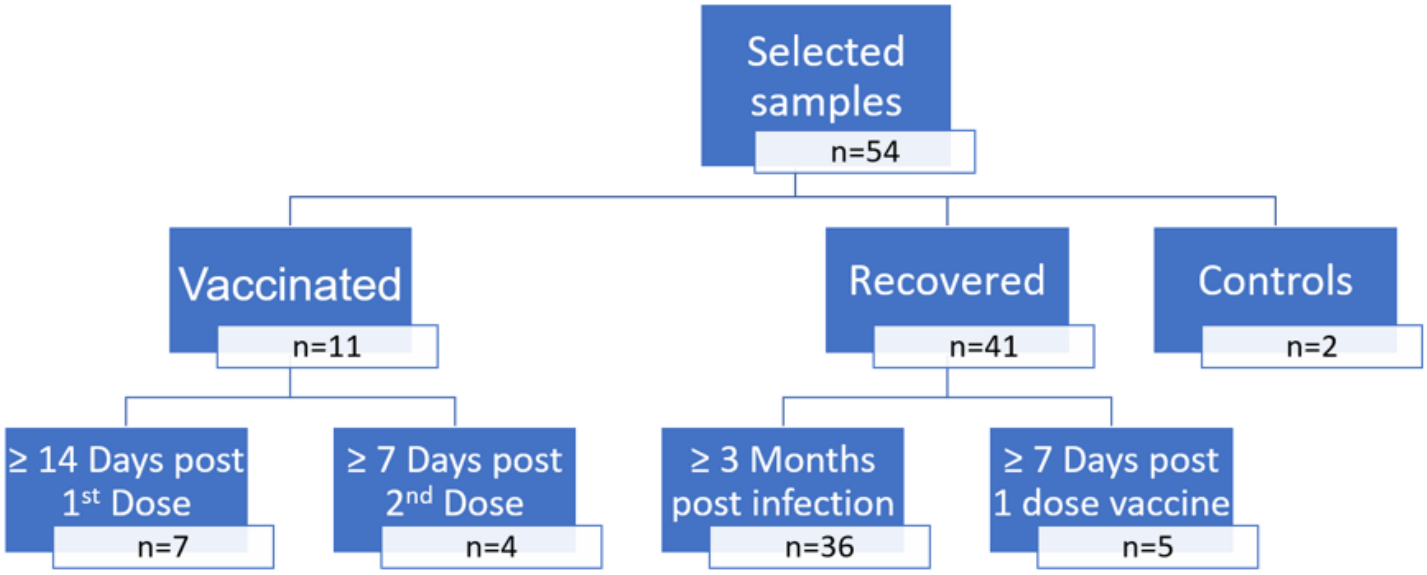
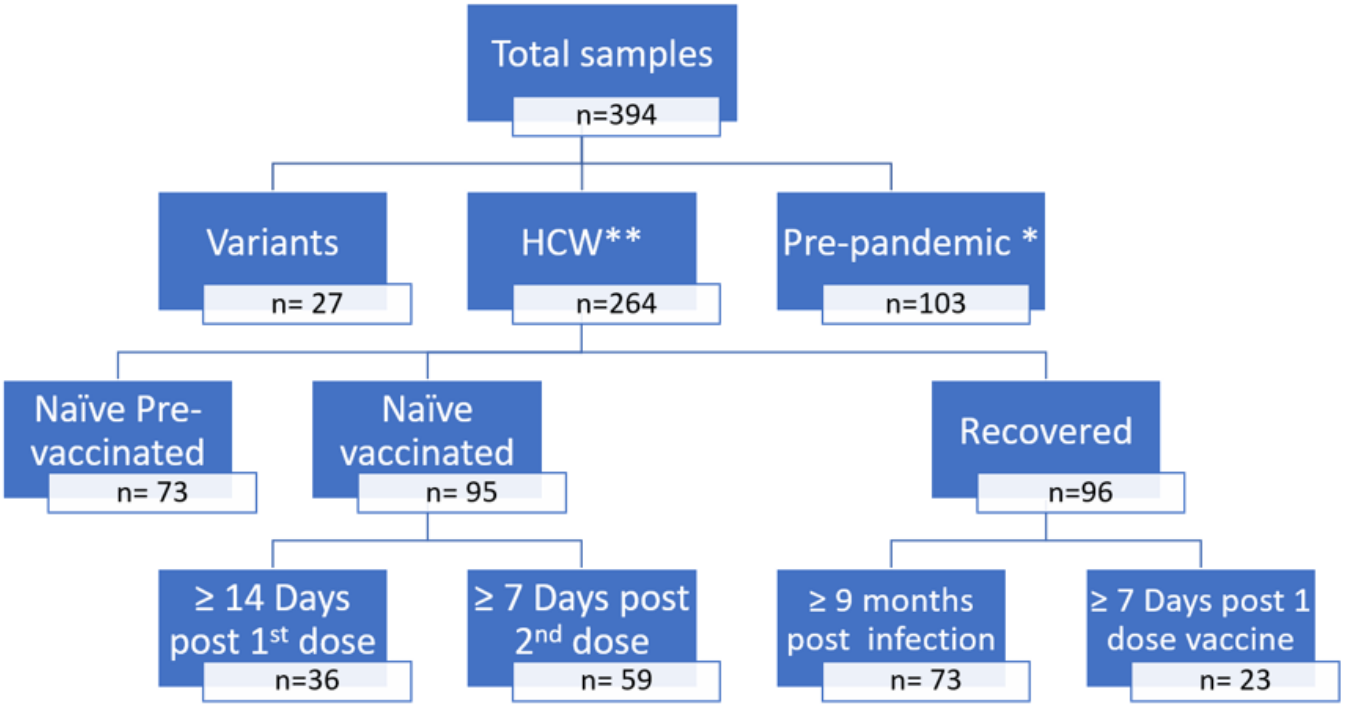


Figure 1

Samples selected to determine thresholds of anti-S eCLIA and sVNT values predictive of neutralizing activity using cVNT as a gold standard..



* Including 3 other HCoV
** HCWs : Healthcare workers

Figure 2

Samples selected to compare anti-S eCLIA and sVNT results

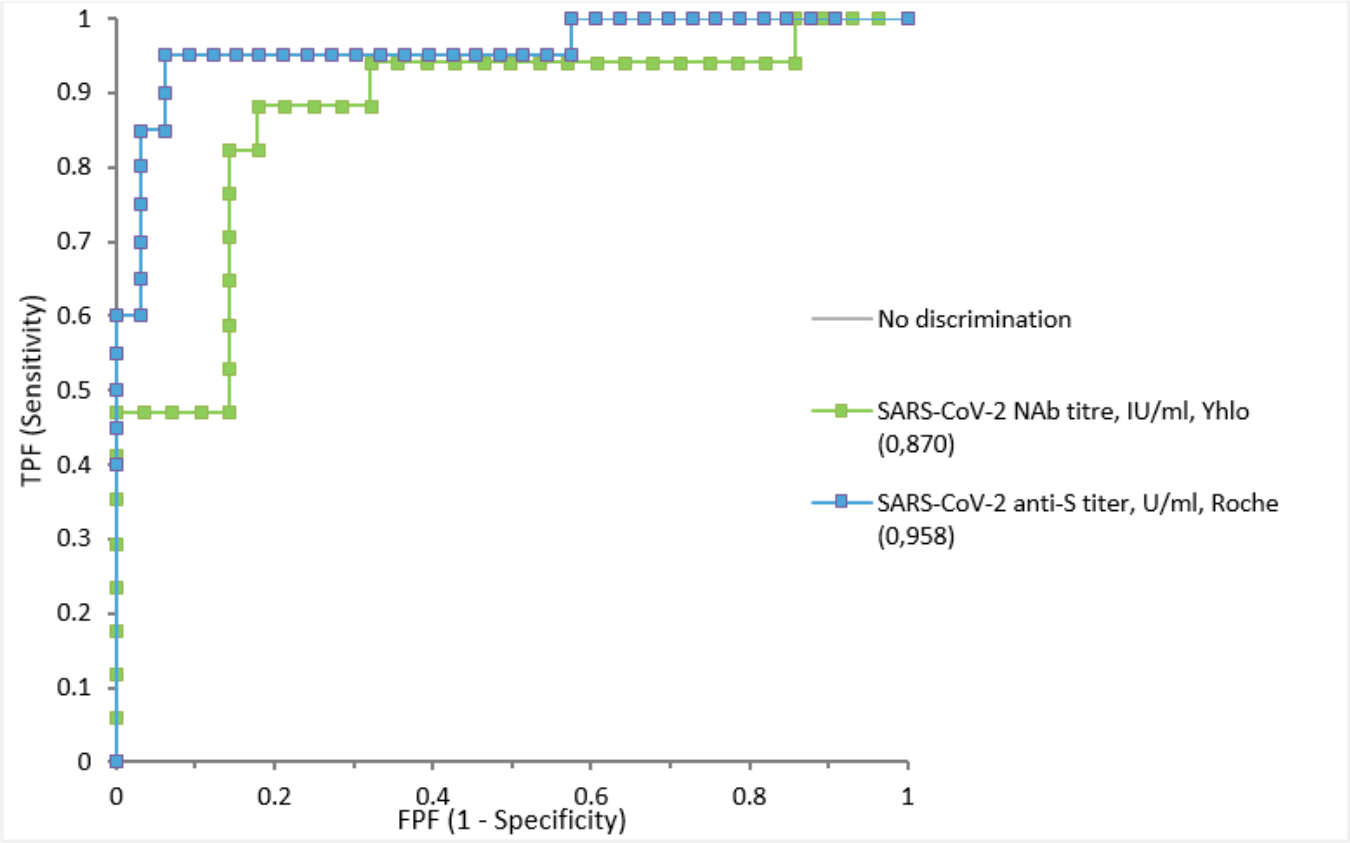


Figure 3

Receiver operating characteristic curve (ROC) for prediction of neutralization titers ≥1:16 by cVNT based on the anti-S eCLIA and sVNT, AUC (Area under the curve); anti-S eCLIA : 0.958 (95% CI 0.889-1.011); sVNT : 0.870 (95% CI 0.752-0.988) ($p<0.0001$).

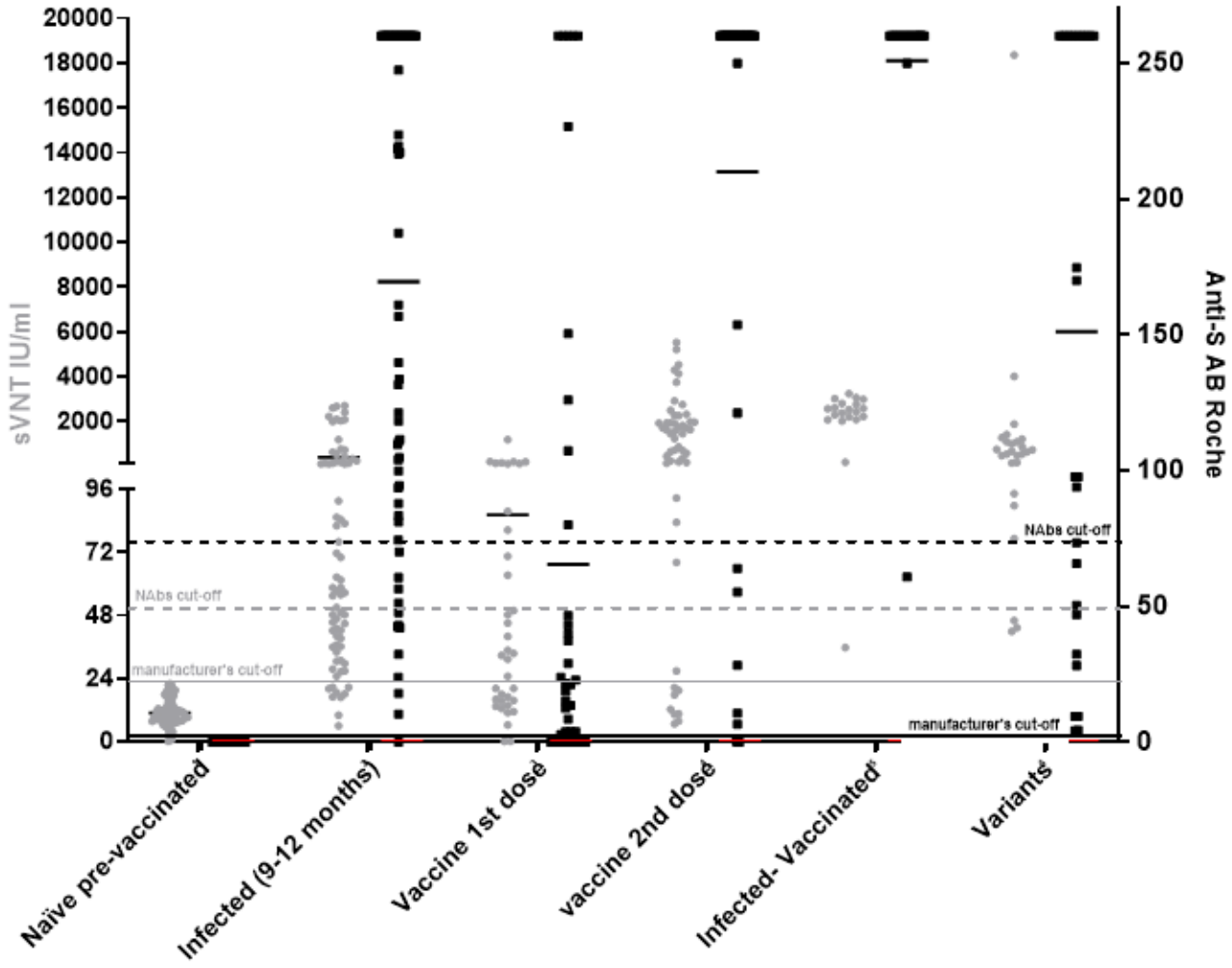


Figure 4

anti-S eCLIA (black dots) vs sVNT (grey dots) results. Cut-offs provided by manufacturers and cut-offs established with cVNT are represented in full and dotted line respectively. Means of titers for each population and each assay are represented by a dash.