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REVIEW

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Advances in Neutralization Assays for SARS-CoV-2

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has triggered a global health emergency and brought disaster to humans. Tremendous efforts have been made to control the pandemic, among which neutralizing antibodies (NAbs) are of specific interest to researchers. Neutralizing antibodies are generated within weeks after infection or immunization and can protect cells from virus intrusion and confer protective immunity to cells. Thus, production of NAbs is considered as a main goal for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines and NAbs may be used for patient treatment in the form of monoclonal antibodies. Neutralization assays are capable of quantitatively detecting NAbs against SARS-CoV-2, allowing to explore the relationship between the level of NAbs and the severity of the disease, and may predict the possibility of re-infection in COVID-19 patients. They can also be used to test the effects of monoclonal antibodies, convalescent plasma and vaccines. At present, wild-type virus neutralization assay remains the gold standard for measuring Nabs, while pseudovirus neutralization assays, Surrogate virus neutralization test (sVNT) and high-throughput versions of neutralization assays are popular alternatives with their own advantages and disadvantages. In this review article, we summarize the characteristics and recent progress of SARS-CoV-2 neutralization assays. Special attention is given to the current limitations of various neutralization assays so as to promote new possible strategies with NAbs by which rapid SARS-CoV-2 serological diagnosis and antiviral screening in the future will be achieved.

1 | INTRODUCTION

The current global outbreak of coronavirus disease 2019 (COVID-19) poses a serious threat to human health. Monoclonal antibodies (mAbs) and vaccines are considered as the two most promising strategies to control this pandemic.[1] The virus that causes the disease is called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It is an enveloped positive-stranded RNA novel coronavirus and contains a genome that encodes spike (S), envelope (E), membrane (M) and nucleocapsid (N) structural proteins, 16 non-structural proteins and 5-8 accessory proteins.[2]

The S trimers protruding on the surface of the virus composed of 1273 amino acids.[3] There is a furin cleavage site on it. Furin can cut the S protein into two subunits S1 and S2 via this cleavage site.[4] The S1 subunit is highly immunogenic and contains two functional domains, the N-terminal domain (NTD) and, more importantly, the receptor-binding domain (RBD) (Figure 1). The S1 domain covers the upper part of the S protein, with a relatively small (~22 kDa) RBD at the tip. RBD is an important site that contains multiple conformational neutralizing epitopes that are used by the virus to bind to the target cell receptor angiotensin-converting enzyme 2 (ACE2).[5] The RBD on each S trimer is dynamic; two of three RBD are predominantly in the 'down' conformation where the receptor-binding site is inaccessible, but RBDs seem to flip like hinges randomly, and when RBDs change to the 'up'

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conformation for a short time, the ACE2 binding sites are presented.[6,7] For instance, the receptor-binding motif (RBM), a neutralizing epitope, is the core of the RBD. The RBM is covered by the adjacent residues, which is inaccessible in the 'down' conformation but becomes exposed in the 'up' conformation.[8] Some antibodies can block the fusion between SARS-CoV-2 and host ACE2 through occupying the RBM. Thus, the antibodies against RBM have the strongest neutralizing activity.[9-12] At present, many therapeutic drugs, treatment methods and vaccines have been developed based on the RBD.[13-15]

The S2 subunit which is buried under the S1 protein is conserved as compared with S1, and it contains a transmembrane region (TM), a cytoplasmic tail, a fusion peptide (FP) and two heptapeptide repeat domains (HR1 and HR2), mediating fusion between the virus and host cell membranes.[16] During the infection process, SARS-CoV-2 first binds to the host cell through the interaction between the RBD of S1 and host ACE2. FP domain of S2 subunit is inserted into host membrane, to expose HR1 and HR2 domains, and then a six-helical bundle, the fusion nucleus, is formed, which makes the lipid bilayers of cells and viruses close to each other, therefore promotes the fusion between viruses and cell membranes.[17] A few of epitopes on S2 protein may elicit neutralizing antibodies (NAbs).[18] These epitopes may be considered to reduce the possibility of viral escape mutants when prophylactic and therapeutic reagents are developing. SARS-CoV-2 utilizes the cell surface serine protease TMPRSS2 to promote viral entry.[19] Besides, current studies have shown that another receptor called Neuropilin-1 on the host cell can also act as an auxiliary binding to the S protein of SARS-CoV-2 to help the fusion,[20] which also can be a candidate for drug targets to inhibit SARS-CoV-2 and helpful for the long-term control of COVID-19.

2 | NABS INDUCED BY SARS-COV-2

More and more pieces of evidence show that COVID-19 patients can produce different levels of antibodies that specifically bind to various structural proteins of SARS-CoV-2 soon after the onset of the disease.[21,22] However, only a small subset of these antibodies that target neutralizing epitopes on the surface proteins of SARS-CoV-2 are NAbs.[23,24]

NAbs are a type of antibodies produced by B lymphocytes when pathogenic microorganisms invade the human body. NAbs can inhibit viral infection during the whole virus replication cycle, especially in the process of viral attachment and the entry to host cells.[25] NAbs can facilitate virus particle aggregation, reduce the number of host cells that virions attach to; degrade the virus or inhibit virus internalization through endocytosis; inhibit viral metabolic events (replication or transcription), etc,[26] among which the most important inhibitory effect is that NAbs can block the infection by interfering with the virus-receptor-binding or interaction when the virus is attaching to the target cell.[26,27] Therefore, not only the level of total antibodies, but NAbs, should be measured in clinical trials of vaccines.[14,28]

The main target of NAbs against SARS-CoV-2 is the RBD. Existing studies have shown that a batch of anti-SARS-CoV-2 mAbs isolated from patients, such as BD23, B38, H4, B5, P2B-2F6 and CB6, exert their antiviral activity via blocking the binding between the RBD and the target cell ACE2.[9-12] Several studies have showed that NAbs interacted with RBD mainly through the heavy chain of the antibody molecule.[9,29] Ju et al[9] superimposed the crystal structures of RBD-P2B-2F6 and RBD-ACE2 and found that P2B-2F6 and ACE2 have different binding angles to RBD and there were

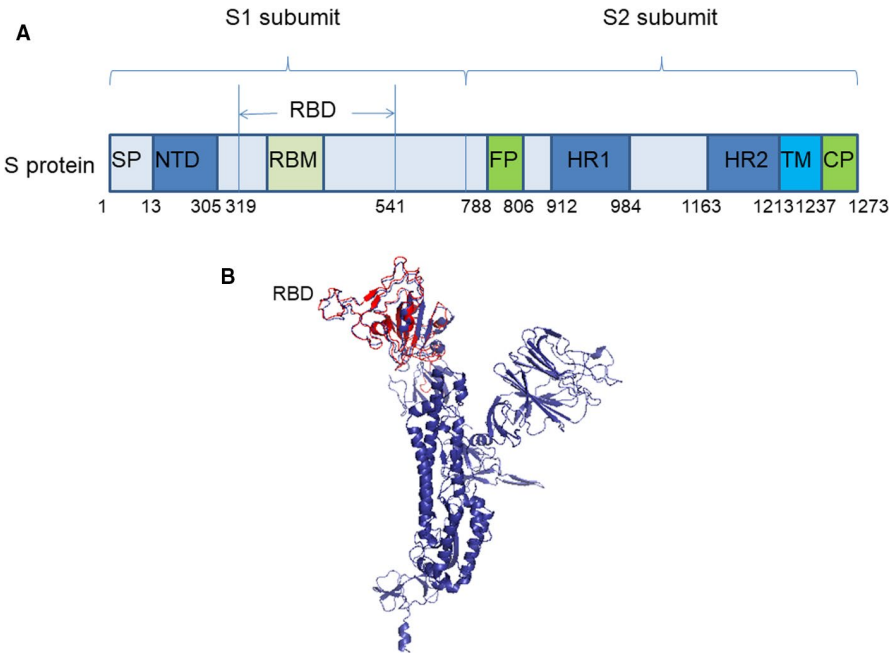

FIGURE 1 Schematic diagram of SARS-CoV-2 spike protein structure. (A) Abbreviations: fusion peptide (FP), heptad repeat region 1 (HR1), heptad repeat region 2 (HR2), receptor-binding domain (RBD), receptor-binding motif (RBM), signal peptide (SP), transmembrane region (TM) and cytoplasmic tail. (B) Overview of the crystal structure of SARS-CoV-2 spike monomer. The RBD is shown in red colour

some overlapping areas between the two structures, mainly in the light chain of P2B-2F6 and a six-residue region of ACE2. Not only are the binding affinity and sites of P2B-2F6 similar to RBD-ACE2, but also P2B-2F6 can bind to both upward and downward conformations of RBD, which has strong competitiveness, unlike ACE2 that only binds to the upper conformation of RBD. Although some other mAbs can bind to the RBD epitope of SARS-CoV-2, they do not disrupt the RBD-ACE2 interaction.[12,30] Besides, mAbs can also target non-RBD regions on the S1 or S2 protein; however, the neutralization ability of these NAbs is weaker than those that target the RBD region.[31,32]

In short, NAbs can reduce the infectivity of the virus by blocking the binding of the virus to the cell receptor or interfering with the virus fusion and are promising candidates for providing prophylactic and therapeutic protection against COVID-19.

3 | WHAT CAN WE GAIN FROM NEUTRALIZATION ASSAYS

3.1 | Understand the NAbs immune responses of infected persons

Although there have been studies on the antibody response of COVID-19 patients, the understanding of the protective immunity of SARS-CoV-2 is still limited. In some recent studies, the rapid decline of NAbs within a few weeks has been a concern, which is obvious in individuals with asymptomatic infection or mild symptoms.[33-38] There have also been reports of some cases of re-infection, increasing concerns about susceptibility to re-infection.[39-42] However, in general, antibody levels will always decrease after the acute infection period. Most of the responses of effector B cells induced by infection are short-lived, while the antibody level needs to be maintained by longer-lived plasma cells and memory B cells.[24,43-45] Therefore, in the early stage, the decline of NAbs level should not cause concern. The key point is to know what level and how long antibody titre will be stable for a long time after natural infection or vaccination by using neutralization assays, so as to prevent subsequent infection and provide references for immunization.

As shown in Table 1, many research groups have used pseudovirus or live virus neutralization assays to monitor the NAbs responses of patients infected with SARS-CoV-2. As expected, the neutralizing activity was significantly correlated with the titre of anti-S protein antibodies.[22,46,47] Moreover, the early development kinetics of NAbs are similar. Even if there is a certain degree of decline in the short term, the NAbs responses of most recovered patients can persist stably in the next few months,[33,36,37,43,48-52] and they may not need to be vaccinated in 9 months or even longer.[53] Wajnberg et al[51] investigated more than 30 000 COVID-19 patients with mild to moderate symptoms and found that the anti-SARS-CoV-2 S protein NAbs titre remained relatively stable for at least 5 months. In a recent study in Wuhan, China, blood samples were tested from approximate 9000 households in 100 local communities for three times. A total of 6.92% of the population developed antibodies against SARS-CoV-2, with only a small fraction of this population (39.8%) seroconverting to have NAbs. However, once NAbs are produced, regardless of symptoms, the titre can be relatively stable for at least 9 months.[53] Wang et al[37] found that the overall trend of NAbs titre was lower in the 7th to 10th day after the onset of symptoms, increased in the 2nd to 3rd week and then slowly decreased after reaching the peak, and the median titre decreased by about 35% after three months, and the NAbs titre of most patients remained at the middle and low level (ID_{50}, 500-999). In the study of Wu et al,[52] most of 175 patients had moderate to high antibody levels on the day of discharge (ID_{50}, 1000-2500), and the plasma of 47 patients was collected, and it was found that there was no significant difference in NAbs levels between the day of discharge and the follow-up two weeks later ($P = .25$).

Some patients in the recovery period showed no neutralizing activity, which indicates that non-neutralizing antibodies or cellular responses unrelated to antibody production may also help patients recover to a certain extent.[46,64] Moreover, the antiviral response is coordinated by humoural and cellular immunity, and stronger and lasting specific T-cell response and memory B cells can help to supplement NAbs and resist virus infection.[22,33,52,65-67]

In summary, based on the currently available data, there is no need to worry too much about rapid decline of NAbs; however, because the attenuation of NAbs is not linear, it cannot be extrapolated from the existing short-term data, so it is too early to draw a conclusion about the duration of NAbs. Therefore, it is necessary to track the NAbs levels of patients on a large scale over a longer time period.

The associations between the NAbs titres measured by neutralization assays and clinical outcomes have been explored. The results showed that the SARS-CoV-2 NAbs responses were stronger and lasted longer in patients with severe infection but appeared later than in patients with mild to moderate disease.[52,63,68,69] In contrast, asymptomatic patients have low levels of specific IgG, long time persistence of viral RNA and are unable to produce strong NAbs.[63,70] This population may still have a risk of suffering re-infection and should be considered for vaccination. The strength of humoural immune response produced by the immune system may depend on the level of virus or some inflammatory indicators during host-pathogen interaction. The NAbs titres of severe patients were clearly higher, presumably because their original viral load was higher, leading to a stronger neutralizing reaction in vivo.[52] The association between NAbs titres

TABLE 1 NAbs responses to SARS-CoV-2 infection

Sample size	NAbs titre	Detection time	Factors affecting neutralizing activity	Type of neutralization assay	Ref
173	Peak levels: 6.7%, low (ID_{50} < 500); 73.3%, medium-low (ID_{50}, 500-999); 20%, medium-high (ID_{50}, 1000-2500)	Within 3 mo after symptom appearance	IgG (r = .410), SCF (r = .616), TNF-TRAIL (r = .514), M-CSF (r = .454)	VSVΔG pseudovirus neutralization assay	[37]
149	33% of the samples NT_{50} < 50, 79% NT_{50} < 1000, and the geometric mean of NT_{50} = 121 (arithmetic mean = 714)	Average 39 d after symptom appearance	Anti-RBD and anti-S IgG antibodies, age, duration of symptoms and severity of symptoms, gender	Luciferase-HIV pseudovirus neutralization assay	[45]
14	NAT_{50}: 0-2000	NA	The number of nucleocapsid protein specific T cells (R^2 = .5771)	Luciferase-HIV pseudovirus neutralization assay	[22]
125	1200->12 800	NA	Positive correlation with age, negative correlation with IL-17A and IFN-γ concentration	Enhanced green fluorescent protein-VSV pseudovirus neutralization assay	[54]
59	NA	NA	Severity of disease	Luciferase-VSVΔG pseudovirus neutralization assay	[55]
343	NT_{50}: 20-3240	From the onset of symptoms to the 75th day	Anti-RBD IgG concentration (r = .87)	Luciferase-HIV pseudovirus neutralization assay	[56]
50	Test group: 6 (12%) had a NAbs titre of <50, 17 (34%) <450; the peak geometric mean of NAbs titre was 982 Control group: NA	>29 d	Severity of disease, fever, oxygen	Test group: luciferase-HIV pseudovirus neutralization assay Control group: MN	[57]
160	The proportion of individuals with an $ID_{50} \geq 100$ was 79%, 92% and 98% 13-20, 21-27 and 28-41 d after the onset of symptoms, respectively	41 d after symptom appearance	Dry cough, high BMI, hypertension	Luciferase-HIV pseudovirus neutralization assay	[58]
35	Test group: IC_{50} of severe cases <100-13 710 (average value 2545), non-severe cases <100-1463 (average 491) Control group: IC_{50} of severe cases is 926-30 175 (average 10 701), IC_{50} of non-severe cases <100-6884 (average value 1485)	>1 mo after symptom appearance	Disease severity, age	Test group: VSV-G pseudovirus neutralization assay Control group: true virus neutralization assay	[59]
9	IC_{50} = 307.2-5925.4, median 1483.9	>1 mo after symptom appearance	NA	Pseudovirus neutralization assay	[60]
1	ID_{50} at the 20th day = 2954, ID_{50} at the 91th day = 114	91 d after symptom appearance	NA	Pseudovirus MN	[36]
7	The IC_{100} of 5 patients was 78.8-1500 μg/mL	8-9 d after diagnosis	NA	True virus neutralization assay	[61]

(Continues)

TABLE 1 (Continued)

Sample size	NAbs titre	Detection time	Factors affecting neutralizing activity	Type of neutralization assay	Ref
41	Median plasma dilution, 202; IQR, 113-254	28-35 d after symptom appearance	cTFH1(CCR6−CXCR3+), cTFH2 (CCR6−CXCR3−)	Vero cell-MN assay	62
59	58 of 59 (98.3%) NAbs titre 4->1024	23-47 d after symptom appearance	NA	MN assay	63

Abbreviations: BMI, body mass index; HIV, human immunodeficiency virus; IC_{100}, 100% inhibitory concentrations; IC_{50}, the half inhibition concentration; ID_{50}, 50% inhibitory dose; IgG, immunoglobulin G; IQR, inter-quartile range; M-CSF, Macrophage colony-stimulating factor; MN, micro-neutralization; NA, Not Assessed; NAT_{50}, neutralizing antibody titres; NT_{50}, neutralization titre data; SCF, SKP1-cullin-1-F-box-protein; TNF-TRAIL, tumour necrosis factor alpha-related apoptosis-inducing ligand; VSV, vesicular stomatitis virus; VSVΔG, VSV lacking a G protein.

and clinical indicators or outcomes in COVID-19 patients deserves further investigation.

3.2 | Evaluation of convalescent plasma (CP) therapy, mAbs and vaccines

Before effective drugs and vaccines are developed, convalescent plasma could represent an immediate stopgap treatment against viruses. Passive immunity driven by convalescent plasma can provide NAbs that inhibit infection. In addition, other antibody-mediated pathways, such as complement activation, antibody-dependent cytotoxicity and/or phagocytosis, may also promote the therapeutic effect of convalescent plasma.[71] Patients with high NAbs titres who have recovered from COVID-19 may be a valuable source of donors for convalescent plasma. However, how high the titre of NAbs in donor plasma is, whether plasma therapy is effective or not, and whether the recipient can produce effective specific NAbs after administration, all these issues need to be evaluated.

NAbs titre is a key factor in convalescent plasma therapy. Based on previous SARS-CoV research, plasma with a titre ≥ 1:40, as evaluated by a cytopathic effect (CPE)-based neutralization test, was considered effective.[72] A study of Middle East respiratory syndrome coronavirus (MERS-CoV) infection with small sample size showed that the NAbs titre was required to exceed 1:80 to achieve effective convalescent plasma therapy.[73] For SARS-CoV-2, the US Food and Drug Administration recommends that the NAbs titre of CP should be at least 1:160, as consistent with a retrospective study,[74] emphasizing the potential advantages of using high-titre recovery plasma for early treatment.

Although several small-scale non-randomized studies have shown that CP therapy has a certain effect on reduction of viral load and improvement in clinical symptoms and radiological examinations,[68,73] some recent high-quality large-scale multicentre randomized clinical trials have shown inconsistent results. In a placebo-controlled double-blind multicentre randomized clinical trial, 228 patients with severe COVID-19 received 500 mL of convalescent plasma with 80% inhibitory concentration (IC_{80}) = 1:300, and their mortality or other clinical outcomes on the 30th day were not different from those of the control group.[75] In a randomized trial from China, which was interrupted prematurely, the benefits of CP for clinical treatment of critically ill patients were not observed.[69] However, a randomized clinical trial of CP for elderly patients with mild symptoms showed that early use of high-titre CP (IgG titre > 1:1000) could slow down the progress of COVID-19.[76] Thus, the current clinical trial data are not enough to support or oppose the use of CP.[77]

Passive antibody protection conferred by convalescent plasma can provide rapid but short-term immunity for

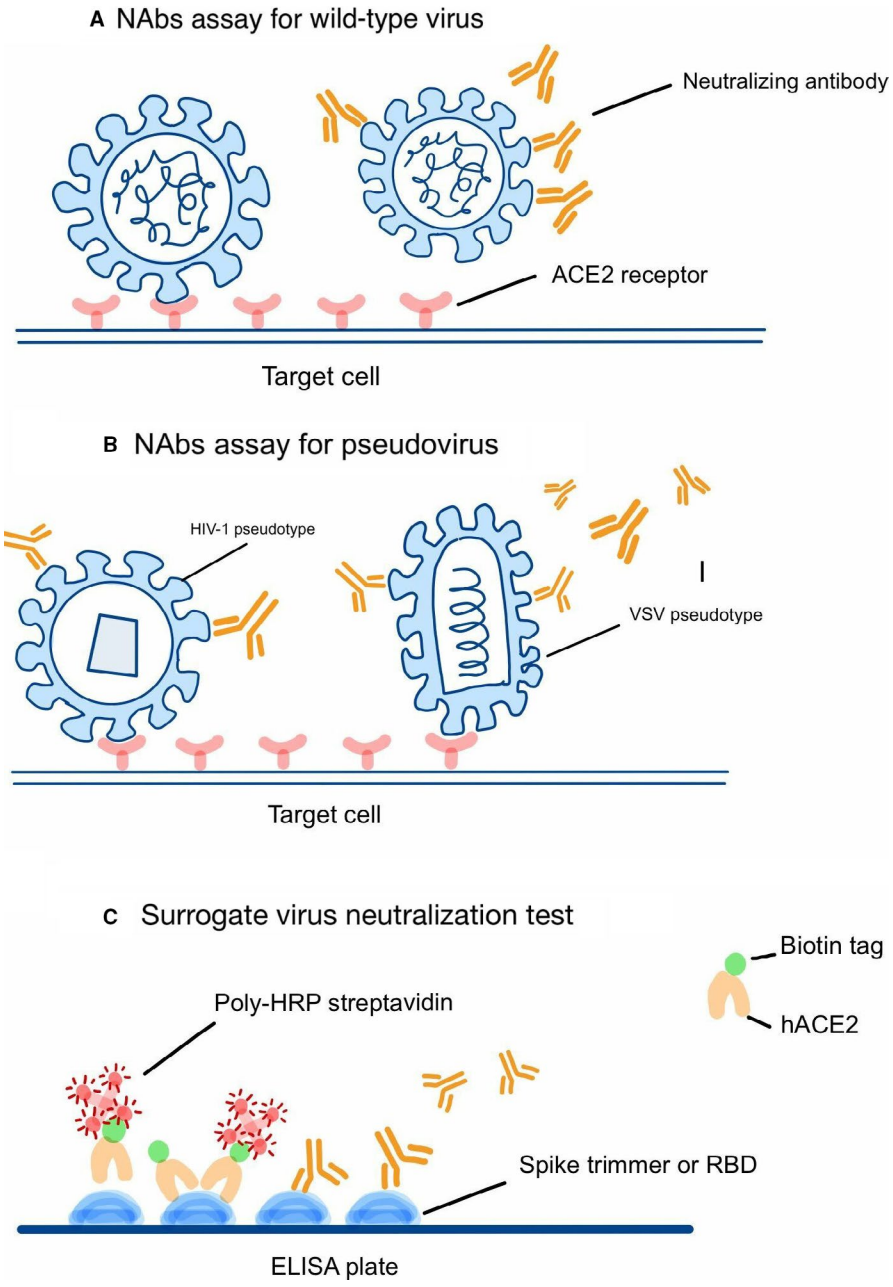

FIGURE 2 The principles of three neutralization assays. (A) Live SARS-CoVs-2 bound to NAbs lose infectivity and inhibit the appearance of CPE. (B) Pseudovirus with SARS-CoV-2 envelope. (C) Using recombinant ACE2 for the detection of spike or RBD epitopes that have not been blocked by NAbs in the OD_{450} (using poly-horseradish peroxidase (HRP)-streptavidin)

susceptible individuals, while vaccines induce long-lasting protective antiviral immunity.[78] In many vaccine trials, the titre of NAbs is usually regarded as an important evaluation endpoint.[79,80] And there are still some key questions to be answered in the development of SARS-CoV-2 vaccine, such as which antigen (epitope) will trigger powerful NAbs in most people.

Besides, we can also use the neutralization assay to do some valuable research on controlling COVID-19, for example, to describe the NAbs response of recovered patients or normal individuals after immunization, including the average titres of asymptomatic, mild and severe patients and the titres of different age groups; to study the kinetics and lifespan of NAbs; to determine the titres of protective NAbs and at what NAbs levels antiviral effects can be achieved. The answers to these questions remain unknown so far.

4 | CURRENT NEUTRALIZATION ASSAYS

4.1 | Live virus neutralization assay

Virus replication requires host cells to supply raw materials, energy and replication sites. After entering the body, the viruses are adsorbed on the surface of the target cells, and then penetrate, unshell and invade the cells to replicate, assemble, exit the infected cell and cause new infections. In the presence of NAbs, they can bind to the viruses and inhibit virus entry by preventing attachment of the virus to the cell, and as a result prevent viral replication and infection within a host (Figure 2A). In the live virus neutralization assay, the viruses and antibodies are mixed and incubated under appropriate

conditions, and then inoculated to sensitive hosts (including animals, chicken embryos and cells). Virus infection, that is the infectivity of the remaining virus to the host, is observed. In the neutralization assay in cell culture, it is mainly to observe the antibodies that can inhibit cytopathic effect or the formation of virus plaques.

Live virus neutralization assays mainly include focus-reduction neutralization test (FRNT),[81] plaque reduction neutralization test (PRNT)[82] and live virus micro-neutralization (MN) assay.[83,84] These assays can be used to detect antibodies or screen antiviral drugs that inhibit SARS-CoV-2 replication in vitro.[85]

PRNT is a traditional method and gold standard for measuring the neutralization ability against viruses. This method quantifies the number of infectious virus particles by amplifying CPE caused by a single virus into 'plaque' by staining, but the final read-out may be affected by the subjective judgement of the operator. PRNT is reliable and requires few specific reagents, but it is usually carried out in low-throughput 6 or 24 well plates(in a few cases also can be carried out in 48- and 96-well format),[50,86-88] which leads to a long time (about 4 days) for analysing samples. Therefore, although PRNT is highly sensitive, it is not efficient to be suitable for large-scale detection of NAbs.

The principle of FRNT is similar to PRNT, but in FRNT, virus is detected using specific antibodies that are conjugated to horseradish peroxidase (HRP), and the results are presented by observing the foci of SARS-CoV-2-infected cells with microanalyser. Compared with PRNT, FRNT can be performed in a 96-well plate, takes less time and requires fewer reagents and cells.[89]

For those indistinct CPE plaques caused by SARS-CoV-2, MN can be selected. Usually, cells are incubated with the immune mixture of antibody and 100 50% tissue culture infectious dose ($TCID_{50}$) virus, and results are observed under a microscope. Antibody concentration is calculated as the inhibition of the virus growth in 50% of wells by Reed-Muench method.[90] For a viral load of 100 $TCID_{50}$, a well that can be observed 'non-infected' with the naked eye almost needs to neutralize all the viruses. When the proportion of neutralized virus is equal to 0.5 $^{(1/inoculum)}$, the IC_{50} can be visually observed.[25,83,91] The purpose of MN is similar to that of PRNT, which aims to directly quantify the virus neutralization in the initial inoculation of SARS-CoV-2, but the result of MN is more sensitive than PRNT, with IC_{50} equivalent to IC_{90} of PRNT.[90]

In short, the live virus neutralization assay is an effective method to evaluate the level of NAbs.[37] Thus, it can be used as a control group in current studies to verify the experimental results. Although the live virus neutralization assay can measure the level of NAbs blocking viral infection, it is expensive and requires well-trained professionals to deal with SARS-CoV-2 in a biosafety level-3 (BSL-3) laboratory. It is time-consuming and labour-intensive, and usually takes 2-4 days to complete. Therefore, this method is not suitable for large-scale serological diagnosis and vaccine evaluation.[23]

4.2 | Pseudovirus neutralization assay

Pseudovirus (or pseudotyped virus) is a recombinant virus particle whose core skeleton and surface protein derive from a variety of viruses. The internal genes are usually changed or modified so that they lose the ability to produce surface proteins on their own; thus, additional plasmids or stable cell lines expressing surface proteins are needed when manipulating the pseudovirus.[92] In order to facilitate reading out, pseudoviruses are usually engineered to carry reporter genes encoding NanoLuc luciferase or green fluorescent protein (GFP). Due to high luminescence brightness and high sensitivity, NanoLuc luciferase reporter is especially useful for the creation of pseudovirus. However, as a shortcoming, the assays using the luciferase reporter need to lyse the cells, add a substrate to detect luminescence and as a result can suffer from high background.[93]

In neutralization assays, the selection of cell lines and virus models will have an impact on the neutralizing activity of antibodies. In most pseudovirus neutralization assays, cell lines of human and animal origin have shown high sensitivity to pseudovirus binding and entry,[94] especially Vero and Huh7 cell lines.[95,96] However, it is worth noting that SARS-CoV-2 has a poor replication effect in the Huh7 cell line in some live virus neutralization assays.[91,97] HEK293T cells transfected to express hACE2 are also used in pseudovirus neutralization assays.[98] Moreover, other factors, such as the amount of inoculation, incubation time and detection methods, will also have a certain impact on the neutralizing activity of the NAbs measured.[90]

When pseudoviruses enter the target cells, they release RNA inside the cells which are reversely transcribed into double-stranded DNA with the retroviral reverse transcriptase and integrase, followed by integrating the gene encoding the luciferase reporter carried by the pseudovirus into the genome of the target cell. The conformational structure of the pseudoviral spike proteins is highly similar to that of the natural viral proteins, which can effectively mediate viral entry into host cells.[99] However, there are inherent differences between pseudoviruses and live viruses. Pseudoviruses are only suitable for studying virus entry and the role of antibodies against the S protein. Because pseudotyped viruses do not cover other parts of the replication mechanism of SARS-CoV-2, factors such as the density and geometry of the S protein on virus particles will also affect the cell entry mechanism and the ability of antibodies to bind pseudovirus particles and neutralize virus infectivity.[100-102] After infection, the number of cells

TABLE 2 SARS-CoV-2 pseudovirus neutralization assays

Type of antibodies testing	Types of pseudovirus	Carrying reporter	Expressing envelope	Infected cells	Time	Relevance to the gold standard	Ref
mAbs304, 306, 309 and 315; COVID-19 convalescent plasma	VSV chimeric virus	eGFP reporter	$S_{\Delta 21}$	Vero-E6	About 3-4 d	$r = .9285$; $P < .001$	81
COVID-19 convalescent sera	VSV	eGFP reporter	Spike protein	Vero-E6	About 7 d	$R^2 = .76$	101
COVID-19 patients and donors' sera		Luciferase reporter	Spike protein	HEK293T	About 4 d	NA	103
COVID-19 convalescent plasma		pLenti-GFP reporter	$S_{\Delta 19}$	HEK293T	About 6 d	NA	4
Human and mice serum samples		Firefly luciferase reporter	Full-length spike protein	293T	About 4 d	NA	95
COVID-19 convalescent plasma		eGFP reporter	Full-length spike protein and $S_{\Delta 18}$	Vero-E6, BHK21- hACE2 and 293 T	About 4 d	$R^2 = .8396$, $P < .0001$	104
Human sera	Treatment group: HIV-1 control group:VSV	Firefly luciferase reporter	Full-length spike protein	HEK 293T/17 cell	5-7 d	ICC = 0.872 (95% CI 0.799-0.92), $P < .0001$	105
COVID-19 convalescent plasma	HIV-1	Firefly luciferase reporter	pcDNA3.1-SARS-CoV-2 spike protein	HEK 293T	About 6 d	$R^2 = .6931$, $P < .005$	96
COVID-19 convalescent plasma, mAbs C121, C144 and C135		Nanoluc luciferase reporter	$S_{\Delta 19}$	HEK 293T	About 4 d	NA	45
mAbs COV2-2196, COV2-2130		Nanoluc luciferase reporter	$S_{\Delta 19}$	HEK 293T	About 3 d	NA	106
COVID-19 patients' sera		pLenti-luciferase reporter	Spike protein	HEK 293T	About 5 d	NA	55
mAbs S309	MLV	Luciferase reporter	Spike protein	Vero-E6 cell or DBT cell	About 6 d	NA	107

Abbreviations: eGFP, enhanced green fluorescent protein; HIV, human immunodeficiency virus; ICC, intra-class correlation coefficient; mAbs, monoclonal antibodies; MLV, murine leukaemia virus; NA, Not Assessed; VSV, vesicular stomatitis virus.

infected by pseudoviruses is directly proportional to the expression of the reporter genes, therefore, allowing quantitative analysis by imaging, flow cytometry or NanoLuc luciferase assay.[100]

At present, several pseudovirus tools have been used for detecting Nabs against SARS-CoV-2, as shown in Table 2, such as vesicular stomatitis virus (VSV), human immunodeficiency virus-1 (HIV-1) and murine leukaemia virus (MLV). HIV-1 and VSV are commonly used vectors of pseudoviruses (Figure 2B). Replication-deficient HIV-1 pseudovirus can carry two-plasmid or three-plasmid, with the *env* gene of HIV knocked out and the *nef* gene replaced by the sequence encoding NanoLuc luciferase protein. The VSV-based pseudovirus can be engineered into two forms; one is replication-deficient VSV lacking a G protein (VSVΔG), and the other is replication-competent VSV/SARS-CoV-2 chimeric virus,[81] all aspects of the virus replication, except for receptor-binding, are mediated by the VSV viral protein. Therefore, it may have different replication kinetics from the authentic SARS-CoV-2.

Virus packaging and infection efficiency are generally the main limiting factors for high-throughput neutralization assays in vitro. Truncated virus particles are more efficient than pseudoviral particles with full-length S protein.[93,104] One advantage of the VSV pseudovirus compared to the HIV-1 pseudovirus is that the former replicates rapidly in the cell, enabling reporter gene expression to be detected within a few hours after infection.[63] At present, the results of neutralization assays using these pseudovirus models correlate well with the live virus neutralization assays, as shown in Table 2. Schmidt et al[93] tested the neutralization sensitivity of three pseudoviruses to COVID-19 convalescent plasma and found that the replication-competent VSV/SARS-CoV-2 chimeric virus was more sensitive than authentic SARS-CoV-2. And also, VSV/SARS-CoV-2 chimeric virus had the closest value of the IC_{50} to authentic SARS-CoV-2 in neutralization assays. In addition, the results of two neutralization assays with replication-competent recombinant VSV chimeric virus are highly correlated with the authentic SARS-CoV-2 neutralization assays ($r = .87$ and .93).[81,101] Several existing neutralization assays using VSV pseudovirus are also highly correlated with the results of live virus neutralization assays. The Pearson correlation coefficient r values range from .82 to .93, with all $P < .0001$.[104,108-110] The r values among neutralization assays using HIV pseudovirus and MN are not quite consistent, which range from .69 to .92 ($P < .05$).[96,105,111] It may be because HIV-1 and VSV pseudoviruses are single-cycle viruses, or their S protein density may be lower than that of authentic SARS-CoV-2. Thus, their sensitivities to neutralization, especially in weakly neutralized plasma, are slightly lower than that of authentic SARS-CoV-2.[90,93] Therefore, it is necessary to select the pseudovirus model according to the purpose of the assay. The single-cycle virus neutralization assays allow to directly read out the proportion of virus prevented from entering in a single round of infection and measure the neutralization activity of the inoculum. Replicating chimeric viruses can be used to measure the ability of NAbs to reduce the growth of virus particles or eliminate viruses. Also, the relationship between pseudovirus neutralization assay and live virus neutralization assay for SARS-CoV-2 should be established.

In summary, as the pseudoviruses are relatively safe and reliable and can be operated in the biosafety level 2 (BSL-2) laboratory, they are widely used to verify NAbs and vaccines,[112] receptor recognition[113] and virus inhibition.[114] Although they overcome the limitations of neutralization assays with live viruses, the assay results are only an approximation of authentic virus neutralization. In addition, despite pseudotype-based neutralization assays that have been used to detect entry inhibitors in vitro, they cannot capture the characteristics of antiviral activities of antibodies in vivo. If conditions permit, a live virus neutralization assay is finally required as a strong verification.

4.3 | Surrogate virus neutralization test (sVNT)

The NAbs detection methods based on cells are complex, time-consuming and require at least BSL-2 facilities. Moreover, the results of assays across laboratories are heterogeneous due to various culture conditions, virus strains and cell lines, so it is difficult to standardize these results without global protocols and standards for assays. At present, the commonly used SARS-CoV-2 ELISA usually measures one or multiple specific binding antibodies such as IgG, IgM and/or IgA. Although there is a certain relationship with NAbs, it does not specifically refer to NAbs.[115,116] An inhibition test can quickly quantify NAbs in samples, which can be used as a substitute test for cell-based neutralization assays within a certain range of use.

Most of sVNTs are based on the principle of blocking the interaction between RBD and ACE2.[87,117-119] The selected specific antigen (usually S protein or RBD, which can be biotinylated[87,118]) is coated on a plate and incubated with test serum, and then added with soluble hACE2 conjugated with HRP and its colorimetric substrate 3,3′,5,5′-Tetramethylbenzidine. Or the plate is coated with ACE2, and soluble RBD is used to compete with antibodies. However, the sensitivity of this mode may be slightly lower than that of solidified RBD-soluble hACE2.[87] The antibody that blocks RBD-ACE2 interaction can be detected by the reduction of HRP luminescence signal or surface plasmon resonance.

TABLE 3 Advantages and disadvantages of the neutralization assays

Detection method	Time to results	advantages	disadvantages
Live virus neutralization assay	2-4 d	Accuracy, gold standard	Lower safety, need to operate in BSL-3/4 laboratory, expensive and time-consuming
Pseudotype-based neutralization assay	3-4 d	More safety, can be performed in BSL-2 laboratory	Complicated procedures
Surrogate virus neutralization test (sVNT)	1-3 h	High throughput, fast and easy, no virus required	Only detect partial NAbs
High-throughput versions of assays	5-24 h	High sensitivity, fast and high throughput	Some need to operate in the BSL-3 laboratory

Abbreviations: BSL, biosafety level; NAbs, neutralizing antibodies; surrogate virus neutralization test (sVNT).

As for the extent to which sVNTs can replace the cell-based neutralization assays, it remains to be explored. At present, only a few of laboratories have verified the relevance with the live virus neutralization assay. For example, Tan et al[117] claimed that the results were R^2=0.86 between sVNT and MN. The sensitivity is over 98%, and the specificity is 100%. Abe et al[87] claimed an overall agreement between sVNT with PRNT, with $R^2 = .6$. The correlation (R^2) between sVNT and pseudovirus neutralization assays ranged from .61 to .84.[87,117-119]

However, Meyer et al.[120] evaluated the sVNT used in the study of Tan et al,[117] and the results showed that it was only moderately correlated to the live virus and pseudovirus neutralization assays (R^2 = .65 and .49, respectively). The sensitivity drops to 80.3%, while maintaining a specificity of 99.2%. This may be due to the different sample titres between the two assays, and the sensitivity of sVNT relies on antibody titres in the sample, which is positively correlated.[90,120] The expression of the final result may also affect the correlation. Meyer et al[120] use 'percentage inhibition', while the IC_{50} used by Tan can better quantify the NAbs response. In the experiment of Sholukh et al,[121] the correlation coefficient (r) was .41-.60 between fifty per cent neutralizing dilution (ND_{50}) of sVNT and cell-based neutralization assay, which was the lowest in all of its correlation analysis; however, when the result is expressed as percentage neutralization, that is, the above percentage inhibition, $r = .59$-.80.

In summary, the sVNTs do not require the use of live virus or pseudovirus, and the detection can be completed in a short time under ordinary experimental conditions. They are more efficient and safer than the above-mentioned two assays. However, they still have some limitations. They can only detect designated antibodies, such as antibodies blocking RBD, not all NAbs.[31,62,106] The synergy between NAbs is difficult to assess. The sensitivity and specificity of sVNTs are lower than cell-based neutralization assays.[121] These shortcomings make sVNTs currently only moderately used as supplementary tests for cell-based neutralization assays.

4.4 | High-throughput versions of neutralization assays

Although the traditional PRNT is considered as a 'gold standard' assay for measuring NAbs levels, it is time-consuming and labour-intensive and has low sample throughput. In recent years, sensitive high-throughput, fluorescence-based neutralization assays have developed. Muruato et al[122,123] conducted a visual fluorescence-based rapid high-throughput neutralization assay to detect the serum samples of COVID-19 patients. Firstly, the virus gene needs to be modified. In that assay, the reporter mNeonGreen gene (or luciferase and mCherry) was inserted into ORF7 of SARS-CoV-2 genome, and then the reporter viruses and serum were incubated in a high-throughput 384- or 1536- well plate. Finally, a high-content imaging reader or a microplate reader is used to detect the luminescent signal for quantitative analysis. It only needs 5-24 hours to detect hundreds of samples. The assays results are highly correlated with PRNT ($R^2 = .85$ and $R^2 = .84$).

Moreover, pseudovirus neutralization assays are easily scalable and also can be used as high-throughput versions of neutralization assays.[110,124] Tsaia et al[124] developed a SARS-CoV-2 neutralization assay based on lentivirus carrying a monomeric infrared fluorescent protein reporter which had the advantage of lower background signals caused by autofluorescence than luciferase or GFP. The assay can be performed in a 384-well plate and directly quantified by flow cytometry in a BSL-2 laboratory, supporting it as a simple, cost-effective and high-throughput version of neutralization assay used for versatile applications, especially for assessing the neutralization sensitivity of virus variants by sera from natural infection or vaccine recipients. In addition, the high-throughput version of neutralization assays is also used for influenza viruses or HIV.[125,126]

Taken together, the emerging high-throughput versions of neutralization assays have remarkable advantages for rapid

serological diagnosis and antiviral screening. Although some of them also need to be operated under BSL-3 conditions, which has certain limitations and insecurity, they are certainly faster and more sensitive than the live or pseudotyped virus neutralization assays. This provides some ideas for the development of neutralization assay in the future; that is, the neutralization assay should be aligned with the more efficient high-throughput test, and be carried out as far as possible without BSL-3, but still have a high correlation with the gold standard.

5 | CONCLUSION

NAbs titre is a key parameter for predicting immunity. Neutralization assays as a powerful tool have been used for COVID-19 diagnosis and vaccine evaluation. Nowadays, a variety of mature methods for the detection of NAbs have been applied in practice and have provided assistance to the development of vaccines and antivirals. However, most of these methods have their limitations (Table 3) and need to be continuously improved. For instance, authentic virus assay requires exposure to viruses, with safety issues. It still remains unclear whether and how the dynamic changes in the NAbs titres are correlated with clinical outcomes. Some ELISAs have been developed with the time-saving and high-throughput features, and may become potential alternatives for detecting and measuring NAbs. More convenient and visible methods, such as high-throughput neutralization assays, need to be innovated by scientists.

Moreover, the natural level of NAbs required to prevent SARS-CoV-2 infection is unknown. Precise evaluation of the protective immunity in the community, both at the individual and population levels, is crucial for guiding decision-makers to reopen the economy and society. An effective neutralization assay should be able to monitor the changes in the protective neutralization titres of COVID-19 patients and vaccinated individuals over time, as well as help to screen effective antiviral and vaccine candidates. Efforts should be made to further improve the sensitivity and specificity of the available neutralization assays as well as development of novel neutralization assays, so as to provide useful strategies for response to the emergence of the outbreaks of infectious diseases like COVID-19.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

AUTHOR CONTRIBUTIONS

Zeliang Chen had the idea for the article. Yuying Lu wrote the first draft of the manuscript. Jin Wang critically revised the workhand. Qianlin Li and Huan Hu searched for references and drew pictures. Jiahai Lu provided some revision opinions for the first draft. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

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