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REVIEW

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Viral infection neutralization tests: A focus on severe acute respiratory syndrome-coronavirus-2 with implications for convalescent plasma therapy

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Summary

Viral neutralization tests (VNTs) have long been considered old-fashioned tricks in the armamentarium of fundamental virology, with laboratory implementation for a limited array of viruses only. Nevertheless, they represent the most reliable surrogate of potency for passive immunotherapies, such as monoclonal or polyclonal antibody therapy. The recent interest around therapy with convalescent plasma or monoclonal antibodies for the Covid-19 pandemic has paralleled the revival of VNTs. We review here the available methods by dissecting variations for each fundamental component of the VNT (i.e., virus type and dose, replication-competent cell line, serum, and detection system).

KEYWORDS
convalescent plasma, high through put neutralizing antibodies, turnaround time, viral neutralization

1 | INTRODUCTION

The magnitude of neutralizing antibody (nAb) responses to SARS-CoV-2 is extremely variable, and a significant fraction of convalescent individuals have comparatively low to undetectable levels of plasma nAb.[1,2] Exact quantification of nAb has implications for studying duration of immunity (acquired either by natural infection or vaccination), and selection of convalescent plasma (CP) donors,[3] or relative potency[4] and durability[5] of monoclonal antibody therapies. In fact, neutralizing potency of sera was found to be greater in patients who went on to resolve infection, compared with those that died from Covid-19,[6] and CP therapy is more efficacious in patients receiving units with highest titres of nAb.[3]

SARS-CoV-2 Spike (S) protein is the main surface protein of SARS-CoV-2 and the target of neutralizing activity: it consists of an N-terminal S1 subunit responsible for virus—receptor binding and a C-terminal S2 subunit responsible for virus—cell membrane fusion. S1 itself consists of an N-terminal domain and a receptor-binding domain (RBD). Most coronavirus nAb target the RBD, while a few target regions in the S2 subunit or the S1/S2 proteolytic cleavage site.[7] By depleting sera of subunit-specific antibodies to determine the contribution of these individual subunits to the antigen-specific nAb response, Steffen et al. demonstrated that epitopes within RBD are the target of a majority of the nAb in the human polyclonal antibody response.[8] Barnes et al. classified nAbs into three categories[1]: VH3-53 hNAbs with short CDRH3s that block angiotensin-converting enzyme

Abbreviations: BSL, biosafety level; Covid-19, coronavirus disease 2019; CP, convalescent plasma; CPE, cytopathic effect; CCID, cell culture infectious dose; CLIA, chemiluminescence immunoassay; CMIA, chemiluminescent microparticle immunoassay; CRNT, chemiluminescence reduction neutralization test; ECDC, European Centre for Disease Prevention and Control; ECLIA, electrochemilunescence immunoassay; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; FFA, focus-forming assay; FFU, focus-forming unit; FRNT, focus neutralization reduction test; MERS-CoV, Middle East respiratory syndrome-related coronavirus; MN, microneutralization; nAb, neutralizing antibodies; PFU, plaque-forming units; PRNT, plaque reduction neutralization test; RBD, receptor-binding domain; RCCL, replication-competent cell line; RLU, relative light units; TCID, tissue culture infectious dose; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TMPRSS2, transmembrane protease, serine 2; VNT, viral neutralization test.

2 (ACE2) and bind only to up RBDs,[2] ACE2-blocking hNAbs that bind both up and down RBDs and can contact adjacent RBDs,[3] hNAbs that bind outside the ACE2 site and recognize up and down RBDs.[9]

S variants that resist commonly elicited nAb are now present at low frequencies in circulating SARS-CoV-2 populations.[10] While vaccines are still under development and reinfection from next pandemic waves is still under observation, CP donor selection represents the most urgent challenge.

To date more than 90,000 patients across the world have already been treated with CP, mostly in non-randomized studies (65,000 in the United States only thanks to expanded access programs approved by FDA[11]). The efficacy of CP therapy is believed to rely on nAb content.[3] Convalescent patients should hence be screened for the presence of nAb levels, and donations collected only from convalescent individuals with high nAb titres. In the setting of Covid-19, several regulatory authorities recommend threshold values, but none of them specifies nAb assay details that could alter test output: FDA says that 'when a measurement of nAb titres is available, we recommend nAb titres of at least 1:160. A titre of 1:80 may be considered acceptable if an alternative matched unit is not available. When the measurement of nAb titres is not available, consider storing a retention sample from the CP donation for determining antibody titres at a later date.[12] The ECDC basically endorse FDA recommendations, suggesting that immunocompromised recipients are transfused with CP units having a titre ≥1:320.[13] As previously said, those thresholds have poor meaning unless details are disclosed, making trial results poorly comparable.

Neutralizing antibody assessment has historically been performed using time-consuming and hazardous methods that required high technical skills. While high throughput platform surrogate tests having substantially shorter turnaround time and good correlations with nAb are heavily under study, old-fashioned methods remain the gold standard for exact nAb quantification. The Covid-19 pandemic has had the side benefit of expediting research on nAb testing upgrades. This manuscript reviews the principle behind classical nAb testing by dissecting each key component (as depicted in Figure 1), and the developments that have been released in the last years.

1.1 | The replication-competent cell line

The virus—serum mixture is generally added onto confluent cell monolayer. In the case of SARS-CoV-2, several cell lines naturally express high levels of ACE2: the—by far—mostly frequently used cells are African green monkey (*Chlorocebus sabaeus*) Vero E6 (a.k.a. Vero 1008, ATCC® CRL-1586™) or Vero CC-81 (a.k.a. Vero CCL-81 or Vero WHO, ATCC® CCL-81™) kidney epithelial cells. At a dose of 3×10^3, 1.5×10^4 or 3×10^4 cells/well monolayers that are 70%, 80%, and 90% confluent, respectively, are generated in 24 h with both cell lines. Vero CCL-81 result in about 2 folds higher foci formation per well relative to Vero E6, despite releasing less viral genome copies as detected by RT-quantitative polymerase chain reaction (qPCR).[14] Alternatively, human lung epithelial cells CALU-3, human hepatoma (Huh7.5 and Huh7), human gastric adenocarcinoma AGS and MKN have proven as effective as Vero cells.[14]

Mammalian cell lines not expressing human ACE2 can be transduced or plasmid-transfected with ACE2 (e.g., human lung epithelial cells A549,[15] human embryo kidney [HEK] 293T[16,17] or 293FT, or BHK21[18,19] or human connective tissue HT1080[20]). Rodent 3T3 and SHHC17 cell lines are instead not permissive.[14]

Stable introduction of the S activating Transmembrane protease, serine 2 (TMPRSS2) further enhanced susceptibility to infection by 5–10 folds.[19,21]

Schmidt et al. have reported that HT1080/ACE2cl.14 and Huh7.5 cell lines are significantly more adherent than 293T-derived cell lines and are hence recommended (for HIV-1 and vesicular stomatitis virus [VSV] pseudotype assays, respectively; see paragraph below) in high throughput situations, as great care is necessary when using 293T-derived cells whose adhesive properties during washing steps are suboptimal.[20]

1.2 | The viral challenge and virus quantification

Intact virions or several different surrogates can be used to represent the viral challenge, as detailed below.

1.2.1 | Intact virions

The challenging dose has variable amounts of virus. Virus quantity can be determined with protein assays (such as haemagglutination assays for influenza viruses, the colorimetric bicinchonic acid assay, or single radial immunodiffusion assay), plaque assays (reported as plaque-forming units [PFU] per ml), endpoint dilution assay (reported as median tissue culture infectious dose [$TCID_{50}$] or cell culture infectious dose [$CCID_{50}$], that is, the amount of virus required to kill 50% of infected host cells or to produce a cytopathic effect [CPE] in 50% of inoculated tissue culture cells).

Plaque assays have both an immunostaining variant (focus-forming assay, with virus quantity reported as focus-forming unit per ml) and, for firefly luciferase-tagged recombinant viruses,[22] a luminometric variant (with quantity reported in relative light units).[17]

More modern methods of virus quantification include transmission electron microscopy, tuneable resistive pulse sensing, flow cytometry, quantitative PCR, or ELISA: they have shorter turnaround times but do not provide information about virus viability. Hence, it is easily inferred that TCID is generally preferred to define the challenging dose,[23] and the challenging value is generally 100 $TCID_{50}$ of input virus per well,[24] presenting a difficulty for viruses which replicate to low titre in cell culture (such as the majority of recent A [H_3N_2] isolates). Assuming that the same cell system is used, that the virus forms plaques on those cells, and that no procedures are added which would inhibit plaque formation, according to Poisson distribution, 1 $TCID_{50}$ is approximatively 0.69 PFU.

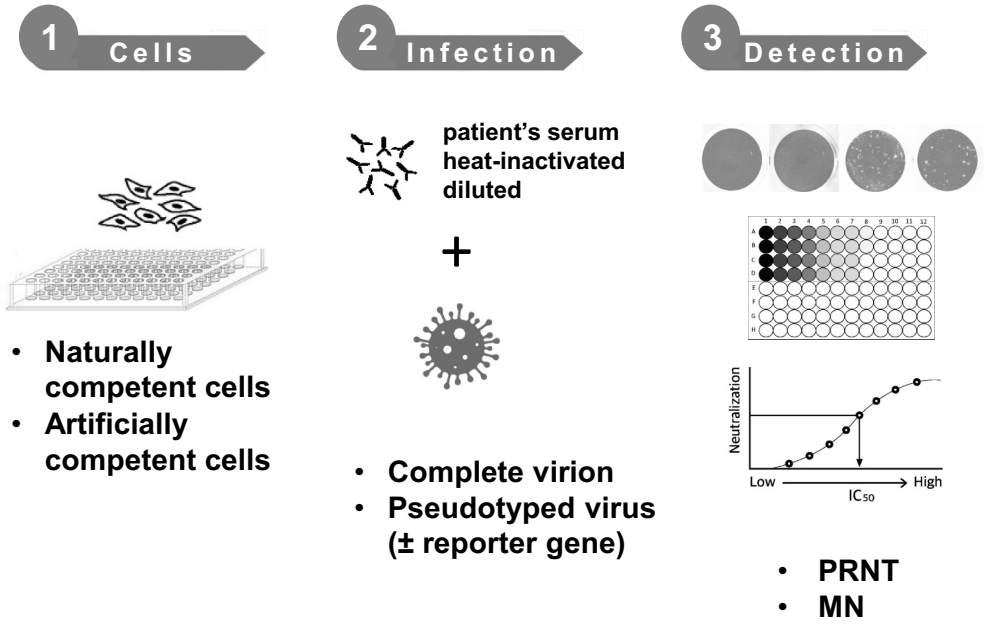

FIGURE 1 Summary of the key components of a viral neutralization assay

1.2.2 | Pseudotyped viruses

SARS-CoV-2 has been classified as a category B pathogen, but biosafety caution applicable to the category A pathogens are encouraged when handling it. Manipulation of SARS-CoV-2 should only be carried out in a biosafety level 3 (BSL3) facility with negative air pressure. Cell culture procedures are carried out in BSL2 and moved to BSL3 when ready for viral infection.[25]

Pseudotyped viruses provide a safe viral entry model because of their inability to produce infectious progeny virus. In the pseudoparticle neutralization test (ppNT; a.k.a. pseudovirion neutralization assay [PsVNA]), a single-cycle, replication-defective virus (e.g., retroviruses such as replication-defective HIV-1, third-generation lentiviral [pLV], or G-protein-deficient VSV[18,20]) is pseudotyped with the surface protein from the virus against which NAb should be measured. This activity can be run in BSL2 facilities. For SARS-CoV-2, the expression of full-length S protein was enhanced over 10-fold by deleting the last C-terminal 18[18] or 19[19,26] amino acids of the cytoplasmic tail, or by codon optimization.[17] Such methodology has previously been used to produce pseudotyped viruses for SARS-CoV-1[27] and MERS-CoV.[28] Modification of a single amino acid in the Furin cleavage site of S (R682Q) enhanced infectious particle production another 10-fold. With all enhancing elements combined, the titre of pseudotyped particles reached almost 10^6 infectious particles/ml.[19] Nevertheless, lower Spike densities in pseudotypes viruses could affect the avidity of bivalent antibodies, particularly those that are unable to engage two S-protein monomers within a single trimer and whose potency is dependent on engaging 2 adjacent trimers.

1.2.3 | VSV pseudotypes

Assembly of the VSV occurs at the plasma membrane and involves budding of virions from the cell surface. During budding, VSV acquires an envelope consisting of a lipid bilayer derived from the plasma membrane and spike proteins consisting of trimers of the VSV-glycoprotein (VSV-G). When the VSV-G is absent and the glycoprotein from a heterologous virus is complacently expressed in cells infected with recombinant vesicular stomatitis virus with protein G deletion (rVSV-dG), the glycoprotein of the heterologous virus could be assembled into the VSV membrane (30). Recently, VSVdG-luc bearing S chimeras has been used to study the cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses.[29] A PsVNA assay for SARS-CoV-2, which consists of pseudotyped VSV bearing the full-length Sprotein of SARS-CoV-2 and Huh7 cell, has been successfully tested.[30] Interestingly, the absence of proof-reading activity in VSV-L polymerase has been exploited to generate virus stocks with greater diversity (especially in S protein) than authentic SARS-CoV-2.[10] The assay provides results in 12–16 h.

Because single-cycle, replication-defective pseudotypes viruses do not allow for any viral spread and this could impact the sensitivity of the VNT, replication-competent VSV/SARS-CoV-2 chimeric viruses have been generated for usage in multicycle replication-based assays.[20]

1.2.4 | Lentiviral pseudotypes

Lentiviral pseudotype bearing the truncated spike protein of SARS-CoV-2 was also constructed and used to study the virus entry and its

immune cross-reactivity with SARS-CoV-1.[20,31,32] In one study, Moloney murine leukemia virus (MMLV) was able to pseudotype the VSV-G glycoprotein efficiently but was unable to pseudotype the SARS-CoV-2 Spike protein.[16] However, in a different study, MMLV was able to pseudotype the SARS-CoV-2 Spike protein.[24] These differences could be due to the constructs used to express the Spike protein with the former study expressing a full-length Spike protein, whilst the latter study expressed a Spike protein with a deletion in the C-terminal 19 amino acid (aa) that could aid better expression as alluded to earlier.[33] In contrast, pLV pseudotyped both glycoproteins efficiently; however, much higher titres of pLV-G particles were produced. Among all the tested mammalian cells, HEK 293Ts expressing human ACE2 (hACE2) were most efficiently transduced using the pLV-S system.[16] The neutralizing activity of both CP and human monoclonal antibodies measured using each virus correlated quantitatively with nAb measured using an authentic SARS-CoV-2 neutralization assay. The assay provides results in approximatively 48 h.

1.2.5 | Engineered viruses

Genetically engineered viruses harbouring a reporter gene that is exploited for the detection system (see dedicated chapter below) have been developed by several research groups: examples include luciferases (firefly, *Renillareniformis* or nanoluciferase[15]), green fluorescent protein[34] or mNeonGreen integration into ORF7.[35] Among luciferases, *Gaussia princeps* luciferase, the smallest known to date, is naturally secreted into mammalian cell culture media, thus avoiding the cell lysis step.[36] The results can be obtained by automatically counting positive cell number at 5–12 h after infection, making the assay convenient and high-throughput. In the case of mNeonGreen, VNT in Vero CC-81 cells have shown high correlation with VNT using intact virions.[37] When a chemiluminescent reporter is used, the assay is called chemiluminescence reduction neutralization test (CRNT).[38]

1.2.6 | Nonviral alternatives

SARS-CoV-2 Spike trimer fused to a constitutively fluorescent protein (Gamillus, isolated from *Olindias formosus*) has also been developed, providing a versatile tool for high-throughput screening and phenotypic characterization of SARS-CoV-2 entry inhibitors.[39]

The Promega HiBiT/LgBiT® system can be exploited for HiBiT-tagged VNT (hiVNT): genome-free virus-like particles are incorporated with a small luciferase peptide (HiBiT) and their entry into LgBiT-expressing target cells reconstitutes NanoLuc luciferase readily detected by a luminometer within 3 h.[40]

1.3 | The patient' serum

It is important to heat-inactivate serum or plasma samples from Covid-19 patients at 56°C for 30 min to 1 h before performing the assay to destroy residual viral particles: this step is less crucial for SARS-CoV-2 because of rare and low-titre viremia. It has been reported that complement deposition on virus envelope may lead to infection-enhancement which may mask the neutralizing effects of Abs contained in serum or plasma samples.[41] Although many laboratories start with a 1:10 dilution, for serum or plasma samples it is recommended starting with a 1:100 dilution to avoid potential impurities that may affect the sensitivity of the assay. Dulbecco's minimal essential medium (DMEM) supplemented with $NaHCO_3$, hydroxyethylpiperazine ethane sulfonic acid (HEPES) buffer, penicillin, streptomycin, and 1% foetal bovine serum, also used as cell culture medium, is typically used for the dilutions. In the case of mAbs, 10 μg is a commonly used starting dose: however, this will depend on the neutralizing capability of the mAb.

Since EDTA chelates calcium and blocks the complement cascade, EDTA-anticoagulated plasma samples cannot be used in VNT that detect CPE induced by complement-dependent cytotoxicity (e.g., PRNT). On the contrary, CRNT can instead use whole blood.[38]

1.4 | The detection system

The classical plaque reduction neutralization test (PRNT) is performed in a 24-well format in duplicate for each serum dilution. The virus-serum-cell mixture is left for 1 h at 37°C in a 5% CO incubator.[42] Then, the supernatant is removed and the cells overlaid with 1% agarose in cell culture medium (generally Minimum Essential Medium with 2% foetal bovine serum). After 3–5 days incubation at 36.5°C in a 5% CO_2, the plates are fixed and stained. Positive and negative controls and a virus back-titration are included in each assay. A microscope is used to detect plaques due to CPE on the replication-competent cell line (RCCL) monolayer.[43] Antibody titres are defined as reciprocal of the highest serum dilution that resulted in >90% ($PRNT_{90}$) or > 50% ($PRNT_{50}$) reduction in the number of plaques. When PRNT is run in 96-well plates it is called microneutralization (MN) assay (or when, pseudotyped viruses are used, pseudotype MN [pMN] assay).[44]

In the alternative focus neutralization reduction test (FRNT) cells, the development of visible spots is dependent on the time it takes for viral protein production to occur (rather than to cause lysis as in PRNT) and for infectious virus to spread to neighbouring susceptible cells. Cells (as explained above, Vero WHO cells are preferred) are hence stained after 24–48 h incubation using an antiviral serum as the primary antibody and a secondary horseradish peroxidase-labelled IgG targeting the Fc of the primary antibody.[14] The signal can be developed using a precipitate forming 3,3′,5,5′-tetramethylbenzidine substrate, and the number of infected cells per well are counted using an ELISpot analyser (e.g., ImmunoSpot® 5 Image analyser, CTL Europe GmbH[45]). Again, antibody titres are defined as reciprocal of the highest serum dilution that resulted in >90% ($FRNT_{90}$) or > 50% ($FRNT_{50}$) reduction in the number of foci. The FRNT is also amenable to a 96-well plate format.

Alternatively, the infrared staining technique (relying on secondary Ab IRDye 800CW to stain virus-infected cells, and DRAQ5TM Fluorescent Probe Solution stain the nucleus) holds the advantage of its capability to measure cell viability (in addition to measuring antiviral activity) using readings at 700 nm (800CW, measuring viral infection) and 800 nm (DRAQ5, measuring cell viability) on the Odyssey Sa Infrared Imaging System.[45]

As previously stated, when the firefly luciferase reporter gene is inserted in the viral construct, cells are lysed and assayed for luciferase expression.[17,22]

The evaluability of the PRNT technique may be further improved by overlaying the cells with cellulose or by using specific antibodies to detect remaining viral antigens in the cells.[46]

1.5 | Correlation with high-throughput serological platforms

Many studies have investigated correlations between PRNT and other serological assays. While several studies only correlated PRNT with in-house ELISAs,[21,47–50] Table 1 summarizes studies comparing PRNT with other marketed serological platforms based on enzymes (ELISA), chemiluminesce (CLIA), eletrochemiluminescence (ECLIA) or chemiluminescent microparticle immunoassays, and targeting different viral antigens in the setting of interventional[65,66] or observational[26,42,46,51–55] trials.

Although several assays correlated better than others, even the best performing serological assays had poor correlation results, implying that anti-SARS-CoV-2 nAb should be titrated using a VNT to optimize CP therapy. One major cause could be that, despite IgM, IgG, and IgA are capable of mediating neutralization, VNT titres correlated better with binding levels of IgM and IgA_1 than IgG.[67] In addition, the quaternary structure of S protein available on infected RCCL is hardly replicated by recombinant antigens bound on solid substrates: for this reasons, alternative high-throughput methods of antibody quantification based on Spike expression in cell lines are being developed,[68] but no correlation studies with VNT have.

In the largest study to date, ROC analysis showed Euroimmune anti-S1 IgG ELISA AUC outperformed 6 different in-house ELISAs and pseudotyped PRNT at predicting PRNT titres >1:100 against the native isolate. A cut-off value of 9.1 S/CO in the Euroimmune ELISA identified 65% of donations above the 1:100 nAb threshold with no false identification of donations below this nAb threshold.[42]

1.6 | PseudoNAb ACE2-competing assays

New-generation, cell-free, protein-based pseudo-nAb assays (a.k.a. surrogate virus neutralization test [sVNT]) have been developed, where cells are replaced by receptors, and the virus is replaced by surface proteins. Among them, a competitive serological assay can simultaneously determine an individual's seropositivity against the SARS-CoV-2 S protein RBD and estimate the neutralizing capacity of anti-S antibodies to block interaction with the human ACE2 required for viral entry.[69] In an ELISA-based assay, Zheng et al. presented natively-folded S protein RBD-containing antigens via avidin-biotin interactions. Sera are then supplemented with soluble ACE2-Fc to compete for RBD-binding serum antibodies, and antibody binding was quantified. A comparison of signals from untreated serum and ACE2-Fc-treated serum reveals the presence of antibodies that compete with ACE2 for RBD binding.[70] This test is performed on the same platform and in parallel with an ELISA for the detection of antibodies against the RBD.[71,72]

An entirely different approach is based on the antibody detection by agglutination PCR (ADAP) methodology. A cell-free neutralization polymerase chain reaction (PCR) assay using SARS-CoV-2 S protein and human ACE2 receptor-DNA conjugates has been developed to quantify nAbs. Briefly, the neutralizing antibodies in the specimen will engage with S1-DNA conjugate in step 1 to decrease S1-DNA binding with ACE2-DNA in step 2. Even this assay can be run in BSL2 and provide results in 2−3 h.[73]

1.7 | Clinical correlates for nAb titres

Sixty percent of 24 hospital personnel with mild Covid-19 developed nAb titres <1:20.[54] Patients with mild Covid-19 disease produced stronger nAb responses than asymptomatic individuals.[67,74] Significantly higher nAb titres were accordingly observed in patients with severe forms versus asymptomatic carriers.[21] An infection without fever had a negative predictive value of 92% for nAb titres >1:200.[56] Aziz et al. reported that a history of reduced taste or smell, fever, chills/hot flashes, pain while breathing, pain in arms/legs, as well as muscle pain and weakness were significantly associated with the presence of nAb in those with mild to moderate infection.[75] Infection with the recently described S protein variant 614G produced higher levels of nAb when compared to viruses possessing the 614D variant (6).

1.8 | nAb decline

While the overall antibody responses for other beta coronaviruses typically declines after 6–12 months,[76] SARS-CoV-specific nAb usually persist for 2 years.[77] In most of Covid-19 inpatients, nAb reached a plateau 2 weeks post-symptom onset and then declined, reaching a low or undetectable level ≥40 days post-symptom onset.[21] In less severe cases, nAb in serum reached a peak about 4 weeks after disease onset but dropped to a lower level about 6 weeks later.[78] An earlier IgG antibody response against the S2 domain of the S protein could better mediate virus neutralization, as previously suggested for SARS-CoV-1 nAb targeting the S2 domain.[26,79–81] Analyses at a 1-month interval on 31 convalescent individuals showed that RBD-specific IgG slightly decreased between 6 and 10 weeks after

TABLE 1 Summary of published correlation studies of PRNT with marketed serological assays (*r*-values indicate Spearman correlation coefficient; x: *r* not available).

					Anti-S1 RBD			Anti-S1			Anti-S1/S2 trimers	Anti-N					Anti-S + Anti-N	
					ELISA (OD ratio)						CLIA (AU/ml)	CMIA (S/CO)	ECLIA (COI)	ELISA (OD ratio)		CLIA (AU/ml)	
					Fortress	Wantai	Beckman Coulter Access®	Euroimmun		Ortho Vitros	DiaSorin	Abbott	Roche Elecsys®	Epitope EDI™	Mikrogen *recom* Well	Diazyme DZ-Lite SARS-CoV-2 IgG CLIA test	YHLO
					Total		IgG	IgG	IgA	IgG	IgG	IgG	Total	IgG	IgG	IgG	IgG
PRNT			**Ref.**	***n***				BEP2000®			LIAISON®	Architect®	Cobas®	BEP2000®		DZ-Lite 3000 Plus®	iFlash 1800
VSV-pseudotyped	Vero E6	$PRNT_{50}$ $PRNT_{90}$	[26]	181				x	x								
England/02/2020 and lentivirus pseudotyped ($r = 0.83$)	Vero E6	$PRNT_{50}$	[42]	51	0.58			0.88									
German isolate	Vero E6	$PRNT_{50}$	[46]	10 + 31				0.51/ 0.92	0.63/ 0.98								
		$PRNT_{90}$						0.85/ 0.88	0.79/ 0.93								
(HIV/NanoLuc)-SARS-CoV-2 pseudotype			[51]	30						0.75		0.72					
VSV/NG/NanoLuc-SARS-CoV-2 pseudotype										0.70		0.69					
M16502 isolate	Vero	$PRNT_{50}$	[52]	47				x			x	x	x	x	x		
n.a.	n.a	n.a	[53]	26				0.69	0.76		0.56	0.45	0.35				
Isolate	Vero E6	$PRNT_{50}$ $PRNT_{90}$	[54]	38		x		x									
SARS-CoV-2/USA-WA1/2020	Vero-E6-TMPRSS2 cells	$PRNT_{50}$	[55]	126				x	x								
Germany/BavPat1/2020	Vero	n.a.	[56]	100		0.72		0.76			x		x				

TABLE 1 (Continued)

					Anti-S1 RBD			Anti-S1			Anti-S1/S2 trimers	Anti-N				Anti-S + Anti-N	
					ELISA (OD ratio)						CLIA (AU/ml)	CMIA (S/CO)	ECLIA (COI)	ELISA (OD ratio)		CLIA (AU/ml)	
					Fortress	Wantai	Beckman Coulter Access®	Euroimmun		Ortho Vitros	DiaSorin	Abbott	Roche Elecsys®	Epitope EDI™	Mikrogen *recom* Well	Diazyme DZ-Lite SARS-CoV-2 IgG CLIA test	YHLO
					Total		IgG	IgG	IgA	IgG	IgG	IgG	Total	IgG	IgG	IgG	IgG
PRNT			**Ref.**	***n***				BEP2000®			LIAISON®	Architect®	Cobas®	BEP2000®		DZ-Lite 3000 Plus®	iFlash 1800
hCoV-19/Italy/UniSR1/2020	Vero E6	n.a.	[57]	46							x		x				
MLV-based pseudotype	ACE2-transduced HeLa	$PRNT_{50}$	[58]	164								x	x			x	
n.a.	Vero	n.a.	[59]	44								x					x
Lombardy isolate	Vero E6	$PRNT_{50}$	[60]	304							x						
SARS-CoV-2//Finland/1/2020	Vero E6	Titre >40	[61]	70				x	x		x	x					
Italian isolate	Vero E6	n.a.	[62]	18								x					
mNeonGreen SARS-CoV-2	Vero E6	$PRNT_{50}$	[63]	47			0.48				0.52		0.37				
2019-nCov/Italy-INMI1	Vero E6	$PRNT_{50}$	[64]	181				x	x								

Reference[60] also reports correlation with Acro Biotech 2019-nCoV IgG and IgM assays and with Xiamen Biotime SARS-CoV-2 IgG and IgM assays, not reported in this table.

Abbreviations: PRNT, plaque reduction neutralization test; VSV, vesicular stomatitis virus.

symptoms onset but RBD-specific IgM decreased much more abruptly. Similarly, a significant decrease in the capacity of CP to neutralize pseudo particles bearing SARS-CoV-2 S wild-type or its D614G variant has been reported.[82]

The magnitude of the nAb response is correlated with disease severity, but this does not affect the kinetics of the nAb response. Whilst some individuals with high peak ID_{50} (>10,000) maintained titres >1000 at >60 days after onset of symptoms, some with lower peak ID_{50} had titres approaching baseline within a 94 days follow-up.[83] Neutralizing activity increased with time after the onset of symptoms, reaching a peak at 31–35 days. At this point, the number of sera having nAb titres ≥160 was about 93% ($PRNT_{50}$) and 54% ($PRNT_{90}$). Sera with high SARS-CoV-2 antibody levels (≥960 in-house ELISA RBD titres) showed maximal activity, but not all high titre sera contained nAb.[48]

Sterling et al. reported that while the specific antibody response to SARS-CoV-2 included IgG, IgM, and IgA, the latter contributed to a much larger extent to VNT titre, as compared to IgG, but declined after just 1 month.[84] In 27 patients Ma et al. estimated that convalescent patients' RBD-specific IgG reach an undetectable level approximatively 273 days after hospital discharge, while the predicted decay times are 150 and 108 days for IgM and IgA, respectively.[85]

Wajnberg et al. reported that more than 90% of infected individuals with mild-to-moderate Covid-19 experience robust IgG antibody responses against S protein, based on a dataset of 19,860 individuals screened at Mount Sinai Health System in New York City, which were stable for at least a period approximating 3 months, and correlated with neutralization of authentic SARS-CoV-2.[86] In another series of 30 patients, Wang et al. reported that SARS-CoV-2-specific nAb titres were low for the first 7–10 days after symptom onset and increased after 2–3 weeks. The median peak time for nAbs was 33 days after symptom onset. nAb titres in 93.3% (28/30) of the patients declined gradually over the 3-months study period, with a median decrease of 34.8% (IQR 19.6%–42.4%). NAb titres increased over time in parallel with the rise in IgG antibody levels, correlating well at week 3 ($r = 0.41$, $p < 0.05$).[87] Similarly, Crawford et al. reported in a series of 34 patients that nAb titres declined an average of about four-fold from one to four months post-symptom onset.[88] The decline in anti-RBD antibodies was not related to the number of donations but strongly correlated with the number of days after symptoms onset ($r = 0.821$).[89]

The rapid decline in nAb may be attributed to the rapid decay of IgM in the acute phase. However, the relative contribution of IgG to nAb increased and that of IgM further decreased after 6 weeks after symptom onset.[12] Accordingly, Lei et al. reported that the titres of neutralizing antibodies in asymptomatic individuals gradually vanished in 2 months.[74]

Gontu et al. reported that robust IgM, IgG, and VNT responses to SARS-CoV-2 persist, in the aggregate, for at least 100 days post-symptom onset. However, a notable acceleration in decline in virus neutralization titres ≥160, a value suitable for CP therapy, was observed starting 60 days after first symptom onset.[90]

2 | CONCLUSIONS

The assays described above are adaptable to high-throughput and are useful tools in the evaluation of serologic immunity conferred by vaccination or prior SARS-CoV-2 infection, as well as the potency of CP or human monoclonal antibodies.

Endorsing specific protocols and disclosing them in guidelines and recommendations will largely facilitate a comparison between clinical trials.

AUTHOR CONTRIBUTIONS

Focosi Daniele designed the paper, searched relevant literature and wrote the first draft. Maggi Fabrizio analysed the literature and created figure and table. Maggi Fabrizio, Mazzetti Paola, and Pistello Mauro critically revised the manuscript.

DATA AVAILABILITY STATEMENT

This manuscript contains no original data.

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