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Review Article

Neutralization assays for SARS-CoV-2: Implications for assessment of protective efficacy of COVID-19 vaccines

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The WHO emergency use-listed (EUL) COVID-19 vaccines were developed against early strains of SARS-CoV-2. With the emergence of SARS-CoV-2 variants of concern (VOCs) - Alpha, Beta, Gamma, Delta and Omicron, it is necessary to assess the neutralizing activity of these vaccines against the VOCs. PubMed and preprint platforms were searched for literature on neutralizing activity of serum from WHO EUL vaccine recipients, against the VOCs, using appropriate search terms till November 30, 2021. Our search yielded 91 studies meeting the inclusion criteria. The analysis revealed a drop of 0-8.9-fold against Alpha variant, 0.3-42.4-fold against Beta variant, 0-13.8-fold against Gamma variant and 1.35-20-fold against Delta variant in neutralization titres of serum from the WHO EUL COVID-19 vaccine recipients, as compared to early SARS-CoV-2 isolates. The wide range of variability was due to differences in the choice of virus strains selected for neutralization assays (pseudovirus or live virus), timing of serum sample collection after the final dose of vaccine (day 0 to 8 months) and sample size (ranging from 5 to 470 vaccinees). The reasons for this variation have been discussed and the possible way forward to have uniformity across neutralization assays in different laboratories have been described, which will generate reliable data. Though *in vitro* neutralization studies are a valuable tool to estimate the performance of vaccines against the backdrop of emerging variants, the results must be interpreted with caution and corroborated with field-effectiveness studies.

Key words Alpha variant - Beta variant - Delta variant - fold drop - fold reduction - Gamma variant - neutralization - SARS-CoV-2 - variants of concern

Accelerated efforts to develop safe and effective COVID-19 vaccines were initiated globally as soon as the sequences of viral genome were released in January 2020[1]. Known platforms such as mRNA, DNA, viral vector, recombinant protein subunit, whole virion-inactivated and virus-like particles were used for expedited vaccine development[2,3]. This led to global availability of at least 10 WHO emergency use-listed (EUL) vaccines by December 2021[4], with efficacy ranging from 51 to 95 per cent[5,6]. The BNT162b2 vaccine was the first to receive the WHO EUL on December 31, 2020[7].

Most vaccines (mRNA, viral vector, DNA and protein subunit) targeted the spike protein encoded by the *S* gene of SARS-CoV-2, while the whole virion was used in the inactivated vaccines[2,8,9]. D614G was the first globally dominant mutation by June 2020 with no effect on neutralization by pre-existing antibodies[10]. The B.1.1.28 variant was reported in a limited outbreak in Denmark in September 2020 in farmed minks with a potential of mink to human transmission[10]. B.1.427/429 mutation was reported from California in June 2020 and was designated as variant of interest (VOI)[11]. In December 2020, the UK COVID-19 Genomic Consortium reported a variant of concern (VOC), 202012/01 (Alpha variant), with several distinct mutations in the spike protein[12]. By November 2021, the WHO designated five VOCs of SARS-CoV-2: Alpha: B.1.1.7 (December 2020, first reported in the UK); Beta: B.1. 351 (December 2020, first reported in South Africa); Gamma: B.1.1.28.1/P.1 (January 2021 and first reported from Brazil); Delta: B.1.617.2 (B.1.617 was reported from India and designated as VOC in March 2021; on June 1, 2021, the WHO re-designated B.1.617.2 as a VOC while B.1.617.1 was labelled as a VOI) and Omicron: B.1.529 (first reported from South Africa and designated as a VOC by the WHO on November 24, 2021)[13].

VOCs have mutations contributing to increased virus affinity to human angiotensin-converting enzyme 2 (ACE2) receptors, increased transmissibility, reduced binding of monoclonals and diminished neutralization by pre-existing antibodies. Sharing of mutations among VOCs has also been reported [N501Y increases virus binding to ACE2, while E484K/E484Q and K417T/N reduce susceptibility to neutralizing antibodies (NAbs)][14,15].

Vaccines elicit humoral and cellular immune response (CMI). All frontrunner vaccines elicited NAb titres (NTs) equal to or higher than convalescent plasma in preclinical and Phase 1/2 clinical trials[9,16-22]. Reported efficacy in clinical trials was beyond the WHO's minimum prescribed criteria of ≥50 per cent against the reference strains[23]. The early vaccines (BNT162b2 from Pfizer, mRNA-1273 from Moderna Inc. and ChAdOx1 nCoV-19 from Oxford/AstraZeneca) were based on the first few SARS-CoV-2 sequences and lacked the D614G spike mutation. CoronaVac (from SinoVac) and BBIBP-CorV (from Sinopharm) were based on early viral isolates from China, while BBV152 (BBIL) used D614G mutant strain of SARS-CoV-2[24]. Since all the VOCs have mutations in the spike protein, to which most of the NAbs are targeted, it is critical to continuously monitor the effectiveness of the existing vaccines against the already known and subsequently evolving VOCs of SARS-CoV-2. This involves laboratory studies that measure fold reduction of NTs in the serum of vaccinated individuals, T cell and B cell assays for CMI responses and also assessment of vaccine effectiveness in real-world settings. Measurement of neutralizing potential of pre-existing antibodies due to natural infection and/or vaccination is a relatively simple method for understanding the protective efficacy of vaccines against VOCs. However, results of these assays widely vary across laboratories due to several reasons. In this review article, we have compiled the available literature on the neutralizing activity of eight vaccines (seven WHO EUL vaccines till November 3, 2021, and Gam-COVID-Vac) against the VOCs of SARS-CoV-2[4]. The possible reasons for variability in results across laboratories have been discussed and means to standardize results and make them comparable have been suggested. The analysis presented in this review is not an attempt to compare results across the studies that are reviewed here. The objectives were (*i*) To understand the neutralization potential of WHO EUL COVID-19 vaccines against SARS-CoV-2 VOCs; (*ii*) highlight the inter-laboratory variability and complexities in interpreting results; and (*iii*) suggest a modality for standardizing results of these assays.

Search strategy

A review was done by searching available literature in PubMed and preprint platforms (medRxiv and bioRxiv) on neutralizing activity of serum from individuals vaccinated with the WHO EUL COVID-19 vaccines against WHO-designated SARS-CoV-2 VOCs, till November 30, 2021 using standard MeSH terms. Available data on the WHO EUL COVID-19 vaccines after completion of full vaccination schedule were included till November 30, 2021. As an exception, Gam-COVID-Vac (which has not been yet granted EUL) was included as it was already being used in the COVID-19 Vaccination Programme of India. All studies meeting the inclusion criteria from all parts of the world irrespective of their sample size and timing of serum sample collection, were considered. Studies analyzing the effect of partial immunization, mixed regimens, third (booster) dose, VOI/variant under monitoring (VUM), using surrogate virus neutralization tests and on non-WHO EUL vaccines were excluded from the analysis.

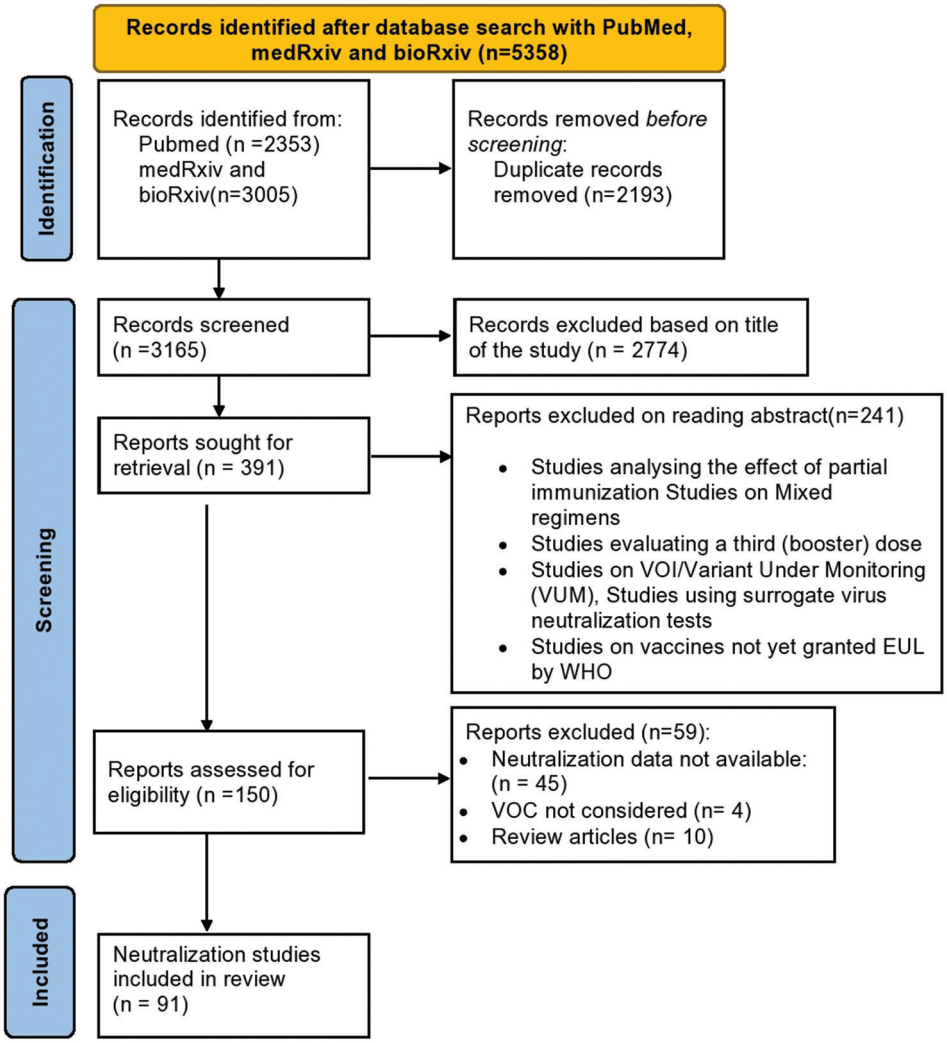

Figure. PRISMA flowchart for selection of articles. VOI, variant of interest; EUL emergency use-listed; VOC, variant of concern.

Our search yielded 91 relevant studies, describing the neutralizing potential of serum samples of individuals vaccinated with BNT162b2 (COMIRNATY, Pfizer-BioNTech), mRNA-1273 (Moderna Inc.), ChAdOx1 nCoV-19 (AZD1222/University of Oxford/AstraZeneca/Covishield), BBV152 (Covaxin, Bharat Biotech), Gam-COVID-Vac (Sputnik V, Gamaleya Institute), CoronaVac (SinoVac), BBIBP-CorV (Sinopharm) and Ad26.CoV2.S (JNJ-78436735, Janssen Pharmaceutical Company, Johnson and Johnson). These studies reported the neutralizing potential against Alpha, Beta, Gamma and Delta VOCs using live virus neutralization (LVNT) or pseudovirus neutralization (PVNT) assays.

The Omicron variant

Till the cut-off date of this study, no neutralization data on the recently designated Omicron VOC were available. However, in December 2021, reports on significantly reduced neutralization titre of pre-existing antibodies against this variant in comparison to the reference strains came into the public domain[25,26]. Individuals fully immunized with CoronaVac vaccine showed no detectable neutralization against Omicron, while 20-24 per cent of serum samples from individuals vaccinated with BNT162b2 could neutralize Omicron, with a 35.7-39.9-fold reduction in LVNT assay (serum collected 56 days after first dose)[25]. However, the Omicron data were not included in our analysis.

The flowchart[27] for study selection is depicted in the Figure.

Major findings

Majority of the studies pertained to the BNT162b2 and mRNA-1273 vaccines with Alpha and Beta being

the most studied VOCs. Initially, the WHO labelled B.1.617 as Delta VOC[28], but later only B.1.617.2 was designated as VOC, whereas B.1.617.1 became a VOI. Therefore, studies conducted before May 2021 could not differentiate between the two sub-lineages of B.1.617[29]. All studies reported NTs against the VOCs as compared to reference strains (early isolates with or without the *D614G* mutation). The neutralization activity of vaccinated serum against the D614G variant was like the D614 strain[30-32]. Observations on the neutralization activity of vaccinated serum samples against the VOCs are described in detail in Tables I-III and summarized below.

mRNA vaccines

BNT162b2: For Alpha VOC, a total of 34 studies reporting NT, by using both PVNT and LVNT assays, were analyzed. Serum samples were collected between 0 week to six months post-vaccination and NT against the Alpha VOC was checked. With PVNT and LVNT assays at 0-4 wk, fold reductions of 0-2 and 0-3.6, respectively, were reported (Table I). Within 3-12 and 4-14 wk timeframe, PVNT and LVNT assays showed fold reductions of 1-2 and 1-3, respectively (Table I). In serum collected six months post-vaccination, no reduction in NT was observed[52] (Table I).

For Beta VOC, a total of 34 studies were reviewed. In serum collected within 0-5 wk timeframe post-vaccination, the fold reductions ranged from 1.5 to 42 and 1.7 to 53 using PVNT and LVNT assays, respectively (Table I). A fold reduction ranging from two to 10.5 was observed between three and 14 wk using PVNT assay whereas it ranged between four and five using LVNT assay at nine weeks and six months, respectively (Table I).

For Gamma VOC, a total of 16 studies were reviewed. A fold reduction of 1-6.7 in serum samples collected between zero and 5 wk using PVNT assay was reported. With LVNT assay, the fold reduction was 0-6.7 and two at 1-4 and 4-14 wk, respectively (Table I).

For 16 studies with Delta VOC, at 1-5 wk timeframe of serum collection, fold reductions of 1.4-5.6 and 1.3-5.8 were reported using PVNT and LVNT assay, respectively. Further, using LVNT assay in post-vaccination serum samples collected between four to 14 wk and three months, a fold reductions of 1.7-2.2 were reported (Table I). Of these 18 studies, four studies with serum collected after dose two of BNT162b2 vaccine demonstrated a fold reduction of 1.46-2.8 against Delta AY.1 variant at 1-4 wk and 2.7 at 12-13 wk, as compared to the reference strain using PVNT assay[38,41,77,109]. Fifteen serum samples of BNT162b2-vaccinated individuals collected two or four weeks after dose two showed a drop of 1.31-fold in PVNT assay with USA WA-1/2020 as reference strain[77]. One study examined NT against VOCs using LVNT with D614G reference strain in the serum collected at 4-14 wk (median nine weeks) and reported a 1.7 and 2.3 fold drop against AY.1 and AY.4.2, respectively[64].

mRNA-1273: For the Alpha VOC, of the 13 studies reviewed, a fold reduction of 0-3 was reported in post-vaccination serum samples collected between one and 11 wk using PVNT assay and 0-2 using LVNT assay on serum collected at two weeks, whereas serum collected between 3 and 6 months showed a fold reduction of two times using both PVNT and LVNT assays (Table I).

For Beta VOC, post-vaccination serum collected between one and 16 wk demonstrated a fold reduction of 2.5-27.7 using PVNT assay. By LVNT assay at two weeks, the fold reduction ranged from 3.8 to 12.4. In serum collected between three and six months, the fold reduction ranged from seven to 9.7 and 4.3 to 6.1 using PVNT and LVNT assays, respectively (Table I).

For Gamma VOC, PVNT assay revealed a fold reduction ranging from 2.4 to 3.8 in the serum collected between one week and six months in five different studies (Table I).

For Delta VOC, 1.8-4 fold reduction was seen in serum collected between one and 11 wk using PVNT assay and three-fold reduction in samples collected between five and seven weeks using LVNT assay (Table I). For Delta AY.1, there was a reduction of 3-3.4-fold in neutralization titres 1-2 wk after dose two and 3.3-fold 11 wk after dose two, as compared to D614G using PVNT assay (Table I).

Five studies included in the analysis examined the fold reduction in NT of mRNA vaccines using PVNT and LVNT assays against Alpha, Beta, Gamma and Delta VOC (Table I). However, the type of mRNA vaccine used has not been specified.

Viral vector vaccines

ChAdOx1 nCoV-19: For the Alpha VOC, a fold reduction of 2.5-8.9 was observed in five studies, using LVNT assay in serum collected between two and five weeks (Table II). For Beta VOC, in four studies reviewed, a four-fold reduction by PVNT was reported in serum collected at two weeks, whereas 3-31.5-fold reduction in samples collected at 2-5 wk was seen by LVNT assay (Table II). For Gamma VOC, LVNT

Table I. Neutralization studies with serum samples from individuals immunized with messenger RNA COVID-19 vaccines that have obtained WHO emergency use listed

Vaccine	Variant	Neutralization method	Serum collection timing after dose 2 (weeks/months)	Number of studies	Sample size	Patient category	Reference strain used for comparison	Neutralization titre reduction (folds)	References
BNT162b2	Alpha	PVNT	0-4 wk	3	5-10	NA	D614G	0-2	33-35
				7	11-20	NA	D614G/D614	0-1.77	36-42
				3	21-40	NA	Wuhan type/ D614G	<1-2.1	43-45
				2	≥50	HCW	D614G	0-1.6	46,47
			3-12 wk	2	9 and 30	NA	D614/D614G	1.11-2.2	32,38
		LVNT	0-4 wk	2	6-10	NA	D614G	0-3.6	33,48
				2	11-19	NA	D614G	0-1.7	49,50
				8	21-40	NA (3)/ HCW (5)	D614/D614G	0-4	51-58
				5	≥40	NA (3)/ HCW (2)	D614/D614G	0-2.6	59-63
			>4-14 wk	2	108 for ICP/30 for NA	ICP (1)/ NA (1)	D614/D614G	1.1-3.4	64,65
			6 months	1	29	HCW	D614G	0	52
	Beta	PVNT	0-5 wk	3	5-10	NA	D614G	3.1-6.8	33-35
				9	11-20	NA	D614G/USA WA-1/2020, Wuhan-Hu-1	1.48-14.1	36,38,39,42,66-70
				5	30-50	NA (2)/ ICP (1)/ HCW (1)	D614G/USA WA-1/2020, Wuhan-Hu-1	5.1-42.4	32,44,68,69,46
				1	≥50	HCW	D614G	5	47
			3-14 wk	3	14-30	NA	D614g/Wuhan-Hu-1/Wild type	2-10.46	32,38,71
			3 months	1	77	ICP	D614G	2.04	72
		LVNT	1-5 wk	3	10-20	NA (1)/ HCW (2)	D614G	10.3-14	33,49,50
			2	10-20	NA (1)/ HCW (1)	Alpha	16-53	49,73	

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Vaccine	Variant	Neutralization method	Serum collection timing after dose 2 (weeks/months)	Number of studies	Sample size	Patient category	Reference strain used for comparison	Neutralization titre reduction (folds)	References
				9	20-50	NA (2)/ HCW (8)	D614/D614G/ Victoria	1.72-20	52-58,60,74
				4	>50	NA (3)/ ICP (1)	D614/D614G	4.2-8.8	59,61,62,65
			9 wk	1	30	NA	D614G	4.9	64
			6 months	1	29	HCW	D614G	4	52
	Gamma	PVNT	0-5 wk	6	15-50	NA (5)/ ICP (1)/ HCW (1)	USA WA-1/2020/D614G	1-6.7	36,39,41,44,46,68
		LVNT	1-4 wk	3	<20	NA (1)/ HCW (2)	D614/D614G/ Victoria	2.3, raised as compared to reference strain in 2 studies	48,50,75
				5	25-60	NA (3)/ HCW (2)	D614G/Victoria	0-2.6	54,56,58,60,62
				1	>100	HCW	D614G	6.7	63
			4-14 wk (median nine weeks)	1	30	NA	D614G	2	64
	Delta	PVNT	1-5 wk (not available for 1 study)	6	15-32	NA (7)/ ICP (1)	D614G/USA WA-1/2020	1.41-5.6	38,41,66,68,76,77
		LVNT	1-5 wk	2	6-10	NA (1)/ HCW (1)	D614G/USA WA-1/2020	1.35-3.3	48,78
				1	10-20	NA	D614G	2-2.6	50
				2	10-20	HCW	Alpha	2.3-8.4	73,76
				2	30-50	HCW	D614G	2.4-20	54,60
				1	30-50	HCW	D614	5	60
				2	>100	NA (1)/ ICP (1)	Wuhan wild type	2.17-5.8	59,65
			4-14 wk (average nine weeks)	1	30	NA	D614G	1.7	64
			3 months	1	77	ICP	D614G	2.19	72

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Vaccine	Variant	Neutralization method	Serum collection timing after dose 2 (weeks/months)	Number of studies	Sample size	Patient category	Reference strain used for comparison	Neutralization titre reduction (folds)	References
mRNA-1273	Alpha	PVNT	1-4 wk	7	5-30	NA	D614G/USA WA-1/2020	0-2	30,33,38,41,79-81
			3-11 wk	3	6-20	NA	Wuhan-Hu-1/ D614G	1-3	32,38,71
			1-16 wk	1	35	NA	D614	2.3	44
			3-6 months	1	24	NA	USA WA-1/2020	2	80
		LVNT	2 wk	3	12-24	NA	D614G/USA-WA1/2020	0-2	33,80,82
			3-6 months	1	24	NA	USA WA-1/2020	2	80
	Beta	PVNT	1-7 wk	7	6-26	NA	D614G/D614/ USA WA-1/2020	2.5-9.7	30-33,38,80-83
			1-16 wk	1	35	NA	D614	19.2-27.7	44
			11 wk	1	8	NA	D614G	4.6	38
			3-6 months	1	24	NA	USA WA-1/2020	7-9.7	80
		LVNT	2 wk	3	12-24	NA	D614G/USA WA-1/2020	3.8-12.4	33,80,84
			3-6 months	1	24	NA	USA WA-1/2020	4.3-6.1	80
	Gamma	PVNT	1-2 wk	4	8-24	NA	D614G/USA WA-1/2020	2.4-3.5	30,41,80,81
			1-16 wk	1	35	NA	D614	2.9	44
			6 months	1	24	NA	USA WA-1/2020	3.8	80
	Delta	PVNT	1-2 wk	3	6-14	NA	D614G	1.8-3.3	38,41,81
			11 wk	1	8	NA	D614G	4	38
		LVNT	5-7 wk	1	15	NA	USA WA-1/2020	3	78
	Alpha	PVNT	2-4 wk	2	80-197	NA(1)/ ICP(2)/ HCW(1)	D614G	1.4-3.1	85,86
		LVNT	2 wk	1	20	NA	D614	1.3	87
	Beta	PVNT	2-4 wk	3	20-197	NA(1)/ ICP(2)/ HCW(1)	D614G/D614	7.99-11.3	85-87
			2-8 wk	1	11	NA	D614G	3.4	88
		LVNT	2 wk	1	20	NA	D614	6.1	87

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Vaccine	Variant	Neutralization method	Serum collection timing after dose 2 (weeks/months)	Number of studies	Sample size	Patient category	Reference strain used for comparison	Neutralization titre reduction (folds)	References
	Gamma	PVNT	2-4 wk	3	20-197	NA(2)/ ICP(2)/ HCW(1)	D614G/D614	1-6.3	85-87
			2-8 wk	1	11	NA	D614G	1.8	87
		LVNT	2 wk	1	25	NA	D614	2.6	58
	Delta	PVNT	2-4 wk	3	20-197	NA(2)/ ICP(2)/ HCW(1)	D614G/D614	2.6-5.4	85-87
			2-8 wk	1	11	NA	D614G	1.4	88
		LVNT	2 wk	2	20	NA	D614/D614G	2-2.2	87,89

LVNT, live virus neutralization assay; PVNT, pseudovirus neutralization assay; NA, participant demographics not available/healthy adults; HCW, health-care workers; ICP, immunocompromised patients; mRNA, messenger RNA

showed a three-fold reduction in NT in a single study in serum collected between two and four weeks post-vaccination[58]. For Delta VOC in four studies, a fold reduction ranging from 4.1 to 11.3, using PVNT assay, was reported in serum collected at two weeks, whereas LVNT assay showed a fold reduction of 3.2-9 in serum collected between two and five weeks (Table II).

Ad26.COV2.S: For Alpha VOC, a total of four studies using PVNT assay showed a fold reduction of 0-4.5 and 1.3 in serum samples collected at 4-12 wk and eight months, respectively. For Beta VOC, a fold reduction of 3-13.6 was reported in serum collected between four weeks and eight months using PVNT assay, whereas 11-fold reduction was reported using LVNT in serum collected at 10 wk (Table II).

For Gamma VOC, using PVNT, a fold reduction ranging from 3.3 to 9.7 was documented in four studies, whereas a single study at eight months reported a fold reduction of 1.4. For Delta VOC, fold reductions of 1.6-7.4 in three studies and 1.7 in a single study at eight months was reported (Table II). AY.1 showed a 5.4-fold reduction in neutralization titre as compared to D614G after 11 wk of vaccination[38].

Gam-COVID-Vac: For Alpha VOC, serum collected between three and 12 wk demonstrated a fold reduction ranging from 0 to 2.9 using PVNT assay whereas LVNT assay at four weeks showed no decline in NT. For Beta VOC, serum collected between three and 12 wk demonstrated a fold reduction ranging from 6.8 to 19.2 using PVNT assay whereas LVNT assay in the serum collected at four weeks showed a 3.1-fold reduction in the three studies reviewed (Table II). For Gamma VOC, a study using serum collected at three and 12 wk reported a 13.8- and 4.2-fold decline in NT, respectively, using PVNT assay[97]. Another study that used LVNT assay reported a 2.8-fold decline in NT[98]. For Delta VOC, a study using serum collected at 3 and 12 wk reported a 5.1- and 3.4-fold decline in NT, respectively, using PVNT assay[97,98]. Another study that used LVNT assay reported a 2.5-fold decline in NT (Table II).

Inactivated whole virion vaccines

CoronaVac: Using PVNT assay in serum collected between two and three weeks, fold reduction ranged from 0.5 to 1.6; 0.3 to 5.3 and 3.9 for Alpha, Beta and Gamma VOC, respectively. LVNT assay in serum collected at two months for Alpha VOC and 2-8 wk for Gamma VOC reported 4-fold and 3.1-7.5-fold reductions in NT, respectively (Table III).

Table II. Neutralization studies with serum from individuals immunized with COVID-19 viral vector vaccines that have obtained WHO emergency use listed, and Gam-COVID-Vac

Vaccine	Variant	Neutralization method	Serum collection timing after dose 2 (weeks/months)	Number of studies	Sample size	Patient category	Reference strain used for comparison	Neutralization titre reduction (folds)	References
ChAdOx1	Alpha	LVNT	2-5 wk	5	10-108	NA (2)/ICP (1)	D614/Victoria	2.5-8.9	51,58,65,76,90
nCoV-19	Beta	PVNT	2 wk	1	13	NA	D614G	4	91
		LVNT	2-5 wk	4	13-108	NA (2)/HCW (1)/ICP (1)	D614G/Wuhan wild type/Victoria	3-31.5	58,65,74,91
	Gamma	LVNT	2-4 wk	1	25	NA	Victoria	Nearly = 3	58
	Delta	PVNT	2 wk	2	18-33	NA	Wuhan-Hu-1	4.1-11.3	69,76
		LVNT	2-5 wk	3	10-108	NA (2)/ICP (1)	Wuhan-1/D614G	3.2-9	65,76,92
Ad26.	Alpha	PVNT	4-12 wk	4	10-25	NA	D614G/D614	0-4.5	38,93-95
COV2.S			8 months	1	8	NA	D614	1.25	95
	Beta	PVNT	4-12 wk	4	10-25	NA	D614G/D614	3.6-13.6	38,93-95
		LVNT	10 wk	1	25	NA	USA WA-1/2020	10.96	93
		PVNT	8 months	1	8	NA	D614	2.96-10.96	95
	Gamma	PVNT	4-12 wks	4	10-25	NA	D614G/D614	3.3-9.71	38,93-95
			8 months	1	8	NA	D614	1.42	95
	Delta	PVNT	4-12 wk	3	10-25	NA	D614G/D614	1.6-7.4	38,93-95
			8 months	1	8	NA	D614	1.72	95
Gam-	Alpha	PVNT	3-4 wk	2	12-40	NA	D614G	0-2.5	96,97
COVID-Vac			3 months	1	40	NA	D614G	2.9	97
		LVNT	4 wk	1	27	NA	D614G	0	98
	Beta	PVNT	3-4 wk	2	12-40	NA	D614G	6.8-19.2	96,97
			3 months	1	40	NA	D614G	9.7	97
		LVNT	4 wk	1	27	NA	D614G	3.1	98
	Gamma	PVNT	3 wk	1	40	NA	D614G	13.8	97
			3 months	1	40	NA	D614G	4.2	97
		LVNT	4 wk	1	27	NA	D614G	2.8	98
	Delta	PVNT	3 wk	1	40	NA	D614G	5.1	97
			3 months	1	40	NA	D614G	3.4	97
		LVNT	4 wk	1	27	NA	D614G	2.5	98

LVNT, live virus neutralization assay; PVNT, pseudovirus neutralization assay; NA, participant demographics not available/healthy adults; HCW, healthcare workers; ICP, immunocompromised patients; mRNA, messenger RNA

Table III. Neutralization studies with serum from individuals immunized with COVID-19–inactivated whole virion vaccines that have obtained WHO emergency use list

Vaccine	Variant	Neutralization method	Serum collection timing after dose 2 (weeks/months)	Number of studies	Sample size	Patient category	Reference strain used for comparison	Neutralization titre reduction (folds)	References
CoronaVac	Alpha	PVNT	2-3 wk	3	20-93	NA (2)/HCW (1)	D614/D614G	0.5-1.6	99-101
		LVNT	2 months	1	44	HCW	D614G	4.05	102
	Beta	PVNT	2-3 wk	2	25 and 93	NA (1)/HCW (1)	D614	0.3-5.27	99,100
	Gamma	PVNT	2 wk	1	93	HCW	D614	3.92	100
		LVNT	2-8 wk	2	20 and 44	NA (1)/HCW (1)	D614G	3.1-7.51	102,103
BBV152	Alpha	LVNT	4 wk	1	38	NA	D614G	0.8	104
	Beta	LVNT	4 wk	1	17	NA	D614G	3	105
	Gamma	LVNT	8 wk	1	42	NA	D614G	1.92	106
	Delta	LVNT	2.5-22 wk	2	17 and 42	NA	D614G	1.49-2.7	105,107
	Delta AY.1	LVNT	8 wk	1	42	NA	D614G	1.88	107
BBIBP-CorV	Alpha	PVNT	2-4 wk	2	25 and 470	NA (1)/HCW (1)	D614	1.4-2.2	99,108
	Beta	PVNT	2-4 wk	2	25 and 470	NA (1)/HCW (1)	D614	2.5-4.6	99,108
	Gamma	PVNT	4 wk	1	470	HCW	Wuhan reference strain	1.9	108

Number of studies in the same category on different cohorts is indicated in brackets, Age wise classification of patient data was not done as the patient cohorts were of varied ages, even the healthy adult cohorts. USA WA-1/2020 is the first SARS-CoV-2 isolated in the United States. Victoria is the first SARS-CoV-2 isolated in Australia. LVNT, live virus neutralization assay; PVNT, pseudovirus neutralization assay; NA, participant demographics not available/healthy adults; HCW, health care workers; ICP, immunocompromised patients; mRNA, messenger RNA

BBV152: A total of four studies using LVNT assay were reviewed. In serum collected at four weeks, a 0.8- and 3-fold reduction in NT was reported against Alpha and Beta VOCs, respectively[104,105]. In serum collected at eight weeks, a fold reduction of 1.9 was reported against the Gamma and Delta AY.1 VOCs[106,107]. For Delta VOC, serum collected between 2.5 and 22 wk showed a 1.5-2.7-fold reduction in NT[105,107] (Table III).

BBIBP-CorV: For serum collected between two and four weeks, fold reductions of 1.4-2.2; 2.5-4.6 and 1.9 against Alpha, Beta and Gamma VOC, respectively, were documented using PVNT assay in two different studies (Table III)[99,108].

In addition, some studies examined the effects of only key mutations in the VOCs on immune evasion. Most of these studies used PVNT with standalone or a combination of N501Y, E484K, K417N mutations against serum of individuals vaccinated with BBIBP-CorV, Ad26.COV2.S, BNT162b2, mRNA-1273, ChAdOx1 nCoV-19 and CoronaVac vaccines[42,110-118]. Results across laboratories for the same and different vaccines reported wide variations. A proportion of serum samples in various studies did not elicit any neutralization against the VOCs[44,74]. Such results have been excluded from our analysis and fold reduction in NT reported here is based on the serum samples that generated NAb across different studies. Overall, a maximum fold reduction in NT was reported for Beta VOC with all three different classes of vaccines analyzed by both PVNT and LVNT assays. However, wide variations were observed across studies, thus highlighting the need for a standardized framework for interpreting results.

Discussion & conclusion

COVID-19 vaccine efficacy may be affected by the SARS-CoV-2 strains as well as platforms chosen for vaccine development, thereby making comparison difficult. mRNA vaccines and Ad26.COV2.S have more stable S protein configuration than the viral vector vaccine ChAdOx1 nCoV-19 or beta-propiolactone treated inactivated vaccines, thus leading to more NAbs in the former group[24,119]. As per our analysis, Beta variant was found to be the most worrisome in terms of NAbs, as evidenced by poor results obtained with *in vitro* studies[33,44,49,50]. This can also be corroborated in real-world settings with the efficacy of the ChAdOx1 nCoV-19 vaccine dropping to 10.4 per cent in its South Africa clinical trial as compared to 70.4 per cent in the UK trial[91,120].

The *in vitro* neutralization studies judging the efficacy of WHO EUL vaccines against VOCs have reported varied results with different neutralization methods. Therefore, it is imperative to carefully interpret these results keeping in mind the following considerations:

***Type of neutralization assay*:** The current gold standard PRNT assay is time-consuming and labour intensive. Therefore, focus reduction neutralization and microneutralization test have been also tried for SARS-CoV-2 neutralization assays, which have a comparatively higher throughput, and require lesser sample volume and reagents. However, an inherent variability across these tests exists, necessitating their normalization against the gold standard test[121-123]. Further, these assays need containment facilities (BSL-3) and trained staff.

On the other hand, PVNT assays require only a BSL-2 facility and are quicker and easier to interpret as compared to LVNT. However, recombinant viruses are engineered with key spike protein mutations singly or in combination and may not represent the prototype strain. Type of pseudovirus backbone may also impact the results. Neutralization is also a reflection of stearic hindrance (morphology of virus), and hence, LVNT and PVNT assays may yield different assessments. Pattern of S protein distribution on the pseudovirion might not reflect the natural state of S protein or its density on SARS-CoV-2 virus particles. In addition, pseudoviruses may only replicate for a single round and may not infect the cells with the same efficiency as their wild-type counterpart[124], thus making it difficult to compare pseudovirus assays across laboratories.

Even in the same laboratory, results of the same vaccine have differed using PVNT assay with different key mutations[125]. Results using LVNT and PVNT assays for the same vaccine and VOCs have also differed within the same laboratory[33]. The BNT162b2 vaccine showed up to 42.4-fold reduction in neutralization against the Beta VOC with PVNT assay, while with LVNT assay, a maximum drop of 10.3-fold was reported as compared to the reference strains[44]. Similar discrepancy in results was observed with the mRNA-1273[80]. Thus, it is necessary to validate pseudovirus neutralizing assays with results from live virus assays. In the absence of a uniformly accepted protocol, neutralization assays have to be interpreted and compared with each other with extreme care.

Initial neutralization titres

Although various studies have reported reduction in neutralization capacity of vaccinated serum against VOCs, the reduced titres may still be adequate to effectively neutralize the virus, if the post-vaccination titres were higher as compared to the convalescent plasma collected at similar time interval[126]. However, it is not possible to make these assumptions as knowledge related to correlates of protection of SARS-CoV-2 vaccines and the cut-off titres which may be considered sufficient for vaccine response, is still evolving. A study on six previously infected healthcare workers vaccinated with one dose of BNT162b2 showed very high NTs after one dose (GMT 9195 for reference strains, 8192 for Alpha variant, 1625 for Beta variant and 2896 for Gamma variant) as compared to convalescent plasma (GMT 456.1 against reference strains, 256.0 against Alpha variant, 8.00 against Beta variant and 71.46 against Gamma variant)[126]. A drop in neutralization titres against VOCs may still offer protection if the post-vaccination neutralization titres are comparatively higher.

***Memory cell response*:** Decaying NTs have been adopted as a convenient method to assess the effectiveness of COVID-19 vaccines against VOCs (Tables I-III). However, other components of the immune system should not be overlooked. Turner *et al*[127] have studied memory B cell response in individuals following COVID-19 infection and vaccination and have highlighted the protective function of long-lived plasma cells in the bone marrow. In their study, though anti-spike antibodies declined over a period of 11 months in convalescent individuals, SARS-CoV-2–specific plasma cells persisted in the bone marrow[127]. They also showed that mRNA vaccines induced persistent antigen-specific B cell response in the lymph nodes for at least 12 wk after secondary immunization[128]. Andreano *et al*[129] compared memory cell response in five individuals previously infected with COVID-19 and five COVID-19 naive vaccinated (BNT162b2) persons, wherein the naive vaccinees produced protective memory B cells against the Alpha, Beta and Gamma VOCs, though this was reduced as compared to the previously infected vaccines. Limited evidence suggests the cross-protective role of memory B cells against SARS-CoV-2 VOCs in both COVID-19–vaccinated naïve and previously infected individuals, with more pronounced effect in previously infected vaccinees.

***T cell response*:** Studies suggest that T cells recognize conserved regions in the spike protein, with CD8+ cells recognizing highly conserved regions as compared to CD4+ T cells[36,51,130]. There is 96 per cent identity among the CD8+ T-cell epitopes in the three VOCs Alpha, Beta and Gamma[130], which enables recognition of VOCs and maintains effectiveness of vaccines against VOCs[36,51,130]. SARS-CoV-2 S-protein specific CD4+ T cell response remains unaffected by its mutations[53]. Robust but reduced CMI response has been reported against SARS-CoV-2 VOCs as compared to the reference strains[131]. Data also suggest that vaccines elicit good and cross-protective T cell response against VOCs[132,133]. Cell-mediated immunity is a relatively conserved and an equally important protective arm which offers cross-protection to SARS-CoV-2 VOCs.

***Timing of serum collection*:** In various studies, serum samples were collected post-vaccination at different time points between day 0 and eight months, leading to a wide variation in results across different studies where the same vaccine was examined against a particular VOC[32,44,46,68,69]. This may be possible due to the difference in quality and strength of the immune response at the starting point. Serum samples collected at the time of administration of the second vaccine dose will have reduced NAbs and will elicit poor response in *in vitro* assays[43]. Similarly, a serum sample collected at eight months post-vaccination will show waning immune response[97]. Further, some individuals may elicit delayed immune response[134]. It is noteworthy that studies which tracked NAb response over long periods of time (six months and above) reported lower neutralizing titres against the reference strains with the passage of time and less pronounced NT reduction against the VOCs as compared to the reference strains[52,95]. Serum samples should be collected at optimal time points in fully vaccinated individuals to ensure that there is adequate NAbs, which may be representative of the immune response following vaccination.

***Sample size*:** The sample size in some studies was as low as <10 individuals[30,38,48,81,94,86,120], and therefore, such studies are less likely to yield reliable information and meaningful conclusions. It is important to include a statistically appropriate sample size with logical assumptions for reliable estimates with narrow confidence intervals and good precision.

***Possible means of standardizing the results*:** Any new neutralization assay must be validated as per the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines by an independent

laboratory with no conflict of interest[135,136]. To express SARS-CoV-2 neutralization results in a standard format, it is desirable that all neutralization titres are expressed in terms of international units in research publications as well as in data submitted to regulatory authorities. This may be achieved by: (*i*) Using the WHO International Standard (NIBSC 20/136) for anti-SARS-CoV-2 immunoglobulin[137]. This has an assigned unitage of 250 International Units (IU) per ampoule of NAb and is validated for use in neutralization assays. NAb assays must be run side-by-side with this standard and results should be adjusted to ensure comparability across assays; and (*ii*) Using Research reagent 20/130[137]. In case of non-availability of the international standard, a relatively less-characterized reference can be used, which may be subsequently validated against the international standard. Data obtained from assays using such reference serum can be compared among laboratories, and the results may be retrospectively standardized against the international standard. An example is the convalescent plasma obtained from a COVID-19 recovered individual (Research Reagent 20/130) and made available as a reference standard.

To better define the efficacy of COVID-19 vaccines, it will be useful to have universally accepted correlates of protection. A modelling study estimated 50 per cent protective neutralization level in immunized serum corresponding to 20.2 per cent of mean convalescent level, equivalent to an *in vitro* neutralization titre of 1:10 to 1:30 or 54 IU/ml approximately[138]. However, internationally acceptable protective titres are yet to be defined by the WHO.

The analysis in this review had limitations as our search was restricted to PubMed, medRxiv and bioRxiv. The studies included were conducted for WHO EUL COVID-19 vaccines in different locations, individuals, age groups and using varying assay methodologies. We could not adjust for confounders and compare results across studies. Studies on mixed vaccine regimens, booster doses, VOI/VUM of SARS-CoV-2, those with surrogate virus neutralization assays or non-WHO EUL vaccine candidates (other than Gam-COVID-Vac) were not included.

To sum up, *in vitro* neutralization assays offer a quick, convenient and useful tool to assess the overall performance of vaccines against SARS-CoV-2 VOCs. However, variability in results of these assays across the world makes these incomparable. The vital considerations for deciphering the results of *in vitro* neutralization assays in the context of COVID-19 vaccines have been discussed, and the importance of using international reference standards to adjust against confounders and make different assays comparable has also been described. This review also highlights that *in vitro* studies may not accurately reflect the *in vivo* response; therefore, wherever feasible, field-effectiveness studies must be undertaken to understand the real-world performance of vaccines. The laboratory and field data must be analyzed comprehensively for decision-making and prioritization of vaccines against the backdrop of emerging variants of SARS-CoV-2.

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