



Sera of patients infected by earlier lineages of SARS-CoV-2 are capable to neutralize later emerged variants of concern

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Abstract

Serum samples of 20 hospitalized coronavirus disease 2019 (COVID-19) patients from Brazil who were infected by the earlier severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lineages B.1.1.28 and B.1.1.33, and by the variant of concern (VOC) Gamma (P.1) were tested by plaque reduction neutralization test (PRNT₉₀) with wild isolates of a panel of SARS-CoV-2 lineages, including B.1, Zeta, N.10, and the VOCs Gamma, Alpha, and Delta that emerged in different timeframes of the pandemic. The main objective of this study was to evaluate if the serum of patients infected by earlier lineages was capable to neutralize later emerged VOCs. We also evaluated if the 4-fold difference in PRNT₉₀ titers is a reliable seropositivity criterion to distinguish infections caused by different SARS-CoV-2 lineages. Sera collected between May 2020 and August 2021 from the day of admittance to the hospital to 21 days after diagnostic of patients infected by the two earlier lineages B.1.1.28 and B.1.1.33 presented neutralizing capacity for all challenged VOCs, including Gamma and Delta. Among all variants tested, Delta and N.10 presented the lowest geometric mean of neutralizing antibody titers, and B.1.1.7, presented the highest titers. Four patients infected with Gamma, that emerged in December 2020, presented neutralizing antibodies for B.1, B.1.1.33, and B.1.1.28, its ancestor lineage. All of them had neutralizing antibodies under the level of detection for the VOC Delta. Patients infected by B.1.1.28 presented very similar geometric mean of neutralizing antibody titers for both B.1.1.33 and B.1.1.28. Findings presented here indicate that most patients infected in early stages of COVID-19 pandemic presented neutralizing antibodies capable to neutralize wild types of all later emerged VOCs in Brazil, and that the 4-fold difference in PRNT₉₀ titers is not reliable to distinguish humoral response among different SARS-CoV-2 lineages.

Keywords: humoral; immune; Brazil; PRNT; SARS-CoV-2; variants

Introduction

The ongoing pandemic of coronavirus disease 2019 (COVID-19) is a major healthcare threat worldwide. While viral RNA-based testing for acute infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the current standard, surveying antibodies is important to determine the past exposure [1]. Despite the relationship between humoral response and clinical protection from SARS-CoV-2 infection remains not fully understood, some studies have confirmed neutralizing antibodies as an immune correlate of protection [2]. The humoral immune response can block infection through neutralizing antibodies, which bind the virus in a manner that prevents host cell infection [3]. The host humoral response against SARS-CoV-2, including IgA, IgM, and IgG response, has been examined mostly by ELISA-based assays using recombinant viral nucleocapsid protein or pseudovirus-based neutralization assays [4]. Coronavirus

infections typically induce neutralizing antibody responses, and virus neutralization assays performed on cell cultures, as plaque reduction neutralization test (PRNT), are considered as gold standard for serological testing and determining immune protection [1]. Although antiviral T cell certainly contribute some degree of protection, strong evidence of a protective role for neutralizing serum antibodies synthesized by B cell memory exists [5]. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection [2].

Since the pandemic began in China in December 2019, thousands of SARS-CoV-2 lineages have emerged worldwide [6]. The variants that presented increased transmissibility, virulence, and decreased response to available diagnostics, vaccines, and therapeutics were defined by the World Health Organization as the variant of concern (VOC) [7]. Following the upsurge of variants,

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several reinfection cases started to be reported worldwide raising questions about the efficiency of humoral response mounted after primary infections to prevent a secondary infection by SARS-CoV-2 [8]. In fact, some recent studies have experimentally demonstrated that monoclonal antibodies, convalescent plasma of individuals infected by earlier lineages, and serum collected from vaccines have a reduced neutralizing capacity when challenged with the recently emerged VOCs of SARS-CoV-2 [9–11].

The cross-reactivity of humoral responses among all different lineages of SARS-CoV-2 including VOCs, as well as the potential use of specific serological methods as PRNT to distinguish lineage infections remain unclear. For instance, monotypic reactions or 4-fold difference in PRNT titers is commonly used to distinguish exposure to closely related flaviviruses and its serotypes [12]. If PRNT titers can distinguish exposure to different SARS-CoV-2 lineages remains unknown. Several lineages of SARS-CoV-2 have been reported in Brazil by the consortium COVID-19 Fiocruz Genomics Surveillance Network of the Brazilian Ministry of Health (<http://www.genomahcov.fiocruz.br/dashboard/>). Among the most important lineages detected in the country were the VOCs Gamma (also referred as P.1), Delta (B.1.617), Alpha (B.1.1.7), and Omicron (B.1.1.529), as well as the Zeta (P.2), B.1, B.1.1.28, B.1.1.33, and N.10. The main objectives of this study were to assess the capacity of sera of COVID-19 patients infected by earlier lineages of SARS-CoV-2 to neutralize later emerged VOCs, and also to evaluate if the PRNT₉₀ is a reliable serologic method to distinguish infections caused by different SARS-CoV-2 lineages.

Materials and methods

Case description

Swab and serum samples were periodically collected from over 70 hospitalized COVID-19 patients that were admitted between May 2020 and August 2021 to the COVID-19 Hospital of the National Institute of Infectious Diseases, Rio de Janeiro, Brazil. Samples were weekly collected from the day of admittance up to 21 days after diagnostic. The index cases were patients aged 18 years and older with no history of vaccination at the time of sampling. All patients had SARS-CoV-2 infection confirmed by real-time reverse transcription polymerase chain reaction (RT-qPCR) in respiratory samples, which consisted of a combination of two nasopharyngeal swabs and one oropharyngeal swab collected in 3 mL of viral transportation medium.

RT-qPCR for SARS-CoV-2

RNA was extracted from respiratory samples using Chemagic Viral DNA/RNA 300 kit H96 in a Janus G3 automated workstation (Perkin Elmer, www.chemagen.com). SARS-CoV-2 was detected by RT-qPCR assays targeting the viral gene E. Reactions were performed using the Kit Molecular SARS-CoV-2 (E/RP) (Bio-Manguinhos/IBMP, Brazil).

Whole-genome sequencing

Positive samples eligible for whole-genome sequencing had RNA extracted using the QIAamp Viral RNA Mini Kit (QIAGEN) or using Chemagic Viral DNA/RNA 300 kit H96 in a Janus G3 automated workstation (Perkin Elmer, www.chemagen.com). The SARS-CoV-2 genomes were recovered by amplification of long segments (2 kb), according to a protocol developed by COVID-19 Fiocruz Genomic Surveillance Network to recover high-quality genomes (Resende, unpublished data, doi.org/10.1101/2020.04.30.069039). Segment libraries were then sequenced in Illumina MiSeq. The

FASTQ reads obtained were imported into the CLC Genomics Workbench version 20.0.4 (QIAGEN), trimmed, and mapped against the reference sequence hCoV-19/Wuhan/WIV04/2019 (GISAID access number EPI_ISL_402124) to obtain the final genome consensus. The SARS-CoV-2 lineage characterization was performed by Pango Network [13]. All genomic and epidemiological data associated were uploaded at the EpiCoV database in the GISAID (www.gisaid.org).

Isolation of SARS-CoV-2 reference lineages used for PRNT

As part of the Brazil's Surveillance Network for Respiratory Illnesses, respiratory samples collected in sentinel units located in different states of the country are routinely sent to the Reference Laboratory. Eligible samples that tested positive for different lineages of SARS-CoV-2 were submitted to virus isolation in Vero E6 or Vero CCL-81 cells at a Biosafety level 3 Laboratory, as previously described [13]. Briefly, 200 μ L of each respiratory sample was inoculated in cell cultures, which were then inspected daily for cytopathic effect (CPE), up to 4 days. For each sample, isolation was attempted in a maximum of three consecutive blind passages. Overall, in positive cultures, CPE started on second day post-infection and viral harvest was performed at the fourth day post-infection. When CPE was observed, culture supernatants were aliquoted in working stocks and an aliquot submitted to RT-qPCR followed by nucleotide sequencing for lineage confirmation. Once confirmed, the consensus sequences were deposited at the EpiCoV data base on GISAID (www.gisaid.org), and one representative of each SARS-CoV-2 lineage sequentially titrated by lysis plaque assay, to form a lineage bank with reference isolates.

Subsets of serum samples of hospitalized patients that were positive for different lineages of SARS-CoV-2 were selected for PRNT. For the patients who had several blood samples collected during hospitalization, the last samples were preferred. Sera were heat-inactivated at 56°C for 30 min to inactivate the complement system. Heat-inactivated serum samples were subjected to PRNT₉₀ in Vero cells (ATCC, CCL 81) maintained in cell culture medium supplemented with fetal bovine serum, sodium bicarbonate, antibiotics, and incubated in 5% CO₂ atmosphere at 37°C. Briefly, inactivated aliquots were screened at a single dilution of 1:10 in 2–3-day-old Vero CCL-81 cells seeded in six-well plates. Exceptionally, samples with low volume were screened at 1:20. Samples that were reactive for SARS-CoV-2 were then tested in duplicates in serial 2-fold dilutions that ranged from 1:10 to up to 1:320 for their ability to neutralize 50–80 plaques forming units (PFUs) by each one of five infectious SARS-CoV-2 reference lineages isolates. The panel of reference isolates used for PRNT included the lineages B.1 (hCoV-19/Brazil/RJ-FIOCRUZ-314/2020, GISAID accession number EPI_ISL_414045), B.1.1.28 (hCoV-19/Brazil/AL-FIOCRUZ-33444-1P/2020, EPI_ISL_2645638), B.1.1.33 (hCoV-19/Brazil/RJ-FIOCRUZ-20136-1P/2020, EPI_ISL_1181430), Zeta (hCoV-19/Brazil/PB-FIOCRUZ-33096-1P/2020, EPI_ISL_1402429), N.10 (hCoV-19/Brazil/MA-FIOCRUZ-6871-1P/2021, EPI_ISL_3828018), and the VOCs Gamma (hCoV-19/Brazil/AM-FIOCRUZ-3521-1P/2021, EPI_ISL_1402431), Alpha (hCoV-19/Brazil/RJ-FIOCRUZ-2624-1P/2021, EPI_ISL_1402430), and Delta (hCoV-19/Brazil/MA-FIOCRUZ-25688-2P/2021, EPI_ISL_2645417). After 48 h of incubation, plates were overlaid with neutral red solution, and after 72 h, PFUs were visualized and counted through a transilluminator. Serum samples were considered reactive to SARS-CoV-2 when a serum dilution of at least 1:10 reduced no <90% of the PFU of SARS-CoV-2, as previously reported [15]. Serum samples that presented PRNT₉₀ titers \geq 320 and had enough volume available were retested

in higher dilutions to reach endpoint titers. Serum samples were considered seropositive to a specific lineage of SARS-CoV-2 when it had PRNT₉₀ titer of at least 10 and were seronegative for all other lineages in monotypic reactions. Additionally, samples that were reactive for a lineage and its reciprocal neutralizing antibody titer was at least 4-fold greater than what was observed for the other tested lineages, were also considered seropositive in heterologous reactions. Specific neutralizing antibodies were titred by PRNT₉₀ and the geometric mean was calculated for comparative analysis among the different lineages of SARS-CoV-2.

Statistical analysis

In each group of patients, the neutralizing antibody titers detected against the originally infecting SARS-CoV-2 lineage were compared with antibody titers elicited to other lineages by means of two-tailed paired t-tests, at 95% confidence level. Analysis was performed in GraphPad Prism 9.4.1 (GraphPad Software, LLC, San Diego, CA, USA).

Ethics

This study was approved by the Ethics Committee of the Fundação Oswaldo Cruz (CAAE 68118417.6.0000.5248) and the Ethics Committee of the National Institute of Infectious Diseases (CAAE 32449420.4.1001.5262). All patients entering the study were required to read and sign an informed consent form.

Results

From a population of patients recruited from May 2020 to August 2021, we were able to obtain a panel of 20 sera of individuals infected by SARS-CoV-2 lineages B.1.1.28, B.1.1.33, the two most common lineages in early pandemic [16] and Gamma, the first VOC to become prevalent in Brazil [17]. All 20 patients had detectable PRNT₉₀ titers for at least one of the eight lineages tested. Of these 20 serum samples, 5 were from patients positive for B.1.1.28, 4 from patients that tested positive for Gamma, and 11 were positive for B.1.1.33 (Table 1). Among five patients infected by B.1.1.28, highest neutralizing antibody titers were observed for B.1.1.28, followed by Alpha, B.1.1.33, B.1, Gamma, Zeta, N.10, and Delta (Fig. 1). Among patients infected by B.1.1.33, similar profile was observed. Neutralizing antibody titer geometric mean was higher for B.1.1.33, followed by Alpha, B.1, B.1.1.28, Zeta, Gamma, and with equally reduced neutralizing antibodies for Delta and N.10 (Fig. 1). From four patients infected by Gamma, highest PRNT₉₀ titers were observed for Gamma, followed by Alpha, B.1.1.33, N.10, B.1.1.28, and Zeta, and with neutralizing antibodies under the level of detection for Delta in all individuals (Table 1).

The 4-fold difference among PRNT titers was not suitable to distinguish infections among the different lineages of SARS-CoV-2 tested. Convalescent serum from patients infected by B.1.1.28 presented a geometric mean of PRNT titer very similar for B.1.1.28, B.1.1.33, and Alpha lineages, and the titer difference was less than 4-fold for B.1, even though the anti-B.1 antibody titer levels were significantly lower ($P=0.02$). In fact, PRNT₉₀ titers for B.1.1.28 in patients infected by the same virus were not even 2-fold greater when compared with B.1.1.33 or the VOC Alpha. Four-fold difference for B.1.1.28 was observed only when compared with the titers of the variants N.10, Zeta, and Delta (Table 1). A similar profile was observed for samples from patients infected by B.1.1.33. In this case, these patients presented similar PRNT titers among B.1.1.33, B.1, and Alpha. The geometric mean of antibody titer in B.1.1.33-induced sera was

less than 4-fold greater for B.1.1.33 when compared with B.1.1.28, Zeta, and N.10. Four-fold or greater B.1.1.33 titers were observed only when compared with Gamma and Delta VOCs. Finally, for the patients previously infected by Gamma, PRNT titers were more than 4-fold higher for Gamma when compared with B.1 and B.1.1.28, Delta, and Zeta. Differences were smaller than 4-fold when compared with B.1.1.33, N.10, and Alpha (Table 1).

Discussion

Besides being considered markers of immune protection, specific neutralizing antibodies have also been used to evaluate viral exposure, and ultimately, for diagnostic purposes. Assays designed to detect neutralization antibodies have been widely used for the diagnostic of different viral groups. The relationship between humoral response to SARS-CoV-2 antigens, especially the spike protein, and clinical protection from COVID-19 remains not fully understood, although some studies have confirmed neutralizing antibodies as an immune correlate of protection [2]. Likewise, it is still not completely understood to what extent the mutations in viral antigens contribute to virus evasion from neutralizing antibodies, and how significant this escape mechanism could be for the general effectiveness of the protective response, especially in the context of vaccinations, reinfections, and the evolution of the pandemic. It has been shown that mutations in the angiotensin-converting enzyme 2 (ACE2) binding site of variants result in an increased affinity for the receptor ACE2, and that changes outside the receptor-binding domain also impact neutralization [18]. In this scenario, we primarily investigated the neutralization profile of sera from patients infected by the most prevalent lineages in early pandemic in Brazil including B.1.1.33 and B.1.1.28, with three later emerged VOCs including Gamma, Alpha, and Delta. Additionally, we investigated if Gamma-induced antibodies were capable to neutralize the early isolates and the VOC, Delta. We observed that, despite the variation in antibody levels, most serum samples of patients infected by early isolates presented some level of neutralizing activity against all VOCs. The reverse situation, in which serum samples from individuals infected with the Gamma lineage were challenged with early pandemic viral isolates also produced detectable neutralization. However, when sera from Gamma-infected individuals were tested with other VOC Delta, the PRNT₉₀ titer was below the limit of detection of the assay (Table 1).

The variant Gamma, which emerged from the B.1.1.28 lineage, contains 17 amino acid substitutions, 10 of which are in the spike protein, including N501Y, E484K, and K417T in the receptor-binding domain, 5 in the N-terminal domain, and the mutation H655Y near the furin cleavage site [19]. These mutations reduce the neutralization capacity of convalescent sera from individuals infected by early isolates [9], and findings presented here suggest that these mutations perhaps interfere also with the efficiency of its neutralizing antibodies for other variants, as Delta.

Despite the small number of patients evaluated, the findings that Gamma-induced immune sera presented low or absent PRNT₉₀ titers for Delta raise concern over the potential risk of reinfections, and consequent prolongation of the pandemic. It is important to mention that the protective role of neutralizing antibodies does not seem to be directly linked to the titers in which they are found, but to timing and kinetics of their production, with studies suggesting a limited role of antibodies in predicting disease severity of the COVID-19, and that the earlier the presence of neutralizing antibodies after infection, the less severe is the disease outcome [20]. It is noteworthy that for most viruses

Table 1: PRNT₉₀ titers of convalescent sera of COVID-19 patients for different SARS-CoV-2 lineages

Patient ID	Lineage of infection	SARS-CoV-2 lineages							
		B.1	B.1.1.28	B.1.1.33	N.10	Zeta	Alpha	Gamma	Delta
COV029	B.1.1.28	80	160	160	40	80	80	80	40
COV161	B.1.1.28	40	160	160	10	10	80	20	10
COV168	B.1.1.28	80	320	320	20	20	≥320	40	10
COV167	B.1.1.28	160	320	160	20	20	≥320	10	40
COV178	B.1.1.28	40	80	80	40	40	80	40	<10
GM (95% CI)	B.1.1.28	69.6 (33.9–143.1)	183.8 (89.4–377.6)	160 (87–294)	22.9 (11.2–47.2)	26.4 (9.9–70.4)	160 (67.7–378.4)	30.3 (11.4–80.9)	10.9 (1.7–71.5)
COV014	B.1.1.33	20	40	160	<10	20	40	10	<20
COV057	B.1.1.33	160	80	80	40	40	80	80	NT
COV008	B.1.1.33	160	80	160	80	80	160	80	20
COV051	B.1.1.33	40	40	160	20	40	160	80	<10
COV170	B.1.1.33	160	160	160	40	40	80	40	20
COV186	B.1.1.33	40	80	160	20	20	80	40	40
COV036	B.1.1.33	640	80	640	40	160	640	80	40
COV101	B.1.1.33	320	160	160	80	160	320	160	40
COV106	B.1.1.33	40	160	160	20	40	80	40	<10
COV150	B.1.1.33	80	320	160	10	20	80	20	10
COV146	B.1.1.33	320	160	320	160	160	320	80	≥320
GM (95% CI)	B.1.1.33	109.6 (52.7–228)	102.9 (66.9–158.3)	181.5 (127.9–257.4)	26.9 (10.9–66.3)	51.5 (29.3–90.2)	132.4 (76.1–230.6)	51.5 (30.5–86.7)	13.3 (3.1–49.8)
COV381	Gamma	10	80	80	80	40	80	160	<10
COV373	Gamma	40	40	40	80	80	80	160	<10
COV385	Gamma	20	40	40	40	40	160	320	<20
COV386	Gamma	20	40	160	10	40	160	160	<10
GM (95% CI)	P.1	20 (8.1–49.2)	47.6 (27.4–82.5)	67.2 (23.4–193.4)	40 (8.4–190.3)	47.5 (27.4–82.5)	113.1 (59.8–213.9)	190.3 (109.6–330.6)	1

For geometric mean calculation purposes, a value of 1 was assigned to titers <10 and <20, and a value of 320 was assigned to titers ≥320. NT, not tested. GM: Geometric Mean. The bold represents the geometric mean values.

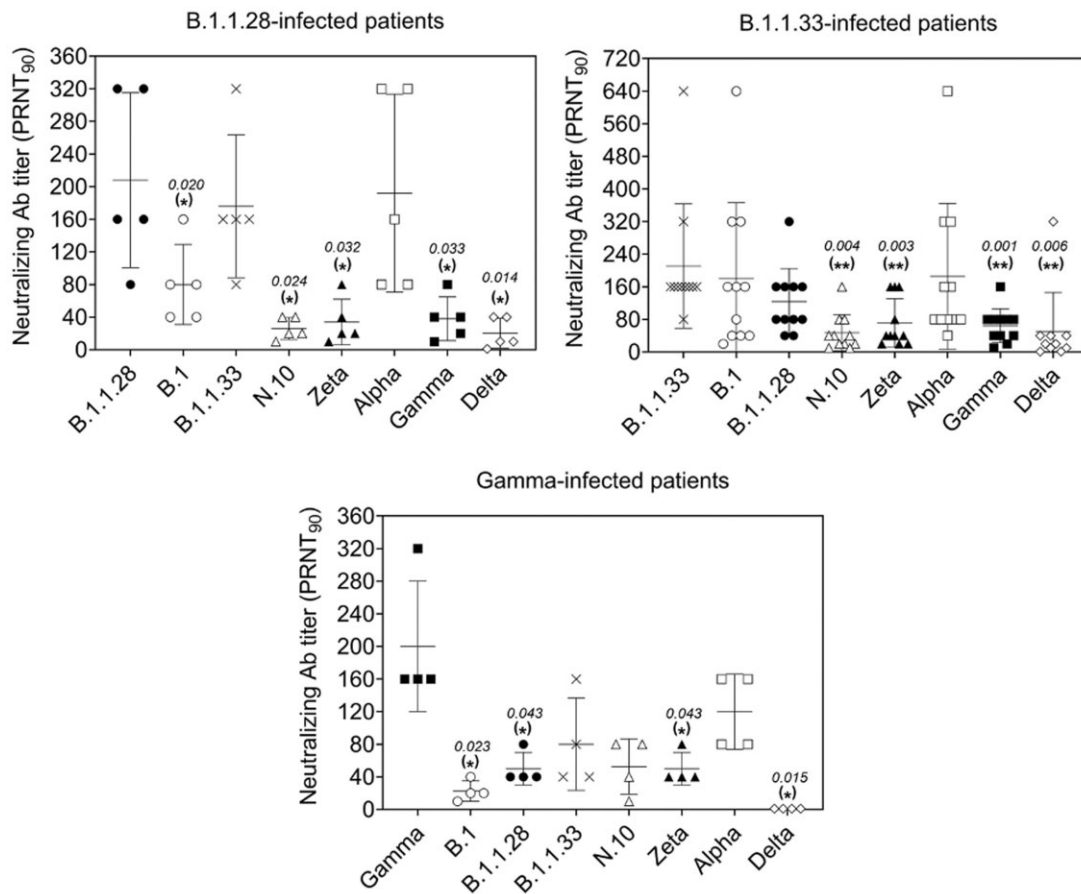


Figure 1. Anti-SARS-CoV-2 neutralizing antibody titers in sera from COVID-19 patients admitted to reference hospital in Rio de Janeiro, Brazil. Samples were obtained from patients infected with the SARS-CoV-2 lineages B.1.1.28 (top left), B.1.1.33 (top right), or Gamma (P.1, bottom panel). Samples were tested for early pandemic lineages (B.1; B.1.1.28, and B.1.1.33), variants of interest (N.10 and Zeta), and VOC (Alpha, Gamma, and Delta). In each group of patients, the neutralizing antibody titers detected for the originally infecting SARS-CoV-2 lineage were compared with antibody titers elicited to other lineages by means of two-tailed paired t-tests, at 95% confidence level. (*) and (**) indicate significant difference in comparison to the antibody levels against the infecting lineage. P-values are indicated to each significant result.

there is no direct correlate of protection in humans, since the studies needed to establish such a correlate in humans are challenging. For instance, protective neutralizing antibody titers have been roughly estimated for yellow fever vaccine by challenge studies in nonhuman primates and hamster models [21, 22]. The threshold of protective neutralizing antibody titers for SARS-CoV-2 and its lineages has not been established. Therefore, lower neutralizing antibodies titers including the samples with titers under the limit of detection as <10 do not necessarily mean susceptibility to a hypothetical secondary challenge. Samples that presented titers <10 were not tested in lower dilutions, and if titers ≤ 9 are protective for each one of the different lineages of SARS-CoV-2 tested remain unknown.

It is noteworthy that the cellular immune response is believed to play an important role in the immune response for SARS-CoV-2 infection [5]. In this study, cellular immune response was not investigated, and only with a combined evaluation of both immune responses for a better understanding of the susceptibility to secondary infections of individuals previously infected by SARS-CoV-2. Despite concerns over the prolongation of the pandemic, the number of reinfections reported worldwide remains limited. The continuous advance of the COVID-19 vaccination has reduced the pace of new infections worldwide and is also expected to mitigate the number of reinfections as well.

The PRNT is the most specific and gold standard serological test for the differentiation of closely related flavivirus infections,

as dengue and yellow fever viruses in convalescent serum samples [23]. Type-specific antibodies can be distinguished using the PRNT, and two or more flaviviruses are distinct from each other by quantitative serological criteria. Four-fold difference between PRNT titers has been used as seropositivity criterion in heterologous reactions for flaviviruses [12, 24]. In this study, despite some genetic and immunogenic differences observed among all lineages of SARS-CoV-2 worldwide, these viruses are closely related and for that reason a PRNT with a highly conservative threshold of 90% neutralization in $\geq 1:10$ serum dilution was used as seropositivity criterion, as previously described [15].

In our group of samples, the 4-fold difference among PRNT titers was not suitable to distinguish infections among all lineages of SARS-CoV-2 tested. Four-fold or greater titers for B.1.1.28 were observed only when compared with the titers of the variants N.10, Zeta, and Delta. The same was observed for B.1.1.33 titers that had 4-fold higher titers only when compared with Gamma and Delta VOCs. Patients previously infected by Gamma presented PRNT titers for Gamma more than 4-fold higher for Gamma when compared with B.1 and B.1.1.28, Delta, and Zeta. However, differences were smaller than 4-fold when compared with B.1.1.33, N.10, and Alpha. Of note, the geometric mean of PRNT₉₀ titers for the Alpha variant was the second highest in all the three groups of patients, which includes samples of patients infected by B.1.1.28 and B.1.1.33 that were collected before the

upsurge of Alpha variant in the UK. Despite using a highly conservative threshold, the difference in PRNT₉₀ titers demonstrated here among all lineages indicates that the 4-fold difference as criterion of seropositivity is not reliable to distinguish lineage infections. The same is true even considering an alternative 2-fold difference as criterion of seropositivity.

In conclusion, findings presented here indicate that most patients infected in early stages of COVID-19 pandemic presented neutralizing antibodies capable to neutralize wild types of all later emerged VOCs in Brazil, and that the 4-fold difference in PRNT₉₀ titers used for other groups of viruses may not be a reliable serologic method to distinguish infection among different SARS-CoV-2 lineages in all cases.

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Conflict of interest statement. None declared.

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