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Cross-Neutralizing Activity Against SARS-CoV-2 Variants in COVID-19 Patients: Comparison of 4 Waves of the Pandemic in Japan

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Background. As of March 2021, Japan is facing a fourth wave of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. To prevent further spread of infection, sera cross-neutralizing activity of patients previously infected with conventional SARS-CoV-2 against novel variants is important but has not been firmly established.

Methods. We investigated the neutralizing potency of 81 coronavirus disease 2019 (COVID-19) patients' sera from the first to fourth waves of the pandemic against SARS-CoV-2 D614G, B.1.1.7, P.1, and B.1.351 variants using their authentic viruses.

Results. Most sera had neutralizing activity against all variants, showing similar activity against B.1.1.7 and D614G, but lower activity especially against B.1.351. In the fourth wave, sera-neutralizing activity against B.1.1.7 was significantly higher than that against any other variants, including D614G. The sera-neutralizing activity in less severe patients was lower than that of more severe patients for all variants.

Conclusions. The cross-neutralizing activity of convalescent sera was effective against all variants but was potentially weaker for B.1.351. The high neutralizing activity specific to B.1.1.7 in the fourth wave suggests that mutations in the virus might cause conformational change of its spike protein, which affects immune recognition of D614G. Our results indicate that individuals who recover from COVID-19 could be protected from the severity caused by infection with newly emerging variants.

Keywords. COVID-19; neutralizing activity; reinfection; SARS-CoV-2; variant.

The coronavirus disease 2019 (COVID-19) pandemic declared by the World Health Organization (WHO) in March 2020 continues to affect all countries around the world. In efforts to control the pandemic, several vaccine platforms have been developed based on the original severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Wuhan-1) as the template, and these vaccines have been shown to be effective in reducing the COVID-19 outbreak [1–3].

However, the evolution of SARS-CoV-2 has continued since its initial emergence. By the beginning of April 2020, a variant bearing a D614G mutation with evidence of increased infectivity had become dominant [4]. The SARS-CoV-2 variant B.1.1.7, first detected in Kent and Greater London in September 2020, has now spread to many countries worldwide, with evidence indicating an increased mortality rate [5, 6]. In addition to D614G and several mutations in other areas of the genome, B.1.1.7 bears 8 mutations in the spike gene including deletions in the N-terminal domain (ΔH69/ΔV70, Δ144) and amino acid substitutions in the receptor binding domain (N501Y) [7, 8].

The SARS-CoV-2 variant B.1.351 was first detected in specimens collected from South Africa in October 2020, and it has rapidly become the predominant variant circulating throughout South Africa [9]. Among the 9 mutations in the spike gene in this variant, there are 3 biologically important mutations: K417N, E484K, and N501Y [7]. Importantly, there is growing evidence that the B.1.351 variant has the ability to escape from the neutralizing antibody elicited by the original SARS-CoV-2 infection and currently available vaccines [7, 10–12].

The SARS-CoV-2 variant P.1, which was first detected in Japan in early January 2021 from 4 individuals with a history of traveling to Brazil, had become the predominant variant circulating in Brazil by January 2021 [13]. It bears 12 mutations in the spike gene, including K417T, E484K, and N501Y [14], which are the same 3 amino acid substitutions found in B.1.351. Interestingly, the P.1 variant showed less resistance to a neutralizing antibody induced by natural infection or vaccination when compared with a similar variant, B.1.351 [15].

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The emergence of these variants poses a tremendous challenge to the control of the SARS-CoV-2 pandemic. In addition, the B.1.351 and P.1 variants carry the E484K mutation, which is responsible for evasion from the monoclonal antibody against the original SARS-CoV-2, further compromising the currently available therapy against this virus [16].

As of May 2021, Japan has experienced 4 waves of the COVID-19 pandemic, beginning in April 2020; the number of total confirmed cases is over 690 000, and there have been more than 11 000 deaths due to COVID-19 in Japan alone [17]. The growth rate of the number of infected individuals in the fourth wave is much faster than that of the first to third waves so far, and there is concern about the possibility of a collapse of the health care system. SARS-CoV-2 genome surveillance has revealed that D614G_KR and its lineages were the predominating circulating viruses responsible for the first to third waves of the pandemic in Japan, but the introduction of the R1 and B.1.1.7 variants in late 2020 has replaced the previously existing strains and may be responsible for the fourth wave [18]. The B.1.351 and P.1 variants have also been detected in Japan, although no trend toward an increasing dominance of these variants has been observed thus far [19].

It is not yet known to what extent the serum of patients previously infected with original SARS-CoV-2 might confer protection against these rapidly emerging variants. In this study, we investigated the neutralizing potency of serum from patients infected during the first to fourth waves of the pandemic against the SARS-CoV-2 variants D614G, B.1.1.7, B.1.351, and P1, using authentic virus. This research is imperative to understand whether individuals who have recovered from COVID-19 could be protected from reinfection by newly emerging variants. This research might also help predict the potency of using plasma from individuals who recovered from the conventional type or any variants of SARS-CoV-2 if convalescent plasma therapy were used for COVID-19 patients infected by the other variants.

METHODS

Diagnosis of COVID-19

COVID-19 diagnoses were based on polymerase chain reaction detection of the SARS-CoV-2 genome in nasopharyngeal swab samples. Disease severity was defined as follows: Symptomatic COVID-19 cases without evidence of pneumonia or hypoxia were classified as mild. Cases in patients with clinical signs of pneumonia were classified as moderate (oxygen saturation as measured by pulse oximetry, ≥90% on room air) or as severe (respirations >30/min, severe respiratory distress, or oxygen saturation <90% on room air). Patients who needed mechanical ventilation were classified as critical.

Definitions of the Waves of the COVID-19 Pandemic in Japan

The period from the first wave to the fourth wave of the COVID-19 pandemic was defined based on the change in the number of infected people on a single day in Japan. The first wave was from March 1 to the end of June 2020; the second wave was from July 1 to the end of October 2020; the third wave was from November 1, 2020, to the end of February 2021, and the fourth wave began on March 1, 2021 [17].

Participant Recruitment

From March 2020 to May 2021, blood samples were collected from patients who became infected with SARS-CoV-2 and were hospitalized at Hyogo Prefectural Kakogawa Medical Center (Hyogo, Japan). We selected serum of convalescent patients with different disease severities who were already confirmed to have neutralizing activity against SARS-CoV-2. In May 2020, the serum of 24 healthy individuals was collected and confirmed to have no antibody against SARS-CoV-2; these sera were used as the negative control group [20]. This study was carried out after written consent was obtained from the subjects or by the opt-out method when it was difficult to get written consent due to disease severity. No statistical methods were used to predetermine the sample size.

Measurement of Neutralizing Activity Against SARS-CoV-2

Neutralization was performed as previously described [21]. Briefly, the neutralizing activity of each serum sample was evaluated by a neutralization assay against each living SARS-CoV-2 variant (D614G, B.1.1.7, P.1, or B.1.351) in a biosafety level 3 laboratory. At 24 hours before the assay, 4×10^4 Vero E6 (TMPRSS2) cells per well were seeded in 96-well tissue culture microplates [22]. A 2-fold serial dilution of heat-inactivated (56°C, 30 minutes) serum was prepared using Dulbecco's Modified Eagle's Medium as the diluent and mixed with a 100-tissue culture infectious dose $(TCID)_{50}$ of virus and incubated at 37°C for 1 hour. After this incubation, the serum-virus mixture was added to Vero E6 (TMPRSS2) cells and incubated at 37°C for 6 days. The neutralizing antibody titer was determined as the highest serum dilution that did not show any cytopathic effects. We confirmed this assay by using the sera of healthy individuals (n = 24) as a negative control, and we observed that none had neutralizing activity.

Preparation of SARS-CoV-2 Variants

We used the SARS-CoV-2 Biken-2 (B2) strain with a D614G mutation as a conventional variant (currently applying for the registration), which was provided by BIKEN Innovative Vaccine Research Alliance Laboratories. The 3 variants B.1.1.7 (GISAID ID: EPI_ISL_804007), P.1 (GISAID ID: EPI_ISL_833366), and B.1.351 (GISAID ID: EPI_ISL_1123289) were isolated and provided by the National Institute of Infectious Disease, Japan.

Each variant was confirmed by the cDNA sequence of the spike gene of each virus.

Statistical Analysis

GraphPad Prism software (version 8.4.3) was used for the statistical analysis and preparation of figures. The Friedman test was used to compare the neutralizing antibody titer among the 4 variants. The Kruskal-Wallis test was used to compare the neutralizing antibody titer among different disease severity groups. Results were considered significant at a P value <.05.

Ethical Approval

This study was approved by the ethical committees of Kobe University Graduate School of Medicine (approval code: B200200) and Hyogo Prefectural Kakogawa Medical Center.

RESULTS

Patient Characteristics

We examined a total of 81 sera of patients with different disease severities who were already confirmed to have neutralizing activity against the B2 strain, which is a D614G variant. The characteristics of the patients are summarized in Table 1. The median number of days between the onset of symptoms and the collection of serum samples (days postonset [dpo]) was 26. Overall, 62% of the patients were male, 38% were female, and the median age was 64 years. The asymptomatic/mildly infected group was comprised of 25 patients, 19 patients were moderate/severe, and the remaining 37 patients were in the critical infection group. The most common medical histories were hypertension and diabetes, in 28.4% of the patients each.

Eleven patients had received antiviral treatment with favipiravir or lopinavir (both for 6 patients and favipiravir for 5 patients), and 42 patients received steroid treatment. A comparison of the 4 waves (Table 1) revealed that the second wave (with 20 patients) contained only 1 critical patient, whereas all 20 patients in the fourth wave were critical and were mostly (75%) male. In addition, antiviral treatment was mainly prescribed for the patients in the first wave, whereas steroids were mainly used in the second wave onward.

Neutralizing Activity Against All Variants in All Patients

Most of the 81 sera had neutralizing activity against the 4 variants, although the activity values varied (Figure 1). The mean neutralizing antibody titer for the D614G variant was 80, and that for the B.1.1.7 variant was 111. The neutralizing titer of B.1.1.7 seemed to be higher than that of D614G, but the difference was not significant. In contrast, the mean neutralizing antibody titer against P.1 was 44, and that against B.1.351 was 21; each of these values was lower than that for D614G, especially in the case of B.1.351 (3.8×, $P < .0001$). The neutralizing activity against B.1.351 was also lower than that against P.1 (2×, $P < .0001$). Interestingly, some sera of individuals showed similar or high neutralizing activity for P.1 compared with D614G (Figure 1).

Neutralizing Activity Against All Variants in Each Wave

From the first wave to the third wave, the neutralizing activity against the B.1.1.7 variant was similar to or slightly lower than that against D614G, whereas it was higher in the fourth wave (increased 4×, $P = .0009$). In addition, the neutralizing activity against B.1.1.7 was also higher than that against the P.1 or B.1.351 variant in the fourth wave. In all waves, the neutralizing activity against the B.1.351 variant was lower than against the other 3 variants (Figure 2).

Table 1. Patient Characteristics in Wave Groups

	All (n = 81)	First Wave (n = 18)	Second Wave (n = 20)	Third Wave (n = 23)	Fourth Wave (n = 20)
Sex, No. (%)					
Male	50 (61.7)	11 (61.1)	9 (45)	15 (65.2)	15 (75)
Female	31 (38.3)	7 (38.9)	11 (55)	8 (34.8)	5 (25)
Age, median (range), y	64 (20–83)	59 (38–79)	68.5 (20–83)	65 (37–78)	64.5 (50–80)
Disease severity, No. (%)					
Asymptomatic or mild	26 (32.1)	6 (33.3)	11 (55)	9 (39.2)	0 (0)
Moderate or severe	19 (23.5)	4 (22.2)	8 (40)	7 (30.4)	0 (0)
Critical	37 (44.4)	9 (50)	1 (5)	7 (30.4)	20 (100)
Medical history, No. (%)					
Hypertension	23 (28.4)	3 (16.7)	4 (20)	6 (26.1)	10 (50)
Previous heart disease	2 (2.5)	1 (5.6)	0 (0)	1 (4.3)	0 (0)
Diabetes	23 (28.4)	3 (16.7)	4 (20)	10 (43.5)	6 (30)
Chronic pulmonary disease	4 (4.9)	1 (5.6)	0 (0)	2 (8.7)	0 (0)
COVID-19 treatment, No. (%)					
Antiviral therapy[a]	11 (13.6)	10 (55.6)	0 (0)	1 (4.3)	0 (0)
Corticosteroids	42 (51.9)	1 (5.6)	5 (25)	16 (69.6)	20 (100)

Abbreviation: COVID-19, coronavirus disease 2019.

[a]Remdesivir or lopinavir.

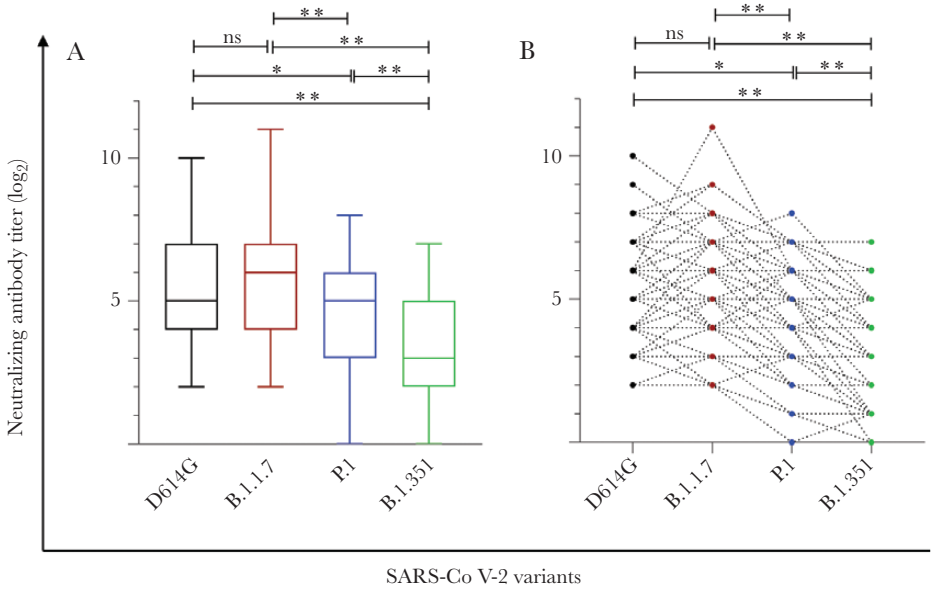

Figure 1. Neutralization activity against SARS-CoV-2 variants. Sera of 81 patients who had recovered from COVID-19 were tested for neutralizing activity against the SARS-CoV-2 variants D614G, B.1.1.7, P.1, and B.1.351. The neutralizing antibody titer is represented by the logarithmic scale of the highest serum dilution that did not show any cytopathic effects. A, Box plot of the neutralizing antibody titers with the minimum, first quartile, median, third quartile, and maximum values. B, Changes in the antibody titer for each patient. The titer of the same patient is connected by a line. The Friedman test was used, and 2-tailed *P* values were calculated. $^{*}P < .05$; $^{**}P < .01$. Abbreviations: COVID-19, coronavirus disease 2019; ns, not significant; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Neutralizing Activity Against Each Variant by Severity

The sera of all the COVID-19 patients showed neutralizing activity against the D614G and B.1.1.7 variants regardless of the severity of the patients' symptoms. A significantly lower neutralizing titer against D614G, B.1.1.7, P.1, or B.1.351 was observed in the serum of the asymptomatic/mild COVID-19 patients compared with the critical patients (4- to 9-fold lower, $P < .0001$) (Figure 3A–D).

Interestingly, almost all the sera from the asymptomatic/mild infected group, with the exception of 3 cases, had neutralizing activity against all tested variants. Three asymptomatic/mild cases and 1 case in the severe infection group with low neutralizing activity against D614G (titer 8 or 16) did not show any neutralizing activity against P.1 or B.1.351 (Figure 3C, D).

DISCUSSION

In Japan, the fourth wave of SARS-CoV-2 arrived in March 2021, and the presence of the variant B.1.1.7 has increased in this wave. It is suspected that the conventional D614G variant has already been almost completely replaced by B.1.1.7. In addition, P.1 and B.1.351 have also been identified in Japan, and there is thus a possibility of a further spread of infection in the future. Given the recent emergence of the B.1.1.7, P.1, and B.1.351 variants, the cross-neutralization of these variants by previous pandemic sera remains to be clarified. To predict and help prevent the further spread of SARS-CoV-2 infection, it is necessary to determine whether the neutralizing activity in COVID-19 patients infected with the D614G variant has similar activity against the newly emerging variants.

In the present study, regardless of the patients' infection time (wave) and disease severity, most of their sera had neutralizing activity against the 4 variants (D614G, B.1.1.7, P.1, and B.1.351), although the neutralizing activity values varied. Some individuals that showed high neutralizing activity against D614G and B.1.1.7, and also had high activity against P.1 and B.1.351, indicating that individuals infected with D614G or B.1.1.7 also could have the neutralizing antibody against P.1 and B.1.351.

Although we observed no significant difference between the neutralizing activity of sera against B.1.1.7 and D614G in all patients, the values of neutralizing activity against P.1 and B.1.351 were lower than against D614G, and the neutralizing activity against B.1.351 in particular was much lower. This means that the neutralizing activity of sera from previously infected patients was also seen against the B.1.1.7 variant but was potentially weaker against the P.1 and B.1.351 variants. As one of the potential explanations for this finding, we note that the N501Y substitution (which is common among these 3 variants) may enhance the binding to ACE2, but its antigenic effects are limited, and it may have little effect on the neutralizing activity of the antibodies [23, 24]. However, the E484K mutation, which is found both in P.1 and B.1.351 but not in either D614G or B.1.1.7, has been reported to affect the binding of serum polyclonal neutralizing antibodies [16].

On the other hand, because P.1 and B.1.351 have similar mutations in their RBD (including E484K, K417T/N, and N501Y), it might be thought that the neutralization of both variants would be affected similarly. However, our present analyses

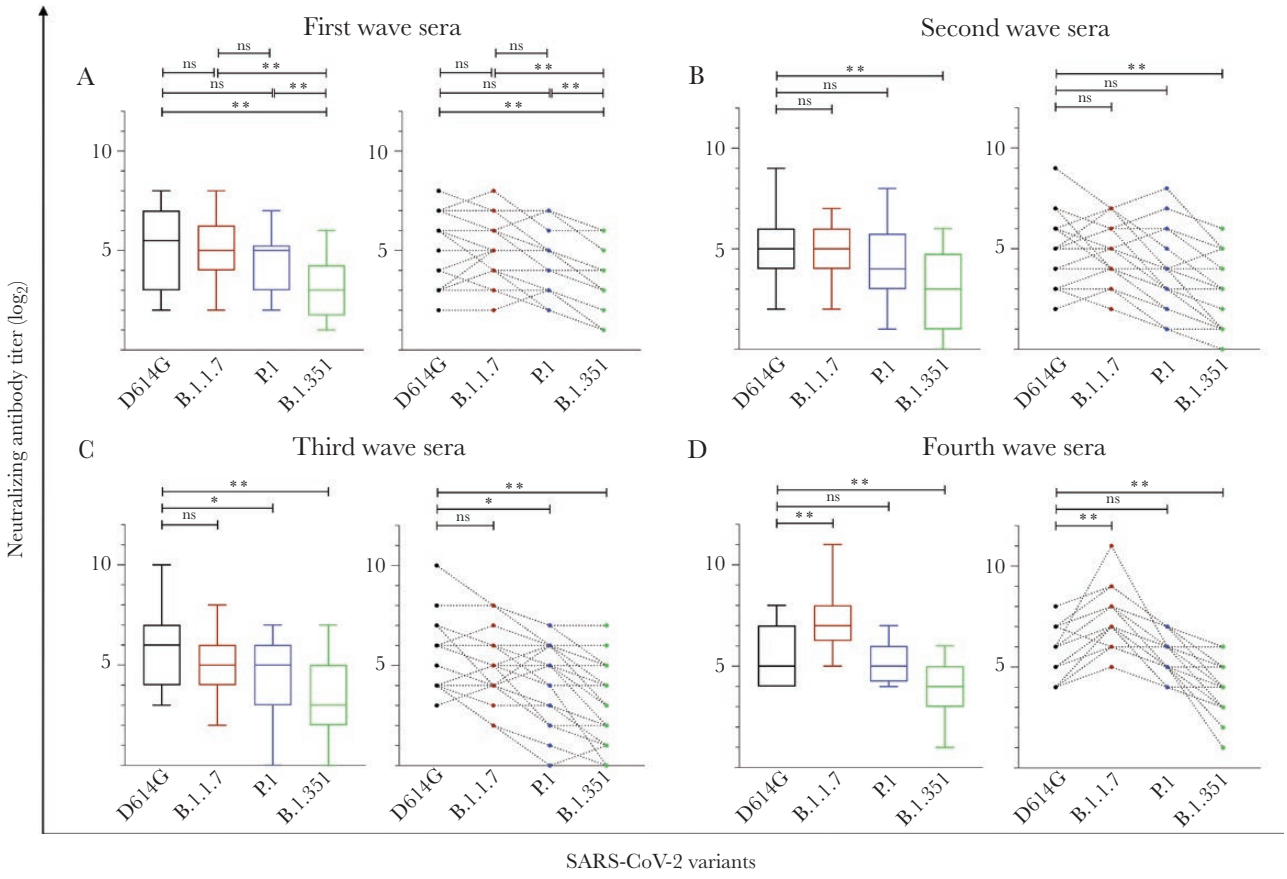

Figure 2. Neutralizing activity against all variants in each wave. The neutralizing antibody titers of sera against D614G, B.1.1.7, P.1, and B.1.351 were compared in the first wave (from March 1 to June 2020) (A), second wave (from July 1 to October 2020) (B), third wave (from November 1, 2020, to February 2021) (C), and fourth wave (after March 1, 2021) (D). The Friedman test was used, and 2-tailed *P* values were calculated. $^{*}P < .05$; $^{**}P < .01$. Abbreviations: ns, not significant; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

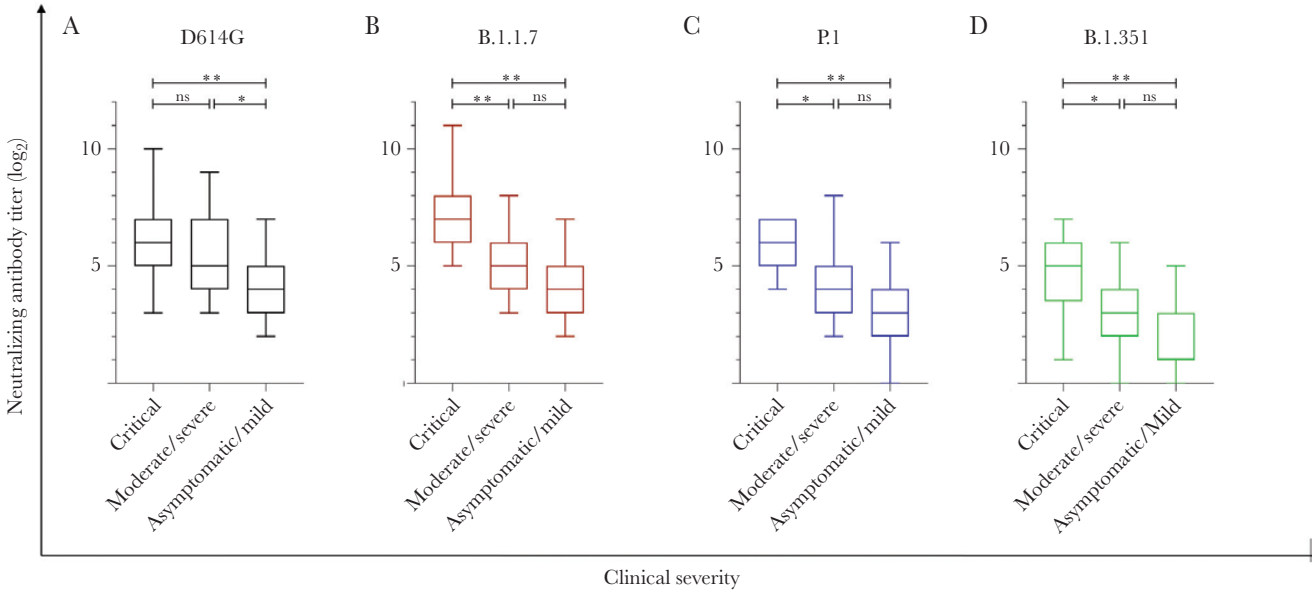

Figure 3. Neutralizing activity against each variant by disease severity. The neutralizing antibody titer against (A) D614G, (B) B.1.1.7, (C) P.1, and (D) B.1.351 in patients' sera with different severity groups. The Kruskal-Wallis test was used, and 2-tailed *P* values were calculated. $^{*}P < .05$; $^{**}P < .01$. Abbreviation: ns, not significant.

demonstrate that while some sera of individuals showed similar or high neutralizing activity against P.1 compared with those against D614G, the activity against B.1.351 was consistently lower than that against D614G, indicating that B.1.351 might avoid neutralization more effectively by means other than mutations of the RBD, such as amino acid deletions (242-244 del) and substitutions (D80A, R246I) in the N terminal domain (NTD) [7, 11, 25].

Interestingly, although we observed that the neutralizing activity against the B.1.1.7 variant seemed to be similar to or slightly lower than that against D614G from the first to third waves in Japan, its activity against B.1.1.7 was higher than that against D614G, P.1, and B.1.351 in the fourth wave, indicating an epidemic of B.1.1.7. In particular, the neutralizing activities against P.1 and B.1.351 were significantly lower than that for B.1.1.7. Regarding this result, some other groups have also reported that antibodies elicited by B.1.1.7 infection exhibited significantly reduced recognition and neutralization of parental (Wuhan) strain or B.1.351 compared with B.1.1.7 [26, 27]. Our results may suggest that the mutations in B.1.1.7 could cause the conformational change of its spike protein, which affects immune recognition for D614G.

The correlation between serum neutralization activity against D614G and clinical severity has been described [28–31], and our present findings revealed a similar correlation for 3 other variants. Even among the asymptomatic/mild patients, all had neutralizing activity against B.1.1.7, and most also had neutralizing activity against P.1 and B.1.351.

Our results suggest that natural infection with each SARS-CoV-2 variant prompts the body to make antibodies that recognize the infecting strain most robustly, with various degrees of cross-recognition of other strains. The efficacy of convalescent plasma therapy remains controversial, but it may be considered to use the convalescent sera induced by conventional strain for high-risk patients infected with B.1.1.7 or P.1 [32–34]. Individuals who have recovered from the infection of fourth wave may not be completely protected against reinfection with the other SARS-CoV-2 variants in the future, especially in asymptomatic or mild cases with low neutralizing activity. However, it is possible that the existing memory B cells (which have neutralizing epitopes that are common to the variants) are stimulated after reinfection by the other variants and expand immediately and function for protection. Additionally, it has been reported that despite the decline of the total antibody titer, the neutralizing potency per antibody against the original SARS-CoV-2 was improved and that the neutralizing potency and breadth against variant of concern(s) (VOCs) increased over time, indicating maturation of the antibody response. Therefore, declining antibody titers alone might not be indicative of declining protection, and it will be important to analyze various indicators other than the antibody titer to understand the prevention of infection, such as cellular immunity [35]. Our findings may indicate that cross-neutralization could work to protect against the induction of severe symptoms when an individual is reinfected by new variants. Further studies are required to address this and many other questions about the variants that continue to arise.

Study Limitations

We did not have data about the infecting variants in our patients, so we could not know the exact percentage of the B.1.1.7 variant in the fourth wave. However, according to a report on the percentage of the B.1.1.7 variant in Hyogo prefecture, only 5% of the cases were positive in February 2021, whereas 80%–90% of the cases examined from March to April were positive. We thus suspect that most (>80%) of the patients in the fourth wave were infected with the SARS-CoV-2 B.1.1.7 variant.

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Author contributions. All authors contributed to the concept of this article. K.F. and L.T. drafted the manuscript; Y.M. provided revisions; K.F., L.T., J.A., M.N., and Y.M. analyzed the data; K.F., L.T., S.Su., and Y.K. performed the experiments; Y.M. and Y.N. supervised the experiments; S.I., Y.T., S.Sa., S.N., T.K., M.Y., and T.N. collected the samples; Y.M. supervised the project. All authors approved the final version of the manuscript.

Patient consent. This study was carried out after written consent was obtained from the subjects or by the opt-out method when it was difficult to get written consent due to disease severity. This study was approved by the ethical committees of Kobe University Graduate School of Medicine (approval code: B200200) and Hyogo Prefectural Kakogawa Medical Center.

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