Neutralizing antibodies from naturally infected individuals against SARS-CoV-2 Gamma and Delta variants in the Paraguayan population

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Abstract

Introduction: Severe Acute Respiratory Syndrome-Coronavirus-2 Virus (SARS-CoV-2) is responsible for Coronavirus Disease 2019 (COVID-19). A substantial number of SARS-CoV-2 infection cases have been reported during the pandemic, and vaccination coverage in some regions, particularly in developing countries, remains very low. SARS-CoV-2 variants of concern (VOCs) have also emerged as some of the most pressing public health issues. In this scenario, it is crucial to know whether COVID-19 convalescent antibodies have cross-neutralizing action against VOCs to contribute to the analysis of the future progress of the pandemic.

Methodology: The plasma of individuals infected with SARS-CoV-2 from June to November 2020 in Paraguay (before the first recorded infections associated with VOCs in the country) was selected. Anti-spike antibodies were determined in plasma samples (n = 626) obtained from this convalescent and unvaccinated group. Using a pseudotyped virus neutralization assay, we then investigated the neutralizing response against D614G variant and Gamma, and Delta VOCs.

Results: IgG antibodies against spike were detected in 85.6% of convalescent individuals. Samples from individuals previously infected by a non-VOC showed a 6.6- and 8.1-fold reduction in neutralizing capacity to the Gamma and Delta variants, respectively, when compared to the D614G variant.

Conclusions: Our findings show that antibodies generated by non-VOC infection have reduced neutralizing capabilities against Gamma and Delta variants that appeared subsequently and might have implications for immunity strategies.

Key words: COVID-19; convalescence; seroprevalence; SARS-CoV-2 variants; neutralizing antibodies.
neutralization by antibodies developed after natural infection or vaccination with the original SARS-CoV-2 [7–10].

Considering these, we evaluated the anti-spike antibody levels in plasma samples obtained from a non-VOCs convalescent and unvaccinated group and the efficacy of the neutralizing capacity of the antibodies generated against ancestral SARS-CoV-2 and Gamma and Delta variants.

Methodology
Plasma samples
Plasma samples were collected between July and December 2020 from qRT-PCR-confirmed COVID-19-recovered individuals 20-60 days after symptom onset who were recovered and participated as plasma donors for the convalescent plasma therapy COVID-19 clinical trial (NCT04747158). The study was approved by the Ethics Committee of Facultad de Ciencias Médicas, Universidad Nacional de Asunción (Approval Number 276.2020). The participants were male and female adults aged 18 years or older (Table 1). Written informed consent was obtained from all participants.

Enzyme-Linked Immunosorbent Assay (ELISA) anti-SARS-CoV-2
Anti-SARS-CoV-2 IgG was measured using enzyme-linked immunosorbent assay (ELISA) (Euroimmun, Germany) according to the manufacturer's instructions. 96-wells microplates coated with the SARS-CoV-2 (Wuhan strain) S1 domain of the spike protein expressed recombinantly in the human cell line HEK293 were used. The samples were diluted with sample buffer to 1:320. The calibrator, positive and negative controls and diluted samples were incubated in a 96-well microtiter plate for 60 min at 37°C, followed by washing. The secondary antibody (peroxidase-labelled anti-human IgG) was added and incubated for 30 min at 37°C. After washing, substrate solution (TMB/H2O2) was added, and incubated for 30 min. Next, the stop solution was added and the optical density at a wavelength of 450 nm (reference wavelength 630 nm) was measured. The results were expressed as the ratio (R) of the patient sample's extinction to the calibrator's extinction. R < 0.8 = negative; R ≥ 0.8 < 1.1 = borderline; R ≥ 1.1 = positive.

Cell Cultures and Plasmids
HEK293T and stable HEK293T-ACE2-expressing cells [11] were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), antibiotics (Sigma-Aldrich, USA), and non-essential amino acids (Gibco, USA) at 37 °C and 5% CO2. HEK293T-ACE2 cells were also treated with puromycin at a final concentration of 1 µg/mL. The plasmids used were as follows: pNL4.3-ΔEnv-FLuc, spike-G614-Δ19 [12], pcDNA3.3_CoV2_P1 (L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I; 18aa deletion in c-terminal tail) (Addgene plasmid #170450; http://n2t.net/addgene:170450; RRID: Addgene_170450) [13] and pcDNA3.3-SARS2-B.1.617.2 (T19R, 156G, 157-158del, L452R, T478K, D614G, P681R, D950N, c-terminal 18aa deletion) (Addgene plasmid # 172320; http://n2t.net/addgene:172320; RRID: Addgene_172320) [14]. pcDNA3.3_CoV2_P1 and pcDNA3.3-SARS2-B.1.617.2 plasmids were gifts from David Nemazee.

HIV-1-Based SARS-CoV-2 Pseudotyped Particles
Pseudotyped viral particles were generated by transfection of pNL4.3-ΔEnv-FLuc and spike-G614-Δ19, pcDNA3.3_CoV2_P1(spike-GammaΔ18), or pcDNA3.3-SARS2-B.1.617.2 (spike-DeltaΔ18) plasmids (molar ratio, 3:2) using the calcium phosphate method [15]. Briefly, 3.2 × 106 HEK293T cells were transfected with 20 µg of the plasmid mix, 2.5 M calcium chloride, and HEPES-buffered saline 2X (0.28 M NaCl, 0.05 M HEPES, 1.5 mM Na2HPO4, pH 7.05). The pseudotyped virus-containing supernatant was collected 48 hours after transfection and centrifuged at 3500 rpm for 5 minutes at room temperature. Viral stocks were aliquoted and stored at ~80 °C. The stocks were serially diluted, and HEK293T-ACE2 cells were transduced. After 48 hours, the firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI, USA) and Fluoroskan FL (Thermo Scientific). The measurement was performed by integrating the signal for 10 seconds with a delay of 2 seconds after the

Table 1. Characteristics of study participants.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (IQR)</td>
<td>41 (33-47)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td>Male 216 (34.50), Female 260 (45.50)</td>
</tr>
<tr>
<td>Time of sampling*, median (IQR)</td>
<td>25 (25-60)</td>
</tr>
<tr>
<td>Anti-spike IgG (%)</td>
<td>Seropositive 85.6, Borderline/Seronegative 14.4</td>
</tr>
<tr>
<td>ELISA R values*, median (IQR)</td>
<td>2.90 (1.81-4.48)</td>
</tr>
</tbody>
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*days after symptoms onset; **positive samples.
substrate was added. The data obtained was used to determine the dilution in which the luminescence reaches 6 relative light units (RLU)/mL. HEK293T cells (lacking the ACE2 receptor) transduced with pseudotyped particles were used as negative controls.

The sample size for the pseudovirus neutralization assay was calculated using the G*Power software.

**SARS-CoV-2 Pseudovirus Neutralization Assay**

Plasma samples were first heat-inactivated at 56 °C for 30 minutes and diluted in DMEM. Fifty microliters of the dilution were mixed with 50 µL of pseudotyped viral particles and incubated for 1 hour at 37 °C. Next, $1 \times 10^5$ HEK293T-ACE2 cells in suspension were added to each well in duplicates. Firefly luciferase activity was measured 48 hours later as described above. An infection without plasma was used as an untreated control. HEK293T cells transduced with pseudotyped viral particles were used as the negative controls.

The following formula was used to calculate the percentage of neutralization: $100 - \left( \frac{\text{RLUs of treated cells}}{\text{RLUs of untreated control cells}} \right) \times 100$. In all assays, the RLUs of untreated pseudotyped virus-transduced HEK293T-ACE2 cells were at least 100-fold higher than the RLUs of untransduced cells.

**Statistical Analysis**

Statistical analyses were performed using the Kruskal–Wallis test. GraphPad Prism software version 6.0 was used for statistical analysis. A $p$ value less than 0.05 was defined as statistically significant.

**Results**

A total of 626 individuals naturally infected with SARS-CoV-2 between June and November 2020 in Paraguay, before the appearance of VOCs, were included in this study. To determine the presence of anti-SARS-CoV-2 antibodies, anti-spike IgGs were measured in plasma from the individuals. The participants were not vaccinated because vaccines were not available at that time. The median age of the 626 participants was 41 (17-65) years, and 216 (34.50%) were female (Table 1). Most participants (~99%) had mild or moderate COVID-19 conditions.

IgG antibodies against spike were detected in 536 out of 626 plasma from convalescent individuals (85.6% had detectable or positive anti-spike reactive IgGs) (Figure 1), at a titer of 1:320.

Next, 13 plasma samples positive for anti-spike antibodies by ELISA were randomly selected and NAb’s by pseudotyped viral particles neutralization assay against the D614G, Gamma (P.1), and Delta (B.1.617.2) variants were determined. ELISA-negative plasma samples were included as controls. We found that plasma from COVID-19-convalescent individuals demonstrated a NAb’s geometric mean titer (GMT) of

![Figure 1](image1.png)

**Figure 1.** Anti-spike antibodies in plasma samples obtained from convalescent individuals.

Distribution of ratio (R) of anti-SARS-CoV-2 (S1-spike) antibodies in plasma samples obtained from convalescent individuals ($n = 626$). The dotted line represents the threshold above which samples were considered positive. Bars represent median and interquartile range.

Changes in 50% pseudovirus neutralization titers (pVNT50) in plasma samples obtained from convalescent individuals against the SARS-CoV-2 D614G, Gamma (P.1), and Delta (B.1.617.2) variants. Geometric mean titers (GMT) of neutralization and differences in GMT between D614G and VOCs are shown. Statistical analyses were performed using the Kruskal–Wallis test. ns = non-significant.
311.8 (95% CI: 206.4 – 471.0) against the D614G variant (Figure 2). Subsequently, neutralization against Gamma and Delta VOCs was analyzed. Samples from previously infected individuals had a 6.6- and 8.1-fold change reduction in neutralizing capacity to the Gamma and Delta variants, respectively, when compared to that against the D614G variant (Figure 2). This study detected no significant differences when comparing neutralizing capacity against Gamma and Delta variants.

Discussion

Individuals naturally infected with SARS-CoV-2 in Paraguay between June and November 2020 were included in this study. The D614G variant was discovered worldwide by June 2020 [16]. The characterization of emerging variants as VOCs was initiated in late 2020 [1]. Epidemiological data on virus circulation in South America show that the D614G variant (lineage B.1) was the main circulating SARS-CoV-2 variant during this period [17] until the appearance of the Gamma variant late in 2020. The first sequence registered in Pango for the Gamma variant corresponds to the one reported in Brazil on October 1, 2020, whereas in Paraguay this variant was reported for the first time on January 31, 2021 [18]. Therefore, the individuals analyzed in this study were unlikely to be infected with SARS-CoV-2 VOCs.

Spike antibody responses are known to be the main target of neutralizing activity [19]. Our findings showed that convalescent plasma after SARS-CoV-2 infection had a seroconversion rate of 85.6%. Anti-spike IgGs were measured at a title categorized as moderate (1:320) [20]. The percentage of positivity was expected to increase to a low titer (1:80 or 1:160). To the best of our knowledge, there is no information related to the percentage of individuals who produce antibodies against SARS-CoV-2 after natural infection in Paraguay. Our observations are in line with a previous report, which stated that ~75-80% of individuals infected with the virus have detectable anti-spike antibodies [21].

The presence of NAbs is a key indicator of the protective immune response in individuals infected with SARS-CoV-2. Samples from previously infected individuals had 6.6- and 8.1-fold changes in neutralizing capacity to the Gamma and Delta variants, respectively, when compared to that against the D614G variant. These observations are in line with previous reports [22–24] and indicate that SARS-CoV-2 Gamma and Delta variants may partially evade neutralization by antibodies present in the plasma samples. A lower neutralizing capacity against VOCs in sera collected from convalescent patients in early 2020 has been reported [22,23]. Moreover, our study detected a reduction in the neutralizing capacity against the Gamma variant, which is similar to that found previously [24].

Interestingly, this study detected no significant differences when comparing neutralizing capacity against Gamma and Delta variants. In contrast, a slightly higher neutralizing capacity (1.17x) of plasma from convalescent individuals against Delta than against Gamma variants has been reported [25]. This could be due, among other factors, to the timing at which the studied individuals were infected.

The evaluation of NAbs responses in various epidemiological contexts is essential for managing the COVID-19 pandemic. In regions where the vaccination rate against COVID-19 is still low, it is important to generate information on the immunological status of unvaccinated individuals who are naturally infected by the virus. These data contribute to the analysis of the future progress of pandemics.

NAbs have been proposed to serve as a correlate of protection against COVID-19 [26]. In addition, other factors must be considered to establish protection against COVID-19, such as time elapsed after infection, cellular immune response, and host genetics [27,28]. However, few epidemiologic studies have provided data on the NAbs response of individuals infected early in the pandemic against VOCs. Despite the limitations of this study, including the small sample size, our findings indicate that the neutralizing capacities of antibodies elicited by non-VOC infections are lower against VOCs that appeared subsequently.

Conclusions

Our findings show that antibodies generated by non-VOC infection have reduced neutralizing capabilities against Gamma and Delta variants that appeared subsequently and might have implications for immunity strategies.

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**Conflict of interests:** No conflict of interests is declared.