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Reduced levels of convalescent neutralizing antibodies against SARS-CoV-2 B.1+L249S+E484K lineage

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1. Introduction

The pandemic of coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is rapidly changing. It is known that, following the infection, a polyclonal set of antibodies against SARS-CoV-2 proteins are produced, playing a crucial role in the acquired immunity against this virus. In particular, antibodies generated against the spike (S) protein are well known for their ability to compromise the host cell receptor binding and membrane fusion. This leads to blocking the entry of the virus into the host cell, and these antibodies are called neutralizing antibodies (Yang and Du, 2021).

However, the adaptive process of SARS-CoV-2 to human host has resulted in the emergence of antigenically distinct and/or more virulent variants with evidence of reduced neutralization by antibodies generated from natural infection or vaccination; a matter of great importance in the process of selecting the antigenic component in vaccine formulation (Harvey et al., 2021).

Whereby, the US government interagency group and Centers for Disease Control and Prevention (CDC), proposed a hierarchical variant classification scheme with three classes of SARS-CoV-2 variants, namely, Variant of Interest (VOI), Variant of Concern (VOC) and, Variant of High Consequence (VOHC). Here, the presence of substitutions or combinations of substitutions at the Spike (S) protein, associated with reduced neutralization by antibodies generated from previous infection or vaccination, is one of the critical attributes to classify as VOI. The escalation to VOC depends on scientific evidence such as laboratory confirmation of significant escape to neutralizing antibodies and/or epidemiological data supporting increased transmissibility and disease severity. Finally, although no VOHC have been identified so far, it is expected that a VOHC has clear evidence of diagnostic failure, a significant reduction in vaccine effectiveness, therapeutics, and more severe clinical disease (CDC, 2021), https://www.cdc.gov/coronavirus/
In February 2020, in the early stages of the pandemic, the B.1 lineage emerged in Europe with the characteristic D614G mutation in the S protein, which distinguishes it from the A and B ancestral lineages (Volz et al., 2021). This lineage rapidly became the most prevalent (Korber et al., 2020), even in South America, where the first introduced cases corresponded to the A.1 lineage with the conserved D614 position (Franco-Muñoz et al., 2020), and several studies provided evidence on the association of this mutation with fitness advantages without significant impacts on the severity of infection or neutralizing antibody titers (Yurkovetskiy et al., 2020; Plante et al., 2020; Volz et al., 2021).

Later, the E484K mutation in the S protein emerged independently in different VOI and VOC, probably by evolutionary convergence (Ferrandez et al., 2021; ECDC, 2021) and was associated with reduced antibody neutralization (Jangra et al., 2021), antiviral drug resistance (FDA, 2021) and a slightly enhancing of ACE2 affinity (Laffeber et al., 2021). In fact, the E484K mutation is a significant genetic marker, and its presence is considered enough to qualify a variant for VOI status (Boehm et al., 2021).

In line with this, SARS-CoV-2 genomic surveillance at Colombia’s National Institute of Health (INS), identified a highly divergent SARS-CoV-2 lineage characterized by the presence of 21 substitutions, including two amino acid changes in the S protein (L249S and E484K). For this reason, it was proposed for lineage reassignment (B.1 + L249S+E484K) and laboratory evaluation of neutralizing antibodies (Laiton-Donato et al., 2021b).

Several methods have been documented for the assessment of neutralizing antibodies against SARS-CoV-2, among them, pseudovirus-based protocols have proved well correlation with neutralization titers (Almahboub et al., 2020; Donofrio et al., 2021; Neerukonda et al., 2021; Riepler et al., 2020), however, the gold standard is the in vitro neutralization using replication-competent viruses because their sensitivity is higher and allow the evaluation of aspects such as viral fitness and the impact of mutations in proteins other than Spike (Desphande et al., 2020; Legros et al., 2021).

Thus, in this work, we determined the neutralizing antibody titers in convalescent sera against B.1 + L249S+E484K and three lineages (A.1, B.1.420, and B.1.111) without the E484K mutation using micro-neutralization assays to evaluate the potential impact of the E484K mutation in this new lineage on the sensitivity to convalescent neutralizing antibodies.

2. Material and methods

2.1. Human subject collection

The samples were collected between March 2020 and February 2021; all subjects enrolled in this research responded voluntarily to an informed consent formulary previously approved by the Ethics Committee of Colombian National Health Institute (CEMIN) – 2020. This study was conducted in compliance with ethical principles of the Declaration of Helsinki and to the conditions provided by the Ministry of Health - Colombia.

2.2. Cells

African green monkey kidney Vero E6 cells (ATCC CRL-1586™) were used to propagate the SARS-CoV-2 isolates and the neutralization assays. Cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (Lonza®, Catalog No. 12-604Q) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Biowest®, Catalog No. S18b-500), and 100 U/mL penicillin and streptomycin (Lonza®, Catalog No. 17-602F) at 37 °C with 5% CO₂.

2.3. Sample selection and virus isolation

Nasopharyngeal swab specimens from volunteer participants from the five Colombian -regions were collected based on the representativeness and virologic criteria, following the Pan American Health Organization (PAHO) guidance for SARS-CoV-2 samples selection (Laiton-Donato et al., 2021a). Samples with positive real-time RT-PCR for SARS-CoV-2 and PANGO lineage assignment (Rambaut et al., 2020), following the nanopore ARTIC network protocol (Laiton-Donato et al., 2021b), were selected for virus isolation in Vero E6 monolayers.

For this, each sample was diluted 1:2 with DMEM supplemented with 2% FBS. The dilutions were filtered through a 0.2 um membrane and used to inoculate 7.5 × 10⁵ Vero E6 cells seeded the previous day in T-25 flasks. The inoculum was incubated for 1 h at 37 °C in a 5% CO₂ environment. Finally, 4 mL of DMEM medium supplemented with 4% FBS were added, and virus-induced cytopathic effect (CPE) was examined daily for up to 7 days. When CPE was observed, culture supernatant was collected and centrifuged at 300g for 5 min at room temperature, distributed in aliquots of 500 µL, and stored in liquid nitrogen (Algaissi and Hashem, 2020). All the procedures handling the infected cell cultures were held in a biocontainment laboratory.

2.4. Phylogenetic analysis

We recovered 1856 sequences from SARS-CoV-2 infections in Colombia from the GISAID database. The sequence dataset was aligned using the MAFFT software v7 (Katoh et al., 2019; MAFFT (2021)) The alignment was manually curated to remove UTRs and correct possible misalignments. Sequences with genome coverage lower than 90 percent were removed, as well as redundant identical sequences. The final aligned dataset contained 400 sequences with representatives from each lineage circulating in Colombia by July 2021 in each region. A maximum likelihood tree reconstruction was performed with the GTR+F+I+G4 nucleotide substitution model using IQTREE. The substitution model was selected according to the lowest BIC score using IQTREE modelfinder (Katoh et al., 2019). Branch support was estimated with 1000 replicates of an SH-like approximate likelihood ratio test (SH-aLRT), and 1000 ultrafast bootstrap replicates.

2.5. Virus titration

Virus titers were determined by the Reed and Muench tissue culture infective dose (TCID₅₀) endpoint method (Reed and Muench, 1938), after three independent assays. Briefly, for titration, 1.5 × 10⁶ Vero E6 cells/mL were plated into 96-well plates 16 to 24 h before infection. Then, the virus stock was diluted serially from 10 – 1 to 10 – 10, and 100 µL of each dilution was added to the respective well (Eight replicates were performed for each dilution) and incubated in 5% CO₂ at 37 °C for 72 h.

2.6. Microneutralization and binding antibody assays

Serum samples were obtained from five convalescent donors with COVID-19 diagnosis confirmed by real time RT-PCR on nasopharyngeal swab specimens at least 15 days before inclusion. A serum bank specimen from 2019 was used as a pre-pandemic negative control. Additionally, SARS-CoV-2 IgG anti-Nucleocapsid antibodies detection on the Allinity ci series (Abbott, Chicago, IL, USA) was done as screening, where relative light units (RLU) >1.4 were interpreted as positive. Serum samples were conserved at −20 °C until the moment of the analysis.

The microneutralization assay was adapted from the methodology published by Algaissi and Hashem (2020). Briefly, the presence of neutralizing antibodies was assessed by means of eight 2-fold dilutions (1:20 to 1:2560) of heat inactivated serum samples against 2000 TCID₅₀ of each variant virus stock in supplemented DMEM with 2% FBS for one h at 37 °C. After that, the suspension was transferred on a monolayer of...
1.5 × 10^4 Vero E6 and incubated at 37 °C with 5% CO₂ for 3 days. The neutralizing titer was determined as the highest serum dilution at which no CPE was observed under an inverted microscope Primovert (Zeiss). Each sample was tested five times, in three independent tests.

2.7. Statistical analysis

For analysis of differences in serum neutralizing antibody titers, data were log transformed and one-way ANOVA using the DMS multiple comparison correction was used for columnated data while two-way ANOVA using the Tukey multiple comparison correction was used for grouped data. Results with p values < 0.05 were considered significant. The data were analyzed in GraphPad Prism (v9.0.2).

3. Results

3.1. Successful isolation of SARS-CoV-2 lineages with and without the Spike E484K mutation

Five SARS-CoV-2 isolates representing four different PANGO lineages were selected for the MN assays (Table 1). Remarkably, the isolate EPI ISL 1,092,007 currently assigned to the B.1 lineage (PANGO v.3.1.7 2021–07–09) was proposed for lineage reassignment (B.1 + L249S + E484K) and laboratory evaluation of neutralizing antibodies in convalescent sera (Laiton-Donato et al., 2021b). The remaining isolates represented SARS-CoV-2 lineages without the E484K mutation as follows; EPI ISL 49,816 representing the A1 lineage, it was the only isolate without the characteristic D614G mutation in the S protein obtained in this study. The other isolates correspond to the B.1.420 (EPI ISL 52,696 B1.420) and B.1.111 (EPI ISL 526,971 and EPI ISL 794,659) lineages. Regarding B.1.111 isolates, although both share the mutation pattern characteristic of the B.1.111 lineage (Spike D614G, NS3 Q57H and NSP12 P323L), several divergent genome wide mutations were observed between those isolates, for example, EPI ISL 794,659 have two additional mutations at the S protein (Spike T859I and W152R) (Table 1).

Viral stocks of the five isolates titrated by the Reed and Muench method yielded similar titers reaching 10^5 TCID₅₀/mL (Table 1). Thus, the E484K mutation in B.1 + L249S + E484K appears not to affect the viral titer in Vero E6 cells (Table 2) or the virus ability to induce a cytopathic effect (Fig. 1A).

3.2. Phylogenetic analysis reveals B.1.111 sublineages

Given the close lineage assignment of four isolates as lineage B.1 or sublineages B.1.111 and B.1.420, we performed a maximum likelihood phylogenetic analysis to confirm their lineage and placement within a tree with 400 representative sequences from each lineage circulating in Colombia by July 2021 (Fig. 1B). The lineage assignment of the five isolates was confirmed in the phylogenetic reconstruction. The isolates B.1.111-I and B.1.111-II share a lineage designation but different mutation profiles (Table 1). The major number of mutations in B.1.111-II indicates a greater divergence consistent with the sampling date and the expected accumulation of mutations during the pandemic, as observed in the tree (Fig. 1B). The isolate B.1.111-I is located close to the node defining the B.1.111 lineage, while B.1.111-II is placed in a monophyletic group along with more B.1.111 sequences containing the Spike mutation W152R, which is not as divergent as B.1 + L249S + E484K. Furthermore, in line with the previous report, the B.1 + L249S + E484K sample genome is grouped in a sublineage of the B.1.111 lineage (Laiton-Donato et al., 2021b).

3.3. Reduced neutralization antibody titers against B.1 + L249S + E484K in convalescent sera

We initially characterized five convalescent COVID-19 patients...
previously diagnosed with SARS-CoV-2 infection by RT-qPCR, sampled at a median of 90d (range 30–150d) post-onset of symptoms, using SARS-CoV2 IgG anti-Nucleocapsid antibodies detection kit (Abbott, Chicago, IL, USA). All individuals demonstrated greater reactivity to nucleocapsid protein than pre-pandemic negative control, which was comparable to the obtained MN titers (Table 3). Thus, neutralizing antibodies correlated with nucleocapsid binding antibodies (Fig. 2C).

Then, the neutralizing capacity of these sera was evaluated using eight 2-fold serially diluted sera (1:20 to 1:2560) against A.1, B.1.420, B.1.111, and B.1 + L249S+E484K lineages to determine the MN50 titer of each serum sample. Results from three independent assays evidenced differences between the neutralization titers obtained against the five isolates. Specifically, the neutralizing titers against B.1 + L249S+E484K were 1.5, 1.9, 2.1 and 1.3-fold lower than against A.1, B.1.420, B.1.111-I and B.1.111-II, respectively (Fig. 2A). Furthermore, the neutralizing titers for the B.1.111-II variant were 1.6-fold lower in relation to the variant B.1.111.I (P < 0.0001) (Fig. 2B). Finally, no significant differences between neutralizing antibody titers were observed between A1 and B lineages without E484K mutation (Fig. 2B).

3.4. Molecular epidemiological data support a decrease of B.1 + L249S+E484K cases between March-2020 and July-2021

The spatiotemporal distribution pattern of the most representative SARS-CoV-2 lineages in Colombia between March 2020 and July 2021 shows a significant country level decrease of cases associated with the lineages evaluated in this study (Fig. 3). By January 2021, B.1.111 was predominant and widely distributed across the five Colombian regions, followed by B.1 and B.1.420 (Fig. 3A and B). This scenario changed by July 2021, with a notable decrease in these lineages by two- to three-fold, which were displaced by P.1 and B.1.621. Remarkably, B.1 + L249S+E484K was exclusively distributed in the Caribbean region between March-2020 and July-2021, and the decline of cases by the end of this period suggests a limited community transmission (Fig. 3A and B).

4. Discussion

The ongoing global surveillance programs have revealed the emergence of variants harboring mutations in Spike, the principal target of

Table 2

| Lineage     | Titer (TCID50 /mL) |
|-------------|-------------------|
| A.1         | 3.91 × 10⁵ TCID₅₀/mL |
| B.1.420     | 3.0 × 10⁵ TCID₅₀/mL  |
| B.1.111     | 3.2 × 10⁵ TCID₅₀/mL  |
| B.1 + L249S+E484K | 4.67 × 10⁵ TCID₅₀/mL |
| B.1 + L249S+E484K | 4.53 × 10⁵ TCID₅₀/mL |

3.4. Molecular epidemiological data support a decrease of B.1 + L249S+E484K cases between March-2020 and July-2021

The spatiotemporal distribution pattern of the most representative

Fig. 1. Phylogeny of SARS-CoV-2 lineages selected for the MN assays. A.) Representative image of Vero E6 cells infected with SARS-CoV-2. B.) Maximum likelihood phylogeny of SARS-CoV-2 representative lineages by July 2021. The tree was reconstructed by maximum likelihood with the estimated GTR+F+I+G4 nucleotide substitution model for the dataset of 400 genomes. The scale represents nucleotide substitutions per site. The interactive phylogeny and map are available at https://microreact.org/project/fNyvqQHyecKrwPjKFMDo6xD/48df9688.
neutralizing antibodies. This study shows the neutralizing activity of natural infection-elicited antibodies against four SARS-CoV-2 lineages, including B.1 + L249S+E484K.

The E484K substitution, located at the receptor-binding domain (RBD), is continuously and independently occurring in emerging SARS-CoV-2 VOCs and VOIs across all over the world (CDC, 2021; ECDC, 2021).
We also observed a slightly higher neutralizing antibody titre against B.1.111-I compared with B.1.111-II (Fig. 2); this could be explained by the presence of two additional mutations in the S protein, T859I and E484K mutation. Hence, surveillance of emerging lineages with the E484K mutation should be considered, especially as high-affinity antibody titers against B.1.1.7, B.1.351, and B.1.111-II have been described in B.1.111-I compared with B.1.111-II (Fig. 2), which could be explained by the presence of additional mutations in the S protein that increase the transmissibility rate associated with this new lineage. While the MN assays unequivocally evidenced reduced neutralization antibody titers against B1-L249S+E484K in convalescent sera (Fig. 3A), molecular epidemiological data indicate that there is no increase in the transmissibility rate associated with this new lineage (Fig. 3). Hence, surveillance of emerging lineages with the E484K mutation, and/or other specific genetic markers associated with greater virulence, should be carried out in individuals with previous infection or vaccination.

A limitation of this study is the small number of convalescent samples tested, and the absence of samples from vaccinated individuals, future work with more subjects, including vaccinated individuals will help to determine the clinical impact of this mutation, as well as its role in the effectiveness of currently approved vaccine strategies.

5. Conclusion

These results suggest the emergence of a new SARS-CoV-2 lineage with the ability to escape from neutralizing humoral immunity. As the virus continues to adapt to the human host, the accumulation of these mutations on aspects such as the immune response against natural infection or vaccination is unknown. Consequently, it is necessary the intensification of the genomic surveillance programs and the refinement of protocols for the evaluation of point and multiple mutations and their association with the escape from neutralizing antibodies.

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CRediT authorship contribution statement

Diego A. Álvarez-Díaz: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. Katherine Laiton-Donato: Investigation, Data curation, Formal analysis. Orlando Alfredo Torres-García: Validation, Formal analysis, Data curation. Hector Alejandro Ruiz-Moreno: Software, Formal analysis, Writing – original draft. Carlos Franco-Munoz: Data curation.

Maria Angie Beltran: Investigation, Marcela Mercado-Reyes: Conceptualization, Methodology, Funding acquisition, Supervision. Miguel German Rueda: Conceptualization, Methodology, Funding acquisition, Supervision. Ana Luisa Munoz: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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