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One-year surveillance of COVID-19 reinfection cases in Ecuador

Tesis en torno a una hipótesis o problema de investigación y su contrastación

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DEDICATORIA

A mis padres Mónica y Rómulo, mis hermanos Ronny y Doménica, mi novia Jennifer, a mi familia y amigos.

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RESUMEN

Durante la pandemia de COVID-19, ha habido reportes confirmando casos de reinfección por SARS-CoV-2. Estos se definen como una nueva infección, con un virus diferente, en individuos que ya se han recuperado de un episodio previo de la enfermedad. La alta tasa de mutaciones contribuye al aparecimiento de algunas variantes capaces de evadir parcialmente la protección inmunitaria adquirida después de una infección natural. A pesar de que la incidencia de reinfecciones se ha estimado por debajo del 1%, la vigilancia continúa en todo el mundo. Los reportes en Latinoamérica son en su mayoría casos aislados, que no permiten dilucidar la frecuencia del evento en la región. En este estudio reportamos una incidencia del 0.2% de reinfecciones por SARS-CoV-2 en el Ecuador, después de un año de vigilancia. Los individuos susceptibles fueron seleccionados de una base de datos de pacientes positivos por RT-qPCR. Cuatro casos fueron confirmados por secuenciamiento del genoma viral mediante la plataforma MinION. El análisis filogenético descartó que variantes de interés (VOI) o preocupación (VOC) causaron los eventos. Los picos de incidencia mensual de reinfecciones fueron alcanzados después de intervalos de cinco meses y coincidían con olas de contagio en el país, lo que sugiere que el aparecimiento de casos de reinfección está relacionado con la alta exposición al virus durante brotes. Reportamos el estudio más prolongado de monitoreo de reinfecciones por SARS-CoV-2 y contribuimos al esfuerzo global de vigilancia genómica del virus.

Palabras clave: COVID-19, SARS-CoV-2, reinfección, incidencia, secuenciamiento, Ecuador.

ABSTRACT

There have been many reports confirming SARS-CoV-2 reinfection cases during the COVID-19 pandemic. These are defined as a new infection, with a different virus variant, in individuals who had already recovered from a previous episode of the disease. The high mutational rate of the virus contributes to the emergence of some variants able to partially evade immunity protection acquired after natural infection. Despite the incidence of reinfection has been estimated to be under 1%, surveillance continues worldwide. Reports in Latin America are mostly isolated cases, that cannot permit elucidate the frequency of the event in the region. Here we show an incidence of 0.2% of SARS-CoV-2 reinfection in Ecuador, after one year of surveillance. Susceptible individuals were selected from a database of RT-qPCR-positive patients. Four cases were confirmed by sequencing the viral genome through the MinION platform. Phylogenetic analysis discard that variants of interest (VOI) or concern (VOC) caused the events. Monthly surveillance of reinfections showed that the peaks of incidence were reached within intervals of five months and coincided with periods of ongoing outbreaks in the country, which suggests that the emergence of reinfection cases is related to higher exposure to the virus during outbreaks. We report the longest study monitoring SARS-CoV-2 reinfection and contributing to the global effort of genomic surveillance of the virus.

Keywords: SARS-CoV-2, COVID-19, reinfection, incidence, sequencing, Ecuador.

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PARTE I: INTRODUCCIÓN GENERAL

Origin and phylogeny of SARS-CoV-2

The ongoing COVID-19 pandemic was officially announced in March 2020; however, cases arose before in late 2019. In December 2019, there were reports of patients presenting pneumonia of unidentified etiology in Wuhan, China. By January 2020, the causal agent was identified as a coronavirus and was named nCov-2019 and renamed as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Phylogenetic analysis of the viral whole-genome sequence revealed that the virus belongs to the family *Coronaviridae*, genus *Betacoronavirus*, and subgenus *Sarbecovirus*. Coronaviruses are divided into four genera, based on their genetic and serologic characteristics: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. The former two are mostly found in mammals, while the others are found in birds and some cetaceans (Singh & Yi, 2021). Human infecting coronaviruses are mostly *beta-CoV*, including two potentially lethal: SARS-CoV and MERS-CoV. Other *beta-CoV*s cause self-limited infections, like *alpha-CoV*, associated with the common cold.

Coronaviruses had caused outbreaks over the last two decades, most of them were isolated at the regional level. Two viruses caused important incidents: in 2003 the severe acute respiratory syndrome coronavirus (SARS-CoV) and during 2012 the Middle East respiratory syndrome coronavirus (MERS-CoV). Research determined that bats were reservoirs of these viruses, and transmission to humans could have happened through secondary hosts, namely civets and camels, respectively (de Wit et al., 2016). These facts founded the hypothesis that SARS-CoV-2 originated from zoonosis by natural selection in two possible hosts: in an animal reservoir or directly in humans (Singh & Yi, 2021).

Coronaviruses isolated in bats of the genera *Rhinolophus* have been the closest relatives to human-infecting SARS-CoV-2. The coronavirus strain RaTG13, founded in the horseshoe

bat *Rhinolophus affinis* in 2013, was the closest relative known for SARS-CoV-2. Now, a group of strains named BANAL, isolated in the caves of Laos, are the more alike found (Temmam et al., 2021). The genome of these strains shares 96% similitude with the viral sequence of SARS-CoV-2 (Tang et al., 2020). Studies have estimated that the divergence time between RaTG13 and SARS-CoV-2 possibly took place from 18 to 71 years ago (Chaw et al., 2020). The Bayesian analysis confirmed that the Most Recent Common Ancestor (MRCA) likely existed in 1969 (Boni et al., 2020). Furthermore, there has been suggested the existence of an intermediate host, as seen for SARS-CoV and MERS-CoV. This presumption is founded in amino acid similarity (> 90%) of structural proteins E, M, N, and S of coronavirus isolated in the pangolin *Manis javanica* with those of SARS-CoV-2 (Xiao et al., 2020). Moreover, when the receptor-binding domain (RBD) from S protein was compared by phylogenetic analysis, pangolin coronavirus was closer to SARS-CoV-2. This suggested a recombination scenario between an unidentified strain with the pangolin coronavirus in the region RBD, before the divergence of human SARS-CoV-2 (Vale et al., 2021). These hypothetical strain remains to be founded, which could finally dismiss the lab origin hypothesis of SAR-CoV-2.

Genome structure and replication cycle of SARS-CoV-2

SARS-CoV-2 genome shows a high similarity with SARS-CoV (75-80%), which allowed the study of its structure and functionality based on previously published data. SARS-CoV-2 has an RNA single-stranded positive-sense genome (ssRNA+) ranging 26-32 Kb and is enveloped. The larger size of its genome allows genes rearrangements as necessary during new host adaptation (Su et al., 2016). The genome encompasses mostly coding genes like seen in other coronaviruses (Singh & Yi, 2021). Just two untranslatable regions (UTR) flank the genome in the 5' and 3' edges and contain *cis*-acting RNA secondary structures needed for RNA synthesis (V'kovski et al., 2021). Coding genes are open reading frames (ORF) able to

produce structural and non-structural proteins (NSPs). Structural proteins are mainly involved in virion assembly and attachment to host cells during infection. They are the spike protein (S) able to attach to the receptor angiotensin-converting enzyme 2 (ACE2) from the human host, and hence is one of the most variable structures of the virus; the envelope protein (E) involved in virion assembly and budding; the membrane protein (M) also functional during assembly and genome packaging; and the nucleocapsid protein (N) implicated in the replication cycle and evasion of host anti-viral response. Ten ORF genes encode 23 NSPs with important roles during the replication cycle and as accessory proteins. The latter are modulators of host immune response, like ORF3b, ORF6, and ORF9b encoding interferon antagonists (Liu et al., 2014); and ORF8 mediating immune evasion (Y. Zhang et al., 2020). Meanwhile, the other NSPs are mainly encoded by the largest gene of the SARS-CoV-2 genome (*Orf1ab*) which is translated into two polyproteins (pp1a and pp1ab) and cleavage by two viral cysteine proteases (papain-like protease, PL^pro; chymotrypsin-like protease). This produces the viral replication and transcription complex (RTC), including enzymes for RNA synthesis, proofreading, and modification.

The replication cycle of SARS-CoV-2 begins with the attachment of the S protein with the ACE2 receptor expressed in most human cells but importantly in the upper respiratory epithelia (Wölfel et al., 2020). Efficient entry of the virus relies on a cell surface serine protease (TMPRSS2) which promotes fusion and uptake (Shang et al., 2020). Within the cytoplasm, the uncoated genome is translated to the RTC complex by host ribosomal machinery. Genome replication takes place in compartments that prevent exposure to immune sensors and provide an appropriate concentration of molecules for RNA synthesis. They are the double-membrane vesicles (DMVs), convoluted membranes (CMs), and small open double-membrane spherules (DMSs), located and interconnected in the perinuclear space (Snijder et al., 2020). Replication first produces a full-length ssRNA in a negative sense, which is used as a template for

transcription and replication. The transcription process is discontinuous and generates several sub-genomic RNA sequences (sgRNA) that are templates for functional monocistronic mRNAs. The low fidelity rate of RNA-dependent RNA polymerase is surpassed by the ExoN protein with proofreading activity. New virions are assembled in the endoplasmic reticulum (ER)-to-Golgi intermediate compartment (ERGIC) where are translocated the newly produced structural proteins and genomic RNA. Host proprotein convertase furin cleavages amino acids, in between S1-S2 coding subunits, to pre-activate the S protein and increase its affinity to cellular receptors (Shang et al., 2020). A similar cleavage site is seen in a few human coronaviruses (HCoV) and MERS-CoV, but not in other members of the subgenus *Sarbecovirus* (Hoffmann et al., 2020). Finally, virions are secreted by exocytosis via the lysosomal traffic pathway.

COVID-19 reinfection

The length of immunity protection, acquired after COVID-19 natural infection, has not been elucidated. Some studies proposed immunity protection lasting for six months after natural infection (Biobank UK, 2021; Lumley et al., 2021). This waning protection could predispose individuals to reinfection with a different SARS-CoV-2 agent. This scenario was suspected due to evidence seen for other coronaviruses, which can cause reinfections in animals (Sariol & Perlman, 2020). Moreover, the immunity protection against human coronaviruses, SARS-CoV and MERS-CoV, dissipates after three to five years (Poland et al., 2020). In August 2020, the first case of COVID-19 reinfection confirmed by sequencing was reported in Hong Kong (To et al., 2020). Since then, many reports were published worldwide, confirming the feasibility of reinfection with SARS-CoV-2. Nevertheless, the cause of reinfection is not clear and would have implications in long-term vaccine efficiency.

COVID-19 reinfection cases must be distinguished of reactivation or persistent viral shedding, which are caused by the same viral agent isolated during the first infection episode. These two last cases are uncommon and are mostly evidenced in immunocompromised individuals with comorbidities (Desimmie et al., 2021). Reactivation (also known as relapse) usually takes place in the first four weeks after the primal infection and is evidenced by the reemergence of COVID-19 symptomatology (Tang et al., 2021). Meanwhile, SARS-CoV-2 persistence could be or not be accompanied by clinical symptomatology, due to being caused by a replicating virus or by its remanent non-replicating particles (Desimmie et al., 2021). The duration of viral particle shedding varies between different body sites and the immunity status of the individual. On average, the viral RNA could be detected after two weeks from symptoms onset (Cevik et al., 2021). However, it has been detected after 83 days, and even, in 104 days in pregnant women (Li et al., 2020). Prolonged shedding of the virus has been linked with severe outcomes, being on average 19.8 days (Fontana et al., 2021). This prolonged detection of SARS-CoV-2 does not necessarily mean that the patient is infectious, as the median duration of infectiousness has been estimated to be 8 days after symptoms onset (Bullard et al., 2020; van Kampen et al., 2021).

WHO has proposed a protocol for the detection of COVID-19 reinfection cases (PAHO/WHO, 2020). This takes into accounts reinfections with at least 90 days in between, if the patient is asymptomatic, or 45 days if presents symptomatology. Suspected cases need to be confirmed by sequencing the viral agents isolated during both episodes of infection. By definition, reinfection must be caused by different viruses, this could be confirmed by phylogenetic and mutations analysis. The phylogenetic analysis must show that viruses are from different clades or lineages. Nonetheless, some reports have confirmed that reinfection could be caused by viruses from the same lineage (Sevillano et al., 2021). In this situation, the mutational analysis must confirm the reinfection when the nucleotide differences between

viruses must exceed the mutational rate expected during persistent viral shedding (2-3 nucleotides per month) (Borges et al., 2021).

PARTE II: ARTÍCULO CIENTÍFICO

One-year surveillance of COVID-19 reinfection cases in Ecuador

Introduction

The outbreak of COVID-19 was declared as a public health emergency of international concern by the World Health Organization (WHO). One year and a half later, the pandemic has not been fully controlled as positive cases and fatal outcomes keep producing epidemic waves. The high evolutive rate of SARS-CoV-2 (Day et al., 2020) contributes to the emergence of more fit variants with increased transmissibility that are now spread worldwide. Mutations have reduced vaccine effectiveness and may have enhanced virus transmissibility and disease severity (Vasireddy et al., 2021). Moreover, new variants could partially evade immunity protection acquired through natural infection and cause reinfections (Prévost & Finzi, 2021). SARS-CoV-2 reinfection is defined as a new infection, with a different viral variant, in individuals who had already recovered from a previous episode of COVID-19. To confirm reinfection, viral genomic sequences must show two different variants associated with both episodes and rule out a relapse of the first infection or persistent viral shedding (Yahav et al., 2021). Sequencing technology is not widespread which could result in underreported reinfection cases.

Clinical presentation of SARS-CoV-2 reinfections has different features and outcomes which difficult the identification of suspected cases. For this reason, the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC), and the European CDC, have proposed parameters for the identification of cases. Criteria of WHO consider an interval between infections more than 90 days or 45 days if the patient develops symptomatology (PAHO/WHO, 2020). This agrees with those proposed by CDC (CDC, 2020) but not with ECDC, which takes into account only infections with over 60 days-interval

(ECDC, 2021). Few studies have reported the frequency of reinfection based on epidemiological data (Abu-Raddad et al., 2021; Hall et al., 2021; Hansen et al., 2021; Harvey et al., 2021) and report low frequency (under 0.7%), which could be biased due to the lack of confirmation through genomic sequencing.

In Latin America, there are some reports of confirmed reinfections (Díaz et al., 2021; Fonseca et al., 2021; Prado-Vivar et al., 2021; Ramírez et al., 2021) however, they are mostly case reports which do not elucidate the reinfection frequency in the region. In this study, we describe cases identified in Ecuador after one year of surveillance. Our results contribute to the global genomic surveillance of SARS-CoV-2 and its implications in reinfections.

Materials and methods

Sample collection and diagnostics.

Samples included in the study were collected by clinics or hospitals from different provinces of Ecuador and tested at the Microbiology Institute of Universidad San Francisco de Quito (IM-USFQ) from May 2020 to May 2021. Demographic and clinical information for each patient was retrieved at the same time of sample collection and was derived to IM-USFQ. Specimens were oropharyngeal (OP), or nasopharyngeal (NP) swabs taken from symptomatic and asymptomatic patients. Swabs were collected in a 1.5 mL sterile tube with 1X DNA/RNA Shield (Zymo, USA) which inactivates the virus and preserves its genomic material. Samples were immediately transported to the laboratory at 4°C in a sealed container. Diagnostic for SARS-CoV-2 was based in RT-qPCR targeting the *ORF3a* and *N* genes by the Veri-Q PCR 316 kit (MiCo BioMed, South Korea), or targeting the *E* gene using the LightMix® SarbecoV E-gene kit (TIB Molbiol, Germany). The cycle threshold (Ct) value to identify positive results

was based on manufacturers' instructions. Positive samples were stored at -80°C for future analysis.

Selection of patients and ethical considerations.

Identification of suspected reinfection cases was based on WHO recommendations (PAHO/WHO, 2020) with some modifications. The process is represented in the flow diagram in **Figure 1**. From the IM-USFQ database of positive samples collected in all the provinces of Ecuador, we selected those patients who have more than one positive specimen. After that, we selected those who met our suspected reinfection inclusion criteria that consider people with an interval between infections of over 45 days. To prevent exclusion of possible reinfections occurring in less than 45 days, we included those cases where the second RT-qPCR test showed evidence of higher viral loads by a lower Ct-value than the first test. In this study, we included a reinfected patient previously reported by our laboratory (Prado-Vivar et al., 2021). Patients' data were managed anonymously after the assignation of identification codes. This study is part of a country-wide project that aims to monitor the SARS-CoV-2 variants in Ecuador which was approved by the Bioethics Committee of Universidad San Francisco de Quito (CEISH No. P2020-022IN) and by Ministerio de Salud del Ecuador (MSP-CGDES-2020-0121-O).

SARS-CoV-2 whole-genome sequencing.

The RNA extraction and cDNA preparation was done as described previously (Prado-Vivar et al., 2021). Briefly, the Quick RNA viral w/zymo-Spin IC (Zymo, USA) kit was used for RNA extraction. Retro-transcription to cDNA was carried out in line with the ARTIC network protocol (Quick, 2020). The cDNA obtained was stored at 4°C for the next step. We used a long-read sequencing approach through MinION™ (Oxford Nanopore Technologies, UK) following the ARTIC network protocol (Quick, 2020) specific for SARS-CoV-2

sequencing. Details were described elsewhere (Prado-Vivar et al., 2021). In brief, target enrichment was performed using multiplex-PCR, with primer schemes V1 and V3 (Quick, 2020; Tyson et al., 2020), over the cDNA previously prepared. Amplification was assessed by agarose gel electrophoresis and the product was quantified using Qubit dsDNA HS assay kit (Invitrogen, USA). After normalization, the library was prepared following manufacturer instructions by using the Rapid Barcoding Kit (SQK-RBK004), and the Native Barcoding Kit (NB-114) with Ligation Sequencing Kit (LSK-109). The genomic library was loaded into a MinION flow cell (FLO-MIN106D) and sequenced by software MinKNOW v4.2.10. The real-time monitoring of the sequencing process was carried out by RAMPART software v1.2.0 (Rambaut, 2020). After sequencing, we used the Porechop algorithm v0.2.4 for demultiplexing and adapter removal. Medaka v1.4.3 was used for variant calling and to create consensus sequences mapped against the Wuhan-Hu-1 reference genome (NC_045512.2).

Data and phylogenetic analysis.

Clades and linages assignment was performed using Nextclade v1.5.2 (clades.nextstrain.org), GISAID CoVsurver, and Pangolin v3.1.5 (pangolin.cog-uk.io). COVID-19 genome annotator (giorgilab.unibo.it/coronannotator; Mercatelli et al., 2021) was used to identify nucleotide mutations and amino acid substitutions on each sequence relative to the reference genome. We also identified the SNVs (Single-Nucleotide Variations) that each sequence had when compared with its corresponding pair. We report this number as total SNVs, after excluding common mutations. The non-confirmed SNVs, because one of a couple of sequences had N nucleotides (missing data), were excluded as well. Then, we built a phylogenetic tree to ensure that the pair of viral sequences from each reinfection had a different immediate ancestor. First, we identified seventy SARS-CoV-2 genomes published on Nexstrain (nextstrain.org) from Ecuador and then we retrieved their consensus sequences from

the GISAID repository (Shu & McCauley, 2017). The Wuhan-Hu-1 reference genome (GenBank accession: MN908947.3) was included for rooting the phylogram. The sequences were aligned along with reinfection sequences using MAFFT online program (Katoh et al., 2019). Regions of the alignment, containing N nucleotides from the reinfection sequences, were eliminated through AliView v1.27 (Larsson, 2014). Finally, the phylogram was estimated by the Maximum Likelihood method under a GTR nucleotide substitution model and 1000 bootstrap replicates using IQ-TREE online tool (Trifinopoulos et al., 2016). The phylogram figure was obtained and annotated using the Iroki Phylogenetic Tree Viewer (iroki.net; Moore et al., 2020).

Statistical analysis and graphs were produced in RStudio v.1.4.1103 with the packages: ggplot2 and ggpubr. The confidence interval of incidences was estimated by Wald's test with continuity correction. Comparison of Ct-values from first and second infection samples was performed according to the Wilcoxon test for paired samples. A p-value < 0.05 was considered statistically significant.

Results

Suspected reinfection cases.

Since May 2020, the IM-USFQ diagnostic database registers 7,633 patients with positive tests; 569 of them (7.45%) had more than one positive sample. From this subset, we selected suspected reinfection cases. Fifteen patients (0.20% [95% CI 0.12-0.32]) met our inclusion criteria, being our incidence of suspected reinfections, and resulting in thirty samples stored at -80°C. Their information is shown in **Table 1**. None of them were vaccinated or reported a second reinfection. Patients' average age was 33.5 years old (median 32; range 19-48), whilst the interval between infections was on average 98 days (median 82; range 34-238).

Only four patients were female (26.67%). Three patients did not live in Quito, the capital city of Ecuador; two patients lived in Manta and one in Tena, cities located in the coastal and Amazon region, respectively. Since December 2020, the IM-USFQ started identifying the SARS-CoV-2 *E* gene instead of *N* and *ORF3a* used previously. On average, the first infections were identified with a Ct-value of 35.1, while the second infection was 26.8 (**Figure 2**). The difference between the Ct-values from the first and second samples was statistically significant ($p = 0.01$). Information about symptoms was not available for all the patients; however, of nine patients none reported severe symptomatology in the first or second infection, according to WHO criteria (WHO, 2020).

Figure 3 shows the incidence rate of suspected reinfection cases per month. The first case was identified in June 2020, meanwhile, the latter was in April 2021. Each month the IM-USFQ processed a different number of tests, reaching the higher frequency in the period between July-October 2020, while the lower was in February-April 2021. We detected at least one suspected reinfection case in nine of the thirteen months of surveillance. The highest point of incidence was reached during March 2021 (0.83% [95% CI 0.23-2.99]) followed by April 2021 (0.75% [95% CI 0.21-2.69]), November 2020 (0.54% [95% CI 0.42-2.74]) and January 2021 (0.34% [95% CI 0.17-1.48]).

Genomic sequencing of SARS-CoV-2 samples.

We were able to obtain informed consent from 6 out of 15 patients suspected of SARS-CoV-2 reinfection. Target enrichment by multiplex-PCR was successful for paired samples from four patients; samples of the remaining two patients could not be amplified, evidenced by the lack of amplification bands in the agarose gel, and were dismissed for sequencing. Nextclade analysis corroborated that resulting consensus genomes fulfilled quality control parameters and were able to be uploaded to the GISAID repository (**Table S1**). **Table 2** shows

clades and lineages assignment results as well as their annotation of mutations. Every reinfection event was caused by a different lineage virus, according to Pangolin classification. Also, all the pairs of sequences had more total SNVs than expected by the persistence of the viral agent, which has been estimated by 2-3 nucleotide changes per month (PAHO/WHO, 2020). Regarding the Wuhan-Hu-1 reference genome, these mutations were mostly non-synonymous SNVs resulting in amino acid substitutions that were on average five per sequence (median 5, range 3-10). The mean number of substitutions for first infection sequences was similar for those of second infections, being five and six respectively. The substitution D614G in the *S* gene was the most frequent event (7/8 genomes) followed by P314L in the *NSP12b* region (5/8 genomes). None of the sequences presented insertions or deletions.

To ensure that viruses causing reinfections were different from those of first infections, we constructed a phylogenetic tree along with consensus genomes reported in Ecuador and the Wuhan reference genome. The resulting phylogram is shown in **Figure 4**. None of the reinfecting viral variants share an immediate ancestor with the first infection virus variant. Also, each variant is grouped with sequences collected in the same period, and none of them show genetic closeness to clades of variants VOC or VOI reported.

Discussion

From May 2020 to May 2021, we carried out monthly surveillance of suspected reinfection cases and obtained only four sequence-confirmed cases. We present the results of the largest surveillance of SARS-CoV-2 reinfections published, where symptomatic and asymptomatic patients were identified by RT-qPCR. This study contributes to the understanding of the immune protection acquired after natural infection.

We report an incidence of 0.20% (95% CI 0.12-0.32) of suspected reinfection cases during 13 months of surveillance, mostly for patients living in Quito. Our results agree with

other studies based on national surveillance done in the UK, Denmark, USA, and Qatar that report an incidence under 0.70% (Abu-Raddad et al., 2021; Hall et al., 2021; Hansen et al., 2021; Harvey et al., 2021). Unlike these reports, our research was conducted during a larger surveillance period, where people at risk of reinfection were identified by the RT-qPCR SARS-CoV-2 positive test. A similar approach was only performed by Hansen et al. in Denmark (Hansen et al., 2021), in contrast with the other studies where immunological assays were used. However, a shortcoming of using immunological assays to identify people recovered by a first SARS-CoV-2 infection is that some patients may not develop protective immunity after the first event (Sevillano et al., 2021) and even after the second one (Ferrante et al., 2021). Our study mainly surveilled adult patients, with less representation of other age groups with a higher risk of COVID-19. Hansen *et al*. reported a higher risk of reinfection in individuals older than 65 years old (Hansen et al., 2021). We observed more cases in male subjects in a ratio of 3:1, in agreement with Abu-Raddad *et al*. findings (Abu-Raddad et al., 2021). This could be explained as working-class individuals are mainly male people, and hence, are more prone to get infected in the working environment (Bienvenu et al., 2020; Kopel et al., 2020). However, a possible biological explanation for this differential risk of reinfection remains to be elucidated.

WHO and CDC parameters for the identification of suspected reinfection cases only consider events with an interval between infections over 45 days, however, we included cases with a shorter interval as other studies have confirmed reinfection cases occurring this sooner, even within ten days (Lee et al., 2020; Rani et al., 2021; Tillett et al., 2021; J. Zhang et al., 2021). We only identified one suspected case reinfected after 34 days that could not be confirmed by sequencing, corroborating that this kind of event is uncommon. In fact, the average interval of our cases was 98 days which agrees with criteria proposed by other institutions as ECDC and PHE (ECDC, 2021; PHE, 2021). Moreover, we observed a

significant decrease in the Ct-value of the second infection in every suspected reinfection case (**Figure 2**), in contrast with the findings of Ringlander *et al.* who report a higher viral titer in the first infections (Ringlander et al., 2021). Low viral titers in the first infection event may not induce appropriate immunity protection against reinfection, nevertheless, as Ct-values depend on many factors (e.g., time of sample collection after symptoms onset), its use as a marker of reinfection could not be suitable.

Confirmation of a reinfection case must be carried on by genomic sequencing of the viral isolates. For this purpose, we used the ARTIC network protocol for Oxford Nanopore MinION technology, a widely distributed protocol recommended by WHO (WHO, 2021). We were able to sequence and confirm reinfection in 4 patients out of 15. The reinfection by different variants was confirmed by the number of SNVs (of each pair) which exceeds the amount expected in persistent viral infection. Also, Pangolin classification placed each virus in different lineages (**Table 2**), which was also confirmed by phylogenetic analysis (**Figure 4**). The *Spike* protein is the most immunogenic component of SARS-CoV-2, and its mutation could promote evasion of the immune protection (Salleh et al., 2021), possibly predisposing to reinfection susceptibility. Besides the substitution D614G, worldwide spread in the virus variants since January 2020 (Isabel et al., 2020), we did not identify another amino acid substitution featured in all the first-infecting variants that could be correlated to reinfection susceptibility. Severeness of symptomatology had been correlated with the development of a stronger immune protection (Wang et al., 2020). Our four sequence-confirmed reinfections presented more intense symptomatology in the second event than in the first one. Milder symptomatology during the first infection might be a marker of a higher risk of reinfection, nevertheless, immunological assays must be performed to confirm this association.

Ecuador was one of the most affected countries during the beginning of the COVID-19 pandemic in South America. The first case was reported in February 2020, being the second

event confirmed in the region. Guayaquil, the main port of the country, was the first epicenter of cases reaching the worst crisis during April. Meanwhile, the first case in Quito was identified in March (Márquez et al., 2020). As the number of cases increased in the city, confinement measures were implemented in early June. Quito overtook Guayaquil as the epicenter in late July (Gutierrez et al., 2021). Our first case of suspected reinfection was identified in Quito during June 2020, precisely at a time when COVID-19 cases were increasing in the city. Since then, we observed peaks of monthly incidence occurring with an interval of five months. The first one in November 2020 (0.5%) and the second one in April 2021 (0.7%), being the former caused by sequence-confirmed cases. These months were identified as periods of ongoing waves of contagious reported in the country (MSP, 2021). Our results suggest that the advent of peaks of monthly incidence may be determined by waves of contagious, where augmented exposure to SARS-CoV-2 could increase the risk of becoming reinfected. Furthermore, the appearance of suspected reinfection cases within an interval of five months might be related to the waning immunity protection acquired after natural infection, which has been expected to last six months (Biobank UK, 2021; Lumley et al., 2021). Nevertheless, we cannot confirm this assumption without immunological data of the patients, hence, more evidence is needed in this regard. Beyond our results, there is just one additional study reporting sequence-confirmed reinfection in Ecuador (Sevillano et al., 2021). This took place in October 2020, 3 months later of the first infection, and both events were caused by viral variants of the same Pangolin lineage (B1.1). Despite the patient being immunocompetent, he did not develop measurable antibodies before the reinfection, however, in both events he had mild symptomatology.

Our study has some limitations. First, our results mainly reflect the situation in Quito and cannot be extrapolated to the whole country. Also, subjects surveilled were mostly adult, with less representation of other age groups. We did not have the complete clinical histories of surveilled patients, as this information was collected by other institutions. Additionally, we

cannot confirm all reinfections by sequencing because we were not able to reach some patients or we did not recover enough RNA in their samples, possibly due to low viral titers.

Conclusions

We report a low frequency of reinfection cases in this retrospective study in Ecuador, after one year of surveillance. Most of the affected individuals were male subjects, but none presented severe symptomatology during the second event. Main peaks of monthly incidence appeared during waves of high transmission within periods of five months. None of the reinfecting viral agents were related to emerging variants of concern or interest. Our results contribute to the global effort of surveillance of SARS-CoV-2 by genome sequencing, which continues to monitor virus variants and their implications in immunity evasion.

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ÍNDICE DE MATERIAL SUPLEMENTARIO

FIGURAS
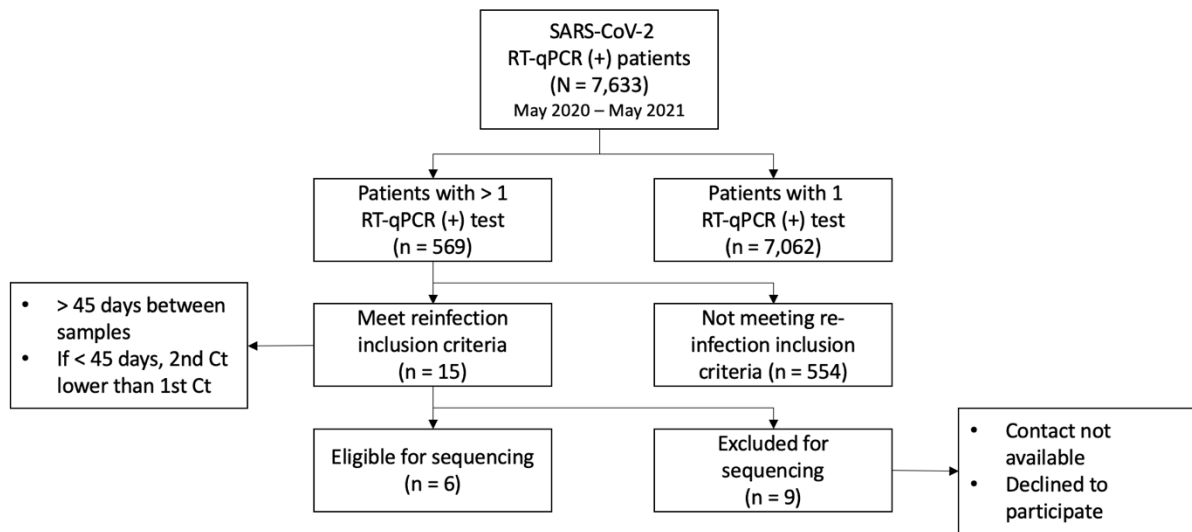
Figure 1. **Flow diagram for selection of suspected reinfection cases.** The database includes tests performed at IM-USFQ from May 2020 to June 2021. Suspected cases were identified first by patients who report more than one positive test, and then by the compliance with inclusion criteria. From the 15 patients who accomplished inclusion criteria, six were considered for sequencing.
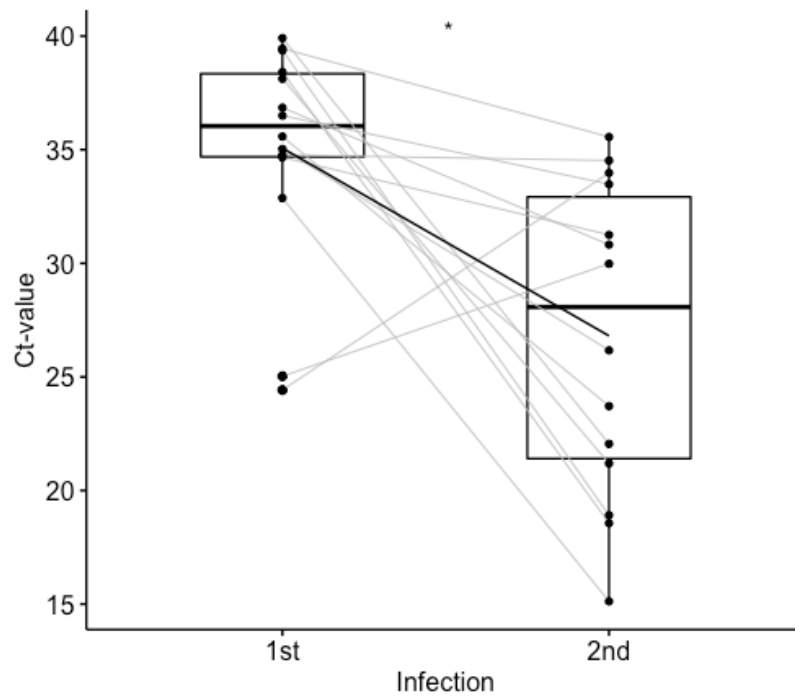
Figure 2. **Comparison of Ct-values from first and second infections**. Paired Ct-values from each case suspected of reinfection is compared. Grey lines represent each case, and the black line indicates the comparison of means. Boxplots shows the interquartile range and median. The asterisk represents the *p*-value from the Wilcoxon test for paired samples.
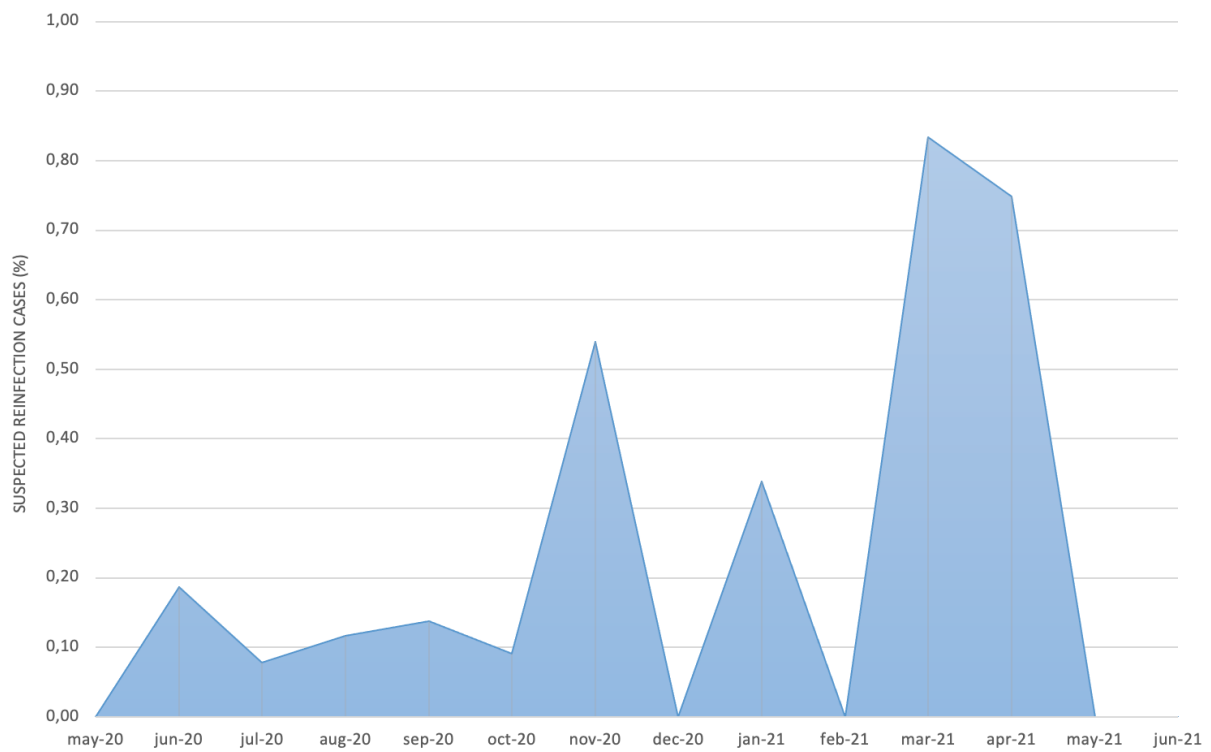
Figure 3. **Monthly incidence of suspected reinfection cases**. The monthly incidence was identified regarding the total SARS-CoV-2 positive tests performed in the same month. Only second infection events are considered for this graph.
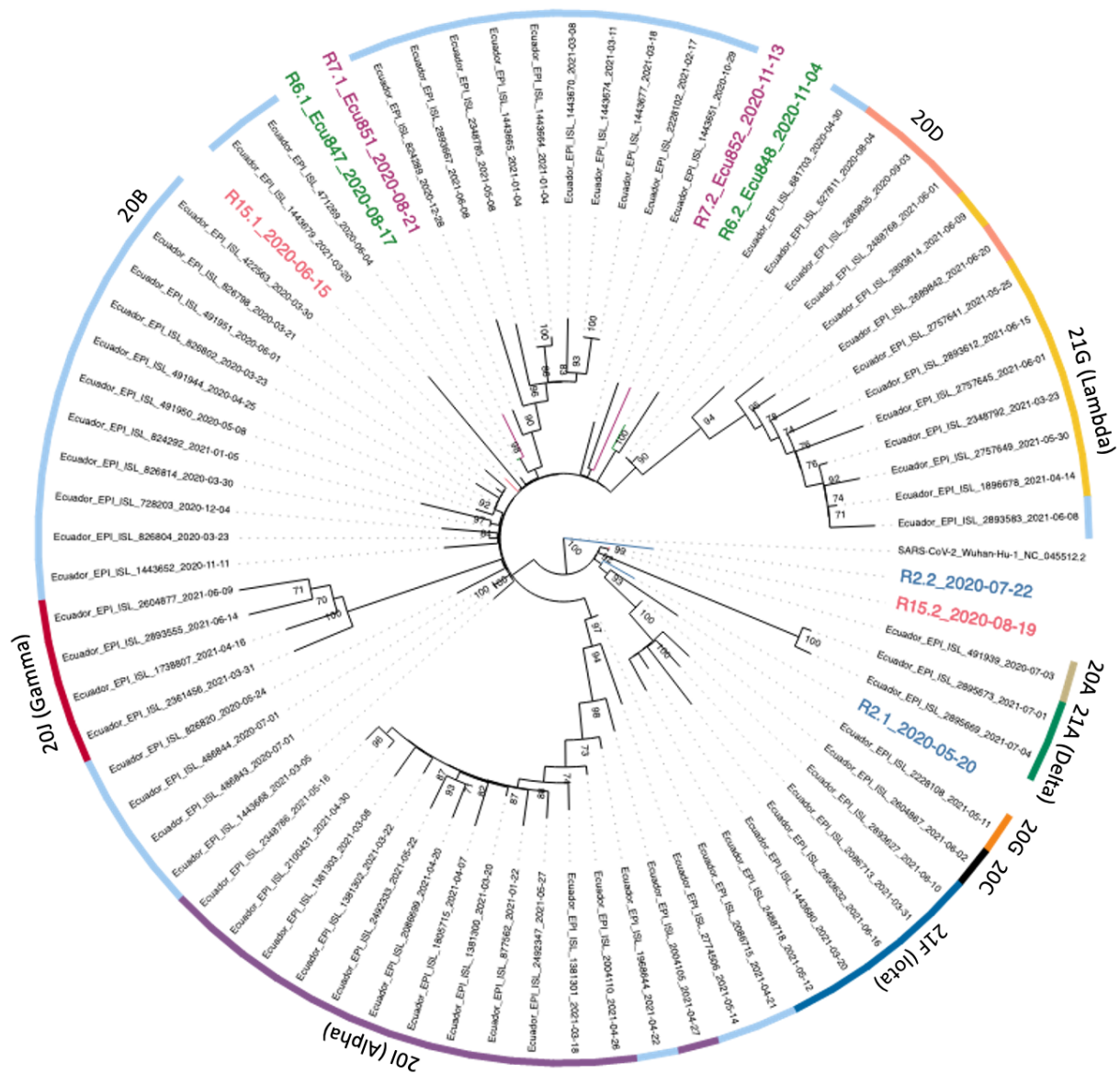
Figure 4. **Phylogram of reinfection sequences and consensus genomes reported from Ecuador**. The tree is rooted to the Wuhan-Hu's SARS-CoV-2 reference genome. Only bootstraps equal to or above 70 are shown. Paired sequences are represented by different colors. The outer ring represents their clades according to Nextstrain classification.

TABLAS

Table 1. Suspected reinfection cases. Each patient and their samples were identified with codes. 'Interval' refers to the days elapsed between infections. 'Negative' means negative tests in between reported by IM-USFQ. 'Type' is the kind of specimen collected: nasopharyngeal (NP) or oropharyngeal (OP). Symptoms severity was defined based on WHO criteria (WHO, 2020). Not reported (N/R) symptoms are also shown. Samples finally sequenced belong to patients highlighted in grey.

| Patient	Age	Gender	Location	Interval	Negative	Code	Type	Ct-value			Symptoms
								N	ORF3a	E	
R1	32	F	Quito	74	No	R1.1	NP	33.36	36.17	-	N/R
						R1.2	NP	31.42	37.62	-	N/R
R2	46	M	Quito	62	Yes	R2.1	OP	-	36.85	-	Mild
						R2.2	OP	30.82	-	-	Moderate
R3	35	M	Quito	61	No	R3.1	NP	39.91	-	-	N/R
						R3.2	NP	22.08	22.02	-	N/R
R4	48	M	Quito	47	No	R4.1	OP	-	39.45	-	Mild
						R4.2	OP	34.77	36.34	-	Mild
R5	24	M	Quito	106	No	R5.1	OP	-	34.66	-	Mild
						R5.2	OP	31.54	30.95	-	None
R6	31	M	Quito	76	Yes	R6.1	OP	35.03	-	-	None
						R6.2	OP	25.84	26.5	-	Moderate
R7	29	M	Quito	82	Yes	R7.1	OP	23.41	25.42	-	None
						R7.2	OP	33.98	-	-	Mild
R8	40	M	Quito	34	No	R8.1	NP	37	36	-	N/R
						R8.2	NP	33.48	-	-	N/R
R9	19	M	Quito	86	Yes	R9.1	NP	-	-	38.42	None
						R9.2	NP	-	-	18.56	Moderate
R10	37	F	Quito	191	No	R10.1	OP	39.37	-	-	None
						R10.2	OP	-	-	18.91	Moderate
R11	-	F	Manta	90	No	R11.1	OP	31.5	34.24	-	N/R
						R11.2	NP	-	-	15.12	N/R
R12	-	M	Manta	90	No	R12.1	NP	37.18	33.98	-	N/R
						R12.2	NP	-	-	23.71	N/R
R13	27	M	Quito	238	Yes	R13.1	OP	21.87	28.18	-	Moderate
						R13.2	OP	-	-	29.98	None
R14	39	F	Quito	169	Yes	R14.1	NP	38.12	-	-	N/R
						R14.2	NP	-	-	21.19	N/R
R15	29	M	Tena	64	No	R15.1	NP	-	-	-	Mild
						R15.2	NP	-	-	-	Moderate

Table 2. Samples of suspected cases sequenced. The resulting assignment of clades and lineages are shown, as well as the total SNVs of each pair, the amount of SNVs expected by persistence, and amino acid substitutions for each sequence.

Sample	Nextstrain clade	GISAID clade	Pangolin lineage	Total SNVs	Expected SNVs	Amino acid substitutions
R2.1	20A	GH	B.1.9	19	4 - 6	NSP12b:P314L, NSP13:S485L, S:D614G, ORF3a:Q57H
R2.2	19B	S	A.1			NSP2:R218C, NSP13:I432T, NSP13:P504L, NSP13:Y541C, ORF8:L84S
R6.1	20B	G	B.1.153	9	5 - 7	S:D614G, N:S2Y, N:R203K, N:P383L
R6.2	20B	GR	B.1.1.231			NSP3:T204I, NSP3:V765F, NSP3:S1443F, NSP4:K399E, NSP4:M458I, NSP5:A234V, NSP12b:P314L, S:D614G, ORF3a:Q38P, N:RG203KR
R7.1	20B	S	A.1	17	6 - 8	NSP3:K839E, S:L452R, S:D614G, ORF7b:M24I, N:R203K, N:P383L
R7.2	20B	G	B.1			NSP2:A205V, NSP12b:P314L, S:D614G, S:T1273I, ORF3a:S40L, ORF3a:T223I, N:R203K
R15.1	20B	GR	B.1.1	7	4 - 6	NSP12b:P314L, S:D614G, ORF3a:G100C, N:RG203KR
R15.2	20A	G	B.1			NSP5:K90R, NSP12b:P314L, S:D614G

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Table S1. GISAID Accession IDs of viral sequences used for the construction of the phylogram. The viral sequences obtained in this study are identified by its corresponding sample code.

GISAID Accession ID	Originating Laboratory	Submitting Laboratory	Authors
EPI_ISL_539783 Sample: R15.1 EPI_ISL_539784 Sample: R15.2	Universidad Regional Amazonica IKIAM	Institute of Microbiology, Universidad San Francisco de Quito	Fabian Aguilar, Katherine Apunte, Andrea Carrera, Nina Espinoza de los Monteros, Giovanna Moran, Marcelo Ortiz, Yeimy Rojas, Sonia Sislema, Carolina Proaño-Bolaños, Belén Prado-Vivar, Sully Márquez, Juan José Guadalupe, Monica Becerra-Wong, Bernardo Gutiérrez, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_516650 Sample: R2.2 EPI_ISL_2492333 EPI_ISL_2100431 EPI_ISL_2228102 EPI_ISL_824292 EPI_ISL_422563 EPI_ISL_824289 EPI_ISL_2086713 EPI_ISL_491939 EPI_ISL_527811 EPI_ISL_728203 EPI_ISL_2086699 EPI_ISL_1896678 EPI_ISL_877562 EPI_ISL_486843	Institute of Microbiology, Universidad San Francisco de Quito		Belén Prado-Vivar, Fernanda Zurita, Sully Márquez, Juan José Guadalupe, Monica Becerra-Wong, Bernardo Gutiérrez, Maria Nataly Velastegui Peralta, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas

EPI_ISL_1805715			
EPI_ISL_1738807			
EPI_ISL_2004105			
EPI_ISL_2086715			
EPI_ISL_2689842			
EPI_ISL_486844			
EPI_ISL_2774506			Rommel Guevara, Sully Márquez, Juan José Guadalupe, Belén Prado-Vivar, Erika Muñoz, Monica Becerra-Wong, Fernanda Zurita, Bernardo Gutiérrez, Tanya Guayasamin, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_2488768 EPI_ISL_525430 Sample: R2.1			Juan José Guadalupe, Sully Márquez, Belén Prado-Vivar, Monica Becerra-Wong, Fernanda Zurita, Rommel Guevara, Bernardo Gutiérrez, Nelson Montalvan, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_2492347 EPI_ISL_2228108 EPI_ISL_2361456			Monica Becerra-Wong, Sully Márquez, Juan José Guadalupe, Belén Prado-Vivar, Fernanda Zurita, Bernardo Gutiérrez, Luis Morales, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_6367762 Sample: R6.1 EPI_ISL_6367763 Sample: R6.2 EPI_ISL_6367764 Sample: R7.1 EPI_ISL_6367765 Sample: R7.2			Rommel Guevara, Belén Prado-Vivar, Mateo Carvajal, Monica Becerra-Wong, Juan José Guadalupe, Sully Márquez, Fernanda Zurita, Erika B. Muñoz, Bernardo Gutiérrez, Rosa Bayas, Stefanie Proaño, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_471269	Hospital Oncológico Solca Núcleo de Quito		Sully Márquez, Belén Prado-Vivar, Juan José Guadalupe, Bernardo Gutiérrez, Marcos Di Stefano, Grace Salazar,

			Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_2004110 EPI_ISL_2689835	Health sciences Department, Universidad Técnica Particular de Loja		Sully Márquez, Juan José Guadalupe, Belén Prado-Vivar, Monica Becerra-Wong, Bernardo Gutiérrez, Rita Rodriguez, Danilo Sosa, Paola Dalgo, Fernando Serrano, Katty Ojeda, Verónica Barragán, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Paúl Cárdenas
EPI_ISL_1381302 EPI_ISL_1381300 EPI_ISL_1968644 EPI_ISL_1381301 EPI_ISL_2604867 EPI_ISL_1443680 EPI_ISL_1443679 EPI_ISL_1443674 EPI_ISL_1443670 EPI_ISL_1381303 EPI_ISL_1443668 EPI_ISL_1443677	Omics Sciences Laboratory	Omics Sciences Laboratory	Derly Andrade Molina, Rubén Armas González, Gabriel Morey León, Darlyn Amaya, Katheryn Sacheri Viteri, Emily Sulay Saltos Montalvo, Paula Juliana Gavilanes Jarrín, Juan Carlos Fernández Cadena
EPI_ISL_1443664 EPI_ISL_1443652 EPI_ISL_1443665 EPI_ISL_1443651	Institute of Microbiology, Universidad San Francisco de Quito		Derly Andrade Molina, Rubén Armas González, Gabriel Morey León, Darlyn Amaya, Katheryn Sacheri Viteri, Emily Sulay Saltos Montalvo, Paula Juliana Gavilanes Jarrín, Sully Márquez,, Fernanda Zurita, Juan José Guadalupe, Monica Becerra-Wong, Belén Prado-Vivar, Bernardo Gutiérrez, Andrea Cunguan, Nabih Dahik, Dayron Brossad, Patricio Rojas-Silva, Gabriel Trueba, Michelle Grunauer, Verónica Barragán, Paúl Cárdenas, Juan Carlos Fernández Cadena
EPI_ISL_2604877	INTERLAB		Derly Andrade Molina, Gabriel Morey León, Darlyn Amaya, Rubén Armas González, Juan Carlos Fernández Cadena
EPI_ISL_491951		INSPI - Charité	

EPI_ISL_491950 EPI_ISL_491944	Instituto Nacional de Investigación en Salud Pública - INSPI		Alfredo Bruno Caicedo, Domenica de Mora Coloma, Andres Moreira-Soto, Anna-Lena Sander, Nina Krause, Maritza Olmedo,Denisses Portugal, Manuel Gonzalez, Silvia Salgado, Alberto Orlando, Alexandra Usiña, Juan Carlos Zeballos, Jan Felix Drexler
EPI_ISL_826814 EPI_ISL_826804 EPI_ISL_826820 EPI_ISL_826798 EPI_ISL_826802	INSPI-CRN DE INFLUENZA Y OTROS VIRUS RESPIRATORIOS	Instituto de Salud Publica de Chile	Javier Tognarelli, Barbara Parra, Loredana Arata, Jaime Lagos, Gisselle Barra, Alfredo Bruno, Domenica de Mora, Solon Narvaez, Jimmy Garcez, Michelle Paez, Martiza Olmedo, Manuel Gonzalez, Patricia Bustos, Rodrigo Fasce, Andres Castillo, Jorge Fernandez
EPI_ISL_2893612 EPI_ISL_2893614 EPI_ISL_2757649 EPI_ISL_2488718 EPI_ISL_2348786 EPI_ISL_2893667 EPI_ISL_2893583 EPI_ISL_2757641 EPI_ISL_2348785 EPI_ISL_2893555 EPI_ISL_2895669 EPI_ISL_2893632 EPI_ISL_2893627 EPI_ISL_2895673 EPI_ISL_2348792 EPI_ISL_2757645		NIC-INSPI	Alfredo Bruno , Maritza Olmedo, Michelle Páez, Jimmy Garcés, Johanna Laines, Lizbeth Patiño, Manuel Gonzalez, Domenica de Mora.
EPI_ISL_681703	Zurita & Zurita Laboratorios	Zurita & Zurita Laboratorios	Gabriela Sevillano, Camilo Zurita-Salinas, Jeannete Zurita