

A Rapid and Consistent Method to Identify SARS-CoV-2 Variants by RT-PCR

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Methodology

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Abstract

Background: Since 2020, the COVID-19 pandemic spread worldwide causing health, economic, and social distresses. Containment strategy rely on rapid and consistent methodology for molecular detection and characterization. The emerging variants of concern (VOCs) are currently associated with increased infectivity, and immune escape (natural defense mechanisms as well as a vaccine). Several VOCs has been detected and include lineage B.1.1.7 first identified in the UK, lineage B.1.351 in South Africa, and lineage P.1 (B.1.1.28.1) in Brazil. Here we validated a rapid and low-cost technique to distinguish B.1.1.7, B.1.351 and P.1 SARS-CoV-2 variants by detecting Spike gene mutations using RT-PCR methodology.

Results: We recruited 77 positive patients affected by Coronavirus Disease-19 (COVID-19). Specific Real-time reverse transcription-polymerase chain reaction (RT-PCR) was employed targeting single nucleotide polymorphisms (SNPs) to screen Spike protein mutations. All data were validated by next generation sequencing (NGS) methodology and using sequence from a public database. Among 77 COVID-19 positive samples we could discriminate with 100% of concordance all the investigated SARS-CoV-2 variants when comparing with NGS method.

Conclusions: PCR-based assays for identification of circulating VOCs of SARS-CoV-2 resulted in a rapid method to identify the specific SARS-CoV-2 variants allowing a better survey of the spread of the virus and its transmissibility in the pandemic phase.

Background

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), the virus causing Coronavirus Disease-19 (COVID-19), was reported for the first time in Wuhan (Hubei Province, China) in December 2019, and has become a major public health. Worldwide in less than 4 months outbreak of COVID-19 was inevitable, has moved from a few affected people in Wuhan to more than 130 million people around the world (Coronavirus Research Center, <https://coronavirus.jhu.edu/map.html>) (Lu et al., 2020; Zhu et al., 2020). Recent characterization from England regarding a SARS-CoV-2 variant of concern (VOC) lineage B.1.1.7, have further exacerbated the pandemic situation. This variant firstly identified in south of England in September 2020 has since then spread in over 30 different countries (Volz et al., 2021). B.1.1.7 strain has 23 novel mutations, this variant had rapidly replaced other SARS-CoV2 lineages occurring in the region and has been established to have greater transmissibility through modelling and clinical correlation studies. (du Plessis et al., 2021; *Investigation of novel SARS-CoV-2 variant Variant of Concern 202012/01 Technical briefing 5*, 2021) At the end of 2020, two others new variants with increased transmission and potential clinical importance emerged, the B.1.351 variant identified in South Africa and the P.1 variant identified in Brazil (Tegally et al., 2021). In Italy on 18th March 2021 the prevalence of the B.1.1.7 England variant was 86.7%, with values ranging between 63.3% and 100% in the individual regions whereas the P.1 Brazilian variant was 4.3% (0% -36.2%) ranging between 0% and 36.2% in the individual regions, at the time of survey the other variants monitored are below 0.5% (www.salute.gov.it). All three SARS-CoV-2 lineages (B.1.1.7, B.1.351 and P.1) share the N501Y mutation, which is of interested among the other

mutations because is involved in the receptor binding mechanism and has been identified as increasing binding affinity to human Angiotensin-converting enzyme 2 (ACE 2) (Leung et al., 2020). In addition to the N501Y mutation P.1 and B.1.351 variants carry both the E484K mutation in the Spike protein's receptor-binding domain (RBD). E484K variants was previously reported to escape from neutralizing antibodies (Weisblum et al., 2020). Because B.1.1.7, P.1, and B.1.351 lineages containing N501Y, E484K and other mutations enabling SARS-CoV-2 virus to spread more quickly in people and evade natural or vaccine-induced immunity, it is important monitoring their prevalence to better understand the SARS-CoV-2 evolution. As today, SARS-CoV-2 lineages are characterized exclusively by sequencing-based technologies and increasing monitoring of variants spreading it would be necessary to develop rapid and low-cost method than NGS platform. In this study a real-time PCR (RT-qPCR) test was developed for rapid identification of B.1.1.7, B.1.351 and P.1 variants in SARS-CoV-2 positive samples.

Results

Genotyping Clinical SARS-CoV-2 samples

To characterize SARS-CoV-2 variants we genotyped 77 SARS-CoV-2 positive swaps samples by qPCR analysis using TaqMan probes. Firstly, we designed D614G and N501Y probes common to all three B.1.1.7, B.1.351, P.1 variants. While seven probes were designed for B.1.1.7 variant (H69_V70del, Y145del, A570D, P681H, T716I, S982A, D1118H); four probes were designed for P.1 variant (K417T, E484K, D138Y, T1027I), and three probes were designed for B.1.351 variant (D215G, E484K, A701V) (Custom TaqMan Assay Design Tool). Results of SARS-CoV-2 variant after qPCR analysis was compared with NGS sequenced data of all 77 SARS-CoV-2 positive samples, and after Pangolin classification (github.com/cov-lineages/pangolin) we showed a concordance of 100% (CI 95% 95.3–100%) between NGS sequenced data and qPCR data for the B.1.1.7, B.1.351, P.1, and other variants not investigated (B.1.177, B.1.258, B.1.545) (Table 1). Considering each specific variant separately the revealed concordance was 100% (48/48; CI 95% 92.6–100%) for B.1.1.7 variant; a concordance of 100% (6/6; CI 95% 54–100%) for P.1 variant; a concordance of 100% (1/1; CI 95% 2.5–100%) for B.1.351 variant (Table 1); and a concordance of 100% (22/22; CI 95% 84.6–100%) in excluding the three variants (B.1.1.7, B.1.351, P.1).

The analysis of mutations by using designed TaqMan probes has brought to no false positive (0/76) neither false negative (0/76). The mean technical concordance for each TaqMan assay probe A570D, D1118H, H69_V70del, N501Y, P681H, S982A, T716I, D614G, Y145del, D138Y, E484K, K417T, T1027I, A701V, D215G was of 97.5% (ranging from 89.7%-93.5).

Table 1
 Concordance of Results of SARS-CoV2 variant characterization between TaqMan probe RT-qPCR and NGS Sequencing

Lineage	N	Concordance qPCR vs NGS
B.1.1.7	48/48	100% (CI95% 92,6%-100)
P.1	6/6	100% (CI95% 54,3%-100)
B.1.351	1/1	100% (CI95% 2,5%-100)
Other Variants	22/22	100% (CI95% 84.6–100)

Comparison between external dataset and SNP genotyping panel

We downloaded 82 FASTA file sequences from NCBI and submitted to Pangolin tools for variant calling. Pangolin submission produced 6 B.1.1.7 calls, 2 P.1 calls, 1 B.1.351 call, 13 A variants, 2 B variants and 58 B.1 variants with other sub-lineages (different from B.1.1.7, B1.351 and P.1). Afterward the 82 FASTA file sequences were submitted to NextClade website for SNPs calling and all data were compared with our designed genotyping panel. The designed genotyping panel would correctly assign B.1.1.7, P.1, and B.1.351 variants download from the external dataset. Moreover, using D418G probe we could also discriminate B.1 lineage from A and B lineage in the 100% of the cases (82/82) since all viral sequence in external database with D418G mutations belong to B.1 head lineage (Supplementary Table 2).

Frequency and Informativity of SNPs marker for SARS-Cov2 variants characterization by qPCR probe

To determine the technical and biological efficiency of each single TaqMan probe assay to discriminate between *SARS-Cov2* variants, we considered the frequency and informativity of the single nucleotide polymorphism markers. We define “informativity” the percentage of difference between the frequency of a single SNP in one *SARS-Cov2* variant respect his frequency in all other *SARS-Cov2* variants (Table 2). In Fig. 2, and Table 2 we showed the frequency and informativity of the single nucleotide marker for each variant group.

We found that the best three informative SNPs for B.1.1.7 are D118H, S982A and T716I with respectively 100%, 98% and 98% of informativity (Table 2); the best two informativity mutations for P1 are K417T and D138Y with respectively 100% and 83%; the best two informativity mutations for B.1.351 are D215G and A701V with respectively 100% and 98% (Table 2).

Table 2
Frequency and Informativity of 15 selected SNPs in 77 SARS-CoV2 genomes.

	Frequency (%)				Informativity* (%)			
	Other variants	B.1.1.7	P.1	B.1.351	Other variants	B.1.1.7	P.1	B.1.351
A570D	0	96%	0	0	0	96%	0	0
D1118H	0	100%	0	0	0	100%	0	0
H69_V70del	0	94%	0	0	0	94%	0	0
N501Y	0	85%	83%	100%	0	0	0	0
P681H	5%	100%	0	0	0	95%	0	0
S982A	0	98%	0	0	0	98%	0	0
T716I	0	98%	0	0	0	98%	0	0
Y145del	5%	83%	0	0	0	79%	0	0
D138Y	0	0	83%	0	0	0	83%	0
E484K	5%	0	100%	100%	0	0	0	0
K417T	0	0	100%	0	0	0	100%	0
T1027I	5%	0	83%	0	0	0	79%	0
A701V	0	2%	0	100%	0	0	0	98%
D215G	0	0	0	100%	0	0	0	100%
D614G	100%	98%	83%	100%	0	0	0	0

***% Informativity VOC=[%frequency VOC-(% frequency other VOCs +% frequency other variants)]**

Discussion

The emergence of SARS-CoV-2 variants of concern (VOC) with the potential for increased transmission, disease severity, and resistance to vaccine induced immunity is of grave concern. A simple screening assay to monitor the emergence and spread of these strains, despite expensive and time-consuming sequencing the virus genome, may be helpful for implementing public health strategies to counter these and future strains. For early detection and prevalence calculation of VOCs (i.e., B.1.1.7, B.1.351, and P.1) PCR-based assays that produce results in a few hours can be useful methods. Several groups are currently developing and evaluating such techniques (Korukluoglu et al., 2021; Vogels et al., 2021). We developed a genotyping panel assay using specific qPCR custom probes able to distinguish with 100% of

concordance respect to the gold standard NGS technology the investigated B.1.1.7, B.1.351, and P.1 SARS-CoV-2 variants (Table 1). The developed custom genotyping panel probes differentiate between the B.1.1.7, B.1.351, P.1 and all B.1 derived variants (all carrying D614G mutation) from the A.1 (Wuhan) lineage. To determine the capacity of the SNPs to discriminate between B.1.1.7, B.1.351, and P.1 SARS-CoV-2 variants informativity of the selected SNPs was calculated. As shown in Table 2 individual SNPs informativity ranged from 79–100%. Bioinformatics analysis of 82 SARS-CoV-2 genomes download from NCBI suggested that our SNPs genotyping panel could correctly assign B.1.1.7, P.1, and B.1.351 and other sub-lineages (different from B.1.1.7, B.1.351 and P.1) variants. All selected SNPs are S protein mutations of concern selected from PANGO lineage reports (<https://cov-lineages.org/index.html>). Substitutions D614G and N501Y are common to all three SARS-CoV-2 variants, while E484K is common in B.1.351 and P.1 variants. All these recently characterized mutations are known to enhance transmissibility of the affected virus (Khan et al., 2021; Korber et al., 2020). Among the 77 sequenced clinical samples we identified an already reported B.1.525 variant, since this new variant was reported on PANGO lineage his frequency in Italy was less than 0,9 % (supplementary table 1). Interesting, B.1.525 shares with B.1.1.7 variant the Y145del mutations and with B.1.31 and P.1 variants the E484K mutation. Additional studies about mutations of B.1.525 variant are required to implement additional mutations to discriminate this variant with qPCR TaqMan probes from other variants.

To date, SARS-CoV-2 variants are mostly screened by NGS methodology of positive samples. Our results show that qPCR methodology using TaqMan probes direct to specific SNPs can be useful and rapid genotyping tool for SARS-CoV-2 positive samples at a low cost. Testing laboratories may also consider designing their own genotyping panel based on regional or national datasets to maximize the virus survey. The robustness of our panel is further increased by use of two probes in the same reaction, one complementary to the WT sequence and one complementary to the mutant sequence. All diagnostic structure qualified to detect SARS-CoV-2 in oropharyngeal swab with this low-cost technique could further screen positive SARS-CoV-2 sample to identify variants, thereby providing valuable genomic data to investigate outbreaks, potentially identifying transmission pathways linking local and regional cases and helping to inform possible interventions. Notably, efficient methods for tracking transmission of certain lineages could be vital in situations where mutations are associated with increased transmission, severity of disease or vaccine failure. The limitation of this study is related to the limited number of samples, especially for P.1 variant counting only 6 patients positive and for B.1.351 variant counting only one sample. To overcome at this problem, we used an external sequenced dataset confirming the validity of our results.

Conclusion

Targeting specific SARS-CoV-2 mutations by qPCR methodology using TaqMan probes is a rapid and consistent method to identify specific SARS-CoV-2 variants. When used on large scale by a wide number of private or public laboratories this method would be helpful to minimize the spread of the recent emergence of several SARS-Cov2 variants allowing a better survey of the spread of the virus in the pandemic phase.

Methods

Sample Collection

Nasopharyngeal/Oropharyngeal swabs collected in Viral Transport Medium were processed in Altamedica laboratory (Rome) for the detection of SARS-CoV-2 virus using approved RT-PCR kits (KHB, Diagnostic kit for SARS-CoV-2). Among these, 77 Viral RNA samples that tested positive for SARS-CoV-2 were randomly selected for this study. The study was conducted with the consent of all the participants, and was approved by the internal Ethics Committee of Altamedica Laboratories, Artemisia S.p.A .

Detection of the SARS-CoV-2 virus by real-time polymerase chain reaction (RT-PCR)

Detection of the SARS-CoV-2 RNA was performed by RT-PCR (KHB, Diagnostic kit for SARS-CoV-2) in accordance with the manufacturer's instructions. The assay targets three genes of the SARS-CoV-2 (N, E and ORF1ab). All samples included in the study had a cycle threshold (Ct) value of the gene targets less than 30 for all genes.

RNA extraction and cDNA synthesis

Viral RNA was extracted from nasopharyngeal swab using automated systems PANA 9600s extractor according to manufacturer's instructions and stored at -80°C until use. SuperScript 2 VILO cDNA Synthesis Kit (Thermo Fisher, USA) was used to reverse transcribe (RT) the SARS-CoV-2 RNA with the following protocol: 4 μL of 5X VILO™ Reaction Mix, 2 μL of 10X SuperScript™ Enzyme Mix, viral RNA (10 ng), and molecular water to a final volume of 20 μL . Retrotranscriptase reaction was performed in Thermal Cycler with the following program: 25°C for 10 min, 42°C for 60 min and 85°C for 5 min. To ensure enough cDNA content for NGS workflow, RNA was quantified it with Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA).

TaqMan RT-qPCR assay

SARS-CoV-2 mutations was genotyping using *TaqMan*® Universal PCR Master Mix and specific custom TaqMan probe. Each qPCR mixture is consisting of 10 μL TaqMan Universal PCR Master Mix, 1ul of TaqMan probe 10x, 1 μL cDNA template and 8 μL of nuclease-free water. The thermal protocol was as follows: an initial enzyme activation step was included at 50°C for 2 min, an incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 1 min annealing and elongation at 60°C . For the use of UMM, which contains Uracil-DNA Glycosylase (UNG), an initial enzyme activation step was included at 50°C for 2 min. All qPCR assays were performed on a QuantStudio 12K Flex Real-Time (Thermo Fisher, USA).

SNPs genotyping panel design for SARS-CoV-2 variants

The trimmed SARS-CoV-2 genome sequences were downloaded from the COVID-19 Genomics UK (COG-UK) consortium website (<https://www.cogconsortium.uk/data/>) and from NCBI SARS-CoV-2 Resources

(<https://www.ncbi.nlm.nih.gov/sars-cov-2/>). Genotyping panel probe was specifically designed to target Spike (S) protein mutation characteristics of each SARS-CoV-2 variants of concern, as reported on PANGO lineage (<https://cov-lineages.org/index.html>). For B.1.1.7 variant we selected D614G, N501Y, H69_V70del, Y145del, A570D, P681H, T716I, S982A, and D1118H substitutions; for B.1.351 variant we selected D614G, N501Y, E484K, D215G, and A701V substitutions; for P.1 variant we selected D614G, N501Y, E484K, K417T, D138Y, and T1027I substitutions. Using Custom TaqMan Assay Design Tool sequence-specific forward and reverse primers were designed to amplify the target sequence region (Thermo Fisher Scientific). Each assay includes two TaqMan minor groove binder (MGB) probes with nonfluorescent quenchers (NFQ): a VIC dye labeled probe to detect the reference sequence and a FAM dye labeled probe to detect the mutation sequence in Spike (S) gene.

Criteria for SARS-CoV-2 variants definition

As criteria to SARS-CoV-2 variant characterization we consider the presence of D614G and N501Y mutations as first filter criteria, as the second step the presence of H69_V70del, Y145del, A570D, P681H, T716I, S982A, D1118H SNPs for B.1.1.7 variant; D215G, E484K, A701V for B.1.351 and K417T, E484K, D138Y, T1027I for P.1 variant.

Libraries Preparation and Sequencing

For each sample target amplification reaction was set up using 10 µL of cDNA, 4.5 µL of 5X Ion AmpliSeq™ HiFi Mix, and 3.5 µL of water; this mixture was splitted into two different tubes and 2 µL of each of the 5X Ion AmpliSeq™ Primer Pool 1 and 2 were added to the corresponding tubes. Reaction of amplification was performed in Thermal Cycler with the following program: 98°C for 2 min, followed by 16 cycles at 98°C for 15 s and 60°C for 4 min. The previous reactions were then combined together and 2 µL FuPa Reagent were added to partially digest the primers (Thermo Fisher Scientific), afterward the mixture was incubated in Thermal Cycler with the following program: 50°C for 10 min, 55°C for 10 min and 60°C for 20 min. Then, 2 µL of diluted Ion Xpress™ Barcode Adapters together with 4 µL of Switch Solution and 2 µL DNA Ligase were added to ligate the adapters to the amplified products, and the samples were incubated with the following program: 22°C for 30 min, 68°C for 5 min, and 72°C 5 min. After ligation, each DNA library was purified with the magnetic beads (Agencourt™ AMPure™ XP Reagent, Beckman Coulter) and then amplified with 50 µL of Platinum™ PCR SuperMix HiFi and 2 µL of Library Amplification Primer Mix using the following conditions: 2 min at 98°C, 5 cycles of 15 s at 98°C and 1 min at 64°C. The amplified libraries were again purified with magnetic beads, and the final concentration of each barcoded cDNA library was determined with Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer recommendations. Barcoded libraries were diluted to 33 pM, pooled in equal volume aliquots, and then loaded on to the Ion Chef™ Instrument (ThermoFisher Scientific) for emulsion PCR, enrichment, and loading into the Ion S5 Prime System (ThermoFisher Scientific). From 20 to 32 Sample are pooled together and sequenced on Ion 530 Chip (ThermoFisher Scientific). Torrent Suite™ software was used to compare base calls, read alignments, and variant calling. Reads were aligned with the Wuhan-Hu-1 NCBI Reference Genome on the Torrent Suite v. 5.10.1. The

following plugins were used: Coverage Analysis (v5.10.0.3), Variant Caller (v.5.10.1.19) and COVID19 AnnotateSnpEff (v.1.0.0), a plugin specifically developed for Sars-Cov-2 that can predict the effect of a base substitution. The software Integrative Genomic Viewer v.2.8.0 (IGV) was used to visualize the TVC (Torrent Variant Caller) bam files to check the consistency of nucleotides calls (Alessandrini et al., 2020)

SARS-CoV-2 variants and mutations characterization

Raw sequence reads were aligned to the complete genome of SARS-CoV-2 Wuhan-Hu-1 isolate (Genbank accession number: NC_045512.2) and classified using the Pangolin COVID-19 Lineage Assigner tool v2.0.7 (github.com/cov-lineages/pangolin). Mutations of external dataset was defined uploading the FASTA sequences by NextClade website (<https://clades.nextstrain.org/>).

External Validation Dataset

All SARS-CoV-2 genomes sequences were retrieved from NCBI Virus (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/>). The criteria for download were the presence of all complete genome sequences and all Italian submitted sequences. The sequences met these criteria were 82. The FASTA file sequences was submitted to Pangolin tools for variant calling and to NextClade website for mutations calling.

Statistical analysis

Standard statistical analyses (average, standard deviation) were performed using Microsoft Excel and GraphPad Prism 8.4.3 for Windows. Visualization graphs in this paper are generated using Matplotlib in Python.

Abbreviations

SARS-CoV-2

Severe Acute Respiratory Syndrome Coronavirus 2

VOCs

variants of concern

COVID-19

Coronavirus Disease-19

Declarations

Ethics approval and consent to participate: The study was approved by the local ethical committee of Artemisia SPA.

Consent for publication: Informed consent was obtained from all subjects involved in the study.

Availability of data and materials: Raw data are available in Supplementary materials, other datasets used and/or analysed during the current study are available from the corresponding author on reasonable

request.

Competing interests: The authors declare no conflict of interest.

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Authors' contributions: MF, and KM conceived the study. MF, KM and MS provide on data elaboration and analysis. MF, and AV performed the laboratory analyses. AM and CG participated in conceiving the study and drafted and revised the manuscript. All authors read and approved the final manuscript.

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Figures

Plots qPCR probes

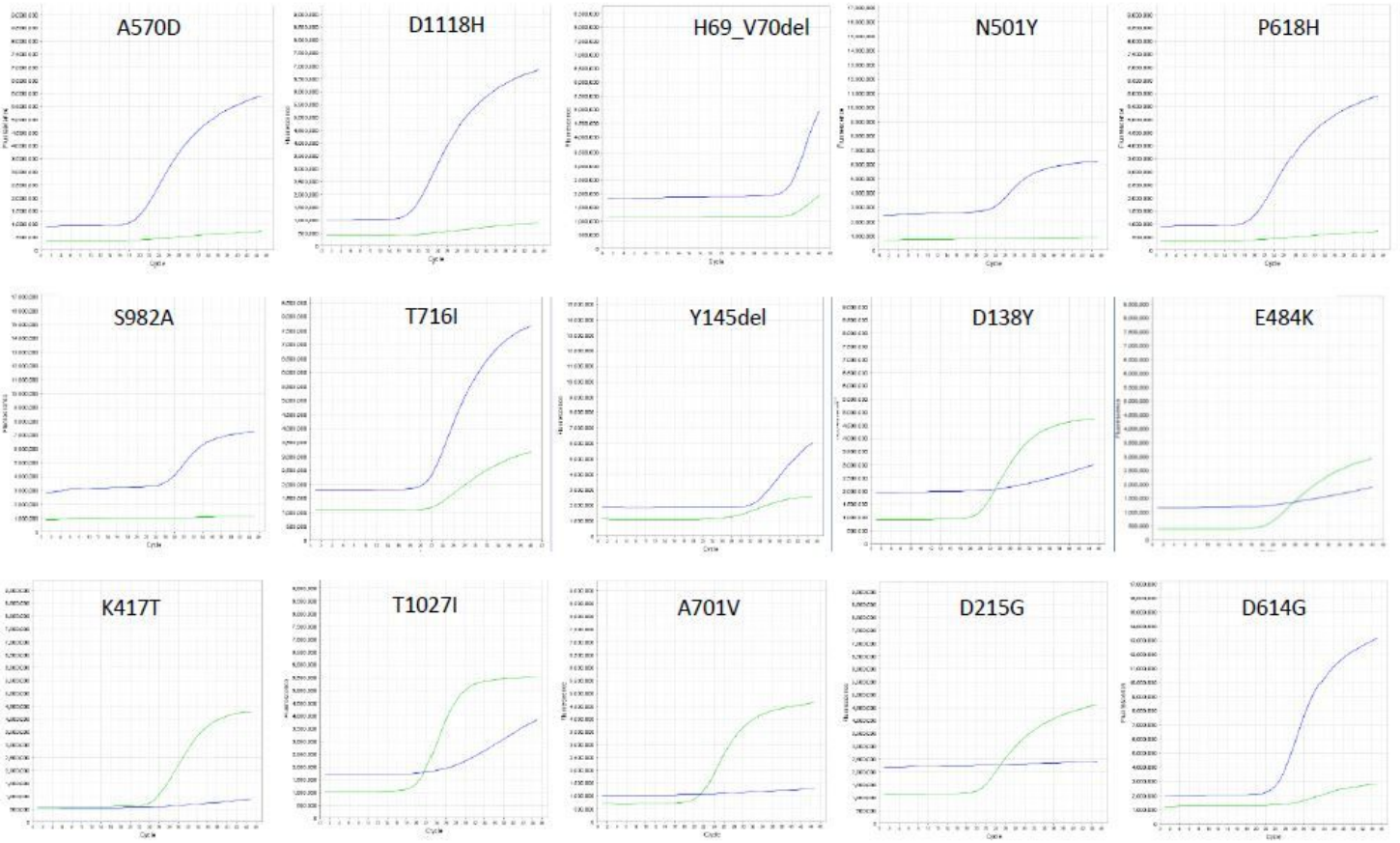


Figure 1

qPCR plots for SARS-CoV-2 B.1.1.7 variant identification. FAM curves (blue) represent mutated sequence, VIC curves (green) represent wild-type sequence (referenced to Whuan sequence).

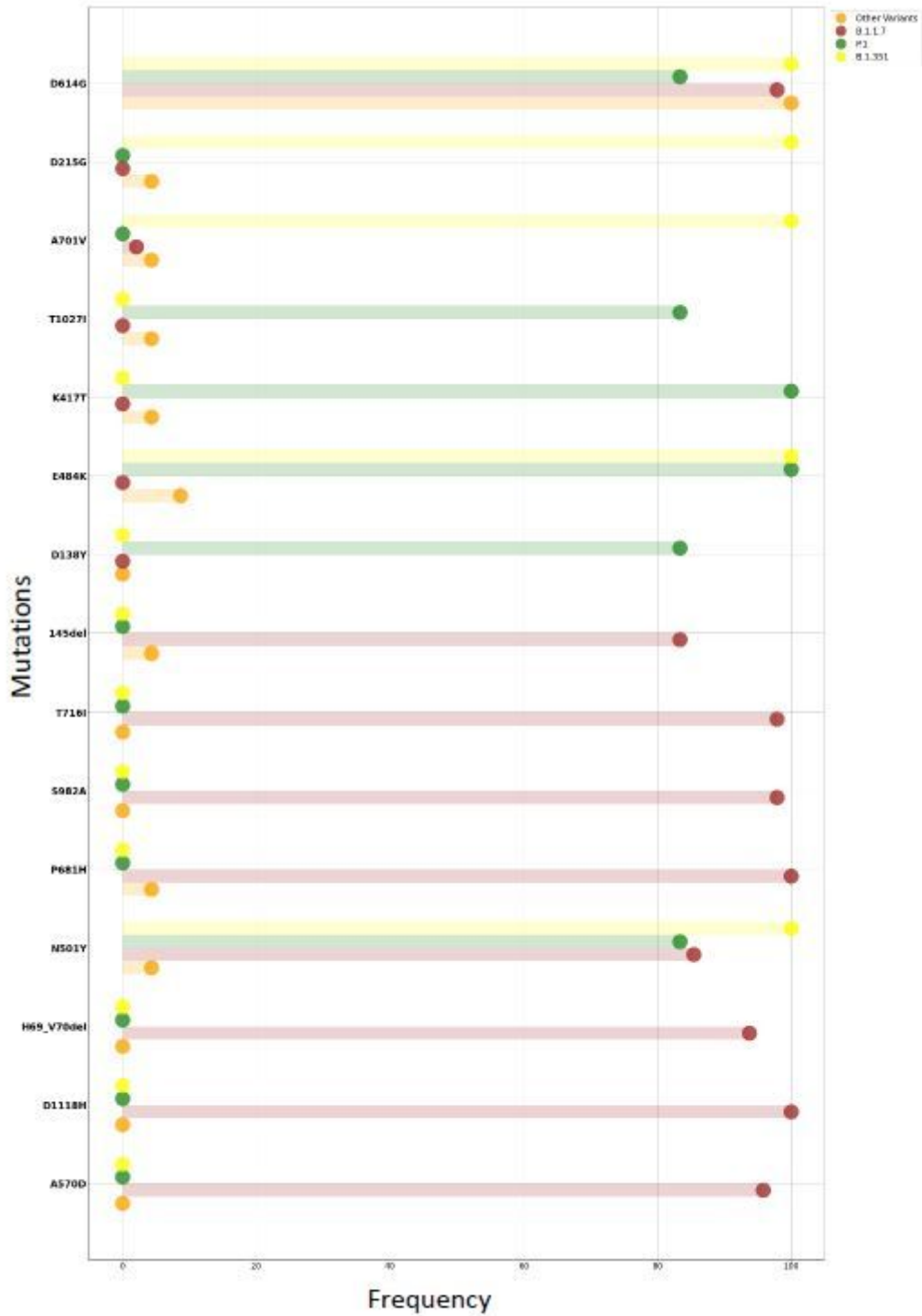


Figure 2

Frequency of mutations for each variant SARS-CoV2 group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.xlsx](#)
- [SupplementaryTable2.xlsx](#)