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Rapid System to Detect the Main Variants of SARS-CoV-2 in Biological Specimens

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Abstract

Since its appearance in late 2019, SARS-CoV-2 has been reported to acquire substitutions more slowly than other RNA viruses, but its tendency to manifest recurrent deletions/mutations in the spike glycoprotein exceeds this slow replacement rate. To date, variants have been identified in many countries, some of which are transmitted efficiently and also present several lineages. The rapid identification of such variants is paramount to quickly implement containment measures. We developed a novel assay using traditional real-time PCR to detect the main reported variants of the spike gene of SARS CoV-2. Primers and probes were designed to detect the following deletions and mutations as well as to cover all lineages known to date (B.1.617, B.1.617.1, B.1.617.2, B.1.617.3 and B.1.618): delta 69:70 and delta 144:145 deletions, which denote the UK variant (VOC 202012/01, now called Alpha); delta 242:244 deletion, which identifies the South African variant (now named Beta); delta 3675:3677 deletion in the ORF1a gene, which denotes the Brazilian variant (now called Gamma); and P681R mutation as well as delta 145:146 and delta 157:158 deletions, which identify the Indian variant (also known as Delta). Our assay will help clinical microbiologists and clinicians to rapidly recognize the presence of variants in biological samples (particularly nasopharyngeal swabs), and it may also be useful for epidemiological purposes in the early selection for successive tracing of patients harbouring virus variants that may be more diffusive and/or not responsive to vaccines.

Introduction

Since the appearance of SARS-CoV-2 in late 2019, people worldwide have presented with severe pneumonia at hospitals (1). Over time, the number of patients has rapidly increased. Community transmission of the virus, as well as antiviral treatments, can promote mutations in the virus, resulting in more virulent and/or more diffusive viruses with potentially higher mortality rates (2, 3). Although most of the emerging mutations do not have a significant impact on the spread of the virus or on its virulence, many others may provide selective advantages, including increased transmissibility, the ability to escape from the host immune response and resistance to antiviral drugs and vaccine effectiveness (2, 4, 5). To date are known at least eleven variants of SARS-CoV-2 named with the letters of the Greek alphabet to simplify their identification (from Alfa to Kappa) (5). Some variants pose an increased risk to global public health, and they are identified as Variants of Concern (VOCs: Alpha, Beta, Gamma and Delta,) in order to prioritize global monitoring and research (6).

The world is still facing these four main variants of SARS-CoV-2 which in detail are: VOC 202012/01 (501Y.V2; UK or Alpha variant); B1.351 (South African variant, or Beta); variant P.1 (Brazilian and Japanese variant or Gamma); and the Indian variant (specifically the B.1.617 lineage), known as Delta (5, 7–15). These variants likely have no impact on the mortality rate but have led to increased transmissibility, especially for the Brazilian and South African variants in which the K417N and E484K point mutations affect the efficacy of vaccines; in fact, a worsening epidemiological situation has been observed in many countries worldwide (7–9). The Delta variant may have been present for some time, but the first B.1.617 genome was recorded on October 5, 2020 in the global database (GISAID) (14). The Delta

variant comprises the B.1.617.1, B.1.617.2 and B.1.617.3 SARS-CoV-2 lineages, which have been increasingly detected in many countries (15). B.1.617 has several mutations (approximately thirteen) that are present in other variants of interest/concern, and it is controversial whether it has antigenic escape (15). To identify and trace these variants, researchers are using the whole-genome sequencing approach, which helps to define the emerging clades and identify single point mutations, but it is time consuming, expensive and available only in large laboratories or in national reference laboratories (16–19). Therefore, for the early identification of such variants, it is desirable to develop a molecular assay that is easy to use and cost efficient.

Here, we present a novel assay based on real-time PCR to detect SARS-CoV-2 variants located in the spike gene. Our assay detects the main deletions/mutations associated with the variants reported above, namely, the UK variant (Δ69-70 and Δ144:145 deletions; called VOC 202012/01), the South African variant (Δ242:244 deletion; also known as 501Y.V2), the Indian variant (P681R mutation as well as Δ145:146 and Δ157:158 deletions) and the Brazilian variant (Δ3675:3677 deletion in ORF1a; called 501Y.V3).

Materials And Methods

Samples

The present study did not include human participants but included leftover samples. For the assay, we used nucleic acids (NCs) extracted from 400 nasopharyngeal swabs (NFWs) routinely processed using a Nimbus instrument (Seegene Inc; Songpa-gu, Seoul 05548, Republic of Korea) and also confirmed using a qRT-PCR of our design as reported by Favaro et al (20). 5 µl of the eluate was used for the assay. NFWs were routinely delivered to the microbiology laboratory of our hospital from March to May 2021. Positive NFWs were established to be positive based on the results obtained using a commercial system (Allplex ^{TM} 2019-nCov Assay-Seegene) and the method described in our previous work (20). NCs were randomly selected among positive NFs and then processed using our assay.

Primers and Probes

The PCR assays used five sets of primers and probes of our own design. Four sets were used for the identification of the specific deletions, and one set of primers and probes, targeting human β–actin, was used as an internal control (IC). Table 1 shows the primer and probe sequences as well as labelling fluorophores for each probe. The primers and probes were synthesized by Metabion International AG (Planegg, Germany) and Bio-Fab (Rome, Italy).

Table 1
List of primers and probes used in our assay

Primers	Sequence
UK Forward Del 69-70	GTT CCA TGC TCT MTC TGG G
UK Reverse Deletion 144:145	GTG GTA AAC ACC CAA AAA TG
South Africa Forward Deleletion 242:244	GGT TTC AAA CTT TAC ATA G
South Africa Reverse Deleletion 242:244	ACC AGC TGT CCA ACC TGA AG
Brazil Forward Deletion 3675:3677	TTA CCT TCT CTT GCC ACT GT
Brazil Reverse Del 3675:3677	CTT ACA AAC TAG TAT CAA CC
Forward Deletion 145:146	GAT CCA TTT TTG GGT GTT TAT AAA
Indian Forward Deletion 157:158	AGT TGG ATG GAA AGT GAG GTT TAT
Indian common Reverse del 145:146/157:158	CTGTTTTCCTTCAAGGTCCATA
Forward Indian mutation P681R	ATC AGA CTC AGA CTA ATT CTC G
Reverse Indian mutation P681R	CAA GTG ACA TAG TGT AGG CAA TG
beta-actin Forward	GAG GGT GAA CCC TGC AAA AG
beta-actin Reverse	CCC TCT AAG GCT GCT CAA TG
Probes	**Sequence & labelling fluorophores**
VAR. Brazil probe	5' Cy5,5 TGC CTG CTA GTT GGG TGA TGC GT 3' BHQ3
UK VAR probe	5' TexasRed TTG GTA CTA CTT TAG ATT CGA AGA 3'BHQ2
South Africa VAR probe	5' Cy5 GTT ATT TGA CTC CTG GTG ATT C 3' BHQ3
Common probe Deletion 145: 146/157-158	5’ Fam CTA GTG CGA ATA ATT GCA CTT TTG A 3’ BHQ1
Indian probe mut P681R	5’ Fam CAC GTA GTG TAG CTA GTC AAT CCA 3’ BHQ1

Primers	Sequence
beta-actin probe	5' HEX GGT GGG GCA GTG GGG GCC ACC TTGT 3' BHQ1

PCR conditions

The PCR working solution contained 5 pmol/μl primers and 2.5 pmol/μl probe (FAM, ROX, Cy5 or Cy5.5), except for the HEX-labelled probe, which was used at 3.0 pmol/μl. The final concentration for each reaction was 500 nM with 250 and 300 nM for primers and probes, respectively. Taq DNA polymerase and reverse transcriptase were both used according to the manufacturer’s instructions (PCR Biosystems Ltd., London, UK).

The real-time PCR conditions were as follows: reverse transcription for 10 minutes at 45°C; RT inactivation/Taq DNA polymerase activation for 2 minutes at 95°C; and 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. An Amplilab real-time machine (Adaltis SRL, Guidonia Montecelio, Italy) was used for the real-time PCR, and the results are shown in Table 2.

Table 2
Possible results of our assay and interpretation criteria for samples with CT ≤ 38

Fluorophores	FAM	ROX	Cy5	Cy5.5	HEX	Variant detected
Interpretation	P681R D145:146 D157:158	S D69:70 144	S D242:244	Orf1 D3675:3677	IC	
Signals on each channel	POS	NEG	NEG	NEG	POS	INDIAN
	NEG	POS	NEG	POS	POS	UNITED KINGDOM
	NEG	NEG	NEG	POS	POS	BRAZIL
	NEG	NEG	POS	NEG	POS	SOUTH AFRICA
	NEG	NEG	NEG	NEG	POS	NEGATIVE
	NEG	NEG	NEG	NEG	NEG	INVALID

Sequence analysis

Amplicons from our assay were sequenced by the Sanger method using the Bio-Fab Research sequencing service (Rome, Italy). The following primers were used: S seq F 5'CCA CTA GTC TCT AGT CAG TGT GT 3' and S seq R 5'GAG AGG GTC AAG TGC ACA GT 3' (this work).

Results

A total of 400 SARS-CoV-2-positive NFs were used in the present study. Using the newly developed assay, 89 NFs (89/400; 22%) were positive for the UK variant, eight NFs were identified as South African variants (8/400; 2%), four NFs were identified as Brazilian/Japanese variants (4/400; 1%) and 24 (24/400, 6%) were Indian variants. The remaining samples were concluded as wild type.

To confirm the nature of the variants identified by our assay, all samples were analysed by sequence analysis.

Seventy-nine samples showed sequences compatible with Δ69:70 and Δ144:145 deletions, while ten samples showed mixed electropherograms with overlapping peaks. Figure 1 shows the UK variant Δ69:70 sequence, while Figure 2 shows a mixed electropherogram, resulting from the presence of two viral genomes (wild type and variant) in the same sample. The presence of two different lineages has previously been reported in the literature (3, 21). Table 2 shows the assay results. Of note, the presence of the Brazilian variant was verified by comparing the signals obtained in the different channels (FAM, ROX, Cy5 and Cy5.5) after the amplification assay. The UK and the Brazilian variants share the ORF1a deletion, while the Δ69:70 and Δ142:144 deletions are only present in the UK variant. Thus, if the PCR showed amplification curves in the ROX and Cy5.5 fluorophore channels (identifying Δ69:70 and Δ142:144 deletions as well as Δ3675:3677 deletion), it indicated that the UK variant was present. If only one signal for the Cy5.5 fluorophore (Δ 3675:3677 deletion) was present, the sample was positive for the Brazilian variant. Moreover, if an amplification curve was observed in the FAM channel, we could conclude that the Indian variant was present, even though the assay could not discriminate between the P681R mutation and the Δ145-146 or Δ157:158 deletion.

Discussion

VOC 202012/01 was the first variant identified in the United Kingdom in December 2020, but it has been traced back to September 2020 (22, 23). VOC 202012/01 is the predominant variant circulating in the UK, and it has become a great concern due to its increased transmissibility (2–23). The UK has implemented stricter nonpharmaceutical interventions (NPIs) to reduce risk of transmission (23, 24). Additionally, community transmission of VOC 202012/01 has been observed in Denmark, and in its response, the country has strengthened and prolonged the measures of containment. In December 2020, the 501Y.V2 variant was first identified in South Africa, and again, it is now one of the most prevalent. This V2 variant is characterized by increased transmissibility, and starting in January 2021, it has been identified in ten

EU/EEA countries (France is at the top), but also in Israel and the UK (24–28). Starting in December 2020, the B.1.617.1, B.1.617.2 and B.1.617.3 SARS-CoV-2 lineages were reported in India, and they have been increasingly detected in many countries (5, 6, 15). Therefore, early identification is extremely urgent to contain the spread of such variants (5, 6, 29). The reference method for identifying variants of SARS-CoV-2 is whole-genome sequencing, but it is expensive, time consuming and limited to use in large laboratories and reference laboratories. Our system has been demonstrated to be a rapid and cost-effective method to detect the main variants of the virus. Our assay is a simple real-time PCR and does not require expensive instrumentation. Furthermore, it is easy to use and can be introduced in any laboratory even in those that may not have advanced sequencing systems available. The advantage of our assay is that every hospital may quickly obtain the result of the variant circulation to promptly implement the infection control measures required to prevent further transmission in their setting (25–29).

Our assay has helped to quickly confirm/exclude the presence of the main SARS-CoV-2 variants, because our test is based on the direct detection of the presence of deletions/mutations, it is not affected by the potential co-presence of the wild-type SARS-CoV-2 virus in the specimens (which indicates a coinfection). Importantly, some of the commercially available tests have based their detection of the variants on the lack of amplification of the S gene. For both instances of a co-infection and re-infection (conditions that may generate the co-presence of two types of virus in the same sample/patient), the commercial tests may show a curve for the spike gene, but the latter belongs to amplification of the SARS-CoV-2 wild type virus, thereby masking the co-presence of a viral variant.

Finally, the results of our assay have provided information for the circulation of such variants in our location. Surprisingly, we found that the UK variant is widespread in our country, but it explains the massive and prolonged second longwave despite the lockdown measures implemented by our authorities (26). Moreover at the time of study also Indian variant was rising, and up to date it is dominant in our country (30). The global and rapid diffusion of SARS-CoV-2, combined with its ability to mutate, provides a terrible example of the prediction of the Nobel Laureate, Joshua Lederberg, who defined the fight against microbes as "*Our wits versus their genes".*

Declarations

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Competing Interests

Carla Fontana has received a research grant by Quintiles/Angelini. Advisory Board: Angelini, Pfizer

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Ethics approval

The study did not include human participants but leftover samples. Specific informed consent are not required (as stated by "Independent Ethics Committee Tor Vergata Polyclinic "on 17 June 2020, approval no.102/20), having based this study on the use of leftover human specimens collected for routine analysis that would otherwise been discarded. The same specimens are "unlinked anonymized materials". This statement is in agreement to FDA "Guidance on Informed Consent for In Vitro Diagnostic Device Studies Using Leftover Human Specimens that are Not Individually Identifiable" April 25, 2006, and "Bioethical ed use di campion biologici umani" Pezzoli P. & Graziani MS biochemical clinic, 2008, vol. 32, n. 3.

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Figures

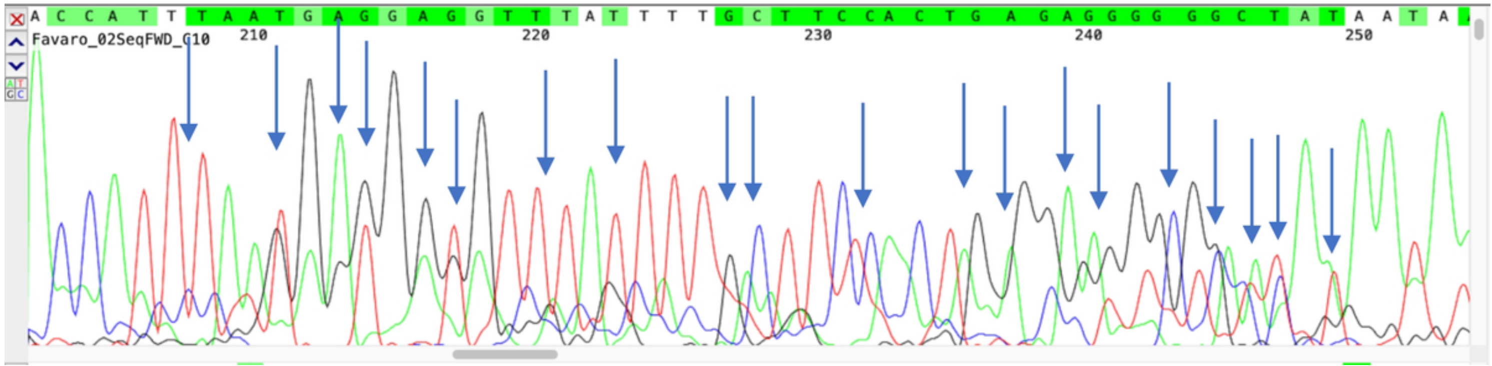

Figure 1

Electropherogram peak analyses showing the sequence of “mixed type sample” blue arrows indicate the presence of overlapped peaks in the eletropherogram due to the co-presence of wild type as well as variant of SARS-CoV-2

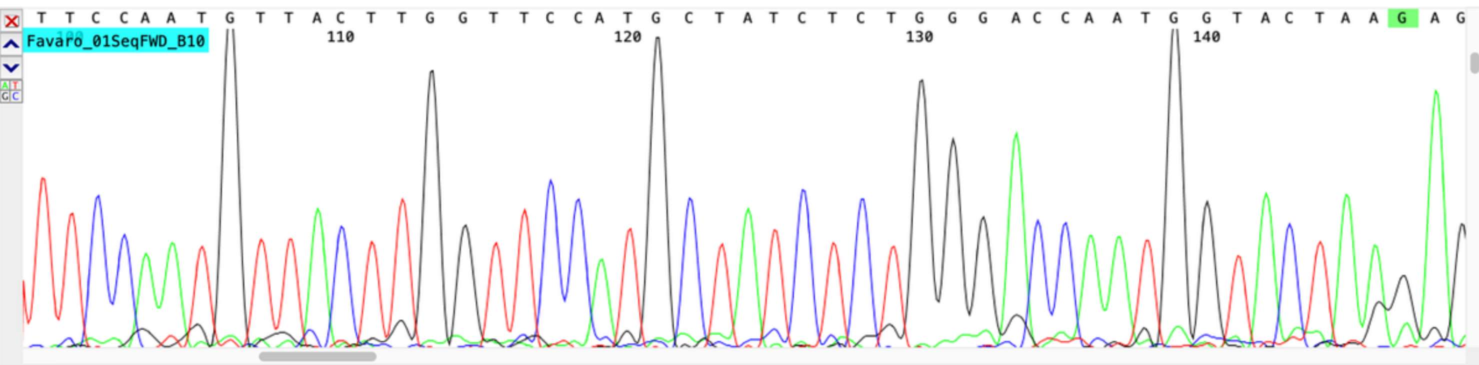

Figure 2

Electropherogram peak analyses showing the sequence of one sample concluded as “UK variant” with the Δ69/70 deletion