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Journal of Clinical Virology 141 (2021) 104906

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Rapid SARS-CoV-2 variant monitoring using PCR confirmed by whole genome sequencing in a high-volume diagnostic laboratory

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ARTICLE INFO

Keywords:
SARS-CoV-2
B.1.1.7
B.1.351
P.1
Whole Genome Sequencing (WGS)
Variants screening

ABSTRACT

Objectives: The emerging SARS-CoV-2 variants of concern (VoC), B.1.1.7, B.1.351 and P.1, with increased transmission and/or immune evasion, emphasise the need for broad and rapid variant monitoring. Our high-volume laboratory introduced a PCR variant assay (Variant PCR) in January 2021 based on the protocol by Vogels *et al.*

Study design: To assess whether Variant PCR could be used for rapid B.1.1.7, B.1.351 and P.1 screening, all positive SARS-CoV-2 airway samples were prospectively tested in parallel using both the Variant PCR and whole genome sequencing (WGS).

Results: In total 1,642 SARS-CoV-2 positive samples from individual patients were tested within a time span of 4 weeks. For all samples with valid results from both Variant PCR and WGS, no VoC was missed by Variant PCR (totalling 399 VoC detected). Conversely, all of the samples identified as "other lineages" (i.e., "non-VoC lineages") by the Variant PCR, were confirmed by WGS.

Conclusions: The Variant PCR based on the protocol by Vogels *et al.*, is an effective method for rapid screening for VoC, applicable for most diagnostic laboratories within a pandemic setting. WGS is still required to confirm the identity of certain variants and for continuous surveillance of emerging VoC.

1. Background

The discovery of a new SARS-CoV-2 variant (lineage B.1.1.7 / N501Y_V1) with increasing incidence in the United Kingdom in the autumn of 2020, highlighted the importance of rapid and comprehensive surveillance of SARS-CoV-2 variants of concern (VoC) [1]. As exemplified by the arrival of the N501Y spike mutation, which was reported to contribute to increased viral transmission [2], rapid detection of VoC has become essential to control the current pandemic. The emergence of other VoC, such as B.1.351 and P.1, further underlined the need for variant monitoring both in pandemic control, but also for adjusted vaccine design.

The B.1.1.7 lineage is characterised by a repertoire of 17 mutations and deletions located in the open reading frame (ORF) 1 a/b, ORF 8, nucleocapsid (N gene) and spike gene regions [1]. B.1.351 and P.1 lineages also have a set of characteristic genetic mutations and deletions. These include both the N501Y and E484K spike mutations, which may be important for increased viral contagion and immune evasion [3]. The gold standard for the detection of VoC is whole genome sequencing (WGS), which is a powerful tool to map and describe the viral genome, including monitoring for current and emerging virus variants and viral genome changes with possible biological significance [4,5]. WGS is increasingly performed in several countries and has given valuable insights into the emergence and dynamics of SARS-CoV-2 variants worldwide. However, sequencing is time and resource demanding and requires extensive data processing. It is therefore less apt for the comprehensive rapid variant analysis needed to contain VoC within a population in a pandemic setting.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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https://doi.org/10.1016/j.jcv.2021.104906
Received 14 April 2021; Received in revised form 24 June 2021; Accepted 28 June 2021
Available online 7 July 2021

Direct PCR-based variant analysis of SARS-CoV-2 is both rapid and inexpensive and can easily be performed on all positive samples after SARS-CoV-2 detection using other methods. Recently, Vogels *et al.* published a protocol for a triplex RT-PCR assay that detects the VoC B.1.1.7, B.1.351 and P.1 by targeting characteristic deletions of the ORF1a and spike gene (Δ69/70) [6]. This assay can be performed with hands-on and running time totalling 4 hours including RNA extraction.

2. Objectives

The PCR assay based on the Vogels protocol (in this paper termed "Variant PCR") was established in our high-volume laboratory at Oslo University Hospital (OUH) in January 2021. To assess whether this assay could be used for rapid B.1.1.7, B.1.351 and P.1 screening, all positive SARS-CoV-2 samples collected within a span of 4 weeks were tested in parallel using both Variant PCR and WGS.

3. Study design

3.1. Cohort and materials

The Department of Microbiology at OUH receives the majority of community and hospital samples for SARS-CoV-2 diagnostics from Oslo and parts of south-eastern Norway. Most samples are combined throat/nasopharyngeal swab specimens. Primary detection of SARS-CoV-2 positive samples was performed by a number of high-throughput diagnostic procedures (see below). All positive samples analysed for the detection of SARS-CoV-2 RNA between 25 January and 19 February 2021, were then prospectively included for both Variant PCR and WGS.

3.2. SARS-CoV-2 detection

For the majority of the samples (community testing), SARS-CoV-2 detection was performed at the *Unit for Large Scale SARS-CoV-2 PCR Diagnostics*, established to process large volumes of specimens. RNA extraction was performed on an automated Tecan Fluent 1080 workstation (Tecan Trading AG, Switzerland) using an in-house extraction protocol developed at the Norwegian University of Science and Technology (Trondheim, Norway) based on a standard method using magnetic beads [7,8]. Bacteriophage MS2 RNA (Merck, Sigma-Aldrich, Darmstadt, Germany) was added before extraction as internal control [9]. Nucleic acid was isolated from 100 µl sample and eluted in 100 µl PCR-grade water. The eluate (5 µl) was analysed by real-time RT-PCR using an Aria Dx Real-Time PCR System (Agilent Technologies LDA, Malaysia) in a reaction volume of 25 µl containing SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, Darmstadt, Germany), 0.4 µM E-gene primers and 0.2 µM probe described by Corman *et al.*, and 0.3 µM MS2 primers and 0.1 µM probe described by Dreier *et al.* [9,10]. All oligonucleotides were synthesized by Tib-Molbiol (Berlin, Germany), except the double quenched E gene probe, which was provided by Integrated DNA Technologies Inc, (Coralville, Iowa, USA). Thermal cycling was performed at 50°C for 15 min for reverse transcription, followed by 95°C for 2 min and then 45 cycles of 95°C for 15s and 58°C for 30s. For samples from in-hospital patients, the detection of SARS-CoV-2 RNA was performed using one of the following assays; the Cobas® SARS-CoV-2 kit on the Cobas® 6800 system (Roche Diagnostics GmbH, Mannheim, Germany), the Aptima® SARS-COV-2 Assay on the Panther® System (Hologic, Inc., San Diego, CA, USA), the Cobas® SARS-CoV-2 & Influenza A/B kit on the Cobas Liat® System (Roche Diagnostics GmbH, Mannheim, Germany), or the Xpert® Xpress SARS-CoV-2/Flu/RSV kit on the GeneXpert Instrument System (Cepheid, Sunnyvale, CA, USA). All positive samples were re-extracted for Variant PCR and WGS analysis, regardless of CT values from the primary detection assay.

3.3. Variant PCR

Variant PCR was performed on positive samples only, using the Vogels protocol [11]. RNA from positive samples was re-extracted using magnetic beads on Tecan Fluent 1080 as described above. Real-time RT-PCR assays in 25 µl reaction volume were performed with the QuantiNova Pathogen master mix (Qiagen, Germantown, MD, USA), using CDC-N1 ("N gene"), Yale-Spike 69/70del ("Δ69/70") and Yale-ORF1a3675-3677del ("ORF1a") primer and probe sets in accordance with the protocol. Thermal cycling was performed at 50°C for 10 min for reverse transcription, followed by 95°C for 2 min and 40 cycles of 95°C for 10s and 55°C for 30s. Results indicated by target failure were interpreted in accordance with the Vogels protocol (Table 1). The N gene was regarded as a positive "pan-SARS-CoV-2" control, and samples with CT values for N1 >35 or undetected (No CT) were defined as inconclusive. Likewise, samples with Δ69/70 and/or ORF1a detected, but with a CT >35 were also defined as inconclusive.

3.4. SARS-CoV-2 whole genome sequencing

All samples subjected to the Variant PCR assay during the study period were sequenced, regardless of N gene CT values. Samples with low CT values were diluted in PCR-graded water to normalise CT value to ≥20. RNA was converted to cDNA with the SuperScript™ IV First-Strand Synthesis System (#18091200, Invitrogen) in 10 µl reactions using 4 µl RNA and random hexamers, according to the manufacturer's instructions, whereby the incubation at 50°C was increased to 30 min. Half of the cDNA (5 µl) was used as input for the library generation. Sequencing libraries were prepared according to the manufacturer's instructions using the Swift Normalase® Amplicon Panel SARS-CoV-2 Additional Genome Coverage (#COVG1V2-96, Swift Biosciences, Ann Arbor, MI, USA) in combination with Swift Normalase® Unique Dual Indexing Primer Plates (#X91384, Swift Biosciences), whereby 4 + 22 PCR cycles were applied during the Multiplex PCR step followed by 9 PCR cycles during the Indexing PCR step. Libraries were pooled and normalised to 4 nM, then sequenced on Novaseq SP flow cells (Illumina, San Diego, CA, USA) with 150 bp paired end reads. On average, 1 million reads per sample were obtained. Base calling during sequencing was performed using RTA v3.4.4. BCL files were first demultiplexed using bcl2fastq v2.20.0.422 and converted to FASTQ format, and the remaining steps were performed independently for each sample. Primer sequences were trimmed from the reads using pTrimmer v1.3.3 [12]. Reads pairs that did not have known primer sequences at the beginning of both reads were discarded. The read pairs were then subjected to quality filtering and adapter trimming using fastp v0.20.1 [13]. Reads were aligned to the Wuhan-Hu-1 reference sequence (NC_045512.2; https://www.ncbi.nlm.nih.gov/nuccore/1798174254) genome using bowtie2 v2.4.0 [14]. The reads at each genomic position were first collated using samtools v1.9 mpileup (http://www.htslib.org/doc/samtools-mpileup.html), then iVar v1.3 was used to call the consensus sequence based on the mpileup output [15]. Positions covered by fewer than ten reads were masked in the consensus sequence. At each position, the nucleotide variant with the highest frequency in the reads was used. The lineages of the samples were assigned based on the consensus sequences, using *Panglin* and *Nextclade* [16–20]. The analysis pipeline is

Table 1
Variant PCR interpretation criteria based on PCR results. Abbreviations for Δ69/70 spike and ORF1a deletions are indicated by Δ69/70 and ORF1a, respectively.

Result	N gene	Δ69/70	ORF1a
Potentially B.1.1.7	CT≤35	No CT	No CT
Potentially B.1.135 or P.1	CT≤35	CT≤35	No CT
Potentially B.1.375	CT≤35	No CT	CT≤35
Other lineages	CT≤35	CT≤35	CT≤35
Inconclusive	CT>35 or No CT	Any value	Any value
Inconclusive	CT≤35	CT>35	CT>35

available at https://github.com/nsc-norway/covid-seq/tree/v7.

4. Results

4.1. Lineages and VoC by variant PCR and WGS

During the study period from 25 January to 19 February 2021, SARS-CoV-2 RNA was detected in 1642 (2%) in a total of 83,801 samples from individual patients. All positive samples were then analysed for VoC using both the Variant PCR and WGS, and results were compared (Table 2). Inconclusive Variant PCR results due to no CT value for the N gene were regarded as technical failures and excluded from the analysis. For simplicity, results are presented using *Pangolin* nomenclature only [20].

The Variant PCR showed 466 (28%) isolates to be potential B.1.1.7 variants, 21 (1%) to be potential B.1.351 or P.1 variants, whereas 190 (12%) samples were inconclusive. WGS results from the same samples showed 394 (24%) to be B.1.1.7, five (0.3%) were B1.351 and no isolates were found belonging to the P.1 lineage. A total of 171 (10%) isolates with valid results from Variant PCR had poor or no sequencing results and thus could not be classified.

Direct comparison of the results from these two methods on a single sample level, showed a high concordance between the Variant PCR and WGS results. 394 (84.5%) potential B.1.1.7 isolates identified by Variant PCR were confirmed by WGS, whereas 56 potentially B.1.1.7 isolates did not produce conclusive WGS results. Sixteen samples defined as B.1.1.7 by Variant PCR were classified as B.1.525 by WGS. Importantly, no isolates identified by WGS as B.1.1.7 had a conflicting identification by Variant PCR. For the 21 potential B.1.351 or P.1 samples found by Variant PCR, 5 (24%) were confirmed by WGS to be B.1.351, whereas 13 (62%) were variants B.1.1.261, B.1.1.74 or B.1.1.318. Of the 11 potential B.1.375 found by Variant PCR, 4 (36%) were identified to be B.1.258, whereas no actual B.1.375 was confirmed by WGS.

Overall, for the samples with valid results from both methods, the Variant PCR missed no VoC. Conversely, all of the samples identified as "other lineages" (i.e., "non-VoC lineages") by the Variant PCR, were confirmed by WGS.

5. Assessment of inconclusive variant PCR results

Of the 190 inconclusive Variant PCR results, 18 (9%) had N gene CT values ≤35 but Δ69/70 and/or ORF1a CT values >35, and 172 (91%) were inconclusive due to N gene CT values >35. Fifty-two inconclusive Variant PCR results produced a valid WGS result. The inconclusive results are presented by N gene CT values in Table 3.

Table 3
CT values for N gene from the Variant PCR assay for inconclusive results using either Variant PCR, WGS or both.

Result for Variant PCR/WGS	CT values (N gene) in Variant PCR Assay	
	≤35	>35
Valid / Inconclusive	171	0
Inconclusive / Valid	3	49
Inconclusive / Inconclusive	15	123

6. Results from the variant PCR during the observation period

Variant PCR was continued also after the study period (follow-up). From 25 January to 7 March 2021, the positive rate for SARS-CoV-2 increased from 2.0% (25 January to 19 February), to 3.1% (20 February to 7 March). Within the same period, the B.1.1.7 variant increased rapidly, becoming the dominant variant within 6 weeks (Fig. 1).

7. Discussion

The rapid identification of VoC is key to control the current SARS-CoV-2 pandemic. In an infection control setting, this information is important for choosing adequate containment measures on an individual and community level. We here show that it is possible to gain this information using a rapid and simple PCR-based variant assay, capable of detecting and differentiating the B.1.1.7 and B.1.351/P.1 variants in a high-volume laboratory, which we confirmed by WGS on a high number of samples. Overall, no VoC was missed by the Variant PCR and the potential non-VoC lineages were true "Non-VoC" lineages defined by WGS.

The Variant PCR incorrectly interpreted several other lineages to be either B.1.1.7 or B.1.351/P1. Interestingly, all these wrongly interpreted lineages had biologically important mutations and were in fact "variants of interest" [21]. Sixteen samples defined as potentially B.1.1.7 by Variant PCR were B.1.525, a variant of interest, lacking the N501Y spike mutation but possessing the biologically important E484K mutation. Similarly, for potential B.1.351 or P.1 lineages found by Variant PCR, several were lineages lacking the N501Y mutation, but possessing the E484K mutation (variants B.1.1.261, B.1.1.74 and B.1.1.318). The spike Δ69/70 deletion, which is present in various linages including B.1.375, was found in 11 samples by Variant PCR. Four of these belonged to the B.1.258, which contain the spike receptor binding motif (RBM) mutation N439K, and recently caused a highly contagious local outbreak in Trondheim, Norway. Of the samples defined as "Other lineages" by Variant PCR, 28 were B.1.160 containing another spike RBM mutation S477N, which also recently caused a highly contagious local outbreak in Norway. The N439K and S477N spike mutations have been associated

Table 2
Lineages (n) defined by both Variant PCR and WGS (*in italic*) of samples from 25 January to 19 February 2021.

		Potential lineage by Variant PCR				
	n=1642	B.1.1.7	B.1.351 or P.1	B.1.375	Other lineages	Inconclusive
		466	21	11	954	190
Lineage by WGS	***B.1.1.7***	*394*	*0*	*0*	*0*	*10*
	B.1.351	*0*	*5*	*0*	*0*	*0*
	P.1	*0*	*0*	*0*	*0*	*0*
	Non-VoC lineages	*0*	*0*	*3*	*817*	*40*
	B.1.525*	*16*	*0*	*0*	*0*	*0*
	B.1.1.261*	*0*	*1*	*0*	*0*	*0*
	B.1.1.74*	*0*	*3*	*0*	*0*	*0*
	B.1.1.318*	*0*	*9*	*0*	*0*	*0*
	B.1.160_ S477N*	*0*	*0*	*0*	*28*	*1*
	B.1.258_ N439K*	*0*	*0*	***4***	*1*	*1*
	Inconclusive	*56*	*3*	*4*	*108*	*138*

*Variants of interest containing E484K, but not N501Y.
**Variants of interest causing local outbreaks in Norway.

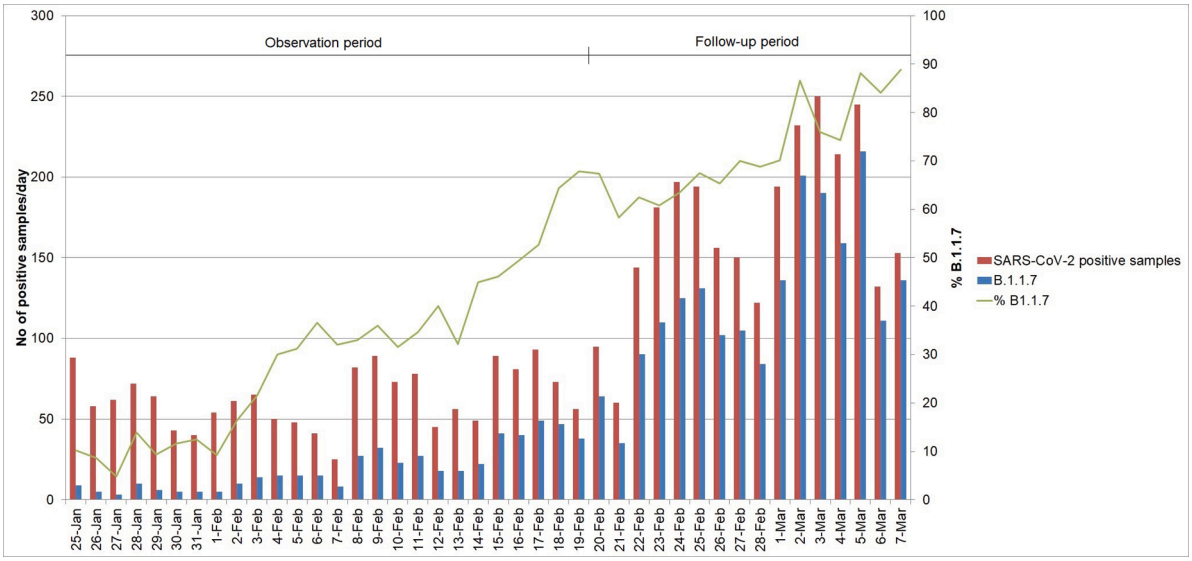

Fig. 1. Total number of SARS-CoV-2 positive samples (red bars), number of samples with the B.1.1.7 variant (blue bars) and % of the B.1.1.7 lineage of all positive samples (green line) on a daily basis from 25 January to 19 February (Observation period) and from 20 February to 7 March 2021 (Follow-up period) using Variant PCR.

with immune evasion or attenuated immune responses, respectively [22, 23].

Vogels *et al.* recommend each laboratory to optimise threshold N gene CT values for calling target failures in Δ69/70 and ORF1a [6,24]. We used the CT cut-off values described in Table 1 and did not have any true false VoC positives. Without these CT thresholds, 41 (79%) Non-VoC lineages would have been wrongly defined as potential VoC by Variant PCR.

A greater proportion of conclusive WGS results may have been achieved by applying a threshold on preanalytical CT input values, as we observed a correlation between low PCR (N gene) CT values and successful WGS (data not shown). However, we were not able to derive a simple CT cut-off value for successful sequencing within the scope of this study, and including all samples ensured the maximum return of conclusive results.

An important limitation of the Variant PCR assay is that it can only detect a predefined set of mutations. Emerging VoC are not detected and would pass unnoticed without a system for sequencing-based surveillance of the whole viral genome. Nonetheless, careful choice of metrics to monitor sequencing coverage and quality, as applied here, are necessary in order to avoid erroneous and misleading lineage assignment using both *Pangolin* and *Nextstrain* bioinformatic toolkits.

Whilst a WGS-based surveillance approach is critical to detect the rise of new variants, rapid monitoring of current VoC remains of utmost importance to limit community spread of SARS-CoV-2. Variant PCR methods thus remain a valuable tool, providing fast results, demanding relatively few human and data resources, and delivering higher throughput. In our hands, the total result time for the Variant PCR including primary SARS-CoV-2 RNA detection was on average 9 hours, compared to 5-6 days for WGS.

There remains a need for parallel continuous surveillance of emerging VoC, with corresponding prompt updates of variant PCR protocols. Still, the current Variant PCR, used within a diagnostic algorithm, can be applied to select samples for WGS. This would enable surveillance of VoC also in countries where WGS facilities are limited. In our case, with a very high incidence of B.1.1.7, "non-B.1.1.7" samples from the Variant PCR are now selected for screening for the emerging VoC B.1.617.2 and variants of interest.

In conclusion, Variant PCR is an effective method for rapid screening for VoC, applicable for most diagnostic laboratories within a pandemic setting. WGS is still needed to confirm the identity of certain variants and for continuous surveillance of emerging VoC.

Credit author statement for the Paper *Rapid SARS-CoV-2 variant monitoring using PCR confirmed by whole genome sequencing in a high-volume diagnostic laboratory*

All authors have actively contributed to Conceptualization, Methodology, Formal analysis, Resources, Writing - Original Draft Review & Editing, Visualization as described by CRediT author statement (see https://www.elsevier.com/authors/policies-and-guidelines/credit-author-statement). Supervision and Project administration was done by Andreas Lind and Mona Holberg-Petersen.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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