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RT-PCR based SARS-CoV-2 variant screening assays require careful quality control

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ABSTRACT

Background: Distinctive genotypes of SARS-CoV-2 have emerged that are or may be associated with increased transmission, pathogenicity, and/or antibody escape. In many countries, clinical and diagnostic laboratories are under mandate to identify and report these so-called variants of concern (VOC).
Objectives: We used an external quality assessment scheme to determine the scope, accuracy, and reliability of laboratories using various molecular diagnostic assays to identify current VOC (03 March 2021).
Study design: Participant laboratories were sent the same five patient-derived samples and were asked to provide their variant detection methods, variant detection results and interpretation of results.
Results: Twenty-five laboratories reported a range of RT-qPCR-based assays to identify specific variations in the SARS-CoV-2 spike protein that are characteristic of three VOC lineages. Laboratories that detected VOC-associated nucleotide mutations at four specific sites had the highest ratio of correct classification. Low template copy number and additional variation in target regions resulted in loss of confidence and accuracy in sample classification.
Conclusions: Melting-curve-based assays to identify genomic variants are less time-consuming and require less bioinformatic analysis compared to partial or whole genome sequencing. However, our results suggest that correct classification of a given genotype/lineage (e.g., a VOC) relies on the ability to detect more than one variant site, adequate template in the sample (i.e., relatively high viral load/copy number) and results may be unclear in certain samples with additional genetic variations. These initial results suggest that some diagnostic laboratories may require additional training to interpret and report complex genetic information about a dynamic emerging virus.

Abbreviations: variant(s) of concern (VOC); Severe acute respiratory syndrome-associated coronavirus 2 (SARS-CoV-2)

Background

Following the emergence and worldwide spread of *Severe acute respiratory syndrome-associated coronavirus 2* (SARS-CoV-2), genomic variation typical of RNA viruses has resulted in the formation of many distinct genotypes [1]. Genomic surveillance of SARS-CoV-2 has allowed the relatively rapid identification of specific variants that have amino acid "signatures" indicative of viruses possessing altered phenotypes, based on prior *in vitro* or *in vivo* studies of related betacoronaviruses [2,3]. Variant viruses with specific spike protein mutations may possess increased transmissibility, increased pathogenicity, and/or escape antibody-mediated immunity, and therefore pose a concern for public health [4,5].

One year after the emergence of SARS-CoV-2, several lineages had been labeled "variants of concern" (VOC). At the time of the study (03 March 2021), local transmission of two had been confirmed from Austria: the Alpha VOC (PANGOlineage B.1.1.7) and the Beta VOC (B.1.351). Both lineages contain a spike protein mutation at site N501, a site that affects receptor binding in SARS-CoV-like viruses and is associated with interspecies transfer [2,3]. Additional mutations, such as E484K, were initially only associated with Beta and Gamma (P.1) VOC,

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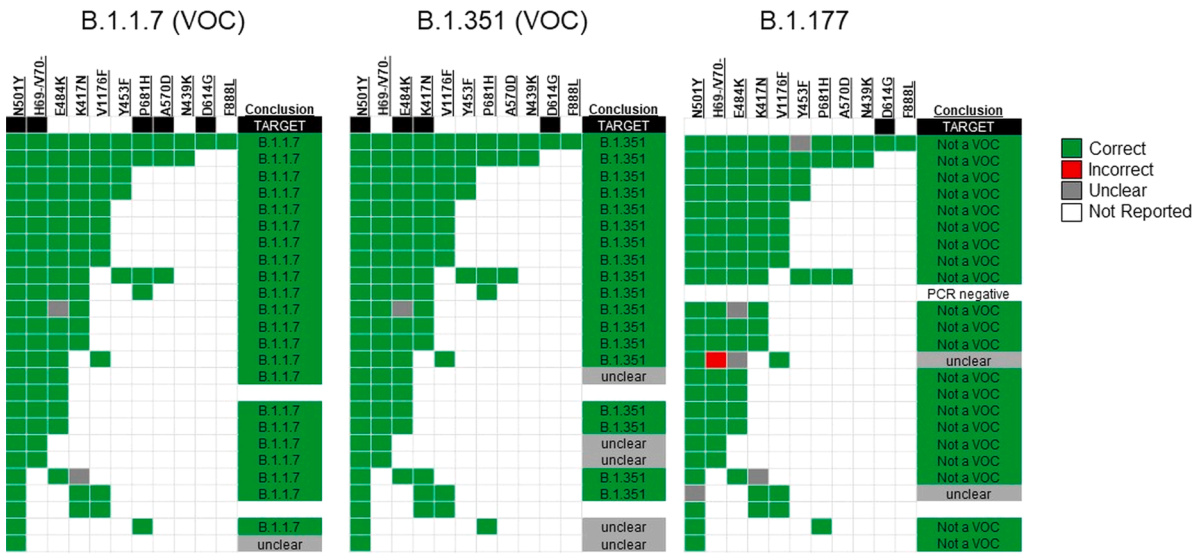

Fig. 1. Summary of SARS-CoV-2 site-specific variant screening assays for three reference samples. The known lineage of each sample is shown above, corresponding to confirmed Austrian patient-derived viral sequences (GISAID: variant of concern [VOC] lineage B.1.1.7 = EPI_ISL_934568; B.1.351 [VOC] =EPI_ISL_1008244; B.1.177 = EPI_ISL_913067). The first row shows the known mutations at each of the indicated sites with black squares in the target sequence. Individual virus spike protein variant sites are listed in columns, sorted by test frequency, and participant laboratories are organized in rows, sorted by number of screening assays, after the sample genotype in the first row. Reported results from individual assays for each of these variant sites and a laboratory-submitted conclusion/interpretation are colored as being correct (green), incorrect (red), unclear (gray) or not done (white, i.e., no information was reported for this assay/conclusion). For example, the B.1.177 sample has the H69-/V70- genotype, and an incorrect result reported a deletion (H69-/V70-, "del69_70") at these sites. One laboratory could not detect the lineage B.1.177 sample by RT-PCR ("PCR negative") and did not report individual assays for this sample.

but were later detected in Alpha [5,6] as well as other non-VOC. The early concerns about the altered phenotype of these VOC have largely been upheld. For example, lineage B.1.1.7 became the predominant strain in many parts of the world in only 4 months [4], and in Austria replaced the predominant non-VOC ("wildtype") lineages B.1.177 and B.1.258. Therefore, many governmental agencies mandated the reporting of VOC in addition to reporting the number of laboratory-confirmed cases.

Due to speed and cost, many diagnostic laboratories have relied on RT-qPCR-based assays to detect specific point mutations, rather than performing partial or whole viral genome sequencing. These assays amplify small (<100 nt) regions of interest with virus-specific primers, and then perform a melting curve analysis. The results are interpreted as present/absent based on prior information about the peak change in melting temperature for either the variant or wild type sequence. This technique has been used for genotyping for many years, over a wide variety of applications in clinical virology laboratories [7,8].

However, the application of this technique to classifying SARS-CoV-2 variants is new and is not yet standardized. We established an external quality assessment scheme for certifying laboratories in Austria for the detection of SARS-CoV-2 in patient samples [9,10]. Following this scheme, we assessed the array of variant sites selected independently by laboratories for genotyping. We were interested in how laboratories interpret and report results based on the scope of their panel and/or encountering aberrant results to gain an indication of the general confidence and knowledge required to classify a dynamic, emerging virus.

Objectives

Using a panel of contemporary viruses, we assessed the scope, accuracy, and reliability of SARS-CoV-2 genotyping analyses to gauge the quality of reported data and inform future recommendations or requirements for certified diagnostic laboratories.

Study design

The general study design, including sample preparation, distribution, and quality control, has been described [9,10]. Five patient-derived samples (one each) were delivered to participant diagnostic laboratories by overnight post with specific instructions on storage conditions (**Supplemental methods**). Laboratories submitted methods used to detect viral genomic RNA and to genotype specific variant sites (as "present", "absent", or "unclear") to an online system (**Tables S2 and S3**). They were required to submit an interpretation for each sample when virus was detected, as a choice between "VOC", "not a VOC", or "unclear, send for sequencing". The participants could additionally provide lineage determination. Results were blinded and analyzed, after which individual reports and summary reports were sent to participants.

The five samples came from patients in Austria and were confirmed by the national reference laboratory for respiratory viruses by whole genome sequencing (**Table S1**). We included one B.1.1.7 sample (EPI_ISL_934568); one atypical B.1.1.7 (EPI_ISL_1191133) with an additional non-synonymous substitution in the spike protein (at G75V) that changes the peak melting temperature of some H69-/V70- detection assays; one B.1.351 sample (EPI_ISL_1008244); and two non-VOC strains common in Austria in late 2020, B.1.177 (EPI_ISL_913067) and B.1.258 (EPI_ISL_913078) (**Table S2**). Notably, B.1.258 contains the H69-/V70- deletion that is also present in B.1.1.7.

Results

Of the 25 participating diagnostic laboratories, 14 unique spike variant-testing combinations were used (Fig. 1). All laboratories screened for the mutation at N501Y, and 20 laboratories screened for the H69-/V70- deletion. Half (n = 13) screened for at least H69-/V70-, K417N, E484K, and N501Y; and eight of the remaining 12 screened for at least three variant sites (**Figure S1**).

Excluding the atypical B.1.1.7 and B.1.258 samples, all laboratories (those reporting complete results) correctly detected the presence/

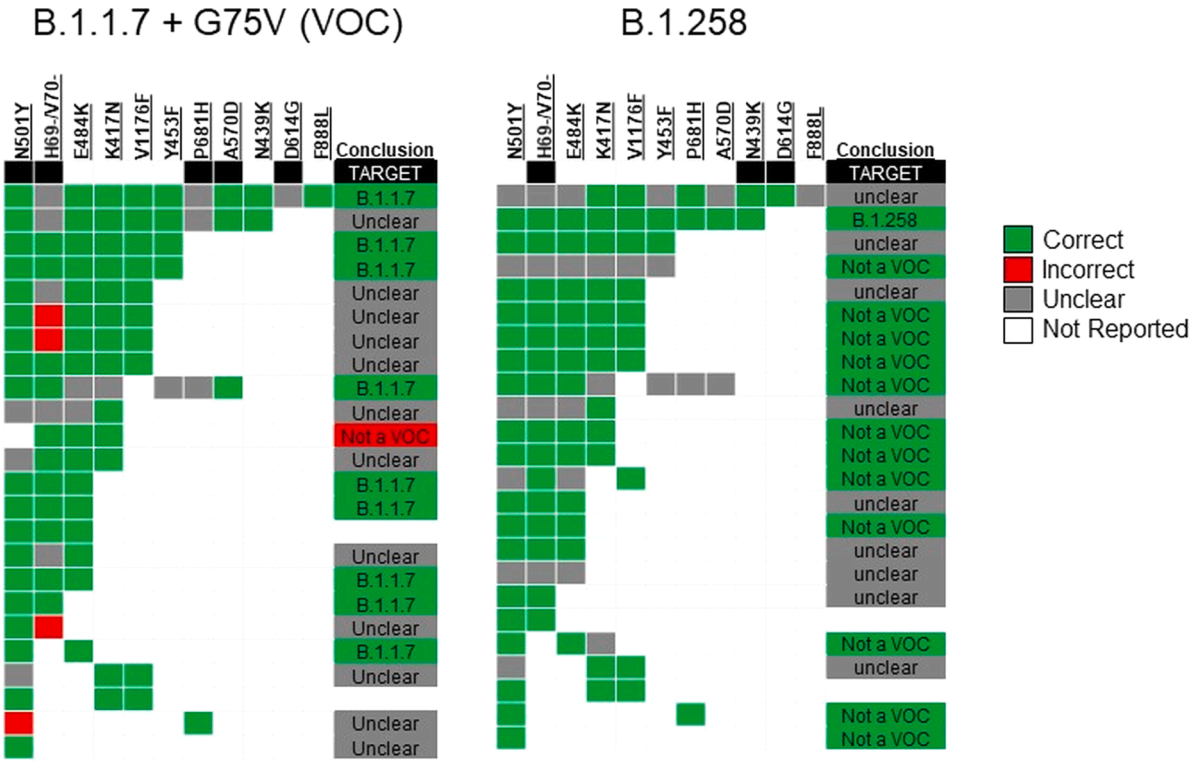

Fig. 2. Summary of SARS-CoV-2 site-specific variant screening assays for two reference samples. The known lineage of each sample is shown above, corresponding to confirmed Austrian patient-derived viral sequences (available in GISAID: an atypical B.1.1.7 variant of concern [VOC] lineage with an additional spike protein substitution at G75V = EPI_ISL_1191133; and B.1.258 = EPI_ISL_913078). The sample genotype (target) is shown in the first row as black squares for sites where known a mutation is present. Individual virus spike protein variant sites are listed in columns, sorted by test frequency, and participant laboratories are organized in rows, sorted by number of screening assays. Reported results from individual assays for each of these variant sites and a laboratory-submitted conclusion/interpretation are colored as being correct (green), incorrect (red), unclear (gray) or not done (white, i.e., no information was reported for this assay/conclusion). For example, the atypical B.1.1.7 has the H69-/V70- ("del69_70") genotype, and three incorrect results reported "wildtype" H69 and V70 for these sites. One laboratory could not detect these two samples by RT-PCR and did not report individual assays for this sample (not shown).

absence of variants ("unclear" in only 8/319 reported individual assays); with the exception of one laboratory falsely reporting the presence of H69-/V70- in the B.1.177 sample (Fig. 1). We did not observe any assay-specific failures (**Table S4**).

Of the 19 laboratories that screened the atypical B.1.1.7 sample for H69-/V70-, five reported "unclear" and three falsely reported deletion-negative for this assay (Fig. 2). Twelve interpreted their cumulative results as "unclear", one reported "not a VOC", and nine identified the sample as B.1.1.7.

Only 22/25 labs detected viral RNA in the B.1.258 sample by RT-PCR – the lowest ratio for any sample – and 14/86 variant detection assays were reported as "unclear" – the highest ratio of "unclear" assays for any sample (Fig. 2). "Unclear" results were reported for 6/22 N501Y assays, 1/16 H69-/V70-, 3/17 E484K, and 1/13 K417N assays. Eleven labs concluded that the sample was "not a VOC", and the remaining laboratories determined "unclear".

Discussion

We challenged the participants with a panel of test samples that reflected the current situation in Austria. We concluded that most routine diagnostic laboratories would be able to detect and classify a virus as a putative VOC, and over half could differentiate between contemporary VOC. All laboratories that included screening for at least four common variant-screening assays reported all three "typical" samples correctly (Fig. 1, **Table S5**). Laboratories screening for less than these four variants in general were less confident about their interpretation: 8/23 interpretations from this group were "unclear", and 5/10 reported "unclear" for the B.1.351 variant sample. Thus, the "unclear" designations were not associated with assay failure, per se, but with lack of information from screening too few mutations. The only incorrect assay reports were from H69-/V70- assays (Figs. 1 and 2, **Table S5**): eight of 19 laboratories did not detect H69-/V70- in the atypical B.1.1.7, likely due to the presence of G75V. This type of failure was demonstrated for other viral genotyping assays [11]. The selection of variant assay panels will be dynamic and must be updated. Guidance from regulatory agencies should consider that assay failure is not a major concern, but clearly there are minimum optimal panel sizes given the array of contemporary variants, and whether VOC should be identified specifically or generally.

In general, many laboratories safely concluded "unclear" and suggested sequencing (or provided no interpretation, n=9). Low template (e.g., the B.1.258 sample) was most likely the reason for assay failure and the highest ratio of "unclear" interpretations (**Table S1**). However, in three instances laboratories inferred the lineage of a sample based on limited information: one (correctly) identified B.1.1.7 based on N501Y and E484; one (correctly) identified "not a VOC" for B.1.258 based on V1176 and H69-/V70-; and one (incorrectly) identified "not a VOC" for the atypical B.1.1.7 based on H69-/V70-, E484 and K417. As more agencies request reporting of viral genotypes to track the spread of VOC, bioinformatics analysis (of whole genome sequencing data) has been identified as the principal bottleneck [12]. Our results suggest that accurate and reliable site-specific variant screening also relies on careful quality control and interpretation, but is suitable for efficiently classifying putative variant samples.

Declaration of 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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2021.104905.

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