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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)
and then perform a melting curve analysis. The results are interpreted as encountering aberrant results to gain an indication of the general melting temperature for either the variant or wild type sequence. This present/absent based on prior information about the peak change in 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].

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]. 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 changes at N501Y, and eight of the remaining 12 screened for the mutation at N501Y, and 20 laboratories screened for the

**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.

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**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 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).

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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