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SARS-CoV-2 Testing in the Community: Testing Positive Samples with the TaqMan SARS-CoV-2 Mutation Panel To Find Variants in Real Time

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ABSTRACT Genome sequencing is a powerful tool for identifying SARS-CoV-2 variant lineages; however, there can be limitations due to sequence dropout when used to identify specific key mutations. Recently, ThermoFisher Scientific has developed genotyping assays to help bridge the gap between testing capacity and sequencing capability to generate real-time genotyping results based on specific variants. Over a 6-week period during the months of April and May 2021, we set out to assess the ThermoFisher TaqMan mutation panel genotyping assay, initially for three mutations of concern and then for an additional two mutations of concern, against SARS-CoV-2-positive clinical samples and the corresponding COVID-19 Genomics UK Consortium (COG-UK) sequencing data. We demonstrate that genotyping is a powerful in-depth technique for identifying specific mutations, is an excellent complement to genome sequencing, and has real clinical health value potential, allowing laboratories to report and take action on variants of concern much more quickly.

KEYWORDS SARS-CoV-2, variants of concern, genotyping, genome sequencing, real time, SNPs, PCR

Viruses mutate, and SARS-CoV-2 is no exception. As the COVID-19 pandemic continues around the world, mutations are naturally occurring, resulting in the emergence of divergent clusters/variants containing sets of mutations. These clusters/variants have differing prevalence in different geographical regions, likely in response to changing immune profiles of the human population (1). Movement of people enabled by global air travel allows these variants to spread and mutate further under differing selection pressures. In the United Kingdom, the Alpha variant (B.1.1.7), first identified in Kent, rapidly swept to dominance by December 2020 (2). In April 2021, the Delta variant (B.1.617.2), first identified in India, rapidly outcompeted the Alpha variant to become dominant in a matter of weeks (3). The geographical location where these variants emerged is not relevant (4); rather, it is the specific mutations present which greatly impact virus characteristics including transmissibility and antigenicity, where mutations of significance have been identified in the SARS-CoV-2 Spike protein of these variants of concern (VOC) that contribute to enhanced transmission and/or immune invasion (5). Other VOC have been identified and characterized, including both Beta (B.1.351, first identified in South Africa) and Gamma (P.1, first identified in Brazil) (5).

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Previously at the University of Birmingham, we set up a SARS-CoV-2 modular testing facility at the request of the United Kingdom Department of Health and Social Care (6). Our PCR assay of choice was the 3-target design (ORF1ab and S and N genes) TaqPath COVID-19 CE-IVD (European CE marking for In Vitro Diagnostic (IVD) medical devices) real-time reverse-transcriptase polymerase chain reaction (RT-PCR) kit, where target areas are unique to SARS-CoV-2 to reduce detection of other coronaviruses and compensate for virus mutations. Initially, we detected all three genes in SARS-CoV-2 samples; however, during November 2020, we along with other laboratories using the same PCR assay started to notice a drop-off in the detection of the S gene and then a rapid rise in this S-gene target failure (SGTF) in early December 2020 (7). From discussions with the University of Birmingham genome sequencing laboratory (as part of the COVID-19 Genomics UK Consortium [COG-UK]) and as confirmed by other laboratories, it was demonstrated that the S gene did amplify, therefore confirming that this gene was still present but was not being detected using the TaqPath COVID-19 assay. This was attributed to a 6-bp deletion (Δ69/70) in the middle of the S gene where the fluorescent specific probe binds, thus negating the probe's ability to fluoresce (8). Simultaneously, these findings of a new VOC were reported by our laboratory and multiple testing facilities across the United Kingdom, where this finding identified the VOC B.1.1.7 (Alpha variant). Although SGTF identification with the ThermoFisher TaqPath real-time RT-PCR assay was not intentional, this observation with this PCR assay allowed us and other COVD-19 testing facilities to use this assay as an accurate epidemiological tool to track the rise, spread, and dominance of this VOC in the United Kingdom. By April 2021, COVID-19 TaqPath PCR testing facilities, including our laboratory, noted an increasing number of samples without SGTF, where the 3 target genes were amplifying, and upon genome sequencing analysis, these samples were identified as the Delta (B.1.617.2) variant (9).

To better understand viral transmission and evolution and to inform public health responses and vaccine development, genomic sequencing is essential. In March 2020, the COVID-19 Genomics UK Consortium (COG-UK) was created for this purpose (10). To date, COG-UK has sequenced over 1,100,000 SARS-CoV-2 samples, providing a vast amount of data to the global COVID-19 response. This yields crucial information about the number of variants circulating in the population and possible lines of transmission; however, sequencing can be time-consuming and costly, and in some cases full coverage of the virus is not possible.

Recently, ThermoFisher has developed genotyping assays to help bridge the gap between testing capacity and sequencing capability to receive results in real time, so in addition to our modular system for COVID-19 testing, we decided to build the ThermoFisher TaqMan SARS-CoV-2 mutation panel into our workflow (Fig. 1). Here, we present our data showing that the TaqMan genotyping assays identify variants in all samples tested with a zero failure rate and that often the assay confirms mutations in a viral isolate that cannot be definitively identified from genome sequence data alone. We conclude that the genotyping assay is an excellent complement to genome sequencing efforts and allows rapid, point-of-testing determination of the presence of any genetic variant for SARS-CoV-2 for which an assay can be designed.

MATERIALS AND METHODS

Patient samples. Five hundred nasopharyngeal Pillar 1 swab samples (testing for those with a clinical need, and health and care workers) in virus transfer medium (VTM) were sent to the University of Birmingham during April and May 2021 from Birmingham Health Trust Hospital. SARS-CoV-2-positive samples from the University of Birmingham lateral flow testing site were also sent and archived. Five Pillar 2 SARS-CoV-2 samples (swab testing for the wider population) previously identified in February 2021 at the University of Birmingham Turnkey laboratory were also utilized. The use of anonymized samples in this study was allowed under ethics gained to aid assay development (NRES Committee West Midlands - South Birmingham 2002/201 amendment number 4, 24 April 2013).

RNA extraction. RNA was extracted from 200 μl of patient sample using the ThermoFisher MagMAX Viral/Pathogen II nucleic acid isolation kits with MagMAX magnetic beads and MS2 phage internal control, using the automated ThermoFisher Kingfisher flex magnetic particle processor (11).

TaqPath COVID-19 assay. Reaction mixtures were prepared using the ThermoFisher TaqPath COVID-19 CE-IVD RT-PCR kit protocol (12). RT-PCR of reaction mixtures was performed using the Applied
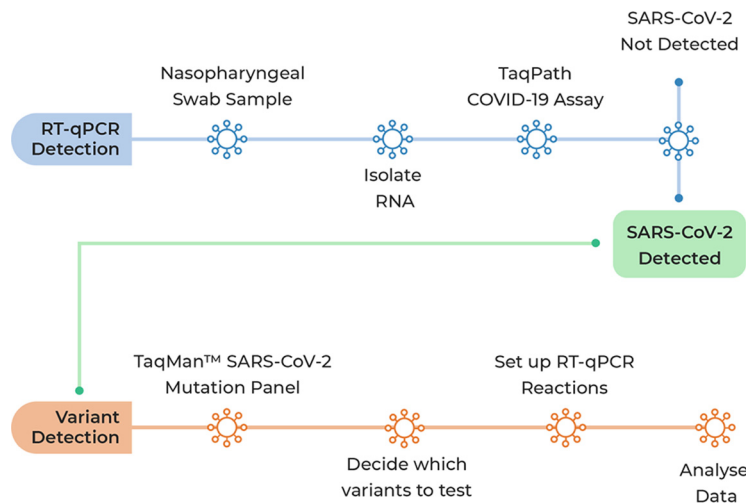
FIG 1 Workflow for ThermoFisher TaqMan SARS-CoV-2 mutation panel assay. Blue workflow, protocol used to detect SARS-CoV-2. Orange workflow, protocol using SNP assays to confirm mutations associated with SARS CoV-2 emerging variants.

Biosystems QuantStudio 5 real-time PCR instrument. Subsequent EDT files were transferred to a computer with QuantStudio Design and Analysis desktop software v2.5.1 for analysis of exponential curves. The TaqPath COVID-19 assay coamplifies three target genes: ORF1ab, N gene, and S gene. Results were classified as positive with respect to at least 2 single-target genes (ORF1ab, N, and S) provided the raw C_T (cycle threshold) values were below 37 for single gene target signals. Bacteriophage MS2 RNA was added to each sample as an internal positive control for each sample and to monitor potential sample inhibition. A negative control (distilled water [dH_2O]) is included on every plate. The SGTF of the TaqPath COVID-19 CE-IVD RT-PCR kit was considered a proxy for the presence of Δ69/70 in the S gene of SARS-CoV-2.

TaqMan SARS-CoV-2 mutation panel workflow. Sample inclusion for the mutation assay required RNA extracts from positive samples (C_T of ≤30, as defined in the manufacturer's protocol, available from ThermoFisher) for the TaqMan SARS-CoV-2 mutation panel workflow. From our pool of positive samples, 185 were randomly selected for the mutation panel assay following the assay workflow (Fig. 1). Samples containing S-gene single-target failure (SGTF) were included in the assay as long as Orf1a and N-gene C_T values were within range as there was no compromise of assay accuracy.

Designing a genotyping panel for the mutation assay. TaqMan probes specific to single nucleotide polymorphisms (SNPs) found in variants known to be circulating widely within the United Kingdom around March to May 2021 were used in this study. S-gene mutations chosen were N501Y, E484K, K417N, P618R, and L452R (Fig. 2). Probes detect both the reference and mutation sequences of SARS-CoV-2. Reporter dye information for the TaqMan SARS-CoV-2 mutation panel is represented in the assay context sequence, which is the nucleotide sequence surrounding the mutation site in the SARS-CoV-2 reference genome (hCoV-19/Wuhan/2019; GISAID EPI_ISL_402124). The variant allele is detected by 6-carboxyfluorescein (FAM) dye, and the reference allele is detected by VIC dye.

The presence of SGTF and N501Y was indicative of the Alpha variant; the Beta variant was defined by the presence of K417N, E484K, and N501Y; and Gamma was defined as E484K and N501Y without the presence of K417N, while the presence of L452R and P681R was indicative of the Delta variant.

Positive controls for both the original SARS-CoV-2 sequence and chosen SNP mutations were used in the assay. The AcroMetrix coronavirus 2019 (COVID-19) RNA control (low positive control), prepared using full-length genomic RNA from SARS-CoV-2, was used as a positive control for SARS-CoV-2. A plasmid control containing mutation sequences for N501Y, E484K, and K417N was used as a positive control for SNP mutations. However, at the time of running these experiments a plasmid control for mutations P681R and L452R was unavailable.

RT-PCR mix was prepared per the mutation assay protocol (13). For samples with C_T values of <30, 5 μL of RNA was added to the reaction mixture, and for samples with C_T values of <16, 2.5 μL of RNA was added to the reaction mixture. Reactions and the real-time PCR program were set up according to the mutation panel assay protocol (13).

Library preparation and sequencing. Library preparation of positive SARS-CoV-2 samples (cycle threshold, <30) was performed using the nCoV-2019 LoCost sequencing protocol version 3 (14), using normalized primers (New England Biolabs) for the V3 ARTIC primer scheme (ARTIC network) (15). Sequencing was performed on a MinION flow cell (R9.4.1) run on a GridION sequencing device (Oxford Nanopore Technologies).

The ARTIC network nCoV-2019 novel coronavirus bioinformatics protocol was used to process the raw sequencing data including genome assembly and variant calling using nanopolish 0.11.3 (15). The genotyping was then performed using a nextflow pipeline (https://github.com/BioWilko/genotyping-pipeline).

FIG 2 ThermoFisher mutation panel assay targets with associated SARS-CoV-2 variants and phenotype.

Briefly, genotypes were called using aln2type (https://github.com/connor-lab/aln2type) utilizing custom variant definition files for each mutation (included in repository), and lineages were called using Pangolin (16).

Data analysis. Results were plotted as allelic discrimination plots using the QuantStudio Design and Analysis v2.5 with the genotyping analysis module.

RESULTS

Allelic discrimination plots show clear discrimination between wild-type samples and mutation samples using QuantStudio Design and Analysis. The Design and Analysis software genotype-calling algorithm was initially designed for diploid organism genotype calling. This is leveraged for the TaqPath assay by amplification and detection of both wild-type (WT) and variant alleles (labeled alleles 1 and 2, respectively). This allows the software to identify samples with a clear amplification curve matching either allele 1 (reference/wild type) or allele 2 (variant) for each specific mutation, respectively (Fig. 3). In some instances, the software will identify heterozygosity (i.e., presence of both wild-type and variant alleles), which indicates the need for further inspection of that sample. Heterozygosity may indicate a mutation at the underlying assay binding site or a truly heterologous sample (e.g., multiple strains present in the sample).

Each probe is labeled with VIC dye to detect the reference (WT) sequence and FAM dye to detect the mutation sequence, which has one nucleotide difference. This allows clear discrimination on a cluster plot between WT and mutation samples, as seen with the AcroMetrix control (reference sequence) and plasmid control containing the mutation sequence (Fig. 3B).

Where input samples have similar C_T values, they appear as clusters on the discrimination plot, as seen in the mut/mut samples (Fig. 3B), or in the case of a range of C_T values, samples are dispersed along/up the axis as seen in ref/ref samples (Fig. 3B and C). We were able to detect samples with C_T values varying from 12 to 29, in respect to ORF1ab gene, N gene, and S gene. In one case we could detect mutations with a C_T value of 33, albeit with slightly reduced sensitivity. Samples with a high viral load cluster in the upper x/y axis, and those with a low viral load cluster in the lower x/y axis.
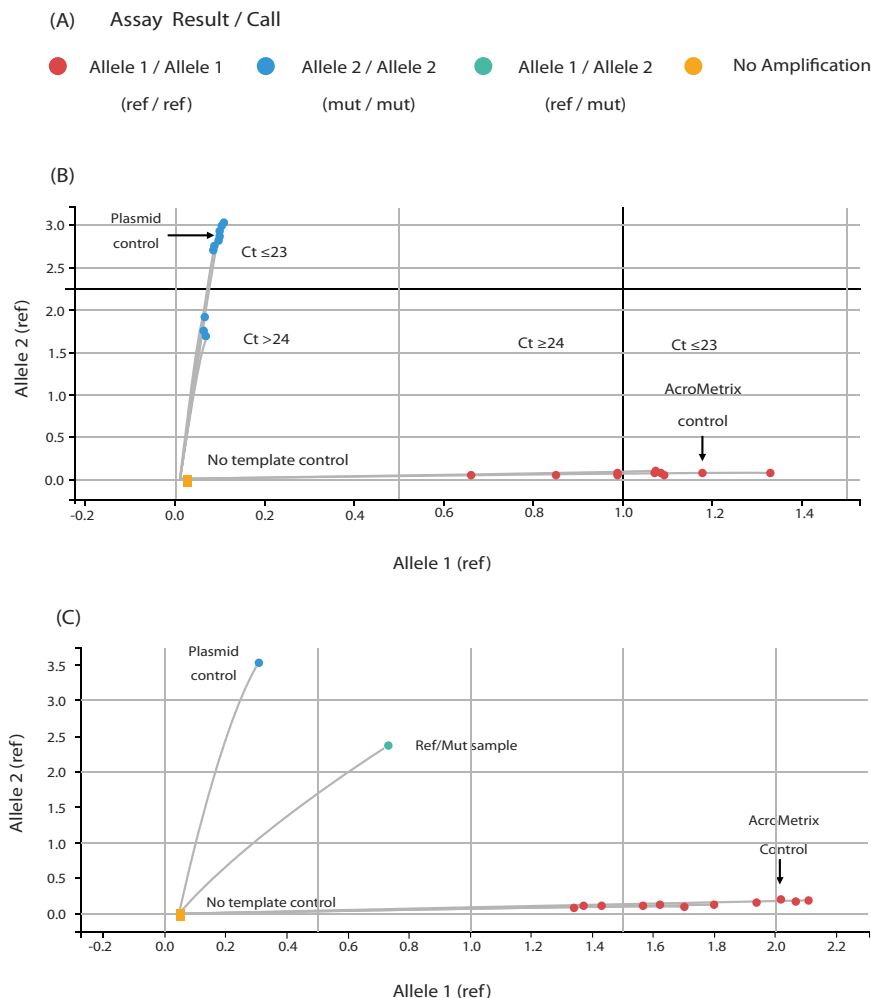
FIG 3 Mutation assay results viewed using QuantStudio Design and Analysis software with the genotyping analysis module. (A) Assay results are "called" in 4 colors according to their outcome. Red indicates allele 1/allele 1 (ref/ref) for WT, blue indicates allele 2/allele 2 (mut/mut) for mutation present, green refers to allele 1/allele 2 (ref/mut), and orange means that results show no amplification. (B) Allelic discrimination plot showing clear discrimination between wild-type (WT) samples (red dots along the x axis) and the mutation samples (blue dots along the y axis) with high and low viral loads. (C) Allelic discrimination plot showing an example of a ref/mut sample (green dots). C_T, cycle threshold. Cutoffs are determined by the QuantStudio Design and Analysis software.

Independent mutations that are located next to one another in the SARS-CoV-2 virus genome, such as P681R and P681H, can complicate genotype analysis, as probes of an assay for one mutation will fail to bind to viral sequences that contain other adjacent mutations. Mutations under the probe can appear as ref/mut and slope away from ref/ref or mut/mut samples, clustering along the x axis, near the no-template controls (NTCs), thus exhibiting weak amplification due to the probes' nonspecific activity (Fig. 3C). Genotyping calls can manually be adjusted to "no amp," or two separate assays can be run, such as P681R and P681H, to compare and facilitate accurate genotype analysis. If it is not possible to make a call, then further characterization by genome sequencing will be necessary.

The mutation panel assay is extremely effective at identifying mutations in laboratory samples. All samples run through the mutation panel assay produced a result, with either mutation present, mutation absent, or reference/mutation, indicating that another mutation within that SNP was present. Input samples had various C_T values (C_T, 12 to 29) with regard to each of the three single-target genes ORF1ab, N, and S (see Data Set S1 in the supplemental material). Using the ORF1ab gene as a

FIG 4 Distribution of TaqPath COVID-19 PCR C_T values for the ORF1ab gene. The y axis indicates the number of samples positive for the SARS-CoV-2 ORF1ab gene. The x axis is grouped into ranges for C_T values up to and including 30. Abbreviations: C_T, cycle threshold; ORF1ab, open reading frame 1ab.

marker for C_T distribution across a C_T value group, good distribution of different C_T values was observed (Fig. 4). RNA quality was not measured, and in some cases, samples had been stored at $-80°C$ for up to 2 mon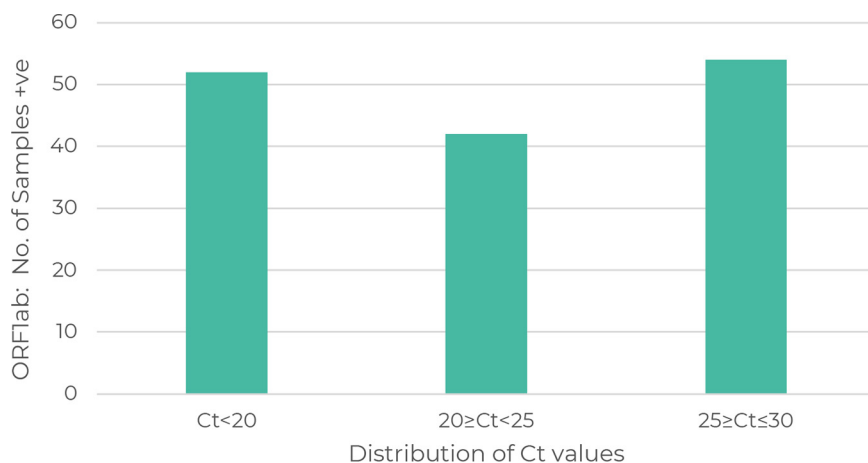ths and through no more than 2 freeze/thaw cycles. However, no effect on the performance of the mutation assay was observed. Furthermore, we also noted that for positive samples with a C_T of ≤16, RNA was added at 2.5 μL instead of 5 μL into the mutation panel PCR, therefore allowing more RNA to be archived.

All samples run through this assay were sent for sequencing onsite at the University of Birmingham as part of COG-UK. This provided us with the ability to compare the mutation panel assay results with those of genome sequencing. By cross-referencing the genome sequencing results for each SNP, we could identify whether the mutation assay correctly called each SNP mutation. Our data show that for all samples, where sequencing data were available, the mutation panel is in 100% agreement (Fig. 5A; also refer to Data Set S1 for the complete data set for all samples run). While Nanopore sequencing may miss an SNP mutation (Fig. 5B), the mutation assay can identify this. This can be due to the RNA quality, the sequencing platform used, or issues with primers required for genome sequencing but highlights the importance of the genotyping assay for rapid identification and subsequent action.

The mutation assay cannot distinguish what the substitution is in "ref/mut" results, which highlights the continued importance of sequencing and updating SNP mutations which can be added when designing an assay panel. The mutation assay cannot be used to identify variant lineages; however, it can, due to the detection of specific mutations, give an indication as to which variant the sample may be and can also exclude the presence of a VOC in a sample based on the absence of key characterizing mutations of significance. This is of particular importance as case numbers rise and sequencing capacity and turnaround time may not be matched. Specifically, for VOC Alpha (B.1.1.7) ref/mut further analysis of genome sequencing data revealed that this variant, although negative for P681R, was positive for P681H. Importantly, it was noted that one ref/mut was also positive for E484K, and clarification from the University of Birmingham arm of COG-UK confirmed that this was a small cluster of B1.1.7 variants that was being monitored and acted on in the Birmingham area.

The mutation panel assay is highly adaptable to newly emerging variants and mutations. The mutation panel designed for this assay was intended to identify samples containing SNPs associated with variants of SARS-CoV-2 widely circulating within the United Kingdom from March 2021 to May 2021; this included mutations found in the table in Fig. 2. Between April and May B.1.617 variant numbers were increasing rapidly and beginning to overtake the B.1.1.7 variant within the UK population. Therefore,

(A)

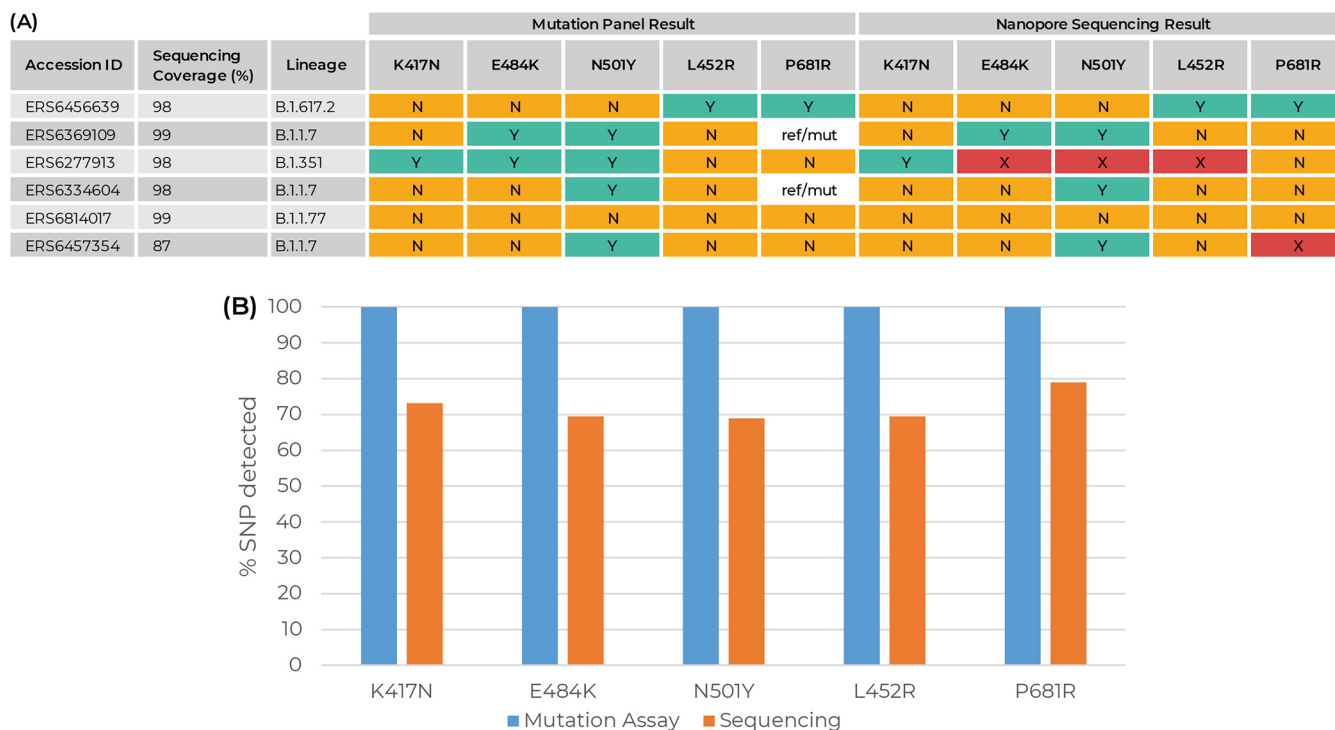| Accession ID	Sequencing Coverage (%)	Lineage	Mutation Panel Result					Nanopore Sequencing Result				
			K417N	E484K	N501Y	L452R	P681R	K417N	E484K	N501Y	L452R	P681R
ERS6456639	98	B.1.617.2	N	N	N	Y	Y	N	N	N	Y	Y
ERS6369109	99	B.1.1.7	N	Y	Y	N	ref/mut	N	Y	Y	N	N
ERS6277913	98	B.1.351	Y	Y	Y	N	N	Y	X	X	X	N
ERS6334604	98	B.1.1.7	N	N	Y	N	ref/mut	N	N	Y	N	N
ERS6814017	99	B.1.1.77	N	N	N	N	N	N	N	N	N	N
ERS6457354	87	B.1.1.7	N	N	Y	N	N	N	N	Y	N	X

(B)

FIG 5 (A) Example of mutation assay results compared with Nanopore sequencing results from a selection of samples run through the assay. All results for all samples run are included in Data Set S1 in the supplemental material. Orange square "N," mutation not present; green square "Y," mutation present; white "ref/mut," mutation present on one allele only. The red square "X" indicates that there was not sufficient coverage of that SNP after sequencing. (B) Percent comparison results of all mutation assay results and corresponding Nanopore sequencing data. Blue bars (mutation assay), percent (%) SNP agreement compared to the corresponding sequencing data. Orange bars (sequencing), percent SNPs identified when sequencing data for each sample were assigned a lineage by Pangolin.

as experiments were being conducted in real time the addition of P618R and L452R assays was essential for rapid identification of B.1.617 variants. Samples that had previously been run for the original assays and sent for sequencing meant that RNA availability was limited. However, freshly isolated samples from May provided the opportunity to run all assays at once.

One hundred eighty-six samples were assayed, with 182 samples run for N501Y, 183 for E484K, and 178 for K417N, and a total of 42 samples were assayed for all 5 mutations; 68.7% of samples assayed were positive for N501Y (WT ref/ref = 31.3%), 2% of samples were positive for E484K (WT ref/ref = 97.2%), 2.8% of samples assayed were positive for K417N (WT ref/ref = 97.2%), 57.1% of samples were positive for L425R (WT ref/ref = 42.9%), and 61% were positive for P681R (WT ref/ref = 19%). A ref/mut "call" was also noted for E484K (1 sample, 0.6%) and P681R (8 samples, 19%).

Lineage was identifiable in 80.1% (Fig. 6) of samples sent for sequencing, which as mentioned previously may be due to the sequencing protocol used and/or RNA quality. From our pool of samples, 65.4% of samples were the B.1.1.7 variant, 18.3% were B.1.617.2, 3.2% were B.1.177, 2.2% were B.1.137, and 1.1% were B.1.351 and B.52, with B.1.177.16, B.1.1.372, B.1.1, and B.1 variants making up 4%. Cases of B.1.617.2 were first identified at the end of April 2021.

DISCUSSION

Genomic epidemiology is a powerful tool for tracking transmission and importation of SARS-CoV–2 as well as assessing the effectiveness of public health measures (1–3). Tracking transmission within the population in real time enables laboratories to report and governments to act. Recently, ThermoFisher has developed genotyping assays to help bridge the gap between testing capacity and sequencing capability to receive results in real time. A previous study from our laboratory demonstrated that B.1.1.7
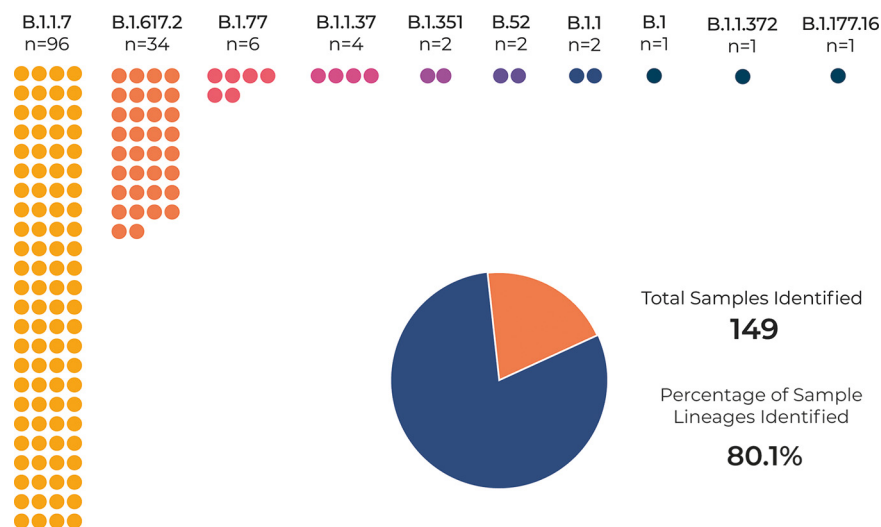
FIG 6 Lineage of sequenced samples identified by Pangolin. (Top and left) Number of samples assigned a SARS CoV-2 lineage. (Bottom right) Percentage of samples assigned a lineage compared to the total number of samples assigned a lineage.

was associated with significantly higher viral loads (17), and had a genotyping assay been available at the time, this would have helped to identify the Alpha variant much more quickly and to identify speed and spread of infection for quicker action and containment.

In our laboratory, an opportunity arose to genotype RNA extracted in real time from positive SARS-CoV-2 samples from Birmingham Trust Hospital, Pillar 2, and the University of Birmingham lateral flow site. Initially using three verified SNP assays from ThermoFisher's Applied Biosystems TaqMan SARS-CoV-2 mutation panel and then expanding to five to include SNPs for the Delta variant, we matched genotypes for specific mutations in variants Alpha (B.1.1.7), Beta (B.1.351), and Delta (B.1.617.2).

Here, we demonstrate that these mutation panels provide robust detection of VOC even if RNA is of low quality and after more than one freeze/thaw step. Importantly, a specific mutation can be identified on the same day that a nasopharyngeal swab tests positive by RT-PCR. Where sequencing data were available, the genotyping assay always matched 100% with the correct lineage.

The ref/mut function is a key to the genotyping assay, as when detected, this will imply an amino acid change for the specific mutation at the genomic site where the primers amplify. This is crucial in alerting researchers to the potential rise of a new VOI (variant of interest) so it can be monitored to see its potential to become a VOC.

Our study confirms that same-day rapid real-time detection of variants present in the population is very achievable—from a swab entering the lab and then being processed through the TaqPath COVID-19 RT-PCR and mutation assay, where results were then available in approximately 5 h. Confirmation of mutations present and lineage identification following genomic sequencing onsite were provided in approximately 72 h; however, when onsite sequencing is not available, turnaround time may increase significantly. The mutation panel is also significantly less expensive than sequencing, at ~£0.45p per reaction, compared to ~£35 per genome when operating at scale. One limitation of the TaqPath assay is the limit of detection, which may impact laboratories wishing to test extremely low-yield samples. This is also an issue for genome sequencing of samples, which in the United Kingdom is possible only on samples with C_T values of <30 on the TaqPath assay.

Rapid identification of VOC enables test-and-trace efforts to identify regional clusters and perform targeted testing to prevent spread of more transmissible variants. Having both PCR setups in our modular testing system and onsite sequencing removes

the logistics, costs, and time of moving samples between testing labs and sequencing labs and reporting the results.

Importantly, the genotyping panel can be updated readily as new SNP mutations are identified via genome sequencing data from COG-UK. Delta variants emerged quickly over a few weeks, and during our study we were able to source very quickly two mutation panels for the Delta variant (L452R and P681R), and on their arrival, we were able to act on the same day.

Genome sequencing can be inconclusive for identifying key mutations found by the mutation panels—we observed several instances where the TaqPath assay identified specific alleles that were not detected via genome sequencing (see Data Set S1 in the supplemental material)—but nonetheless, it is a powerful tool for identifying SARS-CoV-2 variant lineages through phylogenetic trees. However, as previous studies have discussed, there are limitations due to sequence dropout when genome sequencing is used to identify specific key mutations (18–21). The presence of SNPs in the forward and/or reverse primer binding sites may lead to complete or partial lack of amplification. These allelic dropouts specifically affect PCR-based (tile amplicon) targeted sequencing, thus resulting in incomplete genome coverage, especially at smaller amounts, resulting in the loss of both 5′ and 3′ regions that fall outside primer binding positions. This can be mitigated through adjustments to the specific primer scheme used, something that happens at regular intervals for the ARTIC protocol employed worldwide for Nanopore-based SARS-CoV-2 sequencing.

For the study presented here, we demonstrated that genotyping has two major functions. (i) Genotyping is a powerful additional, more in-depth, assay for identifying specific mutations and has real clinical health value allowing laboratories to report and act on VOC much more quickly than genome sequencing. (ii) Genotyping is an excellent additional complement to the already powerful tool of genome sequencing already proven for assigning lineages via phylogenetic trees.

Our data confirm that SARS-CoV-2 genotyping is essential for real-time identification of VOC here now and tracking of those that emerge for informing public health strategy.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, XLSX file, 0.03 MB.

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