Emergency SARS-CoV-2 Variants of Concern: Novel Multiplex Real-Time RT-PCR Assay for Rapid Detection and Surveillance

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ABSTRACT Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread worldwide. Many variants of SARS-CoV-2 have been reported, some of which have increased transmissibility and/or reduced susceptibility to vaccines. There is an urgent need for variant phenotyping for epidemiological surveillance of circulating lineages. Whole-genome sequencing is the gold standard for identifying SARS-CoV-2 variants, which constitutes a major bottleneck in developing countries. Methodological simplification could increase epidemiological surveillance feasibility and efficiency. We designed a novel multiplex real-time reverse transcriptase PCR (RT-PCR) to detect SARS-CoV-2 variants with S gene mutations. This multiplex PCR typing method was established to detect 9 mutations with specific primers and probes (ΔHV 69/70, K417T, K417N, L452R, E484K, E484Q, N501Y, P681H, and P681R) against the receptor-binding domain of the spike protein of SARS-CoV-2 variants. In silico analyses showed high specificity of the assays. Variant of concern (VOC) typing results were found to be highly specific for our intended targets, with no cross-reactivity observed with other upper respiratory viruses. The PCR-based typing methods were further validated using whole-genome sequencing and a commercial kit that was applied to clinical samples of 250 COVID-19 patients from Taiwan. The screening of these samples allowed the identification of epidemic trends by time intervals, including B.1.617.2 in the third Taiwan wave outbreak. This PCR typing strategy allowed the detection of five major variants of concern and also provided an open-source PCR assay which could rapidly be deployed in laboratories around the world to enhance surveillance for the local emergence and spread of B.1.1.7, B.1.351, P.1, and B.1.617.2 variants and of four Omicron mutations on the spike protein (ΔHV 69/70, K417N, N501Y, P681H).

IMPORTANCE COVID-19 has spread globally. SARS-CoV-2 variants of concern (VOCs) are leading the next waves of the COVID-19 pandemic. Previous studies have pointed out that these VOCs may have increased infectivity, have reduced vaccine susceptibility, change treatment regimens, and increase the difficulty of epidemic prevention policy. Understanding SARS-CoV-2 variants remains an issue of concern for all local government authorities and is critical for establishing and implementing effective public health measures. A novel SARS-CoV-2 variant identification method based on a multiplex real-time RT-PCR was developed in this study. Five SARS-CoV-2 variants (Alpha, Beta, Gamma, Delta, and Omicron) were identified simultaneously using this method. PCR typing can provide rapid testing results with lower cost and higher feasibility, which is well within
the capacity for any diagnostic laboratory. Characterizing these variants and their mutations is important for tracking SAR-CoV-2 evolution and is conducive to public infection control and policy formulation strategies.

**KEYWORDS** ΔHV 69/70, E484K, E484R, K417N, K417T, L452R, N501Y, P681H, P681R, SARS-CoV-2, VOC genotyping, epidemiological surveillance, SARS-CoV-2

As of January 2022, more than 340 million cases of COVID-19 have been reported and are associated with over 5 million deaths globally (1) (https://covid19.who.int/). Mutations arise as a natural occurrence of viral replication. When a newly arising mutation confers a competitive advantage with respect to viral replication, transmission, or escape from immunity, that mutation increases in prevalence in the overall virus population (2, 3). Importantly, new SARS-CoV-2 variants of concern (VOCs) may enhance virus transmissibility and/or disease severity, as well as diagnostic and/or treatment failure (4–6). SARS-CoV-2 lineages carrying the amino acid substitution N501Y spread rapidly in the United Kingdom in late autumn of 2020 (7). SARS-CoV-2 lineage with multiple spike mutations in South Africa was first reported in October 2020 and then spread across Western and Eastern Cape provinces, as the B.1.351 variant (8, 9). P.1, referred to as the Gamma variant, was first detected in early March 2020 and spread in Manaus, Brazil (10). B.1.617.2 sublineage, or Delta, was first detected in India in December 2020 and rapidly became the dominant variant in India (9). Currently, SARS-CoV-2 variants are detected mainly by whole-genome sequencing (WGS) (2, 11). Although genome sequencing is the gold standard for identifying SARS-CoV-2 variants, resource and capacity constraints can limit the number of samples that can be sequenced. Therefore, establishing a rapid, accurate, economic, and multisite detection method for SARS-CoV-2 variant identification is an urgent technical need for SARS-CoV-2 infection prevention and control worldwide.

Here, we evaluated the analytical and clinical performance of our real-time reverse transcription PCR (RT-PCR) typing method to qualitatively detect SARS-CoV-2 RNA from wild type (non-VOC) or VOCs (B.1.1.7, B.1.351, P.1, and B.1.617.2), particularly with respect to the VOC B.1.617.2 lineage that is driving the third wave of the Taiwan COVID-19 epidemic. This study also provided preliminary results indicating that the VOC PCR typing method was able to detect the fifth VOC, Omicron (B.1.1.529), which has rapidly spread across the globe.

**RESULTS**

**VOC/VOI genotyping assay design.** VOCs or variants of interest (VOIs) were collected from five dominant SARS-CoV-2 strains (Delta-lineage B.1.617.2 + AY.x, Alpha-lineage B.1.1.7 + Q.x, Beta-lineage B.1.351 + B.1.351.2 + B.1.351.3, GammaBeta-lineage P.1 + P.1.x, and Omicron-lineage B.1.1.529) from GIASIAD, which contains the sequences of more than 4,700,000 isolates identified since 2019 (Fig. 1). Based on these VOCs and VOIs, comparing single nucleotide polymorphisms (SNPs) in the spike region to those in Wuhan-hu-1, nine different TaqMan SNP genotyping assays were designed to detect mutations present in clinical specimens. Locations of forward and reverse sequence-specific primers and dye-labeled probes for the TaqMan SNP genotyping assays are shown in Fig. 2.

**Analytical sensitivity of lab-developed VOC real-time RT-PCR assay.** The limit of detection (LoD) of our lab-developed VOC RT-PCR assay was determined by testing 20 replicates of SARS-CoV-2 gene fragment controls that were serially diluted 2-fold. Using the LightCycler 96 Thermocycler (Roche, Mannheim, Germany), the LoD obtained from the 20 replicate tests was 30 copies/μL for the mutant N501Y and 60 copies/μL for the mutants ΔHV 69/70, K417N, E484K, L452R, and P681R (Table 1). In the AIO 48 open system (LabTurbo, New Taipei City, Taiwan), the LoD had the same performance (Table 1). A result was considered positive if the amplification curve crossed the threshold line within 31 cycles (threshold cycle [C_T] value < 31) (Table S1).
Analytical specificity of lab-developed VOC real-time RT-PCR assay. Targets to differentiate between B.1.1.7, B.1.351, P.1, and Omicron (B.1.1.529) were analyzed over 4,700,000 SARS-CoV-2 genomes on GISAID. In silico analyses showed high specificity of the assays. The analytical specificity of each assay was determined by testing a panel of pathogens which included influenza A, influenza B, rhinovirus, enterovirus, parainfluenza virus subtypes 1 to 3, adenovirus, coronavirus HKU1, and COVID-19-negative samples. All test results were found to be highly specific for our intended targets, with no cross-reactivity observed with other upper respiratory viruses or COVID-19-negative samples (n = 500).

Clinical performance of lab-developed VOC real-time RT-PCR assay. We analyzed 250 clinically positive samples in this study, which were confirmed by the Taiwan CDC central laboratory. Figure 3 depicts the VOC assay interpretation in this study design. These positive samples were further identified as wild type (non-VOC) or VOCs (B.1.1.7, B.1.351, P.1, and B.1.617.2). To confirm that the results were consistent with those of rapid detection of the SARS-CoV-2 VOCs, presumptive cases were also assessed by whole-genome sequencing, and the lineages were confirmed using GISAID software. To verify the accuracy of our lab-developed VOC typing assay, we also used commercial VirSNip SARS-CoV-2 mutation assays for strain surveillance and compared the VOC typing results (Table 2). The findings showed that our novel RT-PCR method exhibited better analytic sensitivity and lower CT values (31 to 34) than those of the commercial kit.

**DISCUSSION**

Nucleic acid-amplification test assays for SARS-CoV-2 VOCs typing employ real-time RT-PCR, which takes anywhere from less than one to a few hours, thus shortening the turnaround time compared with that required for whole-genome sequencing. In this study, we determined the analytical sensitivity of the VOC genotyping assay. Similar LoD values were obtained with two different PCR systems (AIO 48 and LC96), so the assay could be implemented in other diagnostic laboratories using their own resources, equipment, and personnel. We also examined the clinical performance using
COVID-19 patient samples from northern Taiwan. Our findings indicate that the novel RT-PCR method of SARS-CoV-2 VOCs typing is able to identify and differentiate the major four VOCs of SARS-CoV-2 (B.1.1.7, B.1.351, P.1, and B.1.617.2). Our assay is faster and simpler than whole-genome sequencing, which is the gold standard method. With the emergence of the B.1.1.7 lineage, Taiwan confronted a COVID-19 flare-up in May 2021 (12) and later in June 2021. The first imported B.1.617.2 cases were reported by Taiwan Central Epidemic Command Center (CECC), which later led to outbreaks of community transmission (13). To meet this challenge, assays for the detection of SARS-CoV-2 VOCs were designed and implemented in our hospital. These RT-PCR assays

| Thermocycler | Gene target/fluorescent dye | 240  | 120  | 60  | 30  | 15  | 7.5 |
|--------------|-----------------------------|------|------|-----|-----|-----|-----|
| LightCycler 96 | **N501Y/FAM**                | 20/20 | 20/20 | 20/20 | 19/20 | 17/20 | 17/20 | 17/20 |
|              | **ΔHV 69/70/FAM**             | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **E484K/HEX**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **K417N/FAM**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **L452R/FAM**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **P681R/HEX**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
| LabTurbo AIO 48 | **N501Y /FAM**               | 20/20 | 20/20 | 20/20 | 19/20 | 16/20 | 17/20 | 16/20 |
|              | **ΔHV 69/70/FAM**             | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **E484K/HEX**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **K417N/FAM**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **L452R/FAM**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |
|              | **P681R/HEX**                 | 20/20 | 20/20 | 20/20 | 17/20 | 12/20 | 12/20 | ND   |

ND: Not detected.
targeting the nine mutation sites allowed the rapid identification of B.1.1.7, B.1.617.2, and other VOCs, which helped the government rapidly follow up and reformulate more efficient strategies to deal with the emergence of VOCs.

Relying on genome sequencing platforms for identifying SARS-CoV-2 VOCs requires expensive instruments and ample resources, which constitutes a major bottleneck in developing countries. The VOC screening assay design implemented in our study shortens the time required to obtain results, and hence the number of laboratories capable of testing for SARS-CoV-2 VOCs should be expanded.

There were some limitations to our study. First, our designed novel RT-PCR method focuses on identifying currently well-known VOCs. Whole-genome sequencing is thus indispensable to detect new emerging variants when the PCR method fails to obtain results. Second, this PCR assay can be used to screen only known SARS-CoV-2-positive clinical samples for the presence of the major VOCs.

Overall, the rapid implementation of mutation-specific nucleic acid tests to detect four major VOCs should aid public health measures in identifying these emerging

### TABLE 2 Result of SARS-CoV-2 VOC testing by two methodologies

| Spike | Spike variation | Methodology | B.1.1.7 | B.1.351 | P.1 | B.1.617 |
|-------|----------------|-------------|---------|---------|-----|---------|
|       |                | qPCR | TIB | qPCR | TIB | qPCR | TIB | qPCR | TIB |
| N501Y | N501Y          | 49   | 43b | 5     | 5   | 5     | 4   | 1     | 1   |
| HV69/70 | HV69/70      | 49   | 43b | 5     | 5   | 5     | 1   | 1     | 1   |
| E484  | E484K          | 5    | 5   | 5     | 5   | 5     | 5   | 1     | 1   |
| L452  | L452R          | 5    | 5   | 5     | 5   | 5     | 5   | 1     | 1   |
| K417  | K417N          | 49   | 43b | 5     | 5   | 5     | 5   | 1     | 1   |
| P681  | P681H          | 49   | 43b | 5     | 5   | 5     | 5   | 1     | 1   |
| P681  | P681R          | 5    | 5   | 5     | 5   | 5     | 5   | 1     | 1   |

*aTIB, VirSNiP SARS-CoV-2 mutation assays for strain surveillance; qPCR, novel RT-PCR detection of SARS-CoV-2 VOC.

*Reduced number of detectable samples compared to the other methodology; C<sub>T</sub>, value ranged from 31 to 34.
In addition, these VOC assays can provide reliable information at lower viral loads than whole-genome sequencing as described previously (14). We also observed in our own studies that when lower viral loads of SARS-CoV-2 samples were used, we often failed to generate whole-genome sequencing data.

Modern transportation plays a key role in the spread of SARS-CoV-2 and new variants (15), providing a reminder of the necessity of global epidemiological surveillance. Fully vaccinated breakthrough infections have been reported in many countries, involving different SARS-CoV-2 variants (16–20). Travel restrictions can be lifted when high vaccination coverage is reached only if vaccines remain highly effective against VOCs (21). This also highlights the importance of VOC surveillance for government and public health departments and presses for increased vaccination coverage.

While the manuscript was being written, a new SARS-CoV-2 variant, B.1.1.529, rose rapidly in Gauteng province in South Africa (22). Later, B.1.1.529 was announced as a VOC called Omicron by the World Health Organization (23). Our method accurately detected four Omicron mutations on the spike protein (ΔHV 69/70, K417N, N501Y, P681H) with one pass. Using our novel RT-PCR VOC typing technique, we successfully detected the Omicron variant B.1.1.529 (including BA.1 and BA.2) in accordance with whole-genome sequencing (Table S2). It is clear that our RT-PCR assays can not only detect single mutations for identifying currently known VOCs but also detect emerging VOCs. The preliminary conclusion is that our method can rapidly detect B.1.1.529 without sequencing the entire viral genome.

Conclusions. This study describes the development and validation of multiplex real-time RT-PCR assays to detect major mutations of VOCs. Our method demonstrated greater flexibility and more accessibility than those of other diagnostic laboratories and is the first of its kind to detect the five major VOCs, since SARS-CoV-2 VOCs emerged as a global threat worldwide. The molecular-diagnostics protocol developed by us simultaneously tested for B.1.1.7, B.1.351, P.1, B.1.617.2, and B.1.1.529. This should significantly benefit hospitals and health departments and enable effective management and epidemiological surveillance of the COVID-19 pandemic.

MATERIALS AND METHODS

Study design and clinical specimens. This study was registered on February 8, 2021 and approved by the Tri-Service General Hospital Institutional Review Board (approval number C202005041). We tested 750 deidentified nasopharyngeal swab specimens collected from patients confirmed as having COVID-19, including 250 positive and 500 negative specimens. Residual viral transport medium was stored at 80°C. The sample collection periods were between June 2020 and December 2021.

RT-PCR testing for SARS-CoV-2 detection. Sample testing for SARS-CoV-2 was done as described previously (24, 25). Briefly, SARS-CoV-2 RT-PCR testing was performed using the LabTurbo AIO 48 system (LabTurbo, New Taipei City, Taiwan) for detecting the SARS-CoV-2 \( N1 \) and \( E \) genes. A \( C_v \) value of <35 was defined as a positive result for the pathogen. Each sample had an internal control (RNase \( P \) gene). The external control comprised RNA spike-in mix as the positive control and diethyl pyrocarbonate (DEPC) water as the negative control.

Novel RT-PCR assay design. SARS-CoV-2 genomes were downloaded from the GISAID database (26). Primers and probes were designed using the consensus sequences obtained from the sequence alignment for the spike protein region of SARS-CoV-2 (Table 3).

RT-PCR detection of SARS-CoV-2 VOC. The positive SARS-CoV-2 specimens were screened by six multiplex RT-PCR assays. A 20-μL reaction mixture was made up of 5 μL of RNA and 15 μL PCR master mix containing the primer/probe mixture. The master mix was from Luna one-step RT-PCR kit (New England Biolabs). The assay for detection of the N501Y mutation was adapted from a multiplex RT-PCR assay for detection of spike mutants in SARS-CoV-2. The primer and probe overlap the sequences that contain mutant amino acids N501Y and its wild type N501N (Table 3). The assays were performed under the following conditions: reverse transcription at 55°C for 8 min and initial denaturation at 95°C for 1 min, 45 cycles at 95°C for 10 s, and 58°C for 20 s. When the RNA contains the sequence perfectly matching the probe, there will be a signal generated in the channel of the designed sequence. For example, positivity for mutant N501Y is when the FAM channel has a signal, and negativity for N501Y is when the HEX channel has a positive signal. In addition, three separate assays were designed to detect spike mutations and wild type, E484K versus E484E, L452R versus L452L, and K417N versus K417K. For the ΔHV 69/70 assay, positive fluorescence signal means RNA with wild-type sequence. Using a similar strategy, a primer/probe set that targets L452 and P681 was performed under the following conditions: reverse transcription at 55°C for 8 min and initial denaturation at 95°C for 10 min, 45 cycles at 95°C for 15 s and 64°C for 30 s. All six multiplex RT-PCR assays were examined on two different real-time PCR instruments, the Roche LightCycler 96 System and the LabTurbo AIO 48 open system.
| Spike protein | Nucleotide | Amino acid | Forward primer 5'-3' | Reverse primer 5'-3' | Probe 5'-3' | Fluorescence |
|---------------|------------|------------|----------------------|----------------------|-------------|--------------|
| N501          | A23063A    | N501N      | TGTTACTTCTTTACAATCATAT | GAAAGTACTACTACTGTATGG | 5'-/5Cy5/CCAACCCAC/TAO/TAATTGGTGG/3IABkFQ/-3' | Cy5          |
|               | A23063T    | N501Y      | TGTTACTTCTTTACAATCATAT | GAAAGTACTACTACTGTATGG | 5'-/56-FAM/CCAACCCAC/ZEN/TTATGGTGG/3IABkFQ/-3' | FAM          |
| Δ69-70        | 21765-21770| 69-70      | TCAACTCAGCAGCTTCTTAC | TGTTAGGACAGGGTTATCAAAC | 5'-/56-FAM/GTCCCCAGAG/TAO/AAATGGTTTTA/3IABkFQ/-3' | FAM          |
| E484          | G23012G    | E484E      | CAAGTGAATCTTACAGGC  | TTGGAAACCATATGTTAAAG | 5'-/5Cy5/GTAAATGTGT/TAO/TTGAAGGTGT/3IABkFQ/-3' | Cy5          |
|               | G23012A    | E484Q      | CAAGTGAATCTTACAGGC  | TTGGAAACCATATGTTAAAG | 5'-/56-FAM/ATGGTGTTC/TAO/AAGGTTTTA/3IABkFQ/-3' | FAM          |
| K417          | A22812A    | K417K      | TGCAGATTCTTGTAAATGAGG | ATACGCGCAGCTTAAATCATC | 5'-/5Cy5/GCAAACTTG/TAO/AAATGGTTTTA/3IABkFQ/-3' | Cy5          |
|               | A22812C    | K417T      | TGCAGATTCTTGTAAATGAGG | ATACGCGCAGCTTAAATCATC | 5'-/5HEX/GCAAACTTG/TAO/AAATGGTTTTA/3IABkFQ/-3' | HEX          |
| G22813T      | K417N      | TGCAGATTCTTGTAAATGAGG | ATACGCGCAGCTTAAATCATC | 5'-/56-FAM/GCAAACTTG/TAO/AAATGGTTTTA/3IABkFQ/-3' | FAM          |
| L452          | T22917T    | L452L      | TGATAGATTTCAGTTGAAATATCT | AATCTTGGATTAAGTGTGGG | 5'-/5Cy5/GACTTCCTA/TAO/AACAAATCTACTACAGTAAT/3IABkFQ/-3' | FAM          |
|               | T22917G    | L452R      | TGATAGATTTCAGTTGAAATATCT | AATCTTGGATTAAGTGTGGG | 5'-/56-FAM/ACTTCCTAA/TAO/AACAAATCTACTACAGTAAT/3IABkFQ/-3' | FAM          |
| P681          | C23604C    | P681P      | CCCATTGGTCAGCTGATATG | TAGTGATGCAATGATGATGTTG | 5'-/5Cy5/ACTCAAGACT/TAO/AAATCTCTCTCTCG/3IABkFQ/-3' | Cy5          |
|               | C23604A    | P681H      | CCCATTGGTCAGCTGATATG | TAGTGATGCAATGATGATGTTG | 5'-/56-FAM/ACTCAAGACT/TAO/AAATCTCTCAG/3IABkFQ/-3' | FAM          |
|               | C23604G    | P681R      | CCCATTGGTCAGCTGATATG | TAGTGATGCAATGATGATGTTG | 5'-/5HEX/ACTCAAGACT/TAO/AAATCTCTCTCG/3IABkFQ/-3' | HEX          |
We prepared dilutions of the controls (7.5, 15, 30, 60, 120, and 240 copies/μL) using nuclease-free water to assess the limit of detection (LoD) on two different thermocyclers (LightCycler 96 and LabTurbo AIO 48 system). LoD was defined as 95% probability of positive replicates.

**Data availability.** Data are available upon request.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

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**TABLE 4** Purified gene fragment control sequences

| Nucleotide | Amino acid | Selection sequence | Product length |
|------------|------------|-------------------|----------------|
| A23063A    | N501N      | 5’-TTG TAA TGG TGT TGA AGG TTT TAA TGG TTA CTT TCC TTT ACA ATC ATA TGG TTT CCA ACC CAC TAA TGG TGT TGG TTA CCA ACC ATA CAG AGT AGT AGT ACT TTC TTT TGA ACT TCT ACA TGG ACC AGC AA-3’ | 131 bp |
| A23063T    | N501Y      | 5’-TTG TAA TGG TGT TGA AGG TTT TAA TGG TTA CTT TCC TTT ACA ATC ATA TGG TTT CCA ACC CAC TTA TGG TGT TGG TTA CCA ACC ATA CAG AGT AGT AGT ACT TTC TTT TGA ACT TCT ACA TGG ACC AGC AA-3’ | 131 bp |
| 21765–21770| 69-70 wild type | 5’-TCA GGT TTA CAT TCA ACT CAG GAC TGG TGC TTC TCC CTT TTC TCC AAT GTT ACT TGG TGC CAT GCT ATA GAC TCT GTC TGT GGG ACC AAT GGT ACT AAG ATG TTG GAT AAC CCT GTA CCA TTA ATT GAT GAT-3’ (WT) | 131 bp |
| G23012G    | E484E      | 5’-AAC CTT TTT AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC CTT TAC ATG CAT AGT GTT TCC AAC CCA ATG GTG TTA AAG GGT ATG ATT GTT ACT GCC GTG GCC AC-3’ | 132 bp |
| G23012C    | E484Q      | 5’-AAC CTT TTT AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC CTT TAC ATG CAT AGT GTT TCC AAC CCA ATG GTG TTA AAG GGT ATG ATT GTT ACT GCC GTG GCC AC-3’ | 132 bp |
| G23012A    | E484K      | 5’-AAC CTT TTT AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC CTT TAC ATG CAT AGT GTT TCC AAC CCA ATG GTG TTA AAG GGT ATG ATT GTT ACT GCC GTG GCC AC-3’ | 132 bp |
| T22917T    | L452L      | 5’-TGG AAT TCT AAC AAT CAT GAT TCT GAG GGT AAG ATT GCT GAA GTC AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC AAT CAT GG TAT-3’ | 130 bp |
| T22917G    | L452R      | 5’-TGG AAT TCT AAC AAT CAT GAT TCT GAG GGT AAG ATT GCT GAA GTC AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC AAT CAT GG TAT-3’ | 130 bp |
| A22812A    | K417K      | 5’-CTC TGG TCT ACT AAC ATC TAT GCA GCA TAT TTA ATT GAT TCT GAG GGT AAG ATT GCT GAA GTC AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC AAT CAT GG TAT-3’ | 134 bp |
| A22812C    | K417T      | 5’-CTC TGG TCT ACT AAC ATC TAT GCA GCA TAT TTA ATT GAT TCT GAG GGT AAG ATT GCT GAA GTC AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC AAT CAT GG TAT-3’ | 134 bp |
| G22813T    | K417N      | 5’-CTC TGG TCT ACT AAC ATC TAT GCA GCA TAT TTA ATT GAT TCT GAG GGT AAG ATT GCT GAA GTC AGA GAG ATA TTT CAA CTG AAA TCT ATC AGG CCG GTA GCA CAC CTT GTA ATG GTG TTA AAG GGT ATG ATT GTT ACT TCC AAT CAT GG TAT-3’ | 134 bp |
| C23604C    | P681P      | 5’-TAT GAG TGG TAC CAT CCC ATT GGT GCA GTC TGG ATG TAT GACT CAG ACT CAG ACT AAC TCT TCT CTC GGG CCG CCA GGT ATG TTA GCT AGT CAG ACT TCC ATC ATT GCA AAT TGA ACC TAT GCT TGA GTG GCA GAA AAT-3’ | 132 bp |
| C23604A    | P681H      | 5’-TAT GAG TGG TAC CAT CCC ATT GGT GCA GTC TGG ATG TAT GACT CAG ACT CAG ACT AAC TCT TCT CTC GGG CCG CCA GGT ATG TTA GCT AGT CAG ACT TCC ATC ATT GCA AAT TGA ACC TAT GCT TGA GTG GCA GAA AAT-3’ | 132 bp |
| C23604G    | P681R      | 5’-TAT GAG TGG TAC CAT CCC ATT GGT GCA GTC TGG ATG TAT GACT CAG ACT CAG ACT AAC TCT TCT CTC GGG CCG CCA GGT ATG TTA GCT AGT CAG ACT TCC ATC ATT GCA AAT TGA ACC TAT GCT TGA GTG GCA GAA AAT-3’ | 132 bp |

**VOC assay accuracy and analytical specificity.** To verify the assay accuracy in our RT-PCR method, VOCs were also detected using VirSNIP SARS-CoV-2 mutation assays for strain surveillance (TiB Molbiol, Berlin, Germany), which used real-time RT-PCR postmelting curve analysis to detect mutations targeting specific spike protein variations (HV69/70, K417N, L452R, E484K, N501Y, P681H, and P681R). These assays were performed as described previously (27). To testify the analytical specificity of the VOC assay, we obtained samples of known upper respiratory viruses, including influenza A, influenza B, rhinovirus, enterovirus, parainfluenza virus subtypes 1 to 3, and adenovirus as viral cultures from the Taiwan CDC viral infection contract laboratory. Additionally, 500 COVID-19-negative specimens were used to evaluate the analytical specificity of the lab-developed multiplex PCR VOC test performance.

**Analytical validation using gene fragments control.** We used purified gene fragments controls (gBlocks, Integrated DNA Technologies, IDT) of SARS-CoV-2 viral genes for absolute quantification (Table 4). These controls were used to prepare a serial dilution panel with approximately 20 replicates. We prepared dilutions of the controls (7.5, 15, 30, 60, 120, and 240 copies/μL) using nuclease-free water to assess the limit of detection (LoD) on two different thermocyclers (LightCycler 96 and LabTurbo AIO 48 system). LoD was defined as 95% probability of positive replicates.

**Data availability.** Data are available upon request.

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**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.
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Informed consent was obtained from all subjects involved in the study.

We declare no conflict of interest.

REFERENCES

1. World Health Organization. 2022. WHO coronavirus (COVID-19) dashboard. World Health Organization, Geneva, Switzerland. https://covid19.who.int/.

2. Jian M, Jr, Chung H-Y, Chang C-K, Hsieh S-S, Lin J-C, Yeh K-M, Chen C-W, Chang F-Y, Hung K-S, Liu M-T, Yang J-R, Chang T-Y, Tang S-H, Perng C-L, Shang H-S. 2021. Genomic analysis of early transmissibility assessment of the D614G mutant strain of SARS-CoV-2 in travelers returning to Taiwan from the United States of America. PeerJ 9:e11991. https://doi.org/10.7717/peerj.11991.

3. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. 2020. The proximal origin of SARS-CoV-2. Nat Med 26:450–452. https://doi.org/10.1038/s41591-020-0820-9.

4. Boehm E, Kronig I, Neher RA, Eckerle I, Vetter P, Kaiser L, Geneva Centre for Emerging Viruses. 2021. Novel SARS-CoV-2 variants: the pandemics within the pandemic. Clin Microbiol Infect 27:1109–1117. https://doi.org/10.1016/j.cmi.2021.05.022.

5. Bal A, Destrass G, Gaymard A, Stevanovski I, Beukers AG, Dorda D, Gomes C, Lin F, Josset L, the COVID-Diagnosis HCL Study Group. 2021. Two-step strategy for the identification of SARS-CoV-2 variant of concern 202012/01 and other variants with spike deletion H69-V70. September, France, August to December 2020. Eurosurveillance 26. https://doi.org/10.2807/1560-7917.ES.2021.26.3.2100008.

6. Davies NG, Jarvis CJ, Group CC-W, Edmunds WJ, Jewell NP, Diaz-Ordaz K, Keogh RH. CMMID COVID-19 Working Group. 2021. Increased mortality in community-tested cases of SARS-CoV-2 lineage B.1.1.7. Nature 593:270–274. https://doi.org/10.1038/s41586-021-03426-1.

7. Leung K, Shum MHH, Leung GM, Lam TTY, Wu JT. 2021. Early transmissibility assessment of the NS01Y mutant strains of SARS-CoV-2 in the United Kingdom, October to November 2020. Eurosurveillance 26. https://doi.org/10.2807/1560-7917.ES.2021.26.12.2102106.

8. Tegally H, Wilkinson E, Giovanetti M, Izarbaden A, Fonseca V, Gandhari J, Doolabh D, Pillay S, San J, Esmio N, Miliani K, von Gottberg A, Walaza S, Allam M, Ismail A, Mohale T, Glass AJ, Engelbrecht S, Van Zyl G, Preiser W, Petruccione F, Sigal A, Hardie D, Marais G, Hsiao M, Korsman S, Davies MA, Tyers L, Mudau I, York D, Maslo C, Goedal R, Abrahams S, Laguda-Akingba O, Alisoltani-Dekhirodi A, Godzik A, Witzmer CK, Sewell BT, Lourenço J, Alcantara CL, Pond SLK, Weaver S, Martin D, Lessels RJ, Bhiman JN, Williams C, de Oliveira T. 2020. Emergence and rapid spread of a new severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) lineage with multiple spike mutations in South Africa. medRxiv. https://doi.org/10.1101/2020.12.21.20248640. 2020.12.21.20248640.

9. Thye AY, Law JW, Pusparajah P, Letchumanan V, Chan KG, Lee LH. 2021. Emerging SARS-CoV-2 variants of concern (VOCs): an impending global crisis. Biomedicines 9:1303. https://doi.org/10.3390/biomedicines9101303.

10. Faria NR, Mellan TA, Walker C, Claro G, Gandra D, Dantas-Torres F, Crispim MAE. Sales FCS, Hawrylyuk I, McCrone JT, Huls GWJ, Franco LAM, Ramundo MS, de Jesus JG, Andrade PS, Coletti TM, Ferreira GM, Silva CAM, Manuli ER, Pereira RHM, Peixoto PS, Kraemer MUG, Gaburo N, Camilo CCd, Hoeltgebaum H, Souza WM, Rocha EC, de Souza LM, de Pinho MC, Araujo LJ, Malta FSV, de Lima AB, Silva JDdP, Zauli DAG, Ferreira AgdS, Schenkelberg RP, Laydon DJ, Walker PG, Schluter HM, dos Santos AlP, Vitalis MS, do Caro VS, Filho RMF, dos Santos HM, Aguiar RS, Proença-Modena JL, Nelson B, Hay JA, Monod M, Micosaurio U, et al. 2021. Genomics and epidemiology of the P.1 SARS-CoV-2 lineage in Manaus, Brazil. Science 372:815–821. https://doi.org/10.1126/science.abh2644.

11. Bull RA, Adikari TN, Ferguson JM, Hammond JM, Stevanovski I, Beukers AK, Nairn Z, Yeung M, Verich A, Gamaarachchi H, Kim KW, Luciani F, Stelzer-Brajd S, Eden JS, Rawlinson WD, van Hal SJ, Deswos VN. 2020. Analytical validity of nanopore sequencing for rapid SARS-CoV-2 genome analysis. Nat Commun 11:6272. https://doi.org/10.1038/s41467-020-20075-6.

12. Jian M, Jr, Perng C-L, Chung H-Y, Chang C-K, Lin J-C, Yeh K-M, Chen C-W, Hsieh S-S, Pan P-C, Chang H-T, Chang F-Y, Ho C-L, Shang H-S. 2021. Clinical assessment of SARS-CoV-2 antigen rapid detection compared with RT-PCR assay for emerging variants at a high-throughput community testing site in Taiwan. Int J Infect Dis 115:30–34. https://doi.org/10.1016/j.ijid.2021.01.034.

13. Yen AM-F, Chen TH-H, Chang W-J, Lin T-Y, Jen GH-H, Hsu C-Y, Wang S-T, Tang H, Chen SL-S. 2021. Epidemic surveillance models for containing the spread of SARS-CoV-2 variants: Taiwan experience. medRxiv. https://doi.org/10.1101/2021.10.19.21265107.

14. Zelyas N, Pabbajari K, Croxen MA, Lynch T, Buss E, Murphy SA, Shokoples S, Wong A, Kanji JN, Tippels G. 2021. Precision response to the rise of the SARS-CoV-2 B.1.1.7 variant of concern by combining novel PCR assays and genome sequencing for rapid variant detection and surveillance. Microbiol Spectr 9: e0031521. https://doi.org/10.1128/Spectr.00315-21.

15. Hu M, Wang J, Lin H, Ruktanonchai CW, Xu C, Meng B, Zhang X, Carilo A, Feng Y, Yin Q, Floyd JR, Ruktanonchai NW, Li Z, Yang W, Tatem AJ, Lai S. 2021. Risk of SARS-CoV-2 transmission among air passengers in China. Clin Infect Dis. https://doi.org/10.1093/cid/ciaa458.

16. Garpure R, Sami S, Vostok J, Johnson H, Hall N, Foreman A, Sabo RT, Schubert PL, Shepard H, Brown FR, Bricfield B, Ricardin JN, Conley AB, Zielinski L, Malec I, Nuske AP, Chang M, Finn LE, Stainken C, Manug A, Elepe P, Weck M, Green A, Edmundson A, Rechind B, Brown V, Ju, Quinones L, Longenberger A, Hess E, Gumke M, Manion A, Thomas H, Barrios CA, Koczvara A, Williams TW, Pearlowitz M, Assoumou M, Senisse PA, Dishman H, Schardin C, Wang X,
Stephens K, Moss NS, Singh G, Feaster C, Webb LM, Krueger A, Dickerson K, Dewart C, Barbeau R, et al. 2021. Multistate outbreak of SARS-CoV-2 infections, including vaccine breakthrough infections, associated with large public gatherings, United States. Emerg Infect Dis 28:35–43. https://doi.org/10.3201/eid2801.212220.

17. Kroidl I, Mecklenburg I, Schneiderat P, Muller K, Girl P, Wolfel R, Sing A, Dangel A, Wieser A, Hoelscher M. 2021. Vaccine breakthrough infection and onward transmission of SARS-CoV-2 Beta (B.1.351) variant, Bavaria, Germany, February to March 2021. Eurosurveillance 26. https://doi.org/10.2807/1560-7917.ES.2021.26.30.2100673.

18. Shastri J, Parikh S, Aggarwal V, Agrawal S, Chatterjee N, Shah R, Devi P, Mehta P, Pandey R. 2021. Severe SARS-CoV-2 breakthrough infection with Delta variant after recovery from breakthrough infection by Alpha variant in a fully vaccinated health worker. Front Med (Lausanne) 8:737007. https://doi.org/10.3389/fmed.2021.737007.

19. Ioannou P, Karakonstantis S, Astrinaki E, Saplamidou S, Vitsaxaki E, Hamilos G, Sourvinos G, Kosteridis DP. 2021. Transmission of SARS-CoV-2 variant B.1.1.7 among vaccinated health care workers. Infect Dis (Lond) 53:876–879. https://doi.org/10.1080/23744235.2021.1945139.

20. Levine-Tiefenbrun M, Yelin I, Alapi H, Katz R, Herzel E, Kuintel T, Chodick G, Gazit S, Patalon T, Kishony R. 2021. Viral loads of Delta-variant SARS-CoV-2 breakthrough infections after vaccination and booster with BNT162b2. Nat Med 27:2108–2110. https://doi.org/10.1038/s41591-021-01575-4.

21. Priesemann V, Balling R, Bauer S, Beutels P, Valdez AC, Cuschieri S, Czyponka T, Dumps U, Glab E, Grill E, Hotulainen P, Iftekhar EN, Krutzinna J, Lionis C, Machado H, Martins C, McKee M, Pavlakis GN, Perc M, Petelos E, Pickersgill M, Prainsack B, Rocklov J, Schernhammer E, Szczurek E, Tsiodras S, Van Gucht S, Willett P. 2021. Towards a European strategy to address the COVID-19 pandemic. Lancet 398:838–839. https://doi.org/10.1016/S0140-6736(21)01808-0.

22. Callaway E. 2021. Heavily mutated Omicron variant puts scientists on alert. Nature 600:21–21. https://doi.org/10.1038/d41586-021-03552-w.

23. World Health Organization. 2021. Classification of Omicron (B.1.1.529): SARS-CoV-2 variant of concern. World Health Organization, Geneva, Switzerland. https://www.who.int/news/item/26-11-2021-classification-of-omicron-(b.1.1.529)-sars-cov-2-variant-of-concern.

24. Jian MJ, Chung HY, Chang CK, Lin JC, Yeh KM, Chen CW, Lin DY, Chang FY, Hung KS, Perng CL, Shang HS. 2021. SARS-CoV-2 variants with T135I nucleocapsid mutations may affect antigen test performance. Int J Infect Dis. https://doi.org/10.1016/j.ijid.2021.11.006.

25. Chung HY, Jian MJ, Chang CK, Lin JC, Yeh KM, Yang YS, Chen CW, Hsieh SS, Tang Sh, Perng CL, Chang FY, Hung KS, Chen ES, Yang MH, Shang HS. 2021. Multicenter study evaluating one multiplex RT-PCR assay to detect SARS-CoV-2, influenza A/B, and respiratory syncytia virus using the LabTurbo AIO open platform: epidemiological features, automated sample-to-result, and high-throughput testing. Aging (Albany NY) 13:24931–24942. https://doi.org/10.18632/aging.203761.

26. Elbe S, Buckland-Merrett G. 2017. Data, disease and diplomacy: GISAID’s innovative contribution to global health. Glob Chall 1:33–46. https://doi.org/10.1002/gch2.1018.

27. Jian MJ, Chung HY, Chang CK, Lin JC, Yeh KM, Chen CW, Li SY, Hsieh SS, Liu MT, Yang JR, Tang Sh, Perng CL, Chang FY, Shang HS. 2021. Clinical comparison of three sample-to-answer systems for detecting SARS-CoV-2 in B.1.1.7 lineage emergence. Infect Drug Resist 14:3255–3261. https://doi.org/10.2147/IDR.S328327.