Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company’s public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
The application of a novel 5-in-1 multiplex reverse transcriptase-polymerase chain reaction assay for rapid detection of SARS-CoV-2 and differentiation between variants of concern

Hsing-Yi Chung, Ming Jian Jr, Chih-Kai Chang, Chi-Sheng Chen, Shih-Yi Li, Jung-Chung Lin, Kuo-Ming Yeh, Ya-Sung Yang, Chien-Wen Chen, Shan-Shan Hsieh, Sheng-Hui Tang, Ching-Lih Perng, Kuo-Sheng Hung, Feng-Yee Chang, Hung-Sheng Shang

1 Division of Clinical Pathology, Department of Pathology, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
2 Graduate Institute of Medical Science, National Defense Medical Center, Taipei, Taiwan
3 Division of Infectious Diseases and Tropical Medicine, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
4 Division of Pulmonary and Critical Care Medicine, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
5 Center for Precision Medicine and Genomics, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan

A B S T R A C T

Objectives: We have established a novel 5-in-1 VOC assay to rapidly detect SARS-CoV-2 and immediately distinguish whether positive samples represent variants of concern (VOCs).

Methods: This assay could distinguish among five VOCs: Alpha, Beta, Gamma, Delta, and Omicron, in a single reaction tube. The five variants exhibit different single nucleotide polymorphisms (SNPs) in their viral genome, which can be used to distinguish them. We selected target SNPs in the spike gene, including N501Y, P681R, K417N, and deletion H69/V70 for the assay.

Results: The limit of detection of each gene locus was 80 copies per polymerase chain reaction. We observed a high consistency among the results when comparing the performance of our 5-in-1 VOC assay, whole gene sequencing, and the Roche VirSNIP SARS-CoV-2 test in retrospectively analyzing 150 clinical SARS-CoV-2 variant positive samples. The 5-in-1 VOC assay offers an alternative and rapid high-throughput test for most diagnostic laboratories in a flexible sample-to-result platform.

Conclusion: The assay can also be applied in a commercial platform with the completion of the SARS-CoV-2 confirmation test and identification of its variant within 2.5 hours.

© 2022 The Author(s). Published by Elsevier Ltd on behalf of International Society for Infectious Diseases.

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Introduction

The first SARS-CoV-2 variant Alpha was identified in England in late November 2021 [1,2]. Mutations in the viral genome can increase transmissibility, facilitate escape from the human immune system, and alter biologically important phenotypes in a way that confers a fitness advantage to the virus, such as mutations in the spike (S) gene that affect antigenicity [3,4]. The SARS-CoV-2 variants are classified as variants being monitored, variants of interest, variants of concern (VOC), and variants of high consequence [5]. There are two currently circulating SARS-CoV-2 VOCs (Delta and Omicron), and three previously circulating VOCs (Alpha, Beta, and Gamma) (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-classifications.html).

Viral genomic mutations leading to new variants of SARS-CoV-2 are a real challenge in tackling the global COVID-19 pandemic [6–8]. Viruses that are mutated have a high propensity for replication errors, providing them with an advantage; several mutations further occur with each replication cycle, making such viruses more virulent and transmissible [9]. Each characterized variant has mutations in the gene encoding the S protein (Omicron: 30 mutations; Delta: 10 mutations; Alpha: seven mutations and two dele-
tions; Beta: nine mutations and one deletion; and Gamma: >10 mutations), compared to the sequence of the wild type index virus (Wuhan-Hu-1) [10].

As different variants will continue to emerge, identifying positive patients and monitoring their variants is important in the epidemic prevention policy. Whole-genome sequencing (WGS) analysis of positive samples can be used to confirm infection with a specific variant and characterize the variant [11]. WGS is a relatively complex method that can take hours to several days to generate results. Analysis of Sanger sequencing results of the viral S gene is an alternative strategy that can be used to classify the viral lineage of positive samples [12]. However, WGS and sanger sequencing of S protein methods are time-consuming methods and require well-trained laboratory personnel and suitable instruments [13].

Therefore, a more rapid response to identifying emerging SARS-CoV-2 variants is required [14]. We developed a novel, 5-in-1 VOC assay, that can monitor key single nucleotide polymorphisms (SNPs) of the S gene in the S gene, thus identifying the samples that tested positive for SARS-CoV-2 and distinguishing among the specific variants. It could offer correct information on SARS-CoV-2 variants of concerns for the public prevention response to the COVID-19 pandemic.

Materials and methods

Study design and clinical samples

We retrospectively examined the stored residual viral transport medium of nasopharyngeal/throat swab samples, collected from patients between May 2021 and March 2022. A total of 150 positive samples were individually confirmed by detecting the nucleocapsid (N1) and envelope (E) genes of SARS-CoV-2 using quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR), based on a cycle threshold (Ct) value <35, which follows the Taiwan Centers for Disease Control and Prevention (CDC) guidelines [15,16]. The positive sample needs to be reconfirmed by another SARS-CoV-2 RT-PCR system. This retrospective study was registered on February 8, 2021, and approved by the Institutional Review Board of the Clinical and Genomic Research Committee at the Tri-Services General Hospital (approval no.: C202005041). The extracted RNA of positive samples were also further detected for their SARS-CoV-2 lineage by VirSNiP SARS-CoV-2 mutation assays (TIB Molbiol, Berlin, Germany) for confirmation of the variant [17]. The clinical samples were put into our lab-developed 5-in-1 VOC assay on the sample-to-result platform. Simultaneously, the samples were also used to detect four SNPs of SARS-CoV-2 S genes in one PCR reaction for distinguishing the SARS-CoV-2 variants [18]. We also desired a novel 5-in-1 VOC assay, which could confirm the sample with the SARS-CoV-2 by N2 gene [19].

Novel 5-in-1 VOC assay design

To design the 5-in-1 VOC assay, SARS-CoV-2 genome sequences were downloaded from the Global initiative on sharing all influenza data (GISAID). According to the database, the selected SNP loci in the S gene could be used to distinguish between Alpha, Beta, Gamma, Delta, and Omicron variants (Figure 1). Primers and probes were designed using the consensus sequences obtained from the sequence alignment of the SARS-CoV-2 S protein (Table 1). In our design, we selected four key SNPs of S protein: N501Y, P681R, K417N, and deletion H69/V70. We also tested for the presence of N2 to confirm that the samples were positive for SARS-CoV-2, following the US CDC guidelines [20]. The 5-in-1 VOC reagent comprised all primers and probes along with the Luna One-Step RT-PCR Kit components (New England Biolabs). The novel 5-in-1 VOC assay was used with two sample-to-result open platforms, the BD MAX™ System and the LabTurbo™ AIO (LabTurbo, New Taipei City, Taiwan), to determine clinical efficacy.

Reverse transcriptase–polymerase chain reaction detection of SARS-CoV-2 variant of concerns

The samples were screened using the two sample-to-result platforms with our novel 5-in-1 VOC assay. Samples consisted of viral transport media collected from patients. For the BD MAX™ System, 500 μl of the sample was added to the sample buffer tube provided with the BD MAX ExK TNA-3 kit. Subsequently, a 5-in-1 VOC PCR reagent was added at position #3 of the snap-in tubes and the extracted RNA was automatically blended in the same position. RT-PCR was then carried out using the following conditions: reverse transcription at 55°C for 10 min and initial denaturation at 99°C for 2 min, followed by 45 cycles at 99°C for 10 sec and 58°C for 24 sec. A positive result for the target gene was indicated by the presence of an RT-PCR amplification curve and the associated cycle threshold (Ct) value.

For the LabTurbo AIO 48 SP-qPCR automation system, RNA was extracted from 500 μl of input sample using the LabTurbo Viral DNA/RNA Mini kit (Cat. No. LVX480-500). The extracted RNA was then mixed with PCR reagent and PCR was carried out using the following conditions: reverse transcription at 55°C for 8 min and initial denaturation at 95°C for 1 min, followed by 45 cycles at 95°C for 10 sec and at 58°C for 20 sec. A positive result for the target gene was indicated by a Ct value <35.

Evaluation of analytical sensitivity of the 5-in-1 VOC assay with analytical validation using RNA controls

For the 5-in-1 VOC assay, we used RNA controls (Virbell, Spain) of SARS-CoV-2 variants for absolute quantification. The controls included: AMPLIRUN® SARS-CoV-2 B.1.1.7 RNA CONTROL, AMPLIRUN® SARS-CoV-2 B.1.351 (South African variant) RNA CONTROL, AMPLIRUN® SARS-CoV-2 P1 RNA CONTROL, AMPLIRUN® SARS-CoV-2 DELTA (B.1.617.2) RNA CONTROL and AMPLIRUN® SARS-CoV-2 OMICRON RNA CONTROL. Serial dilutions of the control samples (20, 40, 80, 160, 320, and 640 copies/PCR reaction) were prepared using nuclease-free water to assess the limit of detection (LoD), which was defined as a 95% probability of positive replicates.

Evaluation of the 5-in-1 VOC assay specificity

The specificity of the 5-in-1 VOC assay was evaluated against several common upper respiratory tract viruses, including influenza A, influenza B, rhinovirus, respiratory syncytial virus, parainfluenza virus, adenovirus, and coronavirus HKU1. Samples testing positive for these viruses were obtained from the Taiwan CDC viral infection contract laboratory. The samples were analyzed using the 5-in-1 VOC assay on the LabTurbo AIO open platform.

Whole-genome sequencing of SARS-CoV-2

Whole-genome sequences of the SARS-CoV-2 isolates (hcoV-19/TSGH-39 to TSGH-69) were obtained by following the Illumina TruSeq Stranded messenger RNA Library Prep Kit protocol, which enriched for SARS-CoV-2 complementary DNA using multiplex RT-PCR amplicons. The Ovation RNA-Seq System V2 (Nugen Technologies, San Carlos, CA, USA) was used to synthesize complementary DNA, which was then processed into a library for WGS which was performed on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) [21]. Paired-end read assemblies of the whole virus genome sequence were formed using SPAdes assembler with SARS-CoV-2
isoate Wuhan-Hu-1, complete genome (NC_045512.2) to run the genome-guide assembly pipeline.

Comparison of the performance using clinical specimens

To verify the performance of 5-in-1 VOC assay, we used 150 SARS-CoV-2 variant positive samples. Those variants were classified by VirSNiP SARS-CoV-2 mutation assays (TIB Molbiol, Berlin, Germany), which used real-time RT-PCR post-melting curve analysis to detect mutations targeting specific S protein variations. According to the analysis, there are (N501Y, P681R, H69/V70, and K417N). The retrospective samples were detected by 5-in-1 VOC assay on two sample-to-result platforms: BD MAX™ System and LabTurbo™ AIO. Thus, a result was considered positive if the amplification curve crossed the threshold line within 35 cycles (Ct value < 35), and that was defined as a positive result for the SNPs mutation gene. Following the patterns of the mutation gene, the assay could distinguish the SARS-CoV-2 variant according to Table 2 and Figure 2.

Results

Epidemiological features of the patients with the Omicron variant

Clinical information was retrieved for 120 patients infected by the SARS-CoV-2 Omicron variant reported between December 2021 and March 2022. We identified the isolate to be the Omicron variant by using VirSNiP SARS-CoV-2 test kits. After aligning the complete genome sequences of the isolated RNAs against the RNA sequences derived from four SARS-CoV-2 variants (hcoV-19/TSGH-48, hcoV-19/TSGH-49, hcoV-19/TSGH-51, and hcoV-19/TSGH-52)—and associated metadata—hosted on the publicly available database GI-SAID, we identified the isolate to be the Omicron variant.

A summary of the gender, ages, and epidemiological features of the 120 patients is shown in Table 3. The average age and median age were 39 and 35 years old, respectively. All 120 patients had a survival rate of 100%, and 34.2% (41/120) of the Omicron-positive patients were asymptomatic. The top two symptoms associated with infected patients were fever (24.2%) and cough (44.2%). There

| Variant | Total | ΔHV69-70 | K417N | N501Y | P681R |
|---------|-------|----------|-------|-------|-------|
| Omicron BA.1 | 938,638 | 883,355 | 533,329 | 787,532 | 512 |
| Omicron BA.2 | 174,812 | 200 | 154,796 | 148,391 | 9 |
| Omicron BA.3 | 527 | 517 | 264 | 381 | 0 |
| Delta | 4,263,038 | 7,566 | 8,128 | 1,838 | 4,238,601 |
| Alpha | 1,168,479 | 1,108,346 | 156 | 1,141,832 | 2,327 |
| Beta | 40,496 | 22 | 36,703 | 35,323 | 48 |
| Gamma | 121,050 | 28 | 0 | 115,564 | 439 |

Table 1 Primer and probe sequences used in this study.

| Protein region | Fluorescence | Single nucleotide polymorphism mutation | Sequence name | Primer sequence 5`- 3` |
|----------------|--------------|----------------------------------------|---------------|------------------------|
| Spike          | FAM          | N501Y                                  | N501Y-F       | TGTACTTTCCCTTTACATCATATGCCCTTC     |
|                |              |                                        | N501Y-R       | GAAAGTACTACTCTCTTGTGGTGTAACCC     |
|                |              |                                        | N501Y-P       | 5`-/5FAM/CCAACCCAC/G/TAATTGTTG/3lAbRQSp/-3` |
|                | HEX          | P681R                                  | P681R-F       | CACCTGTTGCTAGTATG     |
|                |              |                                        | P681R-R       | TAGCTGAGGCAATGCTAGTGAAC     |
|                |              |                                        | P681R-P       | 5HEX/ACTCAGACT/G/TAATTGTTG/3lAbRQSp/-3` |
|                | Taxus Red    | 69-70                                  | Del69-70-F    | TCAACTCAGAAGTTTACTACAGCTC     |
|                |              |                                        | Del69-70-R    | TGGTAGACAAGCTTAAAC     |
|                |              |                                        | Del69-70-P    | 5`-/5FAM/CCAACCCAC/G/TAATTGTTG/3lAbRQSp/-3`     |
|                | Cy5          | K417N                                  | K417N-F       | TGGAGATGGTATTAGAGG     |
|                |              |                                        | K417N-R       | ATACCGCGGCTTAAACATCT     |
|                | Nucleocapsid | Cy5.5                                  | K417N-P       | 5`-/5Cy5/CCAACCCAC/G/TAATTGTTG/3lAbRQSp/-3`     |
|                |              |                                        | K417N-R       | ATACCGCGGCTTAAACATCT     |
|                |              |                                        | N2-F          | TTAACACATTTGCGCCGAAA     |
|                |              |                                        | N2-R          | GCCCGACATTTGCGACAGGAA     |
|                |              |                                        | N2-P          | 5`-/5Cy5/AACGCGGCTTAC/G/TAATTGTTG/3lAbRQSp/-3`     |

Figure 1. Detailed diagram of SNPs that were used to distinguish the Omicron, Delta, Alpha, Beta, and Gamma variants of SARS-CoV-2.
only 1.7% of patients with the Omicron variant exhibited diarrhea and 0.8% manifested a change in taste.

5-in-1 VOC genotyping assay

SARS-CoV-2 VOCs or variants under monitor corresponded to five dominant SARS-CoV-2 variants (Delta-lineage B.1.617.2, Alpha-lineage B.1.1.7, Beta-lineage B.1.351, Gamma Beta-lineage P.1 and Omicron-lineage B.1.1.529) information of which is hosted on GISAID, that contains the sequence information of more than 4,700,000 isolates identified since 2019 (Figure 1). Differences based on the SNPs in the S region— Wuhan-hu-1—identified using the five-in-one assay could be used to distinguish the variants. According to the information hosted on GISAID, most of the Omicron variants carry the N501Y and K417N mutations, and the P681R mutation could separate Delta from other variants. Our 5-in-1 assay could quickly separate the specimens from Alpha, Beta, Gamma, Delta, and Omicron. Moreover, this assay could distinguish Omicron BA.1 and Omicron sneath-BA.2 based on deletion H69/V70 (Supplementary Figure 1).

Analytical sensitivity of 5-in-1 VOC assay

The LoD of the 5-in-1 VOC assay was determined to be 80 copies/PCR reaction SARS-CoV-2 variant RNA control by testing 20 replicates of serially diluted variant controls (Table 4). This LoD is the same as that obtained using the BD MAX™ and LabTurbo AIO open platforms. According to the results for each gene in the 5-in-1 VOC assay, each variant could be distinguished as depicted in Table 2. For example, when we analyzed 20 Omicron replicate samples at 80 copies of RNA control per PCR reaction, we obtained a positive result for N2, N501Y, K417N, and a negative result for P681R and deletion H69/V70. This is still a good performance in the context of the other SARS-CoV-2 variant RNAs.
Table 3
Clinical features, symptoms, and outcomes of patients with COVID-19 caused by the Omicron variant of SARS-CoV-2.

| Characteristics | SARS-CoV-2 Omicron |
|-----------------|-------------------|
| Total number    | 120               |

| Gender         | Male          | 61 (50.8%) |
|----------------|--------------|------------|
|                | Female       | 59 (49.2%) |

| Age            |               |            |
|----------------|--------------|------------|
|                | <19          | 8 (6.7%)   |
|                | 19-29        | 31 (25.8%) |
|                | 30-39        | 24 (20.0%) |
|                | 40-49        | 13 (10.8%) |
|                | 50-59        | 14 (11.7%) |
|                | 60-69        | 11 (9.2%)  |
|                | ≥69          | 19 (15.8%) |

| Mean           | 44.0         |
| Median (± SD)  | 36.5 ± 21.76 |

| Vaccination    |               |            |
|----------------|--------------|------------|
| Two doses, no., (%) | 52 (43.3%) | 55 (45.1%) |
|                 | AstraZeneca  | 16 (13.3%) |
|                 | Pfizer-BioNTech | 24 (20.0%) |
|                 | Moderna      | 11 (9.2%)  |

| Symptoms       |               |            |
|----------------|--------------|------------|
| No symptoms    | 25 (20.8%)   |
| Fever          | 27 (22.5%)   |
| Cough          | 48 (40.0%)   |
| Sore throat    | 31 (25.8%)   |
| Runny nose     | 12 (10.0%)   |
| Stuffy nose    | 5 (4.2%)    |
| Headache       | 3 (2.5%)     |
| General weakness | 3 (2.5%)    |
| Body soreness  | 4 (3.3%)     |
| GI upset       | 1 (0.8%)     |
| Shortness of breath | 13 (10.8%) |
| Seizure attack | 3 (2.5%)     |

| World Health Organization severity classification | Mild | 60 (50.0%) |
|                                                   | Moderate | 19 (15.8%) |
|                                                   | Severe    | 16 (13.3%) |

| Omicron sub lineage | Omicron BA.1 | 52 (43.3%) |
|                     | Omicron BA.2 | 68 (56.7%) |

| Cycle threshold value | Mean | 18.86 |
|                      | Median (SD) | 17 ± 5.98 |

Table 4
Assessment of the limit of detection for the 5-in-1 variant of concern assay on the BD MAX™ System and LabTurbo AIO System using serially diluted RNA controls.

| Testing platform | Target gene/fluorescence | 120 × | 160 | 80 | 40 | 20 |
|------------------|--------------------------|-------|-----|----|----|----|
|                  |                          | copies/polymerase chain reaction |       |     |    |    |    |
| BD MAX™          | N501Y/FAM                | 20/20 (100) | 20/20 (100) | 20/20 (100) | 19/20 (95) | 7/20 (35) |
|                  | P681R/HEX                | 20/20 (100) | 20/20 (100) | 20/20 (100) | 18/20 (90) | 6/20 (30) |
|                  | 69-70/Texas Red          | 20/20 (100) | 20/20 (100) | 20/20 (100) | 18/20 (90) | N.D.||
|                  | K417N/Cy5                | 20/20 (100) | 20/20 (100) | 20/20 (100) | 17/20 (85) | N.D.||
|                  | N2/Cy5.5                 | 20/20 (100) | 20/20 (100) | 19/20 (95) | 9/20 (45) | N.D.||
| LabTurbo AIO     | N501Y/FAM                | 20/20 (100) | 20/20 (100) | 20/20 (100) | 19/20 (95) | 8/20 (40) |
|                  | P681R/HEX                | 20/20 (100) | 20/20 (100) | 20/20 (100) | 18/20 (90) | 6/20 (30) |
|                  | 69-70/Texas Red          | 20/20 (100) | 20/20 (100) | 20/20 (100) | 17/20 (85) | N.D.||
|                  | K417N/Cy5                | 20/20 (100) | 20/20 (100) | 20/20 (100) | 17/20 (85) | N.D.||
|                  | N2/Cy5.5                 | 20/20 (100) | 20/20 (100) | 19/20 (95) | 14/20 (70) | N.D.||

N.D., not detected; N, nucleocapsid.

* Targets included SARS-CoV-2 spike gene single nucleotide polymorphism mutations or the N2 gene.

* Tested samples included 20 replicates of SARS-CoV-2 variant controls that were serially diluted from 320 to 20 copies/polymerase chain reaction.

* N.D., not detected.

Analytical specificity of the 5-in-1 VOC assay

The analytical specificity of the 5-in-1 VOC assay on the LabTurbo AIO open platform was determined by testing a panel of clinical samples containing the following pathogens: influenza A, influenza B, rhinovirus, enterovirus, parainfluenza virus subtype 1 to 3, adenovirus, and coronavirus HKU1, as well as COVID-19-negative samples. No cross-reactivity with other respiratory viruses was detected using the 5-in-1 VOC assay (Supplementary Table 1).

Clinical performance of the 5-in-1 VOC assay

We analyzed 150 clinically positive SARS-CoV-2 samples in this study, confirming the SARS-CoV-2 positive results of the Taiwan CDC central laboratory. We identified the samples to be the Alpha, Betta, Delta, and Omicron variants by using VirSNiP SARS-CoV-2 mutation assay. The samples were retrospectively analyzed using the 5-in-1 VOC assay on both the BD MAX™ System and the LabTurbo™ AIO open platforms. The results of these analyses demonstrated 100% agreement with the results of the VirSNiP SARS-CoV-2 test kits (Figure 3). To confirm that the results were consistent with those of rapid detection of SARS-CoV-2 VOCs, presumptive cases were also assessed by WGS, and the lineages were confirmed using GISAID software. We selected samples including 19 Omicron variants, three Delta variants, three Beta variants, and five Alpha variants. Furthermore, this novel assay also distinguished Omicron BA.1 and Omicron BA.2 (Supplementary Table 2).

Hence, the 5-in-1 VOC assay shows good concordance with WGS and the VirSNiP SARS-CoV-2 test. In addition, the assay showed no cross-reactivity that is usually observed for other upper respiratory viruses or samples that tested negative for SARS-CoV-2 (N = 500).

Discussion

The US CDC defines a VOC as a variant exhibiting increased transmissibility and associated with different disease severities (https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-classifications.html). Further, such variants are associated with significant recalcitrance to neutralization by antibodies gained from previous infection or vaccinations and reduced efficacy of treatments and/or vaccines. At present, a range of emergent SARS-CoV-2 variants is being identified as VOCs.

Although WGS has been the primary modality for VOC surveillance, sequencing of SARS-CoV-2–positive samples is limited by both laboratory and bioinformatics capacity. Furthermore, turnaround times using WGS may be days to weeks. We provide a PCR-based algorithm for the molecular detection of VOCs that...
could be rapidly executed, providing quick results to inform infection prevention and control and public health measures. PCR may also be more sensitive because WGS is challenging to perform on samples with low viral loads [22]. We also compared the developed assay with a commercial kit (Roche’s VirSNiP) and found good concordance between these two assays. However, commercial kits may not be an affordable option during the COVID-19 pandemic in developing countries. As our 5-in-1 VOC assay is lab-developed, it is economical and hence a suitable assay for developing countries. Although the prevalent VOCs worldwide harbor the S gene SNP mutation N501Y, this mutation is not present in all variants. For example, according to our WGS analysis, many Delta variants do not contain this mutation (Figure 1). Our initial screening PCR targeted N501Y, but because of rising rates of Omicron prevalence, we adjusted our laboratory-developed test to include K417N and deletion H69/V70 in the same PCR reaction. When SARS-CoV-2-positive samples emitted positive signals for K417N, they were considered to be samples of the Omicron variant. Furthermore, the deletion of H67/V70 could distinguish the Omicron subvariant BA.1 and BA.2. However, our lab-developed assay could not distinguish between Beta and BA.2. The Beta variant was identified in September 2020, in Africa. One year later year, in late November 2021, the Omicron variant showed up and World Health Organization designated the variant as VOC [23]. On March 15, 2022, US CDC estimated that 23% of all the then-current COVID-19 cases in the USA were caused by the Omicron BA.2 subvariant. Therefore, using our less time-consuming and economical assay, the lineage a VOC belongs to can be deduced. Given the rapid emergence of new variants, ongoing surveillance is a key step, and laboratories considering a PCR-based algorithm need to adapt the algorithm with the prevalence of changes in VOCs.

In this study, we have developed a novel multiplex RT-PCR assay for use with an automatic platform that would serve as a high-throughput and easy-to-use detection method for SARS-CoV-2. As the novel coronavirus has had a severe impact on human health, economy, and social life, our assay would have a significant impact in improving the lives of affected individuals by enabling early detection of the virus.

**Conclusion**

In this study, we developed a novel multiplex RT-PCR assay for use with an automatic platform to serve as a high-throughput and easy-to-use detection method for SARS-CoV-2, including distinguishing among Alpha, Beta, Gamma, Delta, and Omicron variants. As the novel coronavirus has had a severe impact on human health, the economy, and social life, our assay could have a significant impact in improving the lives of affected individuals by enabling early detection of the virus.

**Declaration of competing interest**

The authors have no competing interests to declare.

**CRediT authorship contribution statement**

Hsing-Yi Chung: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. Ming Jian Jr: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. Chih-Kai Chang: Investigation, Methodology. Chi-Sheng Chen: Investigation. Shih-Yi Li: Investigation. Jung-Chung Lin: Conceptualization, Supervision. Kuo-Ming Yeh: Conceptualization, Supervision. Ya-Sung Yang: Conceptualization, Supervision. Chien-Wen Chen: Conceptualization, Supervision. Shan-Shan Hsieh: Methodology. Sheng-Hui Tang: Supervision. Cheng-Lih Perng: Investigation, Methodology. Kuo-Sheng Hung: Formal analysis, Methodology. Feng-Yee Chang: Conceptualization, Supervision. Hung-Sheng Shang: Supervision, Writing – review & editing.

**Funding**

This work was supported by the Tri-Service General Hospital, Taipei, Taiwan, R.O.C. (grant number: TSGHIRB number: C2020005041), registered on February 8, 2021.

**Ethical approval**

This study was approved by the Institutional Review Board of Tri-Service General Hospital (TSGHIRB number: C2020005041), registered on February 8, 2021.

**Supplementary materials**

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.11.027.
References

[1] Choi JY, Smith DM. SARS-CoV-2 variants of concern. Yongsei Med J 2021;62:961–8. doi: 10.3349/ymj.2021.62.11.961.

[2] Galloway SE, Paul P, MacCannell DR, Johannsson MA, Brooks JT, MacNeil A, et al. Emergence of SARS-CoV-2 B.1.1.7 lineage - United States, December 29, 2020–January 12, 2021. MMWR Morb Mortal Wkly Rep 2021;70:95–9. doi: 10.15585/mmwr.mm7003e2.

[3] Davies NG, Jarvis CI, Edmunds WJ, Jewell NP, Díaz-Ordaz K, Keogh RHC. MID COVID-19 Working Group. Increased mortality in community-tested cases of SARS-CoV-2 lineage B.1.1.7. Nature 2021;593:270–4. doi: 10.1038/s41586-021-03426-1.

[4] Petersen E, Ntoumi F, Hui DS, Abubakar A, Kramer LD, Obiero C, et al. Emergence of new SARS-CoV-2 variant of concern Omicron (B.1.1.529) - highlights Africa’s research capabilities, but exposes major knowledge gaps, inequities of vaccine distribution, inadequacies in global COVID-19 response and control efforts. Int J Infect Dis 2022;114:268–72. doi: 10.1016/j.ijid.2021.11.040.

[5] Ramesh S, Govindaraju M, Parsie RS, Neel L, Shankar T, Patel S, et al. Emerging SARS-CoV-2 variants: a review of its mutations, its implications and vaccine efficacy. Vaccines 2021;9:1195. doi: 10.3390/vaccines9101195.

[6] Chang CK, Jian MJ, Jr. Chung HY, Lin JC, Hsieh SS, Tang SH, et al. Clinical comparative evaluation of the LabTurboTM AIO® reverse transcription-polymerase chain reaction and World Health Organization-recommended assays for the detection of emerging SARS-CoV-2 variants of concern. Infect Drug Resist 2021;15:595–603. doi: 10.2147/idr.s349669.

[7] Chen F, Tian Y, Zhang L, Shi Y. The role of children in household transmission of COVID-19: a systematic review and meta-analysis. Int J Infect Dis 2022;122:266–75. doi: 10.1016/j.ijid.2022.05.056.

[8] Seong H, Hyun HJ, Yun JC, Noh JY, Cheong HJ, Kim WJ, et al. Comparison of the second and third waves of the COVID-19 pandemic in South Korea: importance of early public health intervention. Int J Infect Dis 2021;104:742–5. doi: 10.1016/j.ijid.2021.02.004.

[9] Al-Tawfiq JA, Korotala T, Alhumaid S, Barry M, Alshukairi AN, Temsah MH, Al Mutair A, Rabaan A, Tiripathi R, Gauthret P. Implication of the emergence of the delta (B.1.617.2) variants on vaccine effectiveness. Infection 2021;50:S83–96. doi: 10.1007/s10603-022-01759-1.

[10] Kumar S, Thambiraja TS, Karuppannag K, Subramaniam G, Omicron and Delta variant of SARS-CoV-2: a comparative computational study of spike protein. J Med Virol 2022;94:1641–9. doi: 10.1002/jmv.27526.

[11] Shaibu JO, Onwuamah CK, James AB, Okwurawe AP, Amos OS, Saul OB, et al. Full length genomic sanger sequencing and phylogenetic analysis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in Nigeria. PLoS One 2021;16:e0243271. doi: 10.1371/journal.pone.0243271.

[12] Daniels RS, Harvey R, Ermelot B, Xiang Z, Galano M, Adams L, et al. A Sanger sequencing protocol for SARS-CoV-2 S-gene. Influenza Other Respir Viruses 2021;15:707–10. doi: 10.1111/irv.12892.

[13] Park ST, Kim J. Trends in next-generation sequencing and a new era for whole genome sequencing. Int Neurrol J 2016;20:576–83. doi: 10.5212/inj.1623742.371.

[14] Challen R, Brooks-Pollock E, Read J, Dyson L, Tsaneva-Atanasova K, Danon L. Risk of mortality in patients infected with SARS-CoV-2 variant of concern 202012/1: matched cohort study. BMJ 2021;372:n579. doi: 10.1136/bmj.n579.

[15] Perg CL, Jian MJ, Chang CK, Lin JC, Yeh KM, Chen CW, et al. Novel rapid identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by real-time RT-PCR using BD Max Open System in Taiwan. Peepj 2020;8:e9318. doi: 10.7717/peerj.9318.

[16] Rabaan AA, Tirupathi R, Sule AA, Aldali J, Mutair AA, Alhumaid S, et al. Viral dynamics and real-time RT-PCR Ct values correlation with disease severity in COVID-19. Diagnostics (Basel) 2021;11:1091. doi: 10.3390/diagnostics11061091.

[17] Ong DSY, Koelman JGM, Vaessen N, Breier S, Paltansing S, de Man P. Rapid screening method for the detection of SARS-CoV-2 variants of concern. J Clin Virol 2021;141:104903. doi: 10.1016/j.jcv.2021.104903.

[18] European Center for Disease Prevention and Control Methods for the detection and characterisation of SARS-CoV-2 variants – first update. Sweden: European Center for Disease Prevention and Control; 2021.

[19] Chung HY, Jian MJ Jr, Chang CK, Lin JC, Yeh KM, Yang YS, et al. 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 2021;13:24931–42. doi: 10.18632/aging.203761.

[20] Lu X, Wang L, Sakhivela SK, Whitzaker B, Murray J, Kamili S, et al. US CDC real-time reverse transcription PCR panel for detection of severe acute respiratory syndrome coronavirus 2. Emerg Infect Dis 2020;26:1654–65. doi: 10.3201/eid2608.201246.

[21] Charré C, Ginevra C, Sabatier M, Regue H, Destrai C, Bruin S, et al. Evaluation of NGS-based approaches for SARS-CoV-2 whole genome characterisation. Virus Evol 2020;6:vea0753. doi: 10.1093/varnav/vea0753.

[22] Wang F, Huang S, Gao R, Zhou Y, Lai C, Li Z, et al. Initial whole-genome sequencing and analysis of the host genetic contribution to COVID-19 severity and susceptibility. Cell Discov 2020;6:83. doi: 10.1038/s41421-020-00223-4.

[23] Flores-Vega VR, Monroy-Molina JV, Jiménez-Hernández LE, Torres AG, Santos-Preciado Jr, Rosales-Reyes R. SARS-CoV-2: evolution and emergence of new viral variants. Viruses 2022;14:653. doi: 10.3390/v14040653.