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**Simultaneous Screening of SARS-CoV-2 Omicron and Delta Variants Using High-Resolution Melting Analysis**

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Summary
A novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) strain, the Omicron variant (Pango lineage B.1.1.529), was identified in South Africa in late September 2021. This variant has multiple spike protein deletions and mutations, with 15 amino acid substitutions detected in the receptor-binding domain (RBD). These RBD substitutions are hypothesized to increase infectivity and reduce antibody affinity, which is supported by recent data showing that the Omicron variant spreads faster than the Delta variant (Pango lineage B.1.617.2). Thus, this increase in infectivity should lead to Omicron being the dominant variant and developing screening tests that discriminate between Omicron and Delta variants is urgently needed. In this study, we successfully developed a novel screening assay using high-resolution melting analysis, in which two genotypes at G446/L452 and S477/T478 RBD were determined (G446S/L452 and S477N/T478K for Omicron; G446/L452R and S477/T478K for Delta). Using synthetic DNA fragments, we confirmed both melting point and melting peak shape of the RBD Omicron variant was distinguishable from those of wild-type and the Delta variant. Although this study was conducted without clinical samples, these results suggest that our HRM-based genotyping method can readily identify the Omicron and Delta variants. This simple method should contribute to the rapid identification of SARS-CoV-2 variants and thus prevent potential widespread infection and inflow of the Omicron variant.

Key words SARS-CoV-2; Omicron variant; Delta variant; high-resolution melting; genotyping method; receptor-binding domain
INTRODUCTION

In late December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged from China and caused coronavirus disease 2019 (COVID-19). Although two years have passed, SARS-CoV-2 continues to spread worldwide. The World Health Organization (WHO) reported nearly 269 million confirmed cases and approximately 5.3 million deaths globally as of 12 December 2021 (weekly epidemiological update on COVID-19 – 14 December 2021).

Several SARS-CoV-2 variants with high infectivity or immune escape have emerged and spread rapidly in many countries. Some spike mutations can increase infectivity and reduce antibody affinity. The Delta variant (Pango lineage B.1.617.2), first detected in India in December 2020, has spread worldwide and outcompeted other variants such as the Alpha variant (Pango lineage B.1.1.7). The Delta variant commonly has two amino acid substitutions, L452R and T478K, in the receptor-binding domain (RBD) of the spike protein (Table 1). The newly discovered variant, Omicron (Pango lineage B.1.1.529), was identified in South Africa in late September 2021. The Omicron variant has fifteen amino acid substitutions in RBD (Table 1). This variant has spread faster than the Delta variant in the Gauteng province of South Africa. Moreover, the Omicron variant can evade the immune system of humans that have been infected previously with SARS-CoV-2. Nonetheless, the Delta variant still accounts for more than 99% of COVID-19 new cases uploaded to the GISAID website in the last 60 days (weekly epidemiological update on COVID-19 – 14 December 2021). Therefore, rigorous global monitoring of both the Delta and Omicron variants is warranted.

Next-generation sequencing has been used to diagnose SARS-CoV-2 variants in many countries, including Japan. However, this process takes a few days to determine the SARS-CoV-2 genome sequence of each sample. Thus, a rapid screening method is urgently needed to protect people against novel SARS-CoV-2 variants that are highly infectious. The probe-based PCR assay is a valuable technique that provides rapid detection and high specificity with single-nucleotide resolution, and several probes are available worldwide. However, as the RBD region of the spike protein of the Omicron variant is highly mutated, the reactivity of available specific probes may be affected, which probably increases the risk of false negatives.

Several reports have shown that high-resolution melting (HRM) analysis, a post-PCR technique based on the difference in DNA thermal melting, can identify SARS-CoV-2 variants. Our previous study showed that the HRM-based genotyping method identifies clinical samples containing the Delta variant. In this study, we
developed a rapid screening assay to simultaneously identify mutations found in the Omicron and Delta variants using HRM analysis.

MATERIALS AND METHODS

Preparation of standard RNA fragments: in vitro T7 transcription

The SARS-CoV-2 sequence used was obtained from NCBI (NCBI Reference Sequence: NC_045512.2), the GISAID database (www.gisaid.org), and the Pango nomenclature system (https://cov-lineages.org/lineages.html). Seven RBD DNA fragments (wild type, Delta variant mutant, Omicron variant mutant, G446S single mutant, L452R single mutant, T478K single mutant, and S477N/T478K double mutant; 600–1000 bp in length) with a 5′ T7 upstream promoter sequence were obtained from Eurofins Genomics KK (Tokyo, Japan). *In vitro* T7 transcription was done as described previously. Synthesized single-stranded RNA fragments were used as RT-PCR amplification templates.

Reverse-transcription (RT)-PCR amplification: First PCR

RT-PCR was done as described previously. The primer pairs were as follows: outer forward 5′-TTACAGGCTGCGTTATAG-3′ and outer reverse 5′-ACAAACAGTTGCTGGTGCAT-3′ (Fig. 1). After amplification, the reaction mixture diluted 10,000-fold with water was used as a template for the second PCR and HRM analysis.

HRM analysis: Second PCR

PCR amplification, HRM condition, and HRM curve analysis are the same as those described previously. The second primer pairs (Fig. 1) were as follows: G446–L452 forward 5′-GGCTGCGTTATAGCTTGGAATTCTAACAATCTT-3′ and G446–L452 reverse 5′-TCAAAAGGTTTGAGATTAGACTTCC-3′; S477–T478 forward 5′-TTGTTTAGGAGTCTAACACAACC-3′ and S477–T478 reverse 5′-AAGTAACAATTAAACCTTCAACACCATTACAAGG-3′. As shown in Fig. 1, the G446–L452 forward primer design was based on the N440 coding sequence, and the S477–T478 reverse primer design was based on the E484 coding sequence to avoid the potential influence of N440 and E484 mutations.

RESULTS AND DISCUSSION

One-step RT-PCR for template preparation

The Omicron variant has fifteen amino acid substitutions in RBD, which may
affect PCR amplification, leading to poor fidelity and yield of PCR products. Therefore, positive control RNAs with identical sequences to RBDs from Omicron and Delta variants were prepared (Table 1). We performed nested PCR involving RT-PCR and subsequent HRM analysis to improve the detection limit of HRM analysis. For RT-PCR, the target fragment was amplified using an outer primer set in a single closed tube (Fig. 1). As a positive control RNA for HRM analysis, the SARS-CoV-2 RNA fragments were synthesized by in vitro T7 transcription. After RT-PCR, agarose gel electrophoresis and Sanger sequencing showed that each positive control RNA template was amplified as a specific PCR product (290 bp) (data not shown). These results indicated that our RT-PCR conditions were suitable for the first PCR with an outer primer set.

HRM analysis on standard fragments

After the RT-PCR reaction, HRM analysis as a second PCR was performed using specific primer sets for G446–L452 and S477–T478 sites. In the G446–L452 site, the Omicron variant has the G446S mutation, and the Delta variant has the L452R mutation. In the S477–T478 site, the Omicron variant has the double mutation S477N/T478K, and the Delta variant has the single mutation T478K (Table 1). Initially, G446S and L452R mutations were determined using diluted RT-PCR reaction mixtures. Normalized melting curves and melting peaks for wild-type RBD, Omicron RBD, and Delta RBD are shown in Fig. 2. The melting curve profiles of wild-type RBD, Omicron RBD, and Delta RBD were unequivocally differentiated. Additionally, the Omicron RBD was in good agreement with those of the G446S mutant, and the delta RBD was in good agreement with those of the L452R mutant (data not shown). We next investigated the identification of S477N and T478K mutations by HRM analysis. The melting curve profiles of wild-type RBD, Omicron RBD, and Delta RBD were unequivocally differentiated. Moreover, the Omicron RBD was in good agreement with those of the G446S/T478K double mutant, and the Delta RBD was in good agreement with those of the T478K mutant (data not shown). These results suggest that the current HRM analysis can simultaneously identify the Omicron and Delta variants using one of the G446–L452 and S477–T478 sites. However, both sites would be favorable for increasing assay sensitivity and specificity.

In conclusion, this study succeeded in developing a rapid assay for simultaneous screening of the Omicron and the Delta variants using both G446–L452 and S477–T478 sites. Because the assay does not require a sequence-specific probe (e.g., TaqMan probe), a cost-effective assay can be constructed rapidly using HRM analysis. Nonetheless, this technique still requires future validation using clinical samples from
COVID-19 patients to evaluate the assay sensitivity and specificity.
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Conflict of interest

The authors declare no conflict of interest.
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Fig. 1. Schematic map of primer annealing sites for RT-PCR and HRM analysis.
Fig. 2. Normalized melting curves and melting peaks of positive control RNAs for the G446–L452 site. Normalized melting curve plots (A) and melting peak plots (B) for the G446–L452 site were acquired using standard fragments of the wild-type RBD (dotted line), Delta RBD (dashed line), and Omicron RBD (solid line).
Fig. 3. Normalized melting curves and melting peaks of positive control RNAs for the T478 site. Normalized melting curve plots (A) and melting peak plots (B) for the S477–T478 site were acquired using standard fragments of the wild-type RBD (dotted line), Delta RBD (dashed line), and Omicron RBD (solid line).
Table 1. The RBD amino acid substitutions in Omicron and Delta variants.

| WHO label | Pango lineage | RBD amino acid substitutions |
|-----------|---------------|------------------------------|
| Omicron   | B.1.1.529     | G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H |
| Delta     | B.1.617.2     | L452R, T478K                  |