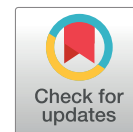


Optimization of multiplex PCR composition to screen for SARS-CoV-2 variants of concern



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ABSTRACT

Background: The ongoing COVID-19 pandemic has led to the emergence of several variants of concern. To rapidly identify those variants, screening samples for whole-genome sequencing (WGS) prioritization could be performed.

Objective: We optimized the polymerase chain reaction (PCR) screening method to identify the mutation in spike and ORF1a regions.

Methods: We adopted primers targeting mutation in spike and ORF1a region from another study. We optimized the PCR screening method using kits readily available in Indonesia. Firstly, we compared N1 and N2 primers as internal positive control. We also compared GoTaq® 1-Step RT-qPCR System and Indonesia TFRIC-19 BioCOV-19 for the multiplex reaction. We used the optimized composition to screen SARS-CoV-2 positive samples from April – June 2021. Samples with spike and/or ORF1a target failure were subjected to whole genome sequencing (WGS).

Results: The results demonstrated the N2 BioCOV-19 reaction as the optimized multiplex PCR composition for spike and ORF1a mutations screening. Whole-genome sequencing has shown that a sample with spike and ORF1a targets failure to be Alpha variant, while other samples with single target failure as non-variants of concern. Therefore, a multiplex RT-PCR composition has been optimized to detect mutation in spike and ORF1a regions.

Conclusion: We have optimized a multiplex RT-PCR composition to detect mutation in spike and ORF1a regions.

Keywords: multiplex, mutation, polymerase chain reaction, SARS-CoV-2, screening

Introduction

One of the main challenges in the COVID-19 pandemic is the emergence of several mutations and variants of concern. At the beginning of 2021, there were four variants of concern (VOC), including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) [1]. Routine PCR diagnostic tests cannot distinguish one variant from another; therefore, whole genome sequencing (WGS) is needed to identify and track the variants. Due to limitations in WGS infrastructure and capacity, genomic surveillance could be lagging [2]. sample prioritization from WGS is required to overcome this limitation. For example, France implemented a two-steps strategy to detect VOC, especially the Alpha variant. Mutation $\Delta 69-70$ in the spike

region of Alpha variant causes amplification failure when using particular PCR kit, such as TaqPath™ (ThermoFisher, USA); therefore, samples with spike gene target failure (SGTF) could be prioritized for WGS [3].

Currently, most of the routine COVID-19 *in-vitro* diagnostics (IVD) RT-PCR kits are multiplex reactions. However, at the beginning of the pandemic, Research-Use Only (RUO) singleplex kits were used. One of the early kits used was GoTaq® 1-Step RT-qPCR System (Promega, USA) utilizing separate N1 and N2 primer pairs designed by CDC, USA. Task Force of the Republic of Indonesia for COVID-19 (TFRIC-19) has designed a BioCOV-19 RT-PCR kit, consisting singleplex reactions for separate N2 and RdRP gene targets. A multiplex reaction

can be created using these singleplex reaction by adding probes with different fluorophores. Several studies have developed multiplex PCR assays for variant screening. One study designed primers and probes targeting mutations in spike and ORF1a regions. The assay is expected to discriminate Alpha, Beta, and Gamma variants from other variants [4]. Therefore, we aimed to optimize PCR screening method using kits available in Indonesia.

Methods

We adopted primers targeting mutation in spike and ORF1a region from another study [4]. Spike mutation targeted was deletion $\Delta 69-70$. ORF1a mutation targeted was deletion $\Delta 3675-3677$, also known as nsp6: $\Delta 106-108$. We optimized the PCR screening method using kits readily available in Indonesia.

Firstly, we compared the use of N1 and N2 primers (IDT, USA) as an internal positive control. We also compared GoTaq® 1-Step RT-qPCR System (Promega, USA) and Indonesia TFRIC-19 BioCOV-19 (BioFarma, Indonesia) for the multiplex reaction. The PCR composition followed each kit's instructions. Modification into multiplex reaction was conducted by adding additional primers and probes targeting spike and ORF1a. For both targets, 0.4 μM of each primer and 0.2 μM of each probe were added [4]. The PCR condition followed the cycles described by each kit.

We used the optimized composition to screen random SARS-CoV-2 positive samples from April – June 2021. Positive samples were obtained from routine diagnostic testing in Biomolecular Laboratory, Arifin Achmad General Hospital of Riau Province. Samples with spike and/or ORF1a target failure were subjected to WGS in National Institute of Health Research and Development (Balitbangkes) and Genomik Solidaritas Indonesia Laboratorium (GSI Lab). Samples subjected to sequencing in the Balitbangkes were sequenced using illumine Miseq and assembled using CLC Genomics Workbench. Samples subjected to sequencing in the GSI Lab were sequenced using Nanopore GridION and assembled using Medaka 1.4.3.

Results

The first optimization was to compare the N1 and N2 primers as an internal control for the multiplex reaction. Figure 1 shows that in the GoTaq® reaction for positive sample, the N1 primer resulted in a higher relative fluorescence unit (RFU) than N2 primers. However, in no-template control reaction, N1 primer reaction resulted in non-specific amplification, which was not observed in N2 primer reaction. In both reactions, spike and ORF1a intensities were relatively comparable. Therefore, N2 was preferred as an internal control for SARS-CoV-2 RNA presence to reduce N-gene non-specific amplification.

The next step was to optimize the reaction using the TFRIC-19 BioCOV-19 kit (BioFarma, Indonesia). The BioCOV-19 kit consists of a singleplex reaction mix with separate N2 and RdRP primers. We compared three multiplex reactions using different internal control, N1 IDT primer, N2 BioCOV-19 primer, and RdRP BioCOV-19 primer. Figure 2 shows that all three multiplexes BioCOV-19 reactions on positive sample resulted in similar spike and ORF1a intensity. The difference was observed in internal control intensity. In the positive sample, the N2 BioCOV-19 primer resulted in the highest RFU, while the RdRP BioCOV-19 primer had the lowest RFU. In no-template control, non-specific amplification was observed in the RdRP reaction but not in the N2 reaction. Therefore, the optimized reaction modified the singleplex N2 BioCOV-19 reaction to multiplex by adding mutation-targeting primers and probes.

We subjected samples with various amplification patterns to WGS to assign the lineage. Sample with no target failure was assigned as B.1.466.2 with no deletions in spike and ORF1a regions (GISAID Accession No.: EPI_ISL_2233109). Sample with double-target failure was assigned as the Alpha variant (GISAID Accession No.: EPI_ISL_2258213). Sample with spike target failure was assigned as B.1.466.2 with H69-V70 deletion (GISAID Accession No.: EPI_ISL_2258218). However, among ORF1a target failure samples, one sample has the deletion resulting in nsp6 106-108 deletion

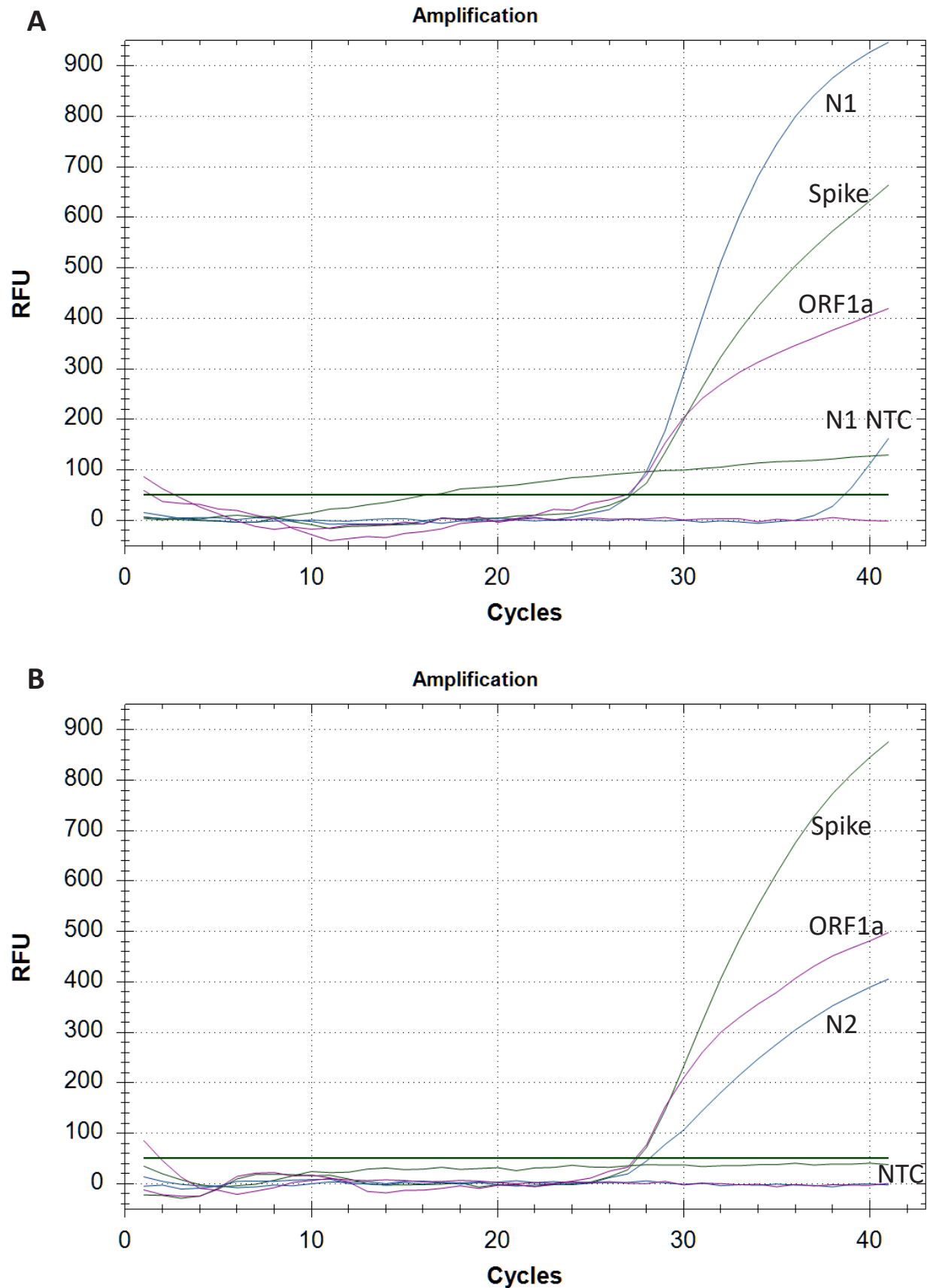


Figure 1. Multiplex PCR using GoTaq® kit showed comparable amplification of spike and ORF1a targets. N gene amplification in positive sample (PS) was higher using N1 primer compared to N2 primer. No-template control (NTC) PCR using N1 primer resulted in non-specific amplification which was not observed in NTC using N2 primer. (A) PS and NTC using N1 primer, (B) PS and NTC using N2 primer.

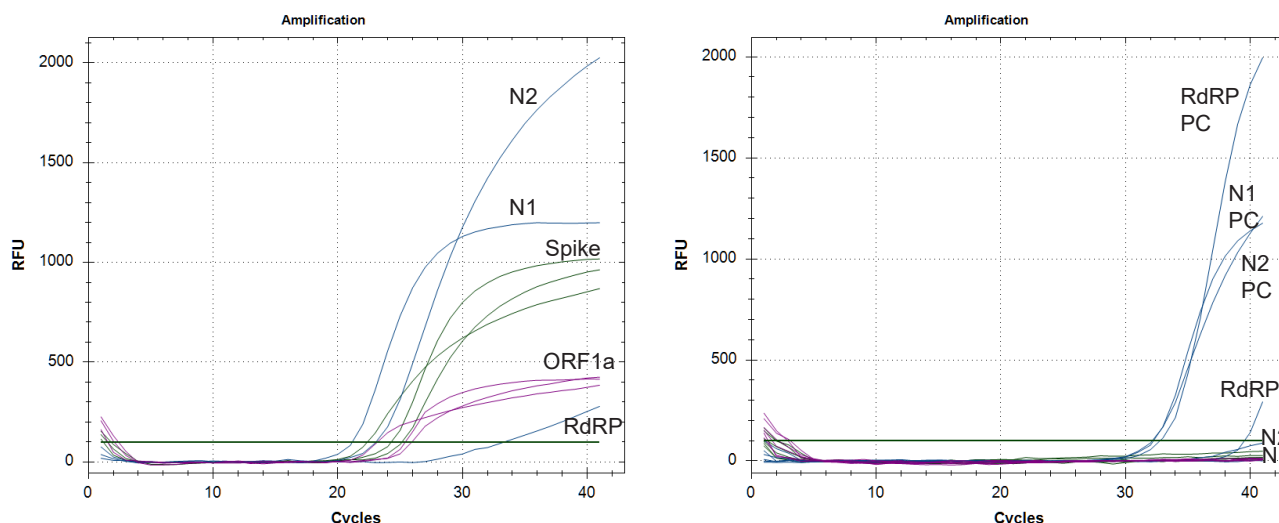


Figure 2. Multiplex PCR using BioCOV-19 kit showed comparable amplification of spike and ORF1a targets. Intensity of N2 BioCOV-19 was higher compared to N1 IDT and RdRP BioCOV-19. Additionally, no non-specific amplification was observed in N2 BioCOV-19 no-template control (NTC) reaction. (A) Positive sample using BioCOV-19 reaction. (B) No-template control and kit-provided positive control (PC) using BioCOV-19 reaction.

(GISAID Accession No.: EPI_ISL_4783668) while another sample does not (GISAID Accession No.: EPI_ISL_2233108). Sample with ORF1a target failure but without nsp6 106-108 deletion has G11291A and T11296G nucleic acid substitution.

Discussion

We have optimized a multiplex PCR reaction to screen for SARS-CoV-2 mutation using a PCR kit from Indonesia. Accordingly, the sample with spike + ORF1a targets failure was assigned as Alpha variant, in line with proposed interpretation [4]. Samples with spike target failure were shown to have $\Delta 69-70$ deletions and assigned as B.1.466.2, the most predominant variant in Indonesia before the Delta outbreak [5]. The spike deletion is not specific to the Alpha variant. A study in Slovakia found that samples with spike target failure were assigned as B.1.258. The B.1.258 lineage with the deletion had a higher viral load and became one of the dominant variants found in Slovakia [6]. The higher infectivity of variant harboring $\Delta 69-70$ mutation may be explained by the increased cleavage on S2 domain of spike protein with the mutation [7]. Therefore, the spike deletion might partly explain the dominance of B.1.466.2 in Indonesia.

Sample with ORF1a target failure was assigned as B.1.470, a variant first detected in Indonesia [5]. Deletion in the ORF1a region of several VOCs was initially predicted to cause ORF1a target failure [4]. However, we found that some of our samples with ORF1a target failure did not have the deletion. Instead, we found G11291A and T11296G substitutions in the probe binding region. The samples have amino acid substitution in nsp6 G107S and nsp6 F108L. The substitution may cause target failure due to the inability of *Taq* polymerase to recognize and perform exonuclease activity for a probe containing mismatched base [8]. The condition has been utilized for real-time PCR allelic discrimination assay for human genotyping [9].

Conclusion

Here we have optimized a multiplex RT-PCR composition to detect mutation in spike and ORF1a regions. This method can be further developed to detect different mutations. The future project will focus on testing primers and probe to detect L452 where the mutation L452R is typical to the Delta variant. Additionally, screening the Omicron variant might utilize this current method due to the presence of both spike $\Delta 69-70$ deletions and NSP6 $\Delta 106-108$ deletions in Omicron. The

screening method can facilitate prioritization and reduce lag in variant detection.

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Declaration of interest

The authors do not have any conflict of interest.

Author contributions

Conceptualization, MS and RAK; Methodology, RAK; Investigation, MS, EA, and RAK; Funding Acquisition, MS and EA; Writing – Original Draft, RAK; Writing – Review & Editing, MS, EA, and RAK

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