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A Case of Whole Genome Analysis of SARS-CoV-2 Using Oxford Nanopore MinION System

Jae-Seok Kim*, Sung Hee Chung, Jung-Min Kim, Hyun Soo Kim, Han-Sung Kim, Wonkeun Song

Department of Laboratory Medicine, Hallym University College of Medicine, Seoul, Korea

Corresponding author: Jae-Seok Kim, jaeseok@hallym.ac.kr

Abstract

The application of whole genome sequencing on the SARS-CoV-2 viral genome is essential for our understanding of the molecular epidemiology and spread of viruses in the community. The portable whole genome sequencer MinION (Oxford Nanopore Technologies, ONT, UK) could be feasibly used in a clinical microbiology laboratory without the need for vast resources or stringent operating conditions. We used the MinION sequencer to analyze the viral genome sequence of one SARS-CoV-2 strain. In June 2020, a nasopharyngeal specimen from one patient was subjected to whole-genome analysis using the nCoV-2019 sequencing protocol ARTIC V2 using the MinION sequencer. The ONT MinKNOW software, RAMPART tool, and Genome Workbench were used. We identified 11 nucleotide variants using the Wuhan-Hu-1 isolate (NC_045512.2) as the reference sequence. There were six nucleotide variants (T265I, F924, Y3884L, P4715L, L5462, and Q6804L) in the ORF1ab region, one variant (D614G) in the S gene, one variant (Q57H) in ORF3a, one variant (P302) in the N gene, and two variants in each the 5'-UTR and 3'-UTR. In this prolonged coronavirus disease 2019 (COVID-19) pandemic season, the MinION system that operates an amplicon-based whole-genome sequencing protocol could be a rapid and reliable sequencer without the need for cumbersome viral cultivation.

Keywords

Nanopore sequencing
SARS-CoV-2
COVID-19
Whole genome sequencing

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), first reported in December 2019 in Wuhan, China, is an RNA virus with a sequence of approximately 30,000 bases, consisting of open reading frames (ORFs) 1-10 that make 16 non-structural proteins and spike (S), envelope (E), membrane (M), and nucleocapsid (N) that make structural proteins [1].

Genomic analysis of SARS-CoV-2 plays an important role in epidemiological analysis and identification of variants [2], and a range of next-generation sequencing (NGS) instruments are available from Illumina (San Diego, CA, USA) and Oxford Nanopore Technologies (ONT, Oxford, UK) [3-7].

Among the NGS instruments, the ONT MinION is small enough to fit in the palm of the human hand and can be analyzed in real-time using the company's cloud-based program, allowing it to be used in laboratory settings where NGS is not routinely performed. In addition, the ARTIC network in the United Kingdom provides various bioinformatic tools, and there is a system that can be used for genomic analysis of the Ebola virus in Africa, and it is also used for genomic analysis of the Lassa virus, yellow fever virus, influenza virus, and SARS-CoV-2 [1,8]. The amount of RNA is adjusted according to the cycle threshold (Ct) value measured during real-time reverse transcription-polymerase chain reaction (RT-PCR), and it is suggested that samples with a Ct value of 18–35 can be used without dilution. ARTIC network's analysis system is based on cDNA synthesis followed by a multiplex PCR with 218 PCR primers to amplify 98 gene sites in two PCR tubes, followed by library construction and analysis with MinION equipment. The MinKNOW software provided by ONT runs on a laptop or personal computer to perform sequencing and generate FAST5 files [8]. Using the ARTIC MinION system provided by the ARTIC network (https://artic.network/ncov-2019), genomic information is obtained by generating files such as BAM files and consensus FASTA. Guppy software is used to perform base calling, demultiplexing, and read filtering, and the RAMPART program is used to check the read generation of each gene site in real time.

This study analyzed the SARS-CoV-2 genome using ONT's Nanopore NGS instrument and the ARTIC MinION system from a SARS-CoV-2 positive specimen in June 2020.

2. Case report

A SARS-CoV-2-positive nasopharyngeal swab was collected from a university hospital in June 2020, and a MinION analysis was performed in July 2020. The patient was a domestic resident and tested positive with the Allplex 2019-nCovV Assay (See gene, Seoul, Korea), with Ct values of 22.1 for the E gene, 24.2 for the RdRp gene, and 26.4 for the N gene.

The test method used was based on the nCoV-2019 sequencing protocol V2 (GunIt V2) released on 9 April 2020, using the V3 primer set (https://www.protocols.io/view/ncov-2019-sequencing-protocolv2-bdp7i5rn?version_warning=no). Due to the small number of samples at that time, the same sample was repeatedly sequenced with five barcodes. Briefly, cDNA was synthesized by reacting 11 μL of RNA with 1 μL of random hexamer (50 μM) and 1 μL of dNTP (10 mM) at 65°C for 5 min, and mixing 4 μL of SSIV buffer, 1 μL of DTT (100 mM), 1 μL of RNase inhibitor, and 1 μL of reverse transcriptase. The PCR reaction was performed with conventional reagents according to the Nanopore protocol (ver. PTC_9096_v109_revE06Feb2020) using 2.5 μL of cDNA and Hot Start High-Fidelity 2X Master Mix 12. After dispensing 5 μL, V3 primer set 1 (110 primers) and set 2 (108 primers) were dispensed 3.7 μL each, and then adjusted to 25 μL by adding distilled water (DW), and then a multiplex PCR was performed.

The PCR reactions were purified by end preparation, barcode ligation, bead purification, etc., and then the amount of DNA was measured, adaptor ligation was performed, bead purification was performed, and the amount of DNA was adjusted and injected into the MinION flow cell.

NGS experiments were performed using the MinKNOW program. The RAMPART program provided by the ARTIC network was used to check the number of reads for each gene site analyzed in real-time, and SARS-CoV-2 isolate Wuhan-Hu-1 (NC_045512.2) was used as a reference sequence.

The FAST5 files obtained from MinKNOW were subjected to base calling and read filtering using Guppy, and then analyzed with the ARTIC-ncov2019 program provided by the ARTIC network to obtain BAM files. For the identification of gene variants with standard sequences, The National Center for Biotechnology

Information (NCBI) Genome Workbench (version 3.6.0) was used for visual analysis.

One sample was experimentally run with five barcodes each, and reads were targeted at approximately 300,000 reads per sample with real-time observation with RAMPART, and the number of reads mapped to the reference sequence (NC_045512.2) was 277,250–414,704. The BAM file generated by ARTIC-ncov2019 was analyzed with NCBI Genome Workbench, and variants were visually read (**Figure 1**). Eleven sequence differences were observed against the reference sequence (**Table 1**), including T265I, F924, Y3884L, P4715L, L5462, and Q6804L in the ORF1ab region, D614G in the S gene, Q57H in the ORF3a region, and P302 in the N gene, and one gene sequence difference was observed in the 5'-UTR and 3'-UTR, respectively.

Table 1. Genomic and amino acid changes of the SARS-CoV-2 positive specimen

Genomic change	Amino acid change	Gene/region
241 C → T		5'-UTR
1059 C → T	T265I	ORF1ab
3037 C → T	F924	ORF1ab
11916 C → T	Y3884L	ORF1ab
14408 C → T	P4715L	ORF1ab
16650 C → T	L5642	ORF1ab
20675 A → T	Q6804L	ORF1ab
23403 A → G	D614G	S
25563 G → T	Q57H	ORF3a
29179 G → T	P302	N
29779 G → T		3'-UTR

Figure 1. Graphic sequence view of NCBI Genome Workbench for D614G variant in S gene. MinION sequencer is known as a next-generation sequencer with less accuracy, but it revealed sufficient accuracy for the mutation with an amplicon-based approach. Abbreviation: NCBI, The National Center for Biotechnology Information

Viral genome analysis plays an important role in epidemiological investigations, the occurrence of variants, and the accuracy of molecular diagnostic tests during outbreaks of novel viral diseases. Gene sequencing is necessary in case mutations occur at the site of attachment of primers or probes used in molecular diagnostic tests or at the site of action of vaccines and antibody therapeutics, and whole genome sequencing (WGS) analysis is useful [1,2,9]. In the case of SARS-CoV-2, it was difficult to characterize the virus in the early stages of the outbreak, but several key variants may have different transmission, prognosis, and vaccine effectiveness, so it is important to understand these variants [2].

MinION NGS is a long-read sequencing device, which is known to be less accurate than Illumina's short-read sequencing device, but it is known that if a sufficient number of reads are read after amplification, there is no problem with accuracy [7]. Since the subject of this study was the second patient identified in our hospital and there were not many samples, the same sample was sequenced repeatedly with different barcodes, and each BAM file was checked with the naked eye.

MinION NGS can process a relatively small number of samples, which is an advantage over other NGS instruments, but it requires PCR amplification with more than 200 primers and NGS reagents, so the analysis is not cheap. In addition, the bioinformatics software required for NGS testing requires familiarity with the Linux environment, Guppy, RAMBART, etc., hence users who are familiar with a graphical user interface environment such as Microsoft Windows may face difficulties initially. However, the ARTIC network describes the experimental and analytical process in relatively great detail, so it can be applied once the basics are learned. The process of reading variants is difficult to handle with the tablet recommended by ARTIC, so we used NCBI Genome Workbench to read variants without difficulty (**Figure 1**). MinION has the advantage of being able to analyze a small number of samples, but it can also handle a large number of samples if necessary. This study was conducted in July 2020 and used the nCoV-2019 sequencing protocol V2 (GunIt V2), but in August 2020, some reagents and procedures were changed to the nCoV-2019 sequencing protocol V3 (LoCost), which is complemented by a high-throughput testing system that can run 11–95 samples.

The G29179T variant in the N gene region has no amino acid change but occurs in the 2019-nCoV-N2-F region of the Centers for Disease Control and Prevention (CDC) PCR primers, which may affect the N gene detection performance of some molecular diagnostic reagents (https://primer-monitor.neb.com/; accessed 23 June 2021) [11]. This variant is mainly reported in Korea, but it has also been reported in the Philippines, Israel, Denmark, Canada, and the United Kingdom, albeit at a lower frequency.

In this study, a positive SARS-CoV-2 specimen was genomically analyzed using MinION and identified as the B.1.497 lineage, which is mainly isolated in Korea. We believe that the MinION NGS instrument is useful for SARS-CoV-2 genomic analysis in places where it is difficult to use NGS instruments or when analyzing small amounts of samples.

3. Conclusion

Genomic analysis of SARS-CoV-2 is necessary to understand its molecular dynamics and to understand community transmission of the virus. The gene sequencing instrument MinION (Oxford Nanopore Technologies, ONT, UK) allows testing in a variety of settings and can be performed in clinical microbiology laboratories with limited facilities and resources. In this study, the genomic analysis of SARS-CoV-2 detected in a patient in South Korea was analyzed using the MinION Genome Sequencer. A single nasopharyngeal swab specimen that tested positive for SARS-CoV-2 at a university hospital in Seoul in June 2020 was sequenced using the MinION instrument and the ARTIC nCoV-2019 sequencing protocol V2.

Variants were identified using software such as ONT MinKNOW, RAMPART, and Genome Workbench. The SARS-CoV-2 standard sequence, Wuhan-Hu-1 isolate (NC_045512.2), 11 genetic variants were observed, where six in the ORF1ab region (T265I, F924, Y3884L, P4715L, L5462, Q6804L), one in the S gene (D614G), one in the ORF3a region (Q57H), one in the N gene (P302), and one each in the 5'-UTR and 3'-UTR. The MinION device, which enables genomic analysis through gene amplification, may be a useful technique for genomic analysis in the absence of culture facilities during viral outbreaks.

Disclosure statement

The authors declare no conflict of interest.

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