

Preplanned Studies

Sequencing the Complete Genome of COVID-19 Virus from Clinical Samples Using the Sanger Method

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Summary

What is already known on this topic?

Coronavirus disease 2019 (COVID-19), a disease caused by a novel human coronavirus named the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or COVID-19 virus, was reported in December 2019. Complete genomes of the COVID-19 virus from clinical samples using next generation sequencing (NGS) have been reported.

What is added by this report?

Here we provide the technical data for sequencing complete genome of COVID-19 virus from clinical samples using the Sanger method. Two complete COVID-19 virus genome sequences (named WH19004-S and GX0002) were obtained from clinical samples of COVID-19 patients, and two single nucleotide polymorphisms (SNPs) in ORF7a (T/C, nt 27,493) and ORF8 (T/C, nt 28,253) of WH19004-S were identified by Sanger sequencing.

What are the implications for public health practice?

The COVID-19 virus genome sequencing by Sanger method reported here could be used to generate data of high enough quality without requirement for expensive NGS equipment, which support sequencing complete genomes from clinical samples and monitoring of viral genetic variations of COVID-19 infections.

In December 2019, a novel coronavirus from patients with pneumonia was identified and subsequently named the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1–3). SARS-CoV-2 has caused a coronavirus disease 2019 (COVID-19) pandemic with high morbidity and mortality. Analyzing the genome of SARS-CoV-2 (also referred to as COVID-19 virus) from clinical samples is crucial for the understanding of viral spread and viral evolution as well as for vaccine development (4–6). Presently, whole genome sequencing of the COVID-19 virus was often generated by next generation sequencing (NGS) (7). Although NGS methods have

many advantages in terms of speed and parallelism, the accuracy and read length of Sanger sequencing is still superior and has confined the use of NGS mainly to resequencing genomes (8).

Here we introduce a detailed method to rapidly obtain COVID-19 virus whole-genome sequence from clinical samples. This method is based on multiple nucleic acid amplified fragments for Sanger sequencing. We applied this method to obtain 2 complete genome sequences of COVID-19 virus from clinical samples of patients with COVID-19.

MATERIALS AND METHODS

Clinical Samples

In this study, broncho-alveolar lavage samples were collected from patients with COVID-19 in Hubei, China. COVID-19 virus RNA was identified as positive (Ct value: 28.78 and 31.86) by a real-time fluorescence-based reverse transcriptase polymerase chain reaction (rRT-PCR) assay as previously reported (7).

Nucleic Acid Extraction and Fragment Amplification

Viral RNA was extracted from 140 µL of sample using QIAamp Viral mini kits (Qiagen, Germany) according to the manufacturer's instructions. RNA was eluted in 80 µL of elution buffer. A total of 38 sets of specific primers covering the whole COVID-19 virus genome were designed (Table 1) according to the reference sequence (WH19004, Accession ID: EPI_ISL_402120) obtained by NGS as previously reported (7). Overlapping fragments were obtained by RT-PCR conducted as follow: 5 µL of extracted RNA were amplified with the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany) and RT-PCR programs were run as follows: 50 °C 30 min; 95 °C for 15 min; 95 °C for 30 s, 50/55 °C 30 s, 72 °C 1/2 min, 40 cycles; 72 °C 5 min. All PCR products were confirmed by gel electrophoresis analysis and sequenced using the Sanger method.

TABLE 1. Primers used for whole-genome sequencing of the COVID-19 virus.

Set	Name	Start [*]	End [*]	Primer sequence (5'→3')
1	1F	64	86	CTCTAAACGAACCTTTAAATCTG
	1R	1,048	1,068	CCATTGAAGGTGTCAAATTC
2	2F	706	729	CGAGCTTGGCACTGATCCTTA
	2R	1,398	1,419	GCAAGACTATGCTCAGGTCCTA
3	3F	950	970	TACTGCTGCCGTGAACATGAG
	3R	2,183	2,203	CCAACCGTCTCTAAGAACTC
4	4F	1,999	2,020	GAGACTCATTGATGCTATGATG
	4R	3,099	3,120	TCAGTACCATACTCATATTGAG
5	5F	2,352	2,374	GTGGAGCTAAACTTAAAGCCTTG
	5R	3,452	3,473	CTCCTCCATGTTTAAGGTAAAC
6	6F	2,846	2,865	ACAGTTGAACTCGGTACAGA
	6R	4,068	4,088	CAATGTCACTAACAAGAGTGG
7	7F	3,884	3,904	CCTAAAGAGGAAGTTAAGCCA
	7R	5,153	5,172	TGGTAGTACTCAAAAGCCTC
8	8F	4,787	4,807	GCTGGTTCCTATAAAGATTGG
	8R	6,146	6,165	ACATCACCATTTAAGTCAGG
9	9F	5,976	5,997	ATTCTTATTTACAGAGCAACC
	9R	7,178	7,200	GAAATGGTAATTTGTATAGTTTC
10	10F	6,977	6,999	GTTTGCCTAGGTTCTTTAATCTA
	10R	8,183	8,204	CTACATCTGAATCAACAAACCC
11	11F	7,985	8,006	CAGGCATTAGTGCTGATGTTG
	11R	9,167	9,188	CTCTAACAGAACCTTCAAGGTA
12	12F	8,966	8,986	AAACTTATAGAGTACACTGAC
	12R	10,166	10,185	CAGATCACATGTCTTGGACA
13	13F	9,900	9,921	ATAAGTACAAGTATTTTAGTGG
	13R	11,114	11,133	GCAGACATAGCAATAATACC
14	14F	10,901	10,922	GGTAGTGCTTTATTAGAAGATG
	14R	12,175	12,196	AAGAACAACCTTCAGAATCACCA
15	15F	12,024	12,043	CCATGCAGGGTGCTGTAGAC
	15R	13,205	13,225	GGATTCTTGATCCATATTGGC
16	16F	12,970	12,991	CAACCTAAATAGAGGTATGGTA
	16R	14,290	14,312	TCCCAATATTTAAATAACGGTC
17	17F	13,775	13,795	CACATATATCACGTCAACGTC
	17R	14,999	15,019	GTGCATCTTGATCCTCATAAC
18	18F	14,756	14,777	ACTTCTCTTTGCTCAGGATGG
	18R	15,989	16,011	TTCAATCATAAGTGTACCATCTG
19	19F	15,929	15,850	GCAAAATGTTGGACTGAGACTG
	19R	17,014	17,036	GCAACATTGCTAGAAAACATC
20	20F	16,832	16,853	CCTTTGAAAAAGGTGACTATGG
	20R	17,956	17,977	GGTCTCTATCAGACATTATGCA
21	21F	17,530	17,549	ATAGGTCCAGACATGTTCT
	21R	18,781	18,801	TTGTAGGTTACCTGTAAAACC

TABLE 1. (Continued)

Set	Name	Start [*]	End [*]	Primer sequence (5'→3')
22	22F	18,487	18,506	ATACCACTTATGTACAAAGG
	22R	19,618	19,639	AAGCCACATTTTCTAAACTCTG
23	23F	19,438	19,459	CCACTAAAGTCTGCTACGTGTA
	23R	20,568	20,589	GTCAATAGTCACTTTGACAACC
24	24F	20,363	20,384	TACATCTACTGATTGGACTAGC
	24R	21,658	21,678	GGGTAATAAACACCACGTGTG
25	25F	19,828	19,850	AAAATACTCAATAATTTGGGTGT
	25R	21,019	21,041	ATAATGAGATCCCATTTATTAGC
26	26F	20,428	20,447	CCTATGGACAGTACAGTTAA
	26R	21,665	21,684	TTGTCAGGGTAATAAACACC
27	27F	21,332	21,354	ATGCAAATTACATATTTGGAGG
	27R	22,539	22,560	GTAATATTAGGAAATCTAACAA
28	28F	22,433	22,449	TGTGCACTTGACCCTCT
	28R	23,345	23,364	CCTGGTGTATAACACTGAC
29	29F	23,123	23,142	CCAGCAACTGTTTGTGGACC
	29R	24,095	24,116	CACAAATGAGGTCTCTAGCAGC
30	30F	23,339	23,360	GGTGGTGTCAAGTTATAACAC
	30R	24,328	24,349	ACTATTAATTTGGTTGGCAATC
31	31F	23,948	23,971	GATTTTGGTGGTTTAAATTTTCA
	31R	25,157	25,176	TTTCCAAGTTCTTGGAGATC
32	32F	24,960	24,981	TCAACAACACAGTTTATGATCC
	32R	26,171	26,192	GGTTCATCATAAATTGGTTCCA
33	33F	25,837	25,857	TGGCATACTAATTGTTAYGAC
	33R	27,033	27,052	GAAAGCGTTCGTGATGTAGC
34	34F	26,815	26,834	CTTCTTTCAGACTGTTTGCG
	34R	27,948	27,968	ACATGACTGTAAACTACATTC
35	35F	27,389	27,406	CGAACATGAAAATTATTC
	35R	28,550	28,568	CGTCACCACCACGAATTCG
36	36F	28,322	28,341	TTTGGTGGACCCTCAGATTC
	36R	29,543	29,561	CCATCTGCCTTGTGTGGTC
37	37F	29,149	29,170	CAGACAAGGAACTGATTACAAA
	37R	29,836	29,857	GAAGCTATTAAAATCACATGGG
38	38F	26,539	26,559	GTACTATTACCGTTGAAGAGC
	38R	27,102	27,122	CCTGTAGCGACTGTATGCAGC
5'-RACE	39R	1,048	1,068	CCATTGAAGGTGTCAAATTTTC
	40R	493	512	GACCATGAGGTGCAGTTCGA
3'-RACE	41F	29,149	29,170	CAGACAAGGAACTGATTACAAA
	42F	29,438	29,459	CAGCAAAGTGTGACTCTTCTTC

^{*} The location on the reference genome, accession ID: EPI_ISL_402120.

5' and 3' Ends of Genome Sequencing

The 5' and 3' ends of the genome were determined by rapid amplification of cDNA ends (RACE) using

the Invitrogen 5' RACE System and 3' RACE System (Invitrogen, USA) according to the manufacturer's instructions. Gene-specific primers for 5' and 3' RACE

PCR amplification were designed to obtain a fragment of approximately 400–500 bp for the two regions. Purified PCR products were cloned into the pMD18-T Simple Vector (TaKaRa, Takara Biotechnology, Dalian, China) and chemically competent *Escherichia coli* (DH5 α cells, TaKaRa), according to the manufacturer's instructions. PCR products were sequenced with use of M13 forward and reverse primers.

Genome Sequence Assembly

All sequencing fragments were assembled using DNASTar software. The open reading frames of the verified genome sequences were predicted using Geneious (version 11.1.5) and annotated using the Conserved Domain Database. Sequence alignment of the COVID-19 virus with reference sequences was done with Mafft software (version 7.450). The SNPs of each sequence were defined as the site's variant from the reference sequence.

RESULTS

Primer Design

The primers were designed in entire genome regions to obtain overlapping amplicons of approximately

1,000–1,200 bp leading to a list of 38 primer pairs. Meanwhile, 5' and 3' terminal sequencing primers were designed to obtain amplicons of 400–500 bp for sequencing (Table 1).

Genomic Characterization

Using DNASTar software all sequencing fragments were assembled, 2 complete sequences named WH19004-S and GX0002 were obtained from the clinical samples (Figure 1). Of which, WH19004-S (29896 nt) is consistent with WH19004-NGS (accession ID: EPI_ISL_402120), except that there are 2 variants in nucleotide (nt) 27,493 (T/C) and 28,253 (T/C) respectively and identified these positions as single nucleotide polymorphisms by alignment with a large number of COVID-19 genome sequences (Figure 2). Nt 27,493 located in ORF7a (amino acid position 34), T or C translated to different amino acid (Ser or Pro), while nt 28,253 in ORF8 (amino acid position 120), no changes in amino acid.

The GX0002 strain (accession ID: EPI_ISL_434534) was 29,892 nt in length, including a 5' untranslated region (UTR) (nt 1 to 265), replicase complex open reading frame 1ab [ORF1ab] (nt 266 to 21,555), S gene (nt 21,563 to 25,384), ORF3a (nt 25,393 to 26,220), E gene (nt 26,245 to 26,472), M gene (nt 26,523 to 27,191), ORF6 (nt 27,202 to 27,387), ORF7a (nt 27,394 to 27,759), ORF7b (nt

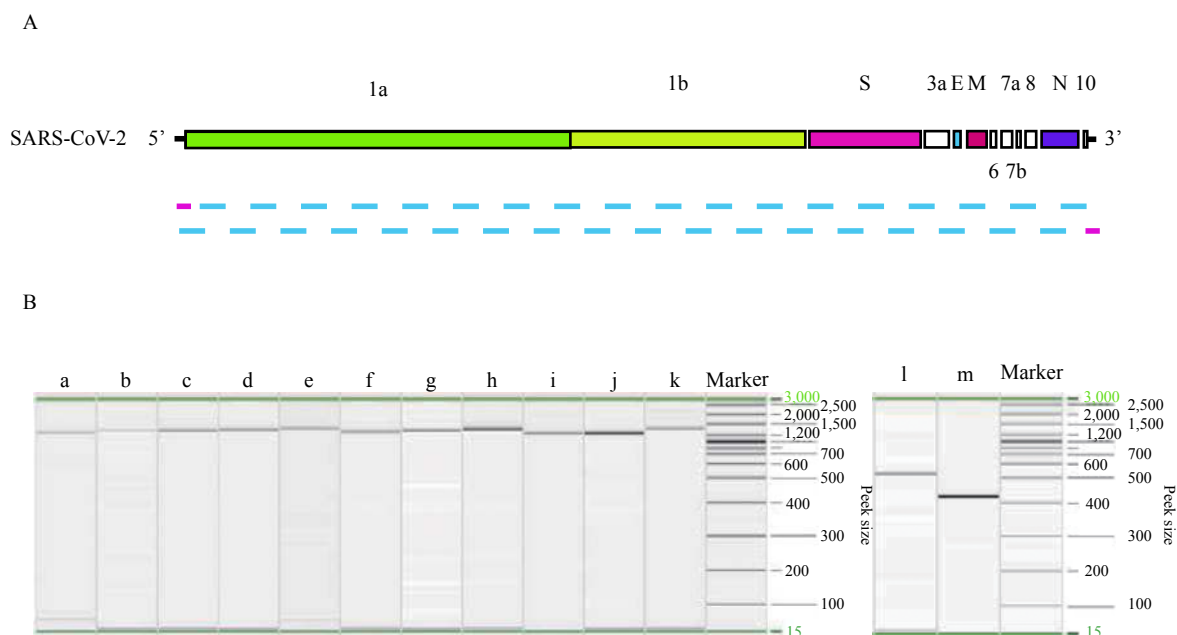


FIGURE 1. COVID-19 virus genome fragment amplification by RT-PCR. A: Schematic representation of the amplificatory fragments of the genome. B: Capillary electrophoresis profiles of RT-PCR products of the obtained partial fragment. a to k: RT-PCR products of the fragment 1–11; l to m: RT-PCR products of the end of 5' and 3' of the genome.

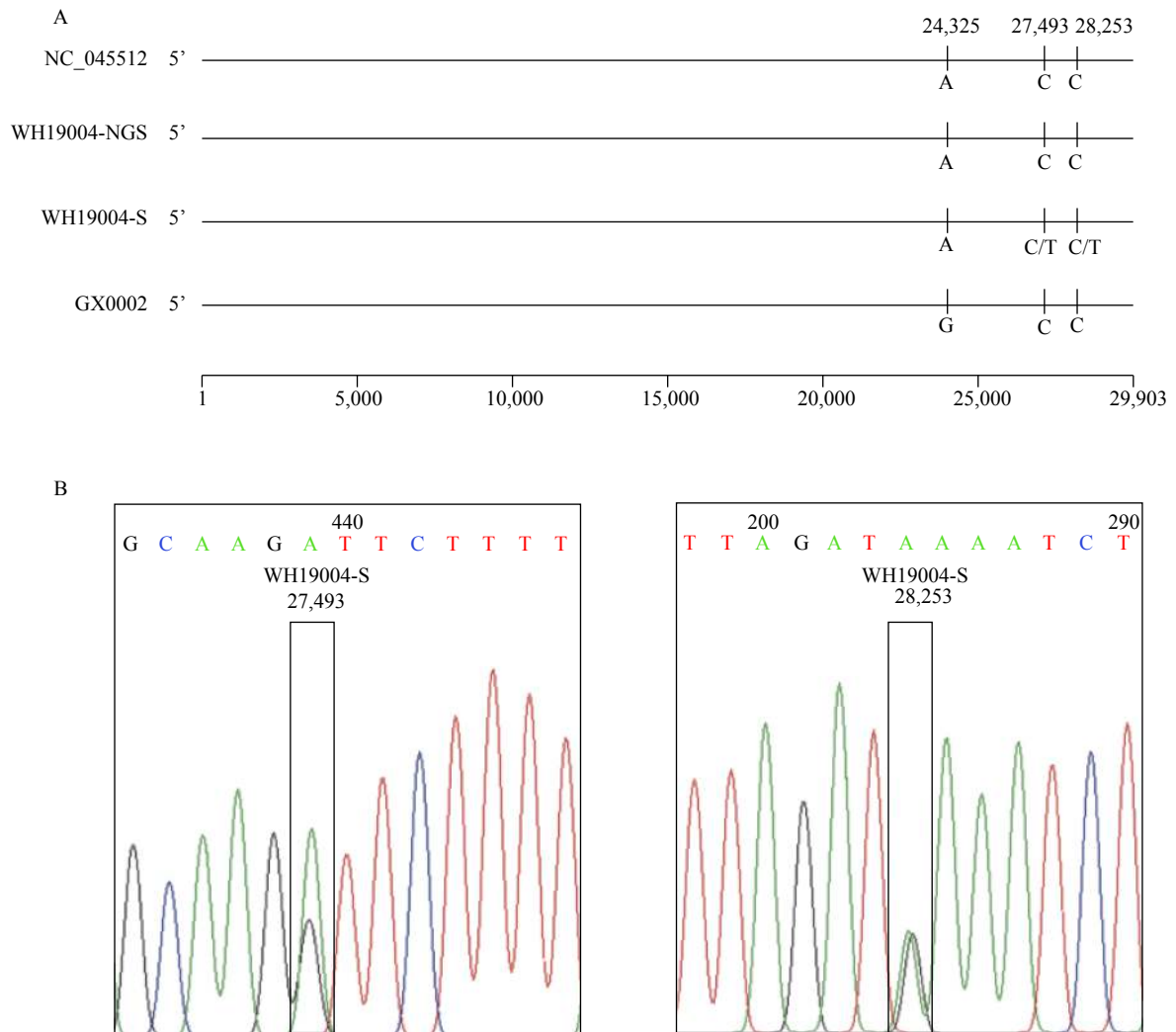


FIGURE 2. Sequence comparison and Sanger sequencing diagram of COVID-19 virus in this study. (A) Sequence alignment of 4 whole-length genomes of COVID-19 virus. (B) Sanger sequencing diagram of WH19004-S by reverse primers 29R and 30R, which shows the SNPs in ORF7 and ORF8 of WH19004-S.

27,756 to 27,887), ORF8 gene (nt 27,894 to 28,259), N gene (nt 28,274 to 29,533), ORF10 gene (nt 29,558 to 29,674), and 3' UTR (nt 29,675 to 29,892). Compared with the reference strain (GenBank no. NC_045512), the GX0002 strain only has a nucleotide variant in nt 24,325 position (G/A) in the S gene and no changes in amino acid (Figure 2).

DISCUSSION

To accelerate our investigation of this virus and the disease it causes, a practical protocol for viral genome research of clinical samples is urgently needed. In this study, we obtained 2 COVID-19 virus complete genome sequences WH19004-S and GX0002 from clinical samples using the Sanger sequencing method.

While NGS is the current mainstream sequencing method with the characteristics of high-throughput, rapidity, etc., it also has some drawbacks such as its relatively short reads. As a result, NGS lacks the capacity to link independent variations on the same nucleic molecule, so it is not well suited to discriminate and phase alleles to their respective parental homolog (9). In addition, the abundance of COVID-19 virus in clinical samples is often low, so the application of conventional NGS requires deeper sequencing of each sample in order to obtain sufficient coverage and depth of the whole viral genome, which increases the time and cost of sequencing. Nevertheless, as one of the earliest sequencing methods, the Sanger method has the characteristics of high accuracy, long reads, no requirement for expensive equipment, etc. Sanger sequencing has been used for analyzing genes where

NGS fails to achieve sufficient depth of coverage or to generate data of high enough quality. Sanger sequencing is also used for confirming NGS variants before they are clinically reported (10). Especially when the general laboratory have common PCR machine and lack of expensive NGS platform, Sanger method is more prefer to be applied. In this study, we identified two SNPs in ORF7a (T/C, nt 27,493) and ORF8 (T/C, nt 28,253) of WH19004-S using Sanger sequencing compared with WH19004-NGS derived from NGS. The SNP in ORF7a of WH19004-S translated to two different amino acid (Ser or Pro). The roles of the SNPs in COVID-19 virus genetic evolution and whether it causes functional changes still need further investigation.

In summary, we reported here a rapid, versatile, and clinic-friendly approach for sequencing the complete genome of COVID-19 virus from clinical samples using the Sanger method, which will facilitate monitoring of viral genetic variations during outbreaks, both current and future.

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