

CHINA CDC WEEKLY



Vol. 2 No. 25 Jun. 19, 2020

中国疾病预防控制中心周报



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ISSN 2096-7071



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Cover Image: A painting from a primary school art teacher in Gansu Province. For professional guidelines on disinfection procedures including the use of gloves and masks, please consult our previously published guidelines.

Preplanned Studies

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

Roujian Lu¹; Peihua Niu¹; Li Zhao¹; Huijuan Wang¹; Wenling Wang¹; Wenjie Tan^{1, #}

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 ^a	End ^a	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	CAGGCATTAGTGTCTGATGTTG
	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	ATGCAAATTACATATTTTGGAGG
	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	CAGACAAGGAAGTATTACAAA
	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	CAGACAAGGAAGTATTACAAA
	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

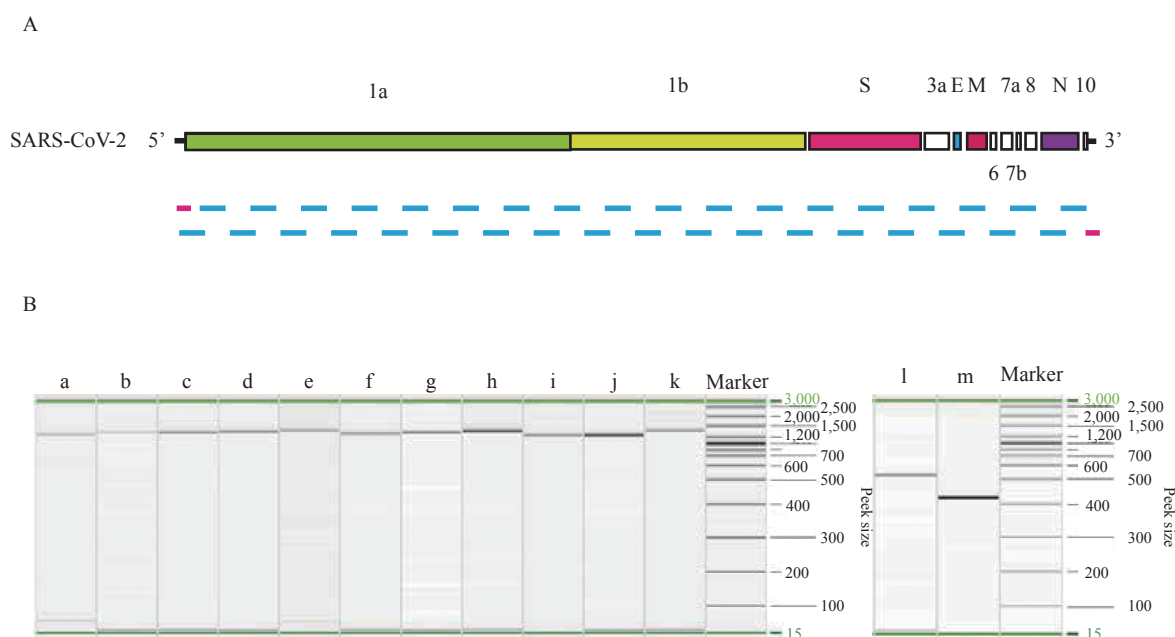


FIGURE 1. COVID-19 virus genome fragment amplification by RT-PCR. A: Schematic representation of the amplificatory fragments of the genome. B: Capillary electrophoresis profiles of RT-PCR products of the obtained partial fragment. a to k: RT-PCR products of the fragment 1–11; l to m: RT-PCR products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

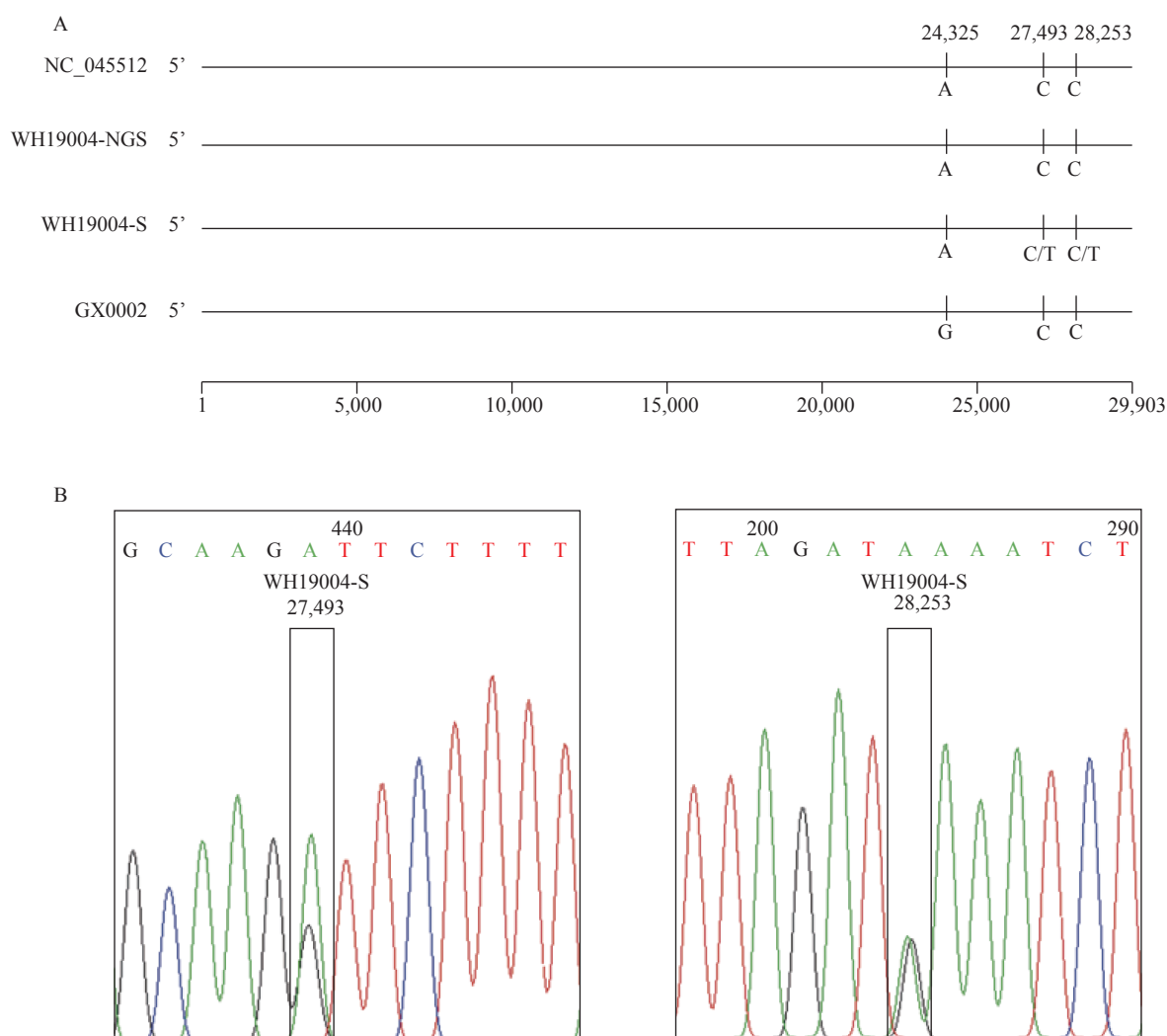


FIGURE 2. Sequence comparison and Sanger sequencing diagram of COVID-19 virus in this study. (A) Sequence alignment of 4 whole-length genomes of COVID-19 virus. (B) Sanger sequencing diagram of WH19004-S by reverse primers 29R and 30R, which shows the SNPs in ORF7 and ORF8 of WH19004-S.

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.

Conflict of interest: No conflicts of interest were reported.

Funding: This work was supported by the National Key Research and Development Program of China (2016YFD0500301).

doi: 10.46234/ccdcw2020.088

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Submitted: April 30, 2020; Accepted: May 06, 2020

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Preplanned Studies

Three Novel Real-Time RT-PCR Assays for Detection of COVID-19 Virus

Peihua Niu^{1,✉}; Roujian Lu^{1,✉}; Li Zhao¹; Huijuan Wang¹; Baoying Huang¹; Fei Ye¹; Wenling Wang¹; Wenjie Tan^{1,✉}

Summary

What is already known on this topic?

A novel human coronavirus, known as SARS-CoV-2 or 2019-nCoV, is the causative agent of the coronavirus disease 2019 (COVID-19). We have released the primers and probes of real-time reverse transcription polymerase chain reaction (rRT-PCR) assays for the laboratory detection of COVID-19 infection.

What is added by this report?

Here we provide detailed technical data and evaluate the performance of three novel rRT-PCR assays targeting the ORF1ab, N, and E genes for detection of COVID-19 infection. The application of rRT-PCR assays among four types of specimens (alveolar lavage, sputum, throat swabs, and stool) from patients with COVID-19 indicated that the mean viral loads detected in sputum were higher than other specimens.

What are the implications for public health practice?

These rRT-PCR assays reported here could be used for laboratory diagnosis of COVID-19 infection with high sensitivity, specificity, and applicability. Sputum rather than throat swabs and stool should be a priority for specimen collection for laboratory detection of COVID-19.

Coronaviruses are single-stranded positive-stranded RNA that have the largest virus genome among RNA viruses (1–2). Coronaviruses are widespread in bats around the world but can be found in many other species as well that are phenotypically and genotypically diverse (1,3). The coronavirus disease 2019 (COVID-19) raised considerable concerns as it was associated with severe acute pneumonia and fatal outcomes (4–5) and thus resembled the clinical presentation of severe acute respiratory syndrome (SARS) observed in 2002 and 2003 as well as Middle East respiratory syndrome (MERS) since 2012 (6). The causative agent of COVID-19 was a novel coronavirus known as SARS-CoV-2 and was previously named 2019-nCoV in China (2,5).

Novel quantitative real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays were developed in rapid response to the emergence of COVID-19 originating in Wuhan, and these assays have been widely used in laboratory detection and written into the national technical guidelines used in China (7). We report here in detail on the technical data and comparative analysis of performance of three rRT-PCR assays targeting three distinct regions of the SARS-CoV-2 genome for detection of COVID-19 infection. Three rRT-PCR assays were further evaluation with several species of clinical specimens from patients with COVID-19.

MATERIALS AND METHODS

Clinical Specimens

A total of 135 clinical specimens were collected from a cluster of patients with pneumonia in Wuhan and Beijing suspected of being infected with SARS-CoV-2. Specimens included alveolar lavage, sputum, throat swabs, and stool. Inactivation of specimen processing was performed in a biosafety level 3 (BSL3) laboratory.

Nucleic Acid Extraction

Nucleic acids were extracted from a 140 µL processed specimen using a QIAamp Viral RNA Mini Kit according to the manufacturer's instructions. Approximately 60 µL of total nucleic acid eluate was recovered into nuclease-free tubes and either tested immediately or stored at -70 °C.

Design of Primers and Probes

Multiple primer and probe sets were designed based on bioinformatics analysis of three complete genomes of SARS-CoV-2 (BetaCoV/Wuhan/IVDC-HB-01/2019, Accession ID: EPI_ISL_402119, BetaCoV/Wuhan/IVDC-HB-04/2020, Accession ID: EPI_ISL_402120) obtained in our lab (5,8). ORF1ab, E gene and N gene sequences were selected as targets

using Primer Premier software version 5 (Applied Biosystems) with the following default settings: primer melting temperature (T_m) set at 60 °C; probe T_m set at 10 °C greater than the primers at approximately 70 °C; and no guanidine residues permitted at the 5' probe termini (Figure 1A, Table 1). Previous reported primer and probe sets for the RNA-dependent RNA polymerase (RdRp) region of pan beta-CoV was designed as a reference experiment (3,9).

Real-Time RT-PCR Assay for Screening of SARS-CoV-2 Infection

Several one step rRT-PCR assays were developed using the OneStep PrimeScript™ RT-PCR kit

(TaKaRa, Japan). Each 25 µL reaction mixture contained 12.5 µL of 2× Master Mix, 0.5 µL of reverse transcriptase/Taq DNA polymerase mixture, 5 µL of RNA extract, 400 nmol/L concentrations of forward primer and reverse primer, and 200 nmol/L of probe. Thermal cycling included 42 °C for 5 minutes, followed by 95 °C for 10 seconds and then 40 cycles of 95 °C for 10 s and 60 °C for 45 s. Each run included one SARS-CoV-2 genomic template control and at least two negative or mock controls (for the extraction and the PCR amplification step) (Table 1).

Assay specificity was determined using high-titer virus stock as well as clinical samples containing known respiratory viruses from collection of our laboratory. Identities and virus RNA concentrations were

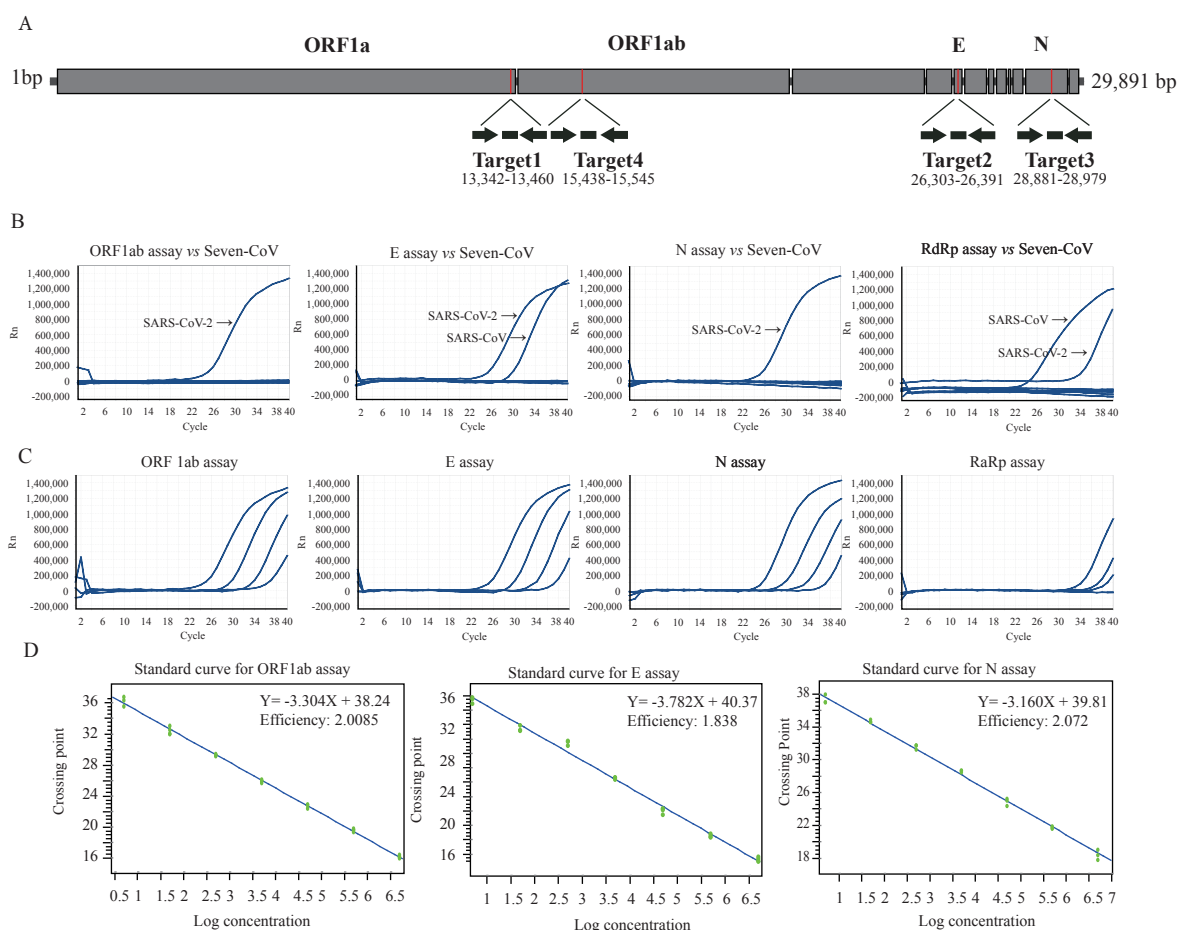


FIGURE 1. Development of three rRT-PCR assays for detection of SARS-CoV-2. (A) Description of specific primers and probes for detection of SARS-CoV-2. Relative positions of amplicon targets on the SARS-CoV-2 genome schematic. ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene; E: envelope protein gene; N: nucleocapsid protein gene. Numbers below amplicons are genome positions according to SARS-CoV-2, accession ID: EPI_ISL_402119, EPI_ISL_402120. (B) Confirmation of rRT-PCR on seven human coronavirus samples using specific probes and primers. (C) Representative amplification plot of developed rRT-PCR assay showing serial dilutions of SARS-CoV-2 RNAs from clinical samples for evaluation of sensitivity of four assays. (D) Determination of detection efficiency and limit of various rRT-PCR assays. Standard calibration curves of Ct and genomic copy number was generated based on rRT-PCR results targeting primer set and SARS-CoV-2 stock with pre-determined genomic copies.

TABLE 1. Primer and probe sets of rRT-PCR assays for the detection of the 2019 novel coronavirus diseases (COVID-19).

Target	Gene	Primer	Sequence (5'→3')	Genome location*	Limit of detection (copies/mL)
Target 1	ORF1ab	Forward	CCCTGTGGGTTTACACTTAA	13342-13460	203
		Reverse	ACGATTGTGCATCAGCTGA		
		Probe	FAM-CCGTCTGCGGTATGTGGAAAGTTATGG-BHQ1		
Target 2	E	Forward	TTCTTGCTTTCGTGGTATTC	26303-26391	664
		Reverse	CACGTTAACAATATTGCAGC		
		Probe	FAM-GTTACACTAGCCATCCTTACTGCGCTTCGA-BHQ1		
Target 3	N	Forward	GGGGAACCTCTCCTGCTAGAAT	28881-28979	667
		Reverse	CAGACATTTTGTCTCAAGCTG		
		Probe	FAM-TTGCTGCTGCTTGACAGATT-BHQ1		
Target 4	RdRp	Forward	GGTCATGTGTGGCGGCTC	15438-15545	
		Reverse	GCTGTAAACAGCTTGACAAATGAAAG		
		Probe	FAM-CTATATGTTAAACCAGGTGGAAC-BHQ1		

*The location on the reference genome, accession ID: EPI_ISL_402119, EPI_ISL_402120.

reconfirmed by specific rRT-PCRs for each virus before the experiment (9–10).

The limit of detection was independently assessed using a SARS-CoV-2 stock with pre-determined genomic copies. The calibration curve for the genomic copy number versus Ct value was obtained from the rRT-PCR machine, which was an ABI 7500 (Applied Biosystems, USA) or a Roche LightCycler 480 (Roche, Switzerland) Real-Time PCR system. Series of four parallel reactions per concentration step were prepared and tested by the respective rRT-PCR.

Clinical samples were considered positive if two or more of the gene targets showed positive results ($C_t \leq 37$ cycles; If the C_t value is between 37–40, the experiment needs to be repeated. The judgment of the final result is based on two consistent experimental results) (7).

RESULTS

Performance of Different rRT-PCR Methods

In the detection of clinical samples using the same gradient dilution, ten-fold serial dilutions of the SARS-CoV-2 RNA were tested to assess the detection limits and dynamic range of our optimized rRT-PCR assays. The detection results of the four rRT-PCR assays were different for clinical samples with gradient dilution. The lower potential limit of detection was approximately 10^{-4} dilution per reaction for ORF1ab assay, E assay, and N assay, and 10^{-3} dilution per

reaction for RdRp assay (Figure 1C). The genomic copy numbers-based C_t values were calibrated on the standard curve for individual rRT-PCR (Figure 1D). The lowest detection limit of ORF1ab assay was 203 copies/mL. The minimum detection limits of E and N assay were 664 and 667 copies/mL, respectively (Table 1).

The rRT-PCR assays were tested with nucleic acid extracts of 6 human coronaviruses, human NL63 coronaviruses (NL63-CoV), human 229E coronaviruses (229E-CoV), human OC43 coronaviruses (OC43-CoV), human HKU1 coronaviruses (HKU1-CoV), SARS-CoV, and MERS-CoV. In addition, nucleic acid extracts of influenza A were also tested. No positive reactions were observed with any of the primer and probe sets of ORF1ab and N genes, while cross-reactions with SARS-CoV were shown using the primer and probe sets of the E gene as well as RdRp (Figure 1B).

Assay reproducibility was tested by using replicate ten-fold serial dilutions of the SARS-CoV-2 RNA and intra- and inter-assay variability evaluated for each dilution point in quadruplicate three different times. The reproducibility for 3 assays (targeting ORF1ab, E, and N) exceeded 90% at the lower copy detection limit (data not shown).

Evaluation with Different Species of Clinical Specimens

The rRT-PCR assay was evaluated using different species of clinical specimens collected from a cluster of patients in Wuhan and Beijing, whom were suspected

of being infected with SARS-CoV-2. Three rRT-PCR targeting ORF1ab, N and E showed consistent detection rates among various species of specimens (Table 2). The mean Ct value detected in sputum was lower than in other species of clinical specimens (alveolar lavage, throat swab, and stool), which indicated the mean viral loads detected in sputum were higher than other specimens. Similar results were also reported recently using a large amount of clinical specimens.

DISCUSSION

To date, several coronaviruses (including SARS-CoV, MERS-CoV, and SARS-CoV-2) that could infect humans have all been beta-coronaviruses (1,8). The risk associated with false-positive PCR results posed a challenge in clinical application. First, the primer dimers and non-specific amplification, in which probe sequences participate, might interfere with experiments. Second, SARS-CoV-2 assays might cross-react with other viruses. In this study, we used 6 human coronaviruses (NL63-CoV, 229E-CoV, OC43-CoV, HKU1-CoV, SARS-CoV and MERS-CoV) and influenza A to test the cross-reactivity. Consequentially, the ORF1ab and N gene-based assay were the most specific, exactly matching target genes of SARS-CoV-2. The E gene was cross-reacted with B lineages of the beta-coronavirus (such as SARS-CoV). We propose that the E gene could be used as a broad-spectrum screening gene for B lineages of the beta-coronavirus, such as SARS-CoV, SARS-CoV-2, bat SARS-like coronavirus, et al. Therefore, we currently prefer to recommend the ORF1ab and N gene-based rRT-PCR assays for detection of COVID-19 infection.

Here, we also verified the sensitivities and detection limits of various rRT-PCR assays. The data showed that similar detection rates and sensitivity were observed among three rRT-PCR assays for ORF1ab, E gene, and N gene targets, which were higher than the RdRp target. We were also noticed that the ORF1ab-based rRT-PCR assay showed the best sensitivity (lowest detection limit).

To determine which type of specimen is suitable for rRT-PCR in clinic, four species of clinical specimens (alveolar lavage, sputum, throat swab, and stool) were collected for evaluation. Our data indicated that the mean viral loads detected in sputum were higher than other specimens, which suggests that sputum should be a priority for collection for laboratory detection of COVID-19 (11–12).

Although an rRT-PCR offers clear advantages over more conventional RT-PCR formats, assay results must still be interpreted with caution. For example, the effectiveness of rRT-PCR for detection of SARS-CoV-2 in clinical specimens had been shown to be greatly influenced by the quantity, type, and timing of specimen collection (11–13). In addition, false-negative results due to poor quality nucleic acids or presence of rRT-PCR inhibitors could also be a concern. False-negative results could also potentially arise from mutations occurring in the primer and probe target regions of SARS-CoV-2 genome. We included multiple genetic targets in our assay based on analysis of SARS-CoV-2 genomes available. Finally, to avoid false-positive results, steps should be taken meticulously to prevent introduction of contaminating viral RNA or previously amplified DNA during preparation of nucleic acid extracts and amplification reactions.

TABLE 2. Detection results of clinical specimen by the rRT-PCR based on molecular targets of ORF1ab, N and E gene.

Target gene		Alveolar lavage n(%)	Sputum n(%)	Throat swab n(%)	Stool n(%)
Total		n=15	n=28	n=53	n=39
ORF1ab	Positive rate (%)	14 (93.33%)	12 (42.86%)	25 (47.17%)	16 (41.03%)
	Mean Ct value	31.11	28.44	32.53	29.93
	Range	26.41–36.25	18.44–36.38	24.22–38.58	22.27–37.51
N	Positive rate (%)	14 (93.33%)	12 (42.86%)	25 (47.17%)	16 (41.03%)
	Mean Ct value	31.45	28.91	33.61	31.14
	Range	26.58–36.98	18.04–37.76	25.73–38.31	23.46–38.11
E	Positive rate (%)	14 (93.33%)	12 (42.86%)	25 (47.17%)	16 (41.03%)
	Mean Ct value	31.33	28.64	33.27	30.46
	Range	26.45–36.55	18.34–36.42	25.03–38.54	22.46–38.30

Conflicts of interest: The authors declare that they have no conflict of interest.

Fundings: This work was supported by the National Key Research and Development Program of China (2016YFD0500301, 2020YFC0840900).

doi: 10.46234/ccdcw2020.116

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Submitted: April 14, 2020; Accepted: April 16, 2020

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Notes from the Field

The Initial Case of COVID-19 — Shulan City, Jilin Province, China, May 8, 2020

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Coronavirus disease 2019 (COVID-19) was designated a global pandemic by the World Health Organization (WHO) on March 11, 2020 (1). After great effort, COVID-19 has been well-controlled in China, but new challenges have emerged due to increasing numbers of imported cases from outside of China. On May 8, the Health Commission of Jilin Province reported a confirmed COVID-19 case of a 45-year-old laundry woman from Shulan City in the northeast of China. This case was suspected to be associated with a possible importation event.

On May 10, genome sequencing was performed at China CDC using the Illumina MiSeq platform. Due to the low viral load in the samples, 98.1% of the virus genome sequence was obtained after sequencing and assembly, which included the key sites of the virus, and the analysis of the potential source of the virus was not affected. Compared with the reference sequence EPI_ISL_402119 (2), which was isolated from Wuhan on January 7, 2020, the virus had mutations at C241T, C3037T, C14408T, and A23403G, and the GGG mutated to AAC at position 28881–28883 of nucleotide of COVID-19 virus, which shows gene mutation characteristics of the L-lineage from Europe and does not have characteristic mutations of the North America branch of the European L-lineage (mutants at C1059T and G25563T) according to the study of Tang et al (Figure 1) (3). Additionally, the genome sequence of such a case also had a mutation at T19839C.

The result of viral genome sequencing provides strong evidence that the first COVID-19 case in Shulan City is related to COVID-19 virus imported from Europe. Although isolation of the imported cases and close contacts are the top priority strategies to interrupt the spread of the virus in China, asymptomatic infected persons may be a potential source of infection for this case or an unknown source of sporadic cases. We suggest that COVID-19 virus

nucleic acid testing for respiratory and stool specimens of the imported cases, close contacts, and inbound passengers whom come from high risk countries, territories, and areas should continue to be strengthened. Only when the nucleic acid tests of respiratory and stool specimens are negative at the same time, the isolation can be ended. Epidemiological investigation, case treatment of asymptomatic infections, and close contact tracing management should be continuously accelerated.

Fundings: This work was supported by National Key Research and Development Program of China (Program No. 2018YFC1200305), National Science and Technology Major Project of China (Project No. 2018ZX10102001, 2018ZX10711001, 2018ZX10713002).

doi: 10.46234/ccdcw2020.115

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Submitted: June 02, 2020; Accepted: June 03, 2020

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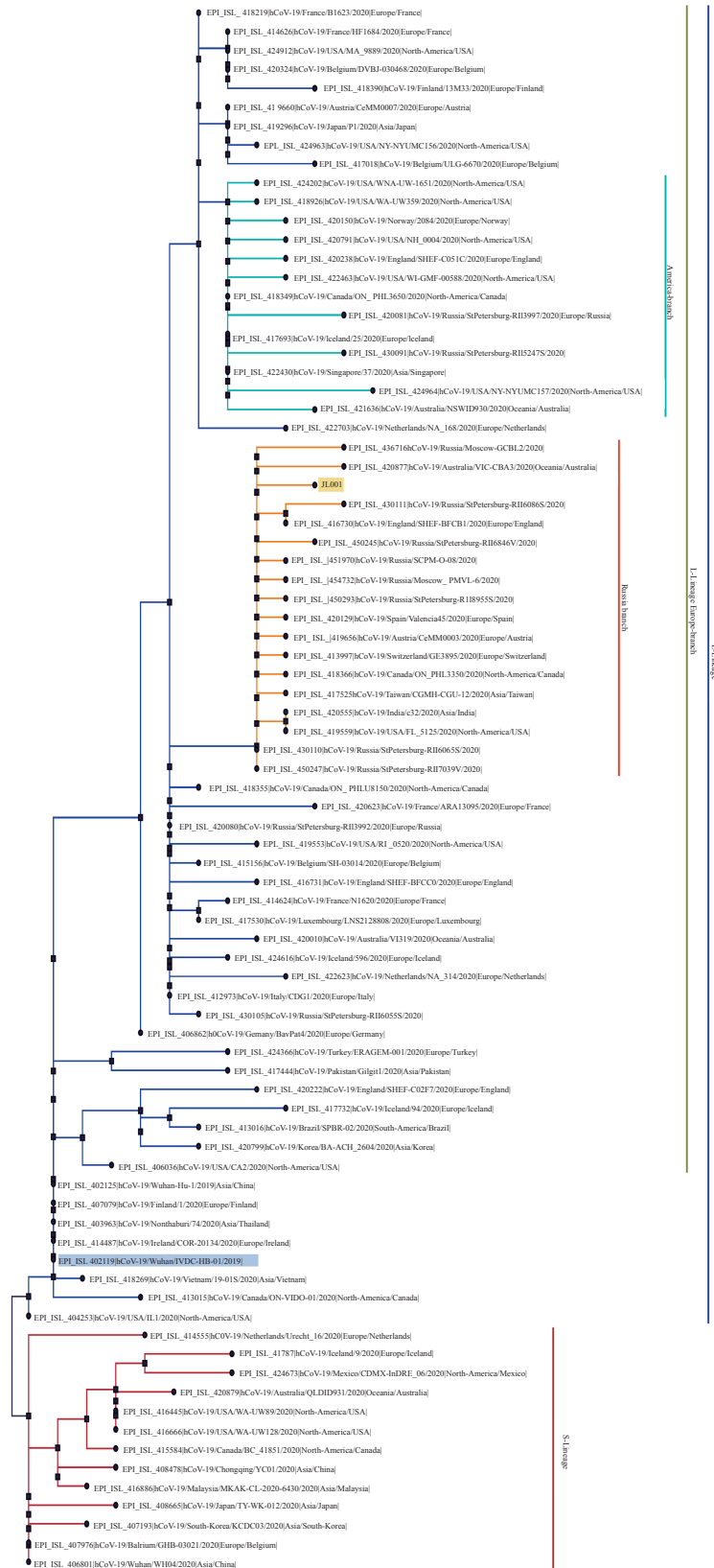


FIGURE 1. Phylogenetic tree based on the genome sequences of representative COVID-19 viruses. The genome of COVID-19 virus of the initial case in Shulan City and those from Wuhan City were highlighted with the shade of yellow and blue, respectively. S- or L-lineage of COVID-19 virus were marked and colored on the right.

Notes from the Field

A Reemergent Case of COVID-19 — Harbin City, Heilongjiang Province, China, April 9, 2020

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As of February 22, a total of 198 confirmed COVID-19 cases were reported in Harbin, Heilongjiang Province, and no new local cases were reported in Harbin over the next 54 days (1). However, on April 9, a newly confirmed COVID-19 case was reported with an unknown source of infection (2). The patient, a 54-year-old male, developed a fever on March 28 with the highest recorded temperature at 37.5 °C. On April 7, the symptoms worsened and included dyspnea, cough, sputum, chest pain, and other symptoms, though he did not experience chest distress, heart palpitations, nausea and vomiting, abdominal pain or diarrhea. He went to the fever clinic of the Harbin Second Hospital and was admitted to the isolation ward with a temperature of 37.2°C. The patient also had a history of diabetes.

The results of a routine blood examination were normal (WBC $5.15 \times 10^9/L$; lymphocyte $1.66 \times 10^9/L$, lymphocyte percentage 32.3%; neutrophil percentage 51.9%, c-reactive protein 36.56 mg/L). Chest computed tomography (CT) showed multiple lymph node shadows in the mediastinum and multiple lamellar ground glass density shadows and mesh shadows in both lungs. On April 7, the throat swabs of the patients were tested by Harbin CDC by using real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) targeting the ORF 1ab and N genes of COVID-19 virus, and the results showed that the nucleic acid of COVID-19 virus of the throat swab was positive. On April 8, Harbin CDC tested his pharyngeal swabs, blood, urine and stool samples again. COVID-19 nucleic acids were detected, but the test for serum antibodies for COVID-19 was negative.

The National Institute for Viral Disease Control and Prevention (IVDC) of China CDC conducted whole-genome sequencing analysis on a pharyngeal swab sample of the patient, which showed 99.97% nucleic acid similarity to the reference strain EPI_ISL_402119, which was isolated from Wuhan on January 7, 2020.

There was a total of eight nucleotide mutations between them. This strain possessed the nucleotide mutations C241T, C1059T, C3037T, C14408T, A23403G, and G25563T, which were consistent with the characteristics of the North American branch of the L lineage from Europe (Figure 1) (3). In addition, sequence alignment also revealed two mutations C26636T and C27213T in the virus genome from this patient. Among them, C1059T, C14408T, A23403G, and G25563T caused amino acid substitutions, which were located on 5'-UTR, ORF1ab, S protein, and ORF3a, respectively. This means that despite amino acid mutations in the virus, its biological characteristics, including its ability to bind to its receptor have not changed significantly.

Epidemiological investigations showed that the patient had no travel, residence, or stay history in Wuhan City and surrounding areas or other communities with confirmed COVID-19 case 14 days before the onset of disease (March 14–March 28). There was no contact with people with fever or respiratory symptoms from Wuhan City and surrounding areas nor from communities with confirmed case reports, nor did the patient have a history of overseas travel.

However, because this patient did not seek medical treatment and isolation immediately after the onset of fever, he still had normal social activities including holding a dinner party. Due to the prevalence of asymptomatic COVID-19 infection, epidemiological investigation and virus tracking surrounding this case became more challenging. Although the local transmission of the COVID-19 virus in the mainland of China had been interrupted, COVID-19 is still an epidemic in many other countries. Many cases of COVID-19 and asymptomatic infections entering China through airports and land ports have been detected (4). Therefore, it is necessary to strengthen COVID-19 surveillance in respiratory tract and stool

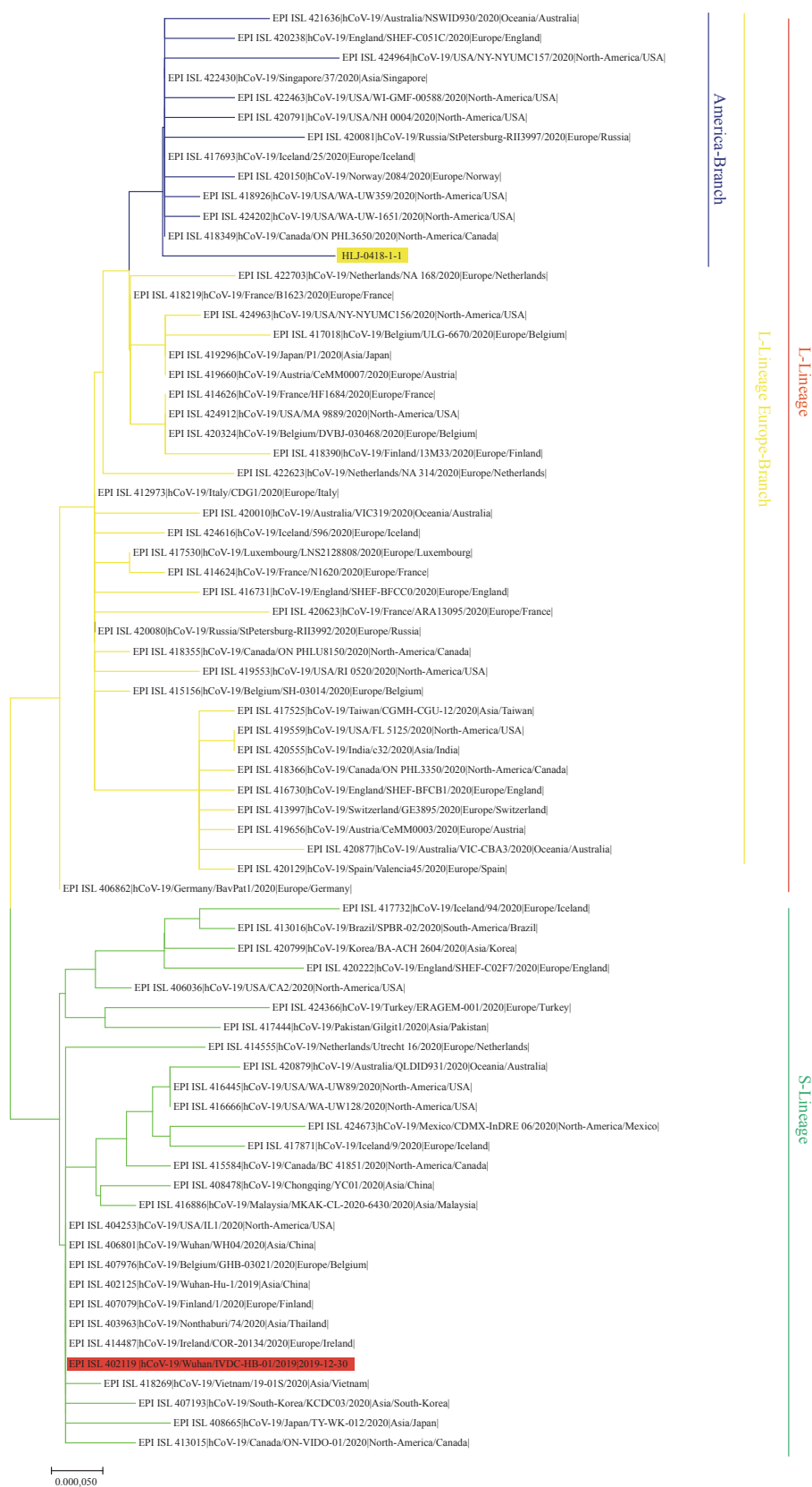


FIGURE 1. Phylogenetic tree based on the genome sequences of COVID-19 virus. The genome of COVID-19 virus of the case in Harbin City and Wuhan City were highlighted in shades of yellow and red, respectively. S- or L-lineage of COVID-19 virus were marked and colored on the right.

specimens among overseas visitors.

Fundings: This work was supported by National Key Research and Development Program of China (Program No. 2018YFC1200305), National Science and Technology Major Project of China (Project No. 2018ZX10102001, 2018ZX10711001, 2018ZX10713002, 2017ZX10104001)

doi: 10.46234/ccdcw2020.127

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Submitted: June 12, 2020; Accepted: June 15, 2020

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Recollection

Fighting Against COVID-19 at the Community Level — Wuhan City, Hubei Province, China, 2020

Canyou Zhang^{1,2}; George F. Gao¹; Zhifeng Wang²; Hao Wu^{3,#}

Since December 2019, an increasing number of cases of pneumonia with unknown etiology (PUE)—later named as coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO)—was identified in Wuhan (1–3), a large city of 11 million people in central China, and the basic reproductive number (R_0) was estimated to be 2.2 (95% CI: 1.4 to 3.9) (4). On March 11, 2020, the WHO formally announced COVID-19 a worldwide pandemic based on the alarming levels of spread and severity and by the alarming levels of inaction (5). Although there is neither a vaccine nor specific medication available, the pandemic was well contained in China by the strict introduction of non-pharmacological interventions (NPIs) (6).

To accomplish this, community-level control and prevention that took measures to “turn off the tap” of COVID-19 cases was key. Through in depth grid management and extensive community mobilization, communities were able to achieve earlier detection, earlier reporting, and reduced risk of transmission. Our field experiences in Wuhan are summarized here, and the accomplishments were also aided by the mobilization of approximately 42,000 health-care workers (HCWs) from all over China that provided an emergency response and addressed major logistical challenges.

Wuhan City has four levels of administrative division including city level, district level, street level, and community level. Community is the most fundamental unit of the society and also the critical battle field to control increasing COVID-19 cases. On February 6, the National Health Commission of China sent a community work group to Wuhan to guide COVID-19 control in communities, and this group consisted of 13 community health specialists and 13 experts from China CDC, which were then made into pairs for the 13 districts of Wuhan.

Community-level Lockdown

At the early stages of the pandemic, there were not enough beds in hospitals for patients and insufficient

nucleic acid test capacity for diagnosis, so many patients later confirmed with COVID-19 were still mobile in their communities and visited hospitals and supermarkets. As a first measure to address the uncontrolled spread in face of logistical problems (“calming the water of the lake”), we recommended on February 9, 2020 in Jiang’an District to close neighborhoods immediately, and we called on residents to stay at home and avoid going outside as much as possible. This policy was extended to the whole city on February 11, and daily necessities and required medical supplies were a priority to be replenished.

City Grid Management and Division of Work

A community was divided into several grid boxes that usually contain 300 to 800 households. The number of households in each grid box depended on the status of neighborhoods it contains: small grid boxes consisted of old neighborhoods without property management, and large grid boxes consisted of neighborhoods with good property management. Wuhan had more than 13,800 grid boxes, and in general, each community had 5 to 7 officers, 5 to 8 coordinators, and several grid workers. The workforce was critically inadequate during the epidemic, so we recommended arranging recruitment of volunteers including government officers, enterprise employees, etc., to participate in grid management. During this process, HCWs from Community Health Service Center played an important role in grid management (Figure 1).

Persuasion at the entrance: Property management Staff and/or volunteers were on duty at the entrance to the neighborhoods to measure body temperature, check identification of all persons who went in and out of the neighborhood, and persuade them to go out infrequently and maintain social distancing. Moreover, they disseminated health promotion materials of COVID-19 and helped with the logistics for managing the residents, and some communities supplied free face

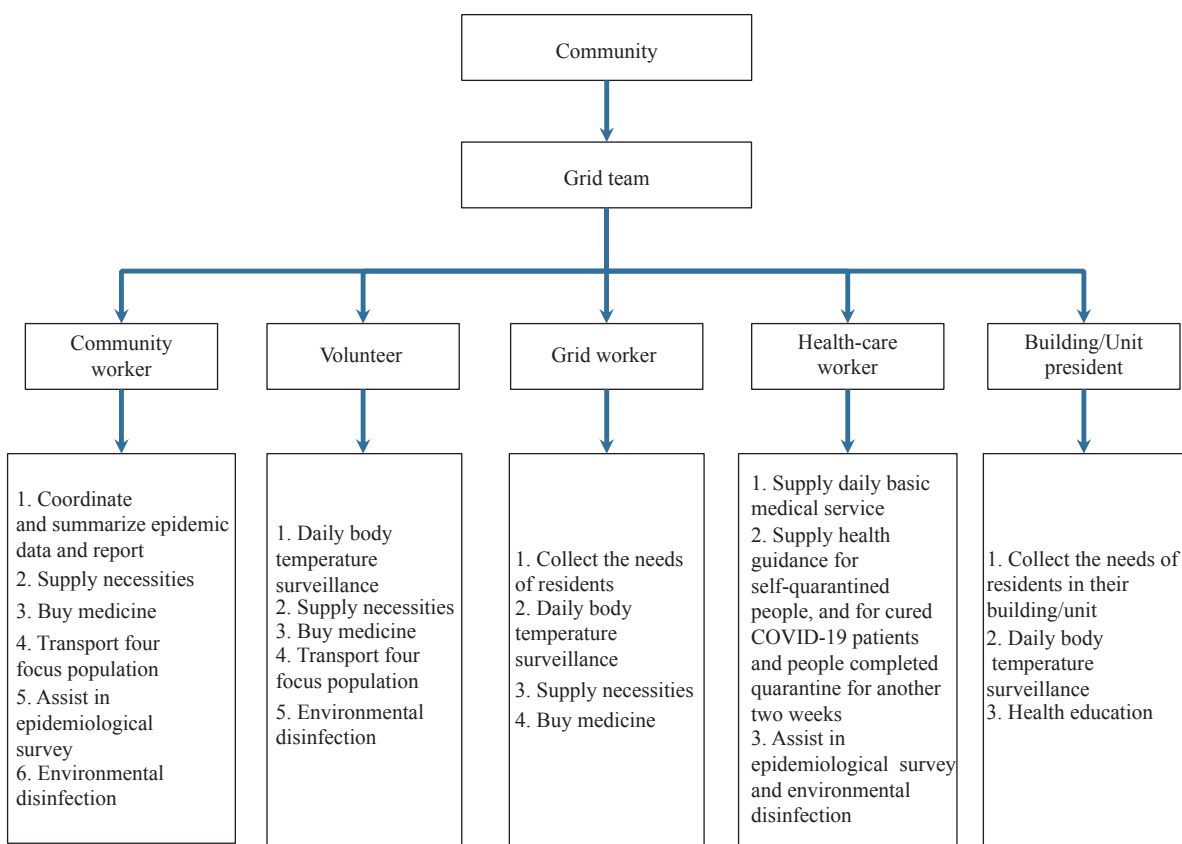


FIGURE 1. Grid team and responsibility division in Wuhan municipality.

masks and household disinfectants. Body temperature measurements at the entrance and other measures also contributed to the COVID-19 case finding and reduced the risk of transmission.

Necessities supply for residents: To reduce direct contact transmission of COVID-19 and to coordinate with the call to “stay at home”, supplying foods and other necessities for residents were an important mission for community workers and volunteers. The residents had a variety of food choices and could shop online individually or buy as groups from supermarket chains via community workers or proprietor group. All goods ordered would be delivered to the entrance of the neighborhood, then the community workers and volunteers informed the residents to get their goods in batches at different times, without direct contact, and while maintaining social distancing. The government also supplied donated foods and/or low price foods for vulnerable people like disabled individuals, seniors living alone, etc. Community workers and volunteers also helped patients with chronic diseases buy medicine or visit hospitals if needed.

Daily screening and specific screening: The grid workers and volunteers worked together to collect the

daily body temperature of all residents through WeChat groups, Weilinli (a WeChat program), mobile phones, and smart calling systems using automated voices. Moreover, the city implemented two specific screenings between February 6–9 and February 17–19 to find 4 focus population, including diagnosed COVID-19 patients, suspected cases, patients with fever, and close contacts, and 6,326 diagnosed COVID-19 patients and suspected cases were found. The community workers or volunteers would transport these people from the community to designated treatment facilities. All diagnosed COVID-19 patients were hospitalized, all suspected COVID-19 patients, patients with fever, and close contacts were quarantined in independent areas to have further tests as soon as possible.

Management of four-focus population: At the early stages of the pandemic, many confirmed COVID-19 cases, suspected cases, patients with fever, and close contacts quarantined themselves at home. We carried out the “five for one” policy where a team with five staff members were responsible for the management of one quarantined person. The five-staff team included a street-level officer, a grid worker, a community worker,

a HCW, and a volunteer. They were in charge of body temperature surveillance, personal protection and family disinfection education, psychological counseling, and life support for self-quarantined people whom would also receive a health package with a thermometer, a face mask, a pen, a registration form, and a set of promotional materials.

After February 17–19, all four focus population were transported out of the community for hospitalization or centralized quarantine. However, the “five for one” policy was still in place for people who came back to community from the hospitals or the quarantine zones, and they would be under medical observation for another two weeks where the community workers would transport them back to the hospital immediately if they developed any symptoms related to COVID-19.

Centralized quarantine: Based on our clinical experience, about 80% of the COVID-19 patients had mild type and self-limited cases. These patients had two choices, self-quarantine at home or centralized quarantine. A total of 14 temporary hospitals reconstructed from hotels, exhibition halls, stadiums, schools, etc., were available to hospitalize mild COVID-19 patients. And all other 3 focus population were also quarantined in requisitioned hotels near where they live.

Health education and promotion: During the outbreak we also face an infodemic where rumors and misinformation were spread. To disseminate COVID-19 knowledge, current control policies, and control measures, many health education and promotion programs were broadcast via television every day. The major information included wearing a face mask, hand hygiene, social distancing, natural ventilation, cough etiquette, etc. Communities displayed posters, distributed paper promotional materials in high visibility places, and broadcast to the community regularly. Some communities even used drones to broadcast this information.

Environmental disinfection and epidemiological survey: The local CDC staff carried out professional terminal disinfection and epidemiological surveys when a newly diagnosed COVID-19 case emerged. At the same time, local CDC would train the HCWs from Community Health Service Center, the staff of property management, and grid workers to assist local CDC staff in finishing epidemiological surveys, reconfirming some critical information of the case and his/her close contacts, and conducting daily environmental disinfection in the community including elevators, corridors, stairways of the building,

door handles, public outdoor fitness equipment, waste containers, etc.

Information Sharing Mechanism

We also recommended building an information sharing mechanism among the communities, community health service center, and local CDC so that information on newly diagnosed COVID-19 cases, suspected cases, patients with fever, and close contacts could be shared promptly and would help recognize high risk populations and decrease delays for hospitalization and quarantine.

Supervision and Consultation

We, the community work group, are supervisors of community-level control and also the consultants of policy makers in Wuhan. We developed control guidelines used by communities, Community Health Service Centers, property management companies, and volunteers. The community work group also offered advice to policymakers of different levels based on evidence obtained in the communities. This advice could be transferred into policy or control measures rapidly by different level of governments.

Conclusion

Measures were focused in the community level with professional epidemiologists as supervisors. All these measures, based on the major transmission mode of COVID-19—droplet transmission and contact transmission—reduce the mobility and contact of people and disrupt transmission routes to reduce R_0 quickly. Meanwhile, guaranteeing living supplies and medication requirements of chronic disease patients are key to allowing residents to stay at home. We believe this can be shared all over the world to tackle the current pandemic.

Contributors: HW, GFG, and CZ conceived and designed the framework. CZ, HW, and GFG reviewed the literature and wrote the first draft. CZ drew the figures. HW, GFG, and ZW reviewed and revised the manuscript. All authors approved the final version.

Acknowledgments: We thank all the members of the community work team.

Conflict of interests: No conflict of interests were reported.

doi: 10.46234/ccdcw2020.126

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Submitted: June 11, 2020; Accepted: June 14, 2020

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Notifiable Infectious Diseases Reports

Reported Cases and Deaths of National Notifiable Infectious Diseases — China, April, 2020

Diseases	Cases	Deaths
Plague	0	0
Cholera	0	0
SARS-CoV	0	0
Acquired immune deficiency syndrome	5,960	1,587
Hepatitis	125,181	37
Hepatitis A	1,187	0
Hepatitis B	101,262	36
Hepatitis C	20,179	1
Hepatitis D	22	0
Hepatitis E	1,732	0
Other hepatitis	799	0
Poliomyelitis	0	0
Human infection with H5N1 virus	0	0
Measles	58	0
Epidemic hemorrhagic fever	540	1
Rabies	15	15
Japanese encephalitis	4	0
Dengue	6	0
Anthrax	16	0
Dysentery	3,695	0
Tuberculosis	85,684	96
Typhoid fever and paratyphoid fever	415	0
Meningococcal meningitis	5	0
Pertussis	479	0
Diphtheria	0	0
Neonatal tetanus	1	0
Scarlet fever	442	0
Brucellosis	5,360	0
Gonorrhea	6,267	0
Syphilis	46,728	11
Leptospirosis	2	0
Schistosomiasis	6	0
Malaria	54	0
Human infection with H7N9 virus	0	0
COVID-19*	995	31
Influenza	15,396	1
Mumps	6,169	0

Continued

Diseases	Cases	Deaths
Rubella	116	0
Acute hemorrhagic conjunctivitis	2,230	0
Leprosy	52	0
Typhus	63	0
Kala azar	26	0
Echinococcosis	362	0
Filariasis	0	0
Infectious diarrhea [†]	58,544	1
Hand, foot and mouth disease	3,001	0
Total	367,872	1,780

[†]The data were from the website of the National Health Commission of the People's Republic of China. The case number and death number do not include the revised additional cases of 325 and the revised additional deaths of 1,290 in Wuhan reported by the Wuhan COVID-19 Epidemic Prevention and Control Headquarters on April 17, 2020.

[†]Infectious diarrhea excludes cholera, dysentery, typhoid fever and paratyphoid fever.

The number of cases and cause-specific deaths refer to data recorded in National Notifiable Disease Reporting System in China, which includes both clinically-diagnosed cases and laboratory-confirmed cases. Only reported cases of the 31 provincial-level administrative divisions in the mainland of China are included in the table, whereas data of Hong Kong Special Administrative Region, Macau Special Administrative Region, and Taiwan are not included. Monthly statistics are calculated without annual verification, which were usually conducted in February of the next year for de-duplication and verification of reported cases in annual statistics. Therefore, 12-month cases could not be added together directly to calculate the cumulative cases because the individual information might be verified via National Notifiable Disease Reporting System according to information verification or field investigations by local CDCs.

doi: 10.46234/ccdcw2020.109

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The inauguration of *China CDC Weekly* is in part supported by Project for Enhancing International Impact of China STM Journals Category D (PIIJ2-D-04-(2018)) of China Association for Science and Technology (CAST).



Vol. 2 No. 25 Jun. 19, 2020

Responsible Authority

National Health Commission of the People's Republic of China

Sponsor

Chinese Center for Disease Control and Prevention

Editing and Publishing

China CDC Weekly Editorial Office
No.155 Changbai Road, Changping District, Beijing, China
Tel: 86-10-63150501, 63150701
Email: ccdcjournal@163.com

CSSN

ISSN 2096-7071
CN 10-1629/R1