

Detection of a Novel Coronavirus (SARS-CoV-2) by Real-Time Reverse Transcription-Polymerase Chain Reaction, China, 2020

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ABSTRACT

Background: During the outbreak of unexplained pneumonia in the city of Wuhan in the late December, 2019, a novel coronavirus named SARS-CoV-2 was identified as the cause of this outbreak.

Methods: A real-time polymerase chain reaction, which targets the orf1ab gene of viral genome, was established to detect and identify the SARS-CoV-2. We used this assay to screen 309 samples from persons with suspected SARS-CoV-2 infection in Wuhan. Then 6 close-phylogenic coronaviruses and 7 viruses which could cause pneumonia were detected. Moreover, 57 clinical samples infected with other viruses and 77 healthy samples were also tested.

Results: The limit of detection of the assay was 6.25 copies per reaction in the detection of cRNA transcribed *in vitro*. The results of detection of throat and fecal swabs from persons with suspected SARS-CoV-2 infection showed throat swabs were more sensitive than fecal swabs during the first 15 days after onset of symptoms (throat: 56.80%, fecal: 30.43%), while the situation was reversed after 15 days (throat: 20.83%, fecal: 27.58%). And matched pair tests suggested the sputum samples had higher virus loads than throat swabs in the patients ($P < 0.05$). There was no cross-reaction when we detected the inactive culture of six other coronaviruses (human coronavirus 229E, NL63, OC43, HKU1, SARS-CoV, MERS-CoV) and seven other viruses (influenza virus A H1N1, influenza virus A H3N2, influenza virus B, parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus). Besides, 27 BALF samples from pneumonia patients infected with human coronavirus 229E, OC43, HKU1 or human adenovirus 7, 30 throat swabs from patients infected with H1N1 and 77 throat swabs from healthy people tested negative by this assay.

Conclusion: The results indicated that the assay specifically and sensitively detected the SARS-CoV-2.

Keywords: SARS-CoV-2; rRT-PCR; Coronavirus

Abbreviations: SARS-CoV: Severe Acute Respiratory Syndrome Coronavirus; MERS-CoV: Middle East Respiratory Syndrome Coronavirus; rRT-PCR: Real-Time Reverse Transcription-Polymerase Chain Reaction.

INTRODUCTION

Since December 9, 2019, several patients with unexplained pneumonia were found to be epidemiologically associated with Huanan sea food market in Wuhan [1,2], central China, where non-aquatic animals, e.g. birds, snakes and rabbits, were also sold before the outbreak. As of December 31, 2019, a 20 survey identified a total of 27 such cases, including 7 severe cases. The number of suspected patients increased to 44 on January 3, 2020 (including 11 severe cases) and to 59 on January 5, 2020 (including 7 severe

cases). These patients displayed hyperthermia, some of whom had dyspnea. Chest radiographs showed invasive lesions in double lungs. However, common respiratory viruses, such as human influenza, avian influenza, human adenoviruses, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), were all detected negative. Metagenomics analysis showed that a novel coronavirus with high homogenous to bat SARS-like coronavirus maybe the causative agent (unpublished).

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Coronaviruses, belonging to the family Coronaviridae, possess a single-stand positive-sense RNA genome with the size ranging between 26-32 kilobases that was the largest genome of all RNA viruses documented thus far. Accumulated evidence shows that many coronaviruses are pathogenic to humans [3-5], though most of infections just cause mild clinical symptoms. However, two exceptions with severe and even fatal cases caused by coronavirus infections are notable [6,7]. One is SARS-CoV that first emerged in Guangdong province in southern China in November 2002 and eventually caused >8,000 human infections and 774 deaths in 37 countries [8,9]. The other case in point is MERS-CoV that was first detected in the kingdom of Saudi Arabia (KSA) in 2012 [10]. A total of 2494 laboratory-confirmed cases of infection with MERS-CoV have been reported since April 2012 [11], including 858 MERS-CoV associated fatalities.

Until March 9, 2020, the novel coronavirus named SARS-CoV-2 has infected over 109,577 people and caused over 3,809 deaths in 105 countries and territories over the world [12]. Therefore, developing a rapid and accurate diagnostic method for detecting SARS-CoV-2 is an urgent priority for controlling the spread of this infection.

In this study, we describe a sensitive and specific real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay for the detection of SARS-CoV-2 and report its use in a survey of more than 309 samples from persons diagnosed as probable SARS-CoV-2 infection during the outbreak in Wuhan.

METHODOLOGY

Design primers and probes

PCR primers and probes were designed using Primer Express Software (Applied Biosystems, Foster City, CA) based on gene sequences of our sequenced virus (WH04|2020-01-05). The specific primers and probe set for orf1a gene was as follows: forward primer, 5'-AGAAGATTGGTTAGATGATGATAGT-3', reverse primer, 5'-TTCCATCTCTAATTGAGGTTGAACC-3' and probe 5'-FAM-TCCTCACTGCCGTCTTGTTGACCA-BHQ1-3'. The probe was labeled with the reporter FAM (6-carboxyfluorescein) and the quencher BHQ1. The human β -actin gene was employed as internal control (Forward primer: 5'-AATGAGCTGCGTGTGGCTC-3', Reverse primer: 5'-GGCTGGGGTGTGAAGGTC-3', Probe: VIC 5'-TTCTCGCGTTGGCCTTGGG-BHQ1-3'). Primers and probes were synthesized by BGI.

The rRT-PCR reaction

We performed rRT-PCR in 30- μ L reaction volumes containing 10 μ L of the RNA dilution, 15 μ L 2X RT-PCR Hot-start PCR Mix, 1.5 μ L RT-PCR Enzyme Mix (HWTS-D002 ; Macro & Micro Test, Jiangsu, China), 0.75 μ L target forward primer (10 μ mol/L), 0.75 μ L target reverse primer (10 μ mol/L), 0.45 μ L target probe (10 μ mol/L), 0.6 μ L IC forward primer (10 μ mol/L), 0.6 μ L IC reverse primer (10 μ mol/L), 0.3 μ L IC probe (10 μ mol/L) and 0.05 μ L water using a fluorometric PCR instrument (Hongshi SLAN-96P, Shanghai, China). Thermal cycling parameters were 20 minutes at 50°C followed by 10 minutes at 95°C and a 45 cycles of amplification (95°C for 15 sec and 60°C for 30 sec); fluorescence was collected during the 60°C step.

Construction of cRNA

A calibration standard was generated by diluting RNA transcription of partial orf1a gene of SARS-CoV-2. The fragment was synthesized based on sequence of orf1a gene of WH04|2020-01-05. The synthesized products were cloned into a pGEM-T Easy vector (Promega Shanghai, Shanghai, China) and then linearized using a specific DNA restriction enzyme. RNA transcribed *in vitro* was generated by the linearized plasmid DNA and the RiboMax Express Large-Scale RNA Production System (Promega, Madison, Wisconsin, USA). After digestion of the template DNA with RNase-free DNase I, RNA transcribed *in vitro* was purified with an RNeasy kit (Qiagen GmbH, Hilden, Germany). The purified RNA was quantified spectrophotometrically at 260 nm, divided into aliquots, and stored at -80°C for future use. Ten-fold serial dilutions (5×10^6 to 5 copies/ μ L) and two-fold serial dilutions of RNA (5 to 0.313 copies/ μ L) were subjected to rRT-PCR analyses.

Evaluate cross-reaction

To evaluate cross-reaction with other close-phylogenetic viruses of the family Coronaviridae, human coronavirus 229E, human coronavirus OC43, human coronavirus HKU1, human coronavirus NL63, SARS coronavirus, and MERS coronavirus culture were involved. All of inactive cultures were supplied by national Institutes of Food and Drug Control. Moreover, the following 7 viruses were also be tested: H1N1 (influenza virus A/PR/8/34); H3N2 (influenza virus A/Beijing/30/95); influenza virus B (Hongkong/5/72); parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus. Total RNA was extracted from 140 μ L viral culture supernatant using a QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany). RNA was eluted from the columns with 50 μ L diethyl pyrocarbonate (DEPC)-treated water. All RNA was diluted to approximately 10^6 copies/ml, and concentrations of them were determined using digital PCR [13].

Twenty-seven BALF samples from patients with different viral pneumonia before June, 2019 were also tested, which included 8 diagnosed as human coronavirus 229E, 2 diagnosed as human coronavirus OC43, 2 diagnosed as human coronavirus HKU1, 15 diagnosed as human adenovirus 7. Throat swabs from 30 patients with confirmed H1N1 infection and 77 healthy peoples in Beijing were also involved. Total RNA was extracted from 140 μ L samples using a QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Detection of samples with a diagnosis of suspected SARS-CoV-2 infection

Clinical materials, including 16 sputum, 75 fecal swabs and 218 throat swabs, were obtained by Wuhan BGI medical. All persons had a diagnosis of suspected SARS-CoV-2 infection according to epidemiology data and World Health Organization (WHO) guidance [14]. Total RNA was extracted from 140 μ L samples using a QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) in a type II biological safety cabinet.

RESULTS

Sensitivity and specificity of the assay

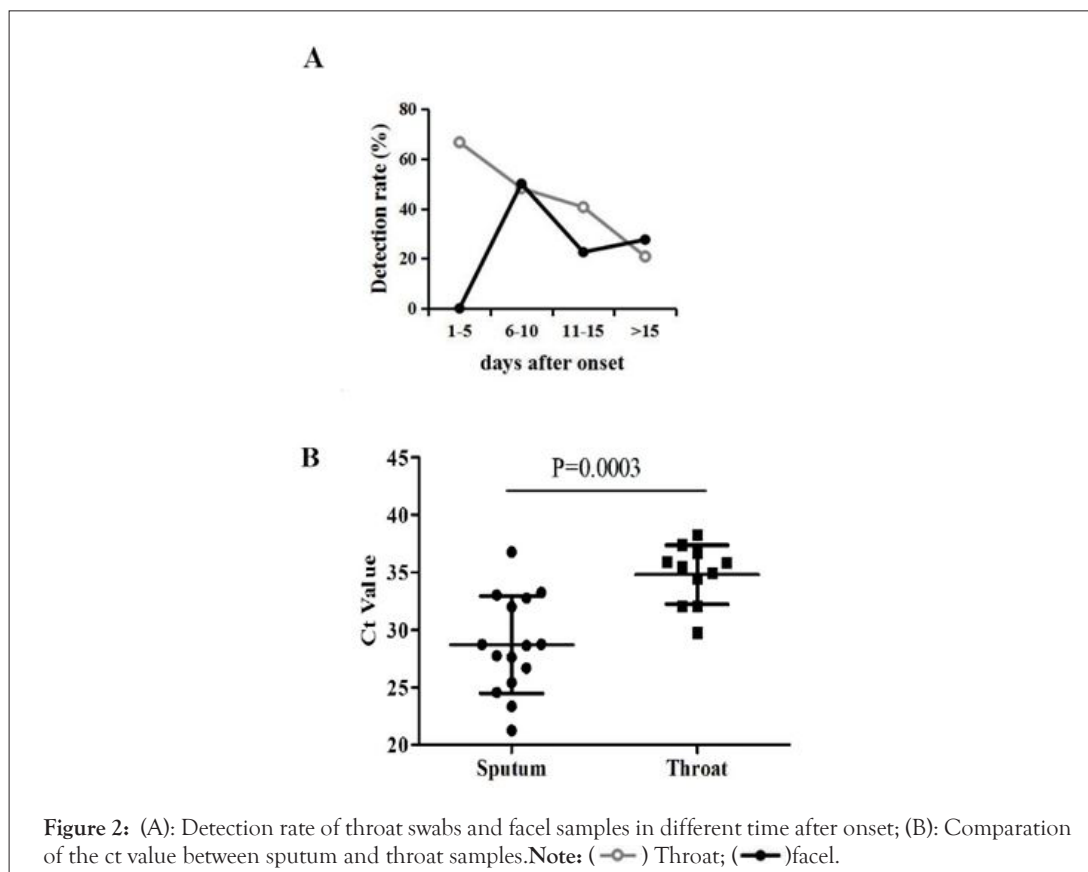
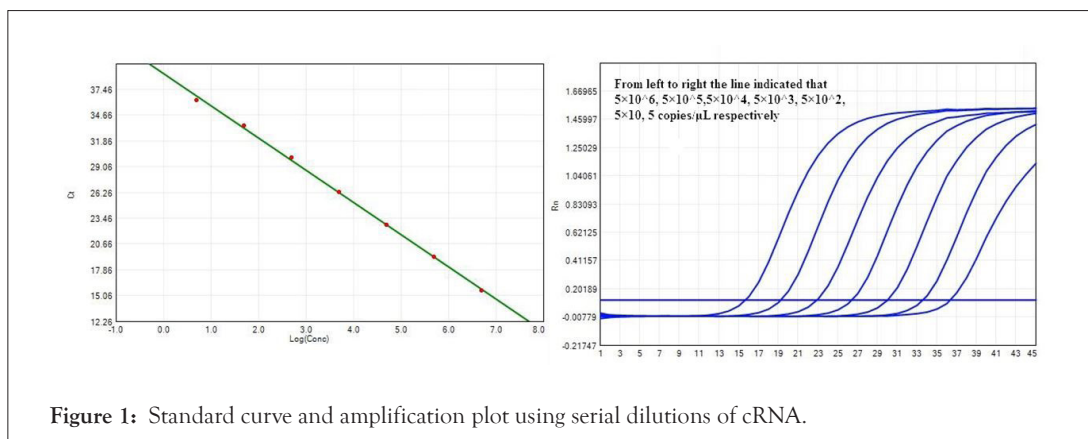
Standard curves of serially diluted RNA transcribed *in vitro* versus threshold cycle were generated to determine both the efficiency of the rRT-PCR and the limit of detection. The assay

exhibited a wide linear range, beginning at 50 copies of target RNA per reaction and extending through 5×10^7 copies per reaction ($R^2=0.9995$) for the assay (Figure 1). To determine the detection limit of the assay, two-fold serial dilutions (5 to 0.313 copies/ μ L) were tested 20 times respectively. The detection rate of approximately 6.25 copies per reaction in the assay were 100% and lower dilutions couldn't be effectively detected.

To evaluate the specificity of our assay, viral RNA of 6 close-phylogenetic viruses and 7 viruses which could cause pneumonia were tested, which all showed negative, suggesting no cross-reaction with any of the 13 viruses. Twenty-seven BALF from pneumonia patients infected with other human coronavirus and human adenovirus were tested. Thirty throat swabs from patients infected with H1N1 and 77 throat swabs from healthy people were also tested. All samples showed negative results, while all human samples were positive for human β -actin gene, which was employed as a control.

Results of suspected cases

We analyzed 16 sputum, 75 fecal swabs and 218 throat swabs from probable SARS-CoV-2 infection. The average of detection rate in throat samples was 56.80% (96/169) during the first 15 days after onset of symptoms (Figure 2A). In the 10 days that followed, the detection rate declined to 20.83% (10/49). The average of detection rate in fecal samples was 30.43% (14/46) during the first 15 days after onset of symptoms (Figure 2A). In the 10 days that followed, the detection rate declined to 27.58% (8/29). The results showed the throat swabs are higher sensitive than the fecal swabs in early stage. However, the fecal swabs were higher sensitive than the throat swabs in late stage. The sputum and throat swabs from 16 patients with severe pneumonia were also employed. Fifteen of 16 sputum tested positive with the average of ct value 28.71 ± 4.23 . Eleven of 16 swabs tested positive with the average of ct value 34.82 ± 2.57 . It seems that the sputum has higher virus loads than throat swabs in the patients ($P<0.05$, Figure 2B).



DISCUSSION

When the dilution of cRNA were tested, the detection limit of the assay in this study showed high sensitivity with approximately 6.25 copies per reaction. There was no cross-reaction when we detected the inactive culture of six other coronaviruses (human coronavirus 229E, NL63, OC43, HKU1, SARS-CoV, MERS-CoV), the same situation presented in the detection of seven other viruses (influenza virus A H1N1, influenza virus A H3N2, influenza virus B, parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus). Besides, 27 BALF samples from pneumonia patients infected with human coronavirus 229E, OC43, HKU1 or human adenovirus 7, 30 throat swabs from patients infected with H1N1 and 77 throat swabs from healthy people were all used to evaluate the specificity of the assay, and results of their tests all showed negative.

CONCLUSION

When the suspected COVID-19 cases were tested by this assay, different sample types all achieved checkout such as throat swabs, fecal swabs and sputum. The results of these suspected cases suggested that throat swabs were more sensitive than fecal swabs during the first 15 days after onset of symptoms (throat: 56.80%, fecal: 30.43%), while the situation was reversed after 15 days (throat: 20.83%, fecal: 27.58%). Moreover, matched pair tests of 16 cases indicated that the sputum had significantly higher virus loads than throat swabs ($P < 0.05$).

The results presented here in this study indicated that the assay specifically and sensitively detected the SARS-CoV-2, which could be considered for the diagnosis of COVID-19 in clinic use.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the ethics committee of Wuhan BGI Medical. The case samples used in this study were obtained from patients with their written informed consent.

CONSENT TO PUBLISH

All of the authors have read this version of the article and consented to publish.

AVAILABILITY OF DATA AND MATERIALS

The gene sequences of our sequenced virus (WH04|2020-01-05) had been deposited in the China National Genebank (accession number: CNA0007335).

COMPETING INTERESTS

There is no competing interest.

FUNDING

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AUTHORS' CONTRIBUTIONS

HHF, HGW and WJC conceived and designed the study. HLW, JYS, HJ and LLY acquired the data. HGW, HLW, JYS, HJ and LLY analyzed and interpreted the data. JYZ, HHF, LC and LCL

contributed reagents, materials and analysis tools. HGW and WJC wrote the paper. All authors have read and approved the final manuscript.

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