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Methods Article

Conventional Reverse Transcription-Polymerase Chain Reaction Assay as an Alternative, Low-Cost, and Reliable Method for the Detection of Coronavirus Disease 2019

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Abstract

Background: SARS-CoV-2 disease is a highly contagious infection causing a large number of deaths in susceptible individuals throughout the world. In this study, a low-cost, sensitive, and easy-to-perform conventional polymerase chain reaction (PCR)-based RNA detection method was evaluated to diagnose the infection, which was feasible at a laboratory with minimal molecular infrastructure.

Methods: From 4 July to 31 August 2020, a total of 277 nasopharyngeal/oropharyngeal swab samples consisting of 72 samples from hospitalized patients with a severe respiratory infection and 205 suspected patients in Isfahan, Iran, were tested using probe-based rtRT-PCR and conventional PCR assays.

Results: A total of 160 clinical samples were tested by rtRT-PCR using the E gene. The sensitivity and specificity of the conventional PCR method were determined to be 100%. Furthermore, out of 117 clinical samples evaluated by the probe-based RT-PCR using the N gene, 74.4% of the samples were positive. Moreover, the duplex PCR method using the N gene and RNase P as an internal control reference gene showed that 68.4% of the samples were positive. Therefore, the tested PCRs could detect positive samples with a sensitivity of 92.55% and a specificity of 100%.

Conclusion: According to the results, this method is a simple, inexpensive, and valuable alternative as well as a suitable procedure for the laboratory diagnosis of SARS-CoV-2 infection.

Keywords: COVID-19, Reverse transcription-polymerase chain reaction, Diagnosis

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Background

The coronavirus disease 2019 (COVID-19) has spread rapidly throughout the world (1-3). There is difficulty in estimating the mortality rate of COVID-19 caused by the novel SARS-CoV-2 due to the uncertainties about testing strategies and expensive laboratory tests for the exact diagnosis (4-6). Real-time reverse transcription-polymerase chain reaction (rtRT-PCR) is the gold standard for rapid diagnosis of COVID-19, providing high specificity and sensitivity; however, it is expensive or difficult to perform in many laboratories around the world (4,7,8). There are many rtRT-PCR-based molecular methods developed for the diagnosis of COVID-19 that target different viral genes including the nucleocapsid (N), polyprotein, spike (S), and envelope (E) gene regions of the RNA virus. These tests have frequently been used to screen symptomatic patients and asymptomatic virus carriers (9,10,11). Similarly, several COVID-19 diagnostic kits are now accessible from different local or international companies (12). However, publicly accessible data on these diagnostic kits are unclear, and in some cases, the sequence information of the primer/probe oligonucleotides is not released, making it difficult to verify and validate the quality and sensitivity of primers (13). Furthermore, studies conducted in different countries reported problems with the reliability of the existing COVID-19 available kits (1,13,14). Laboratory diagnosis of COVID-19 is a rate-limiting step, and there is an urgent need to increase the capacity of laboratories in both developed and low-income countries to control infection and screen patients, healthy individuals, and those with mild symptoms (5,9). Therefore, alternative nucleic acid detecting tests with lower cost and easier availability of reagents are essential, especially when

large scale screening in pandemic conditions is urgent (4). In the present study, a low cost, sensitive, and easy to develop conventional RT-PCR method was developed for the detection of COVID-19 at a laboratory with minimal facilities.

Materials and Methods

Sample Collection

The design of this study is a methodological approach to evaluate an experimental method with a diagnostic-application. This study included 277 clinical specimens consisting of 72 samples from hospitalized patients with a severe respiratory infection and 205 samples from suspected patients who were in contact with affected individuals or indicated some clinical signs of COVID-19 and referred to healthcare centers affiliated to Isfahan University of Medical Sciences, Isfahan, Iran, from 4 July to 31 August 2020. The nasopharyngeal/oropharyngeal swab samples were collected and transferred to tubes containing 2 mL of virus transport medium. The samples and experiments were processed at the core facility laboratory at Isfahan University of Medical Sciences, Isfahan, Iran. The study protocol was approved by the Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (IR.MUI.RESEARCH.REC.1398.778).

RNA Extraction and rtRT-PCR

The RNA was extracted using a viral RNA isolation kit (BehGene Biotech, Iran) according to the manufacturer's instructions. Then, the RNA extracts were stored at -20 ˚C before use. Moreover, 5 µL of the extracted RNAs was subjected to probe-based one-step rtRT-PCR for the detection of SARS-CoV-2 according to the manufacturer's protocol (Kogene Biotech, China). The amplification was performed with a cycle of 30 minutes at 50 ˚C for reverse transcription. The initial denaturation was performed at 95 ˚C for 10 minutes, followed by 45 cycles at 95 ˚C for 15 seconds and 60 ˚C for 60 seconds using a Light Cycler 96-well system (Roche Diagnostics, Manheim, Germany). According to the manufacturer's protocol, a cycle threshold (CT) of < 40 in both genes was considered positive. The selection of samples was carried out according to the CT. Accordingly, the samples were divided into two different groups of 160 samples with CT ranging from 13.15 to 37.98 (mean value of 26.82) (group 1) and 117 samples with CT ranging from 16.46 to 39.6 (mean value of 28.65) (group 2).

Conventional PCR Assay

The experiments were conducted to detect the E and N genes in two different groups using 160 and 117 samples, respectively. The primers used to detect SARS-CoV-2 were based on the E and N genes (14). Regarding the design and optimization of RNase P primer sets, RNase P gene sequences were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/). In silico analyses and criteria for the selection of RNase P primers were verified, and the accuracy and efficiency of the RNase P primer set were confirmed through PCR amplification of the positive control to optimize the PCR conditions. The PCR amplification for the N and RNase P genes was conducted in a 16 µL volume containing 7.5 µL of 2X master mix (Ampliqon, Denmark), 0.75 µL of each primer (10 pg/µL), 0.75 µL of enzyme, and 5 µL of RNA. The PCR amplification for the E gene was conducted in a 15 µL volume containing 7.5 µL of 2X master mix (Ampliqon, Denmark), 0.75 µL of each primer (10 pg/µL), 0.75 µL enzyme, and 5 µL RNA. Amplification conditions were optimized in the Applied Biosystems. The sensitivity and specificity of the two amplification methods, rtRT-PCR (Light Cycler 96, Roche, Germany), Germany and conventional PCR assays were compared to detect COVID-19. Additionally, the PCR products were subjected to 1.5% agarose gel electrophoresis. Table 1 shows the sequences of primers and PCR conditions for

Table 1. The Primer Sequences and PCR Conditions for the Amplification of the N, E, and RNase P Genes

	Primer	Sequence (5′→3′)	Size (bp)	PCR Conditions			
Duplex PCR	N	F: CACATTGGCACCCGCAATC	128	Reverse transcription	50°C	30 min	1
		R: GAGGAACGAGAAGAGGCTTG		Denaturation	95°C	5 min	1
	RNase P	F: AGATTTGGACCTGCGAGCG	244	Denaturation	94°C	15 s	45 Cycles
				Annealing	58°C	30 s	
				Extension	72°C	20 s	
		R: GTGAGATGGATCCGAGACAATAA		Final extension	72°C	2 min	1
PCR	E	F: ACAGGTACGTTAATAGTTAATAGCGT	113	Reverse transcription	50°C	30 min	1
				Denaturation	95°C	5 min	1
				Annealing	94°C	15 s	45 Cycles
				Extension	57°C	30 s	
		R: ATATTGCAGCAGTACGCACACA		Extension	72°C	30 s	
				Final extension	72°C	2 min	1

the amplification of the N, E, and RNase P genes.

Statistical Analysis

The data were recorded using Microsoft Excel 2007 (Microsoft Corp, Redmond, WA, USA) and analyzed in SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Flow diagram depicting the methods and results is shown in Figure 1. The samples were divided into four different age groups of under 20 years (n = 8), 20-39 years (n = 90), 40-59 years (n = 93), and over 60 years (n = 86). Overall, 40 cases out of 72 hospitalized patients aged > 60 years, and the majority (54.1%, n = 150) of study population were male. The mean CT values for E and N genes in the commercial probe-based assay were 26.82 and 28.65, respectively. Among 124 negative samples selected by the probe-based rtRT-PCR, conventional PCR assay showed 7 positive samples using E gene and seven positive samples using N gene (Tables 2 and 3). Out of 160 clinical samples tested by RT-PCR using the E gene, 73 and 87 samples were positive and negative, respectively. Of 117 clinical samples evaluated by RT-PCR using the N and Ranse P genes, 87 samples were positive and 30 samples were negative. Furthermore, the duplex PCR method using the N gene and RNase P as an internal control reference gene showed that 80 and 37 samples out of 117 samples were positive and negative, respectively. The RT-PCR method using the E gene showed the same positive and negative reactions as conventional PCR. The sensitivity values of the conventional PCR assay (assuming the Probe-based rtRT-PCR assay as a reference) were 100% and 92.5% using E and N genes, respectively. All inconsistencies between the probe-based and conventional PCR experiments were observed at CT values higher than 37 for the probe-based reference assay. The results of the conventional PCR that assessed E, N, and RNase P genes revealed fragments with the sizes of 113, 128, and 244 bp, respectively. No other fragments with different sizes were observed in this study (Figure 2A, B).

Discussion

In this study, a traditional PCR method was used to detect

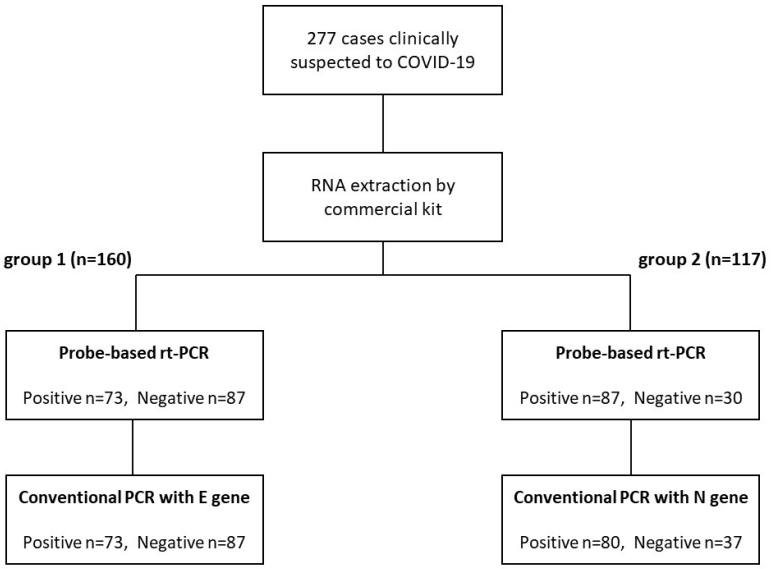

Figure 1. Flow Diagram Showing Methods and Results of the Present Study

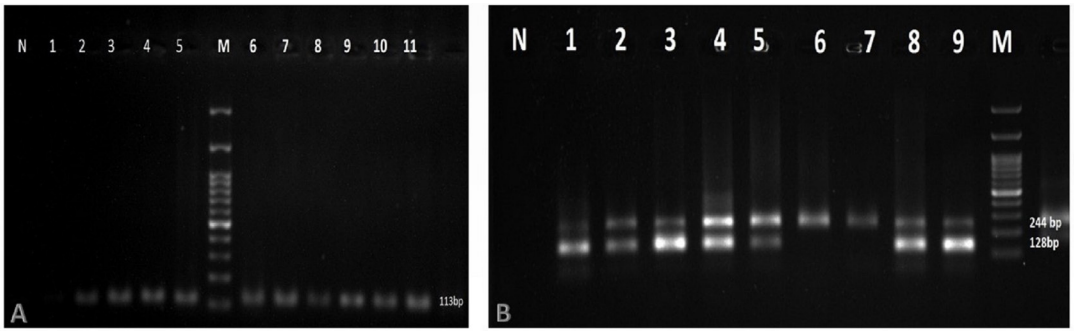

Figure 2. (A) The Amplified Fragment of 113 bp Observed Using the SARS-CoV-2-Specific E Gene Primers. Lane M is 100 bp ladder molecular size marker. **(B)** The results of the conventional PCR that assessed N and RNase P genes revealed fragments with the sizes of 128 and 244 bp, respectively. Lane M is 100 bp ladder molecular size marker.

Table 2. Results of Probe-Based rtRT-PCR for Conventional PCR Using E Gene

Probe-Based rtRT-PCR	Conventional PCR Using E Gene	
	Positive (n = 73)	Negative (n = 87)
Positive (n = 73)	66	7
Negative (n = 87)	7	80

Table 3. Results of Probe-Based rtRT-PCR for Conventional PCR Using N Gene

Probe-Based rtRT-PCR	Conventional PCR Using N Gene	
	Positive (n = 80)	Negative (n = 37)
Positive (n = 87)	73	14
Negative (n = 30)	7	23

SARS-CoV-2 in patients, which compared to probe-based rtRT-PCR as the standard method, was able to detect E and N genes by 100% and 92.5%, respectively. In our conventional PCR method, a further reduction in cost was achieved by optimizing PCR conditions with preserved specificity versus probe-based method. This technique indicates that the development of rapid and reliable diagnostic tools is critical for detecting the infected people, in addition to the isolation and treatment of the patients with a large-scale test in the pandemic era (15). The probe-based rtRT-PCR method is the gold standard assay for the diagnosis of COVID-19; however, it is not accessible in most of the laboratories in developing countries due to the high-cost and global demands for supplies, reagents, and trained laboratory staff (5,7,15). This simple and inexpensive method represents a valuable alternative and suitable procedure for the wider use of SARS-CoV-2 testing. The conventional PCR is useful for diagnosis and screening in regions where laboratory capacities are limited and also may be useful during the COVID-19 pandemic in situations where resources are constrained with preserved specificity and high sensitivity. Conventional PCR protocol allows the clear distinction of each amplicon in a single gel and visualization of spurious primer-dimer formation. There are many commercial kits available with specific primer set sequences that may contain unoptimized primer sets producing false-positive results. Mollaei et al (16) reported that a significant difference in the analytical sensitivity between the studied primer sets (ORF1ab, N, E, and RdRp primers) in rtRT-PCR kits was observed.

In the current study, the unoptimized primer sets were found producing long and short dimer bands (Figures 2A, B), which could be a potential source of false-positive results in RT-PCR based protocols. In the present study, the conventional PCR method was assessed using E, N, and RdRp genes. The subsequent findings indicated an acceptable correspondence with the commercial PB rtRT-PCR.

Conclusion

The proposed low-cost duplex PCR method is an alternative suitable approach for detecting SARS-CoV-2 in clinical samples, mainly in laboratories where the reference probe-based rtRT-PCR is not accessible.

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Authors' Contribution

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Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

This study was approved by the Research and Ethics Committee of Isfahan University of Medical Sciences, Isfahan, Iran (IR.MUI.RESEARCH.REC.1398.778). Furthermore, written informed consent was obtained from the participants for the publication of this report.

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