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Coronavirus Pandemic

One-step nested RT-PCR for COVID-19 detection: A flexible, locally developed test for SARS-CoV2 nucleic acid detection

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Abstract

Introduction: Due to the coronavirus pandemic, identifying the infected individuals has become key to limiting its spread. Virus nucleic acid real-time RT-PCR testing has become the current standard diagnostic method but high demand could lead to shortages. Therefore, we propose a detection strategy using a one-step nested RT-PCR.

Methodology: The nucleotide region in the ORF1ab gene that has the greatest differences between the human coronavirus and the bat coronavirus was selected. Primers were designed after that sequence. All diagnostic primers are species-specific since the 3´ end of the sequence differs from that of other species. A primer set also creates a synthetic positive control. Amplified products were seen in a 2.5% agarose gel, as well as in an SYBR Green-Based Real-Time RT-PCR.

Results: Amplification was achieved for the positive control and specific regions in both techniques.

Conclusions: This new technique is flexible and easy to implement. It does not require a real-time thermocycler and can be interpreted in agarose gels, as well as adapted to quantify the viral genome. It has the advantage that if the coronavirus mutates in one of the key amplification nucleotides, at least one pair can still amplify, thanks to the four diagnostic primers.

Key words: SARS coronavirus; DNA synthesis; pandemics; DNA electrophoresis.

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Introduction

SARS-CoV2, the new coronavirus that emerged in Wuhan, China, in late 2019, quickly developed into a pandemic. The undetected infected persons accelerated its spread in China. It was estimated that up to 86% of infections were undocumented before January 23, 2020 [1] Therefore, identifying those infected is important for health authorities to establish measures that limit the viral spread. Furthermore, the serial detection of the virus in patients can be correlated with their clinical evolution [2,3].

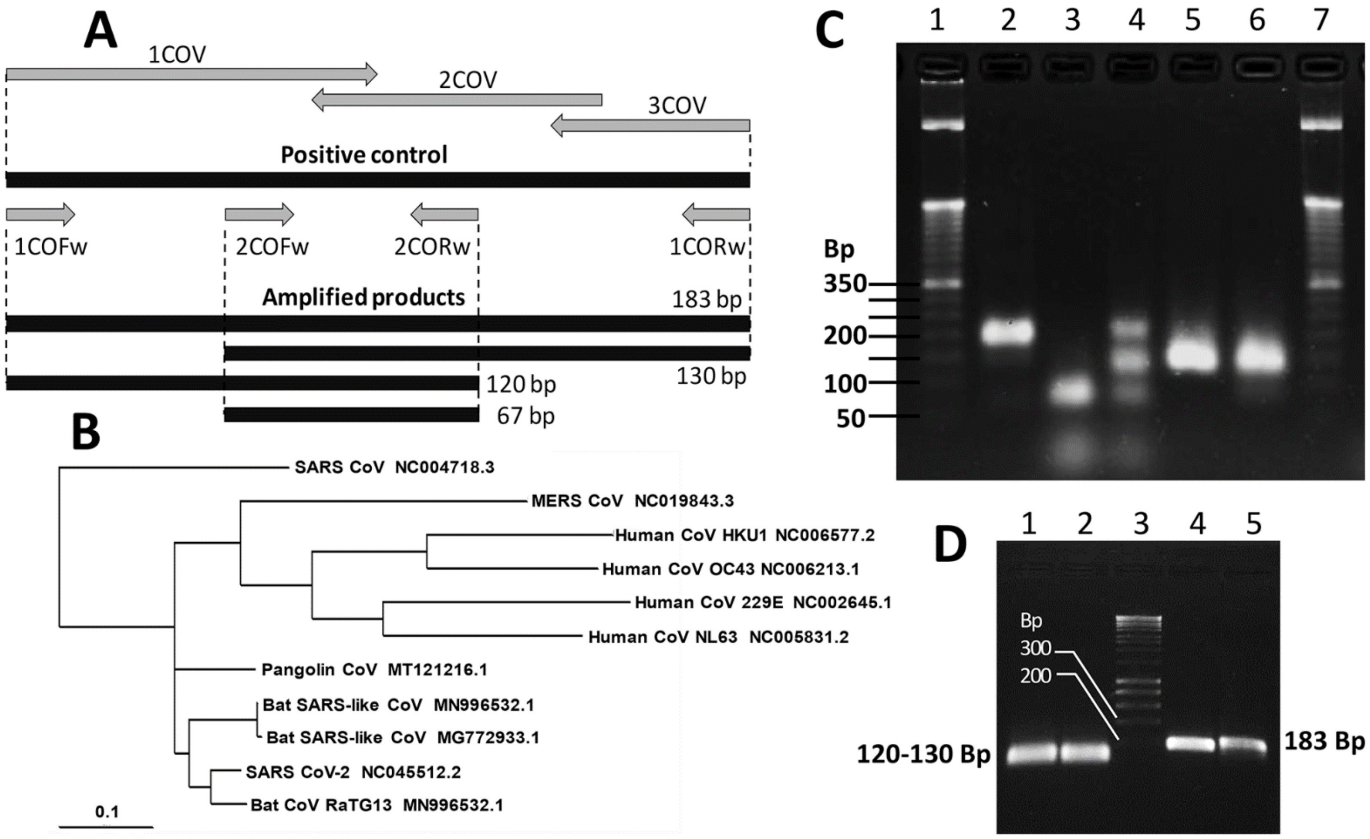
Many laboratory test kits have been developed and used by the Chinese CDC, the American CDC, and other private companies. Virus nucleic acid real-time RT-PCR testing has become the current standard diagnostic method for coronavirus disease (COVID-19) [4]. However, high demand could lead to shortages, making it necessary to have an alternative.

End-point RT-PCR could be such an alternative. Its cost is lower and it is feasible for laboratories that do not have real-time PCR equipment. Among various end-point PCR strategies, nested PCR is highly sensitive and can detect at least one copy of the standard dilution curve. It uses two sets of primers in two successive runs, in which the second set of primers amplifies a secondary target within the first amplified product.

Table 1. Primers sequences.

Primer	Sequence 5-3´
1COV	AGCAAGTTGAACAAAAGATCGCTGAGATTCCTAAAGAGGAAGTTAAGCCATTTATAACTGAAAGTAAACCTTCAGTTGAA
2COV	AGTTTCTTCCAGAGTTGTTGTAACTTCTTCAACACAAGCTTTGATTTTCTTATCATCTTGTTTTCTCTGTTCAACTGAAG
3COV	CAATATAAAGTAACAAGTTTTCTGTGAGGAACTTAGTTTCTTCCAGAGTTGTTGTAACTTCTTCAACACAAGC
1COFw	AGC AAG TTG AAC AAA AGA TCG CTG AG
1CORw	CAA TAT AAA GTA ACA AGT TTT CTG TGA GG
2COFw	ATA ACT GAA AGT AAA CCT TCA GTT GAA
2CORw	CAA CAC AAG CTT TGA TTT TCT TAT CAT

Figure 1. SARS-CoV-2 amplified sequence.



A) The schematic diagram for the amplified products. The upper portion shows the construction of the synthetic positive control from three oligonucleotides (1COV, 2COV, 3COV). The lower portion shows the four primers (1COFw, 2COF2, 2CORw and 1CORw) with their four amplified products. B) Cladogram of the amplified region in SARS-CoV-2 and the homologous sequences of other human coronaviruses, in addition to bat and pangolin coronaviruses. Its closeness to a bat coronavirus and the great difference from other human coronaviruses can be appreciated. The GenBank Reference from where the sequence was extracted is indicated. C) Electrophoresis gel at 2.5% agarose. Lanes 1 and 7: 50 bp DNA Ladder, lanes 2 to 6: amplified products from the synthetic positive control with 1COFw-1CORw, 2COFw-2CORw, all primers, 2COFw-1CORw, and 1COFw-2CORw, respectively. D) Electrophoresis gel at 2.5% agarose. Lanes 1 and 2: amplified products from oropharyngeal and nasopharyngeal samples with all primers; lane 3: 100 bp DNA Ladder; lanes 4 and 5: amplified products from oropharyngeal and nasopharyngeal samples, with only 1COFw-1CORw primers. Upon amplifying the clinical samples by using all the primers, the 120 and 130bp products (that are seen together in the gel) that derived from the nested PCR, predominated over the other bands, being the only ones observed. The 183bp product, amplified using only the 1COFw-1CORw primers, was also very adequately observed.

However, nested PCR is not feasible as a routine test because it is time-consuming and has a high risk for cross-contamination [5]. One-step nested PCR, in which the two successive rounds of amplification are performed in the same reaction tube, is as sensitive as two-step nested PCR [5]. Therefore, we propose using a one-step nested RT-PCR.

Methodology

The project was approved by the ethics committee of the Faculty of Medicine of the University of Colima, Mexico (registered 2020-01-04). To create the diagnostic strategy, a nucleotide region that has the greatest differences between the human SARS-CoV-2 (COVID-19) coronavirus (GenBank: MN908947.3) and the genetically closest bat coronavirus (GenBank: MN996532.1) was selected [6]. That sequence (183 bp, spanning from 3801 to 3983 bp from the GenBank sequence: MN908947.3), located in the ORF1ab gene, is 100% similar to all SARS-CoV-2 complete nucleic acid sequences reported in the GenBank as of June 1, 2020, and has an 86.96% similarity with the bat SARS-like coronavirus isolate sequence. Designed primers are shown in Table 1. The in-silico analysis shows that those primers do not amplify the sequences of the recently reported bat coronavirus RaTG13 (GenBank: MN996532.1), despite having a 93% similarity with the nucleotide region selected for creating the diagnostic strategy. A phylogenic analysis of the selected sequence (from the region amplified with the 1COFw –forward- and 1CORw -reverse- primers in SARS-CoV-2), with the orthological sequences of other coronaviruses, was performed using the GeneStudio™ Pro v2.2.0.0. software (GeneStudio, Inc., Suwanee, GA, USA).

When the 1COV, 2COV, and 3COV primers were placed together in a PCR, they could form the coronavirus sequence and serve as a synthetic positive control in the absence of positive clinical reference samples. The 1COFw and 1CORw primers amplified a 183 bp segment involving the entire region. The 2COF and 2COR primers amplified an internal 67 bp segment (nested PCR). The combination of the four primers produced four amplified products (183 bp, 130 bp, 120 bp, and 67 bp), as shown in Figure 1.

All those primers were specific for SARS-CoV-2 (COVID-19) since the 3′ end of its sequence differs from that of other coronaviruses and makes it species-specific [7]. To corroborate the specificity, a simulated test was performed using MFEprimer software (available at https://mfeprimer3-1.igenetech.com/), which is the only simulation tool that enables the inclusion of genomic (gDNA) and complementary (cDNA) DNA for different species. In addition, it maintains the 3' end pairing rule to indicate that amplification is possible, a control that the NCBI Blast tool and others do not allow [8]. The results of the primers with human DNA in the MFEprimer program showed there were no hairpins, no dimers, and no amplification was detected.

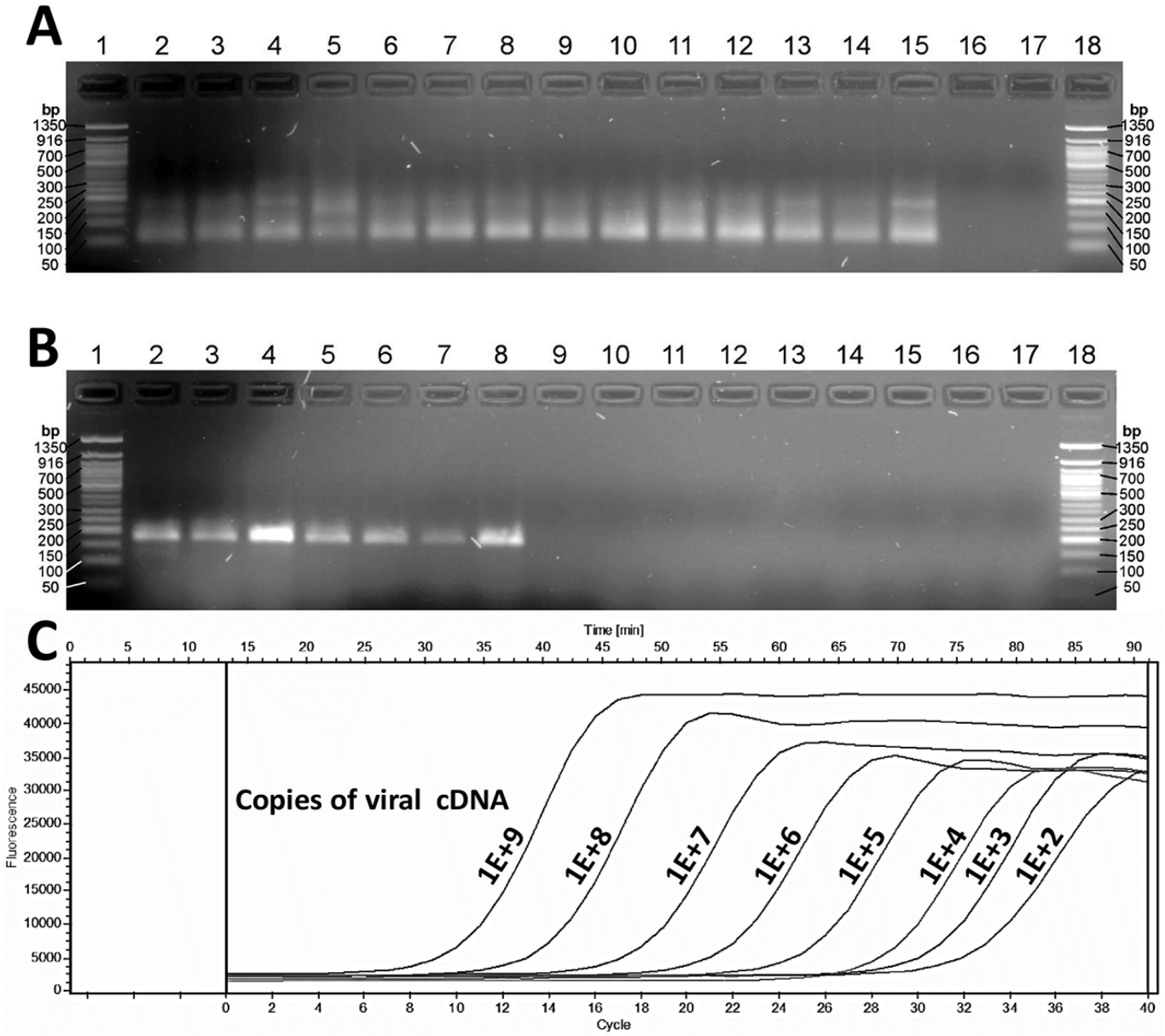
To generate the synthetic positive control, the 1COV, 2COV, and 3COV primers (approximately 19.8 pmol/µL of each) were placed in a 25µL reaction using GoTaq® Green Master Mix (Promega, Wisconsin, Madison, USA), using a programmable thermal cycler (Mastercycler Personal, Eppendorf AG, Hamburg, Germany). The cycling conditions were set as follows: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds. After the reaction, one microliter of that product was re-amplified using the 1COFw and 1CORw primers, under the same conditions. That process produced a fragment of synthetic DNA that served as a positive control for future reactions.

The diagnostic PCR for COVID-19 was performed by placing the 1COFw, 1CORw, 2COFw, and 2CORw (Fw = forward; Rw = reverse) primers (approximately 19.8 pmol/µL) in a one-step RT-PCR reaction (following the manufacturer's instructions), placing 25ng of total RNA from the human nasopharyngeal sample and adding serial dilutions of the synthetic viral cDNA (for the synthetic positive control). Total human RNA was added to the reaction to assess the behavior of the primers in a reaction in the presence of total human cDNA. The reaction was also carried out with each pair of primers (1COFw and 1CORw; 2COFw and 2CORw; 1COFw and 2CORw; 2COFw and 1CORw). The cycling conditions were set as follows: one cycle at 53°C for 5 minutes; 94°C for 10 minutes, 38 cycles at 94°C for 30 seconds, 53°C for 30 seconds, and 68°C for 30 seconds (using a Mastercycler Personal, Eppendorf vapo.protect, Hamburg, Germany). PCR products were loaded, with no purification step, into a 2.5% agarose gel containing GelRed® Nucleic Acid Gel Stain for electrophoresis. The 1COFw and 1CORw primers were also used in a real-time PCR using the innuSCRIPT One-Step RT-PCR SyGreen Kit, (Analytik Jena, Germany), in a thermocycler (Eppendorf RealPlex, Hamburg, Germany), under the following cycling conditions: one cycle at 53°C for 5 minutes; 94°C for 5 minutes, 35 cycles at 94°C for 15 seconds, 53°C for 20 seconds, 68°C for 20 seconds, and 80°C for 15 seconds for capture fluorescence. The last temperature was determined by a melting curve analysis. The amplification efficiency was estimated by the serial-

dilution method (eight dilutions) [9]. The copy number of target sequences was estimated by optical density, according to the exact molar mass derived from the DNA sequence [10].

Clinical sample collection and processing: A total of 18 naso- and oropharyngeal samples were collected with swabs in 2.5 ml of viral transport medium, and stored at -80°C. Those samples were obtained from patients from 3 different states in Mexico (at a distance from each other of up to 1000 km): Zacatecas (central Mexico), Colima (western region with important maritime trading with Asian regions) and Nuevo León (northeast Mexico, bordering the United States), from February to June 2020. The age range of the patients

Figure 2. SARS-CoV-2 amplified in clinical samples using 1COFw and 1CORw primers for endpoint RT-PCR or adapted to a real-time RT-PCR using SYBR Green.



A) Electrophoresis gel at 2.5% agarose with the amplified products for the housekeeping human RNaseP gene of all the clinical samples and controls. The positive amplification (a band of 65 bp) indicated the presence of sufficient nucleic acid and a specimen of acceptable quality. B) Electrophoresis gel at 2.5% agarose with the products of all the clinical samples and controls, amplified using 1COFw and 1CORw. The positive amplification (a band of 183 bp) indicates the presence of the SARS-CoV-2 genome. The lanes for both electrophoresis gels correspond to: lanes 1 and 18: 50bp DNA ladder, lanes 2 to 8: samples positive for SARS-CoV-2 (3 asymptomatic patients, 3 with moderate symptoms, and 1 patient with severe symptoms), lanes 9 to 15: samples negative for SARS-CoV-2, lane 16: RNA extraction control, and lane 17: negative template control. C) Amplification for serial dilutions of cDNA from SARS-CoV-2 synthetic positive control with 1COFw-1CORw primers using SYBR Green-Based Real-Time RT-PCR.

was from 24 to 69 years and there was an equal number of men and women. To establish COVID-19 diagnosis in the patients, viral RNA was isolated from nasopharyngeal specimens, according to the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). The samples were tested for SARS-CoV-2 using the CDC real-time RT-PCR Panel (IDT – Integrated DNA Technologies, Iowa, Coralville, USA), and interpreted according to the manufacturer's instructions. Of the 18 samples tested, 11 were positive and 7 were negative. Positive sample amplification occurred between cycles 16 and 28 (cycle threshold). Of the patients that were positive, 4 were asymptomatic, 5 had moderate symptoms, and 2 presented with severe symptoms. The novel End-Point One-step nested RT-PCR was performed using RNA isolated from those patients for its validation with clinical samples. The RNA from the samples also underwent RT-PCR for the housekeeping human RNaseP gene (the same gene reported by the CDC) to corroborate its quality and the absence of PCR inhibitors (RP-F: AGATTTGGACCTGCGAGCG, RP-R: GAGCGGCTGTCTCCACAAGT, 65 bp).

Results

Figure 1B shows a phylogenetic analysis of the sequence used to detect SARS-COV-2, demonstrating that said region varies greatly between the different coronaviruses. Amplification was achieved, both for generating positive control and for detecting specific sequences of COVID-19. Figure 1C and 1D shows a 2.5% agarose gel electrophoresis, with the products amplified from the combinations of all primers in the synthetic positive control and positive patient controls, respectively. The results for all the patient samples tested for SARS-CoV-2 were concordant between the CDC real-time RT-PCR Panel and the novel End-Point One-step RT-PCR method, even for asymptomatic patients. Figure 2 shows that the 1COFw and 1CORw primers can be used separately for COVIC-19 diagnosis from clinical samples through the End-Point One-step RT-PCR or SYBR Green-Based Real-Time RT-PCR adaptation. Figure 2C shows the amplification of the serial-dilutions of viral cDNA by Real-Time RT-PCR. Lastly, Real-Time RT-PCR had an $R^2 = 0.9947$, with an efficiency of 99.66%. Detection of at least 100 viral cDNA copies was achieved during the End-Point and Real-Time PCR techniques.

Discussion

The novel technique presented herein can be used in specimens by direct RT-PCR, without RNA purification, as previously described, to detect COVID-19 or other viruses, albeit test sensitivity could be affected [11,12]. Although the technique proposes a one-step nested RT-PCR using the four diagnostic primers, it can be performed using only one pair of primers (Fw and Rw) for end-point PCR or adapted to a real-time RT-PCR using SYBR Green for investigations that require quantification of viral genomes. The advantage is that because there are four diagnostic primers, if the coronavirus mutates in one of the nucleotides that is key for amplification (the 3' end of any of the primers), at least one pair will still amplify. Importantly, the synthetic virus sequence should be managed according to the recommendations from laboratories that diagnose by PCR, to reduce the risk of contamination.

Conclusions

A new highly specific strategy was created to detect COVID-19. It can be performed in laboratories that do not have the equipment for carrying out real-time PCR, its cost is lower than that of current techniques, and it provides greater flexibility.

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