Original Article

Rapid Homemade Reverse Transcriptase PCR Detection and Phylogenetic Analysis for *SARS-CoV-2* Based on E and M genes

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Abstract

Background & Objective: Coronavirus disease 2019 spreads worldwide and needs detection systems capable of rapid diagnostic of this virus (*SARS-CoV-2*). The aim of this study is to design the homemade RT-PCR method for the Detection and phylogenetic analysis of this virus.

Material & Methods: The genes selected for diagnosis were *E* and *M* genes for this virus. PCR product was cloned in *pTZ57R/T* plasmid for preparation of positive control. In order to determine the sensitivity of this molecular method, the genes mentioned in the clone *pTZ57R/T* vector and the Limit of detection (LOD) the genes were determined and phylogenetic analysis was performed using partial *E* and *M* gene sequences.

Results: PCR product was observed for E and M genes 156 and 547 bp on the Agarose gel. The LOD of the *E* and *M* gene was 60 and 82 copies. There was also a positive response to the samples of patients who were positive by other methods.

Conclusions: Since this virus is considered to be the cause of a pandemic in different countries all over the world, the present study is very important as a method of rapid and low-cost molecular diagnosis for monitoring this virus. Phylogenetic analysis is necessary for epidemiological studies for the control and prevention of the disease.

Keywords: RT-PCR, Covid-19, Detection, LOD, Phylogenetic Analysis

Introduction

Viruses and Bacteria are naturally present in the environment. Some pathogenic microorganism is associated with human or animal activities. The vaccination or antibiotics reduced risk of these pathogens. However, we are always at risk of new viruses and they could cause genetic changes and are more pathogenic and dangerous (1). Coronaviruses belong to the *Coronaviridae* family of enveloped RNA viruses. They contain a single-stranded, positive-sense RNA, with nucleotide sequence of 27-31 kb that express their replication and transcription complex, including their RNA-dependent RNA polymerase (RdRp) structural protein have been identified: the nucleocapsid protein (N), the membrane glycoprotein (M), the surface peplomer glycoprotein

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(Spike) (S) and envelope (E) (2). Coronaviruses cause common diseases such as colds to respiratory syndrome such as Middle East Respiratory Syndrome (MERS) and Acute Respiratory Syndrome (SARS). Coronaviruses are so zoonoses. In December 2019, coronavirus disease (covid-19) was an emerging infectious disease threat to human health in Wuhan, Hubei Province, China. Initially, it appeared that some patients had a history of attending or working in the wholesale market for fish and seafood. This major virus of this pandemic was severe acute respiratory syndrome coronavirus-2 (*SARS-CoV-2*) (3). A new coronavirus that is 5% genetically related to SARS and is subspecies of Sarbecovirus (4). Currently, the only new coronavirus diagnostic test is a molecular detection method. Therefore, only a limited number of laboratory centers in the world have the ability to perform this test (5). The *E*, *N*, *M*, *S* and *RdRp* genes are used for this virus diagnosis by Molecular Method (6). Designing a homemade molecular detection method based on conventional RT-PCR is very useful for this virus detection in a laboratory that does not have the necessary equipment such as the REAL TIME PCR device. Examination and comparison of tests show that there must be standard methods for diagnosing this virus so that infections caused by this virus can be performed in the shortest possible time with high accuracy and repeatability. Polymerase chain reaction techniques is much more sensitive and reliable and can be easily done in a laboratory. Designing and setting this method based on the virus *E* and *M* genes, it is possible to identify infected and suspected cases of this virus in short time with high sensitivity and specificity. RT-PCR can detect the virus from throat, nasopharyngeal swab as well as from stool sample. One cohort study found that patients even if not having any symptoms still may be the carrier for viruses thus RT-PCR can detect those at genetic level. Another aim of this study was the genetic relation and phylogenetic study of this virus in Qom province which would lead us to understand coronavirus evolution in Qom province in Iran, so by this way probable variations in above-mentioned genes can be traced and in another view by importing these sequences in NCBI data bank and blasting them, origin and identity with other strains can be detected. Another aim of this study was the genetic relation and phylogenetic study of this virus in Qom province.

Materials & Methods

The specific target genes of this virus are *E* and *M*. The required primers for the genes were first collected using sequences recorded in the NCBI database (https://www.ncbi.nlm.nih.gov) and these primers were aligned using the CLC Sequence viewer 8.0 software (CLC bio, Arhus, Denmark). About 15 and 18 sequences for *E* and *M* were extracted from NCBI. The primer sequences were selected based on the conserved regions of this genes, and sequences were determined to check the specificity of primers in the Primer-Blast section. The primer sequence and the length of the amplified fragment are shown that in Table 1.

Table 1. Primer sequence, TM and PCR product

Primer name	Sequence 5'-3'	TM	PCR Product	reference
AM-E-F	TCGTTTCGGAAGAGACAGG	51°C	156 bp	This study
AM-E-R	AAGAAGGTTTTACAAGACTCACGT			
AM-M-F	GTCTTCTACAATTTGCCTATGCC	51°C	547bp	This study
AM-M-R	TCACTGCTACTGGAATGGTCTG			

RNA extraction was performed by Sinapur Kit, Sinaclon Company in (Iran) based on kit protocol. After extraction, the samples were examined based on concentration using nanodrop device (Thermo science company). RNA extraction was synthesized cDNA by Biofact (South Korea) kit. PCR reaction was performed on a volume of 25 (2 Mm of magnesium ion, 0.2 Mm dNTPs, 0.5 Mm of Forward and Reverse primers, 2 µl of 35ng /µl bacterial genome, and one Taq DNA polymerase enzyme 1 unit on 5 µl of cDNA. The PCR program was done for 40 cycles in conditions of initial denaturation at 94°C for 3 minutes, secondary denaturation at 94°C for 35 second, Annealing primers at 51°C for 35, Extension at 72°C for 35 second and the final Extension was used at the 72°C for 5 min. PCR products were electrophoresed on the Agarose gel 2%.

Positive Control

Cloning and positive control preparation

For making positive control, the PCR products for E and M gene were cloned. The product was purified using Sinaclon purification kit (Sinaclon, Iran), and the ligation reaction between *pTZ57R/T* plasmids and the purified *E* and *M* genes were performed according to the working instructions of Sinaclon PCR cloning kit (Sinaclon, Iran), respectively. After preparing competent *E. coli JM107*, the cells were transformed and cultured onto Luria-Bertani medium containing X-gal (40g/ml), IPTG (38.4g/ml), ampicillin (100g/ml) and tetracycline (50g/ml) and incubate overnight at 37°C. The selected colonies received each fragment and confirmed. For final confirmation of the insert receiving clones, enzymatic digestion was done after plasmid extraction by means of Sinaclon plasmid mini Extraction kit (Sinaclon, Iran). The confirmed recombinant plasmids were named pTZ57R/TE and *pTZ57R/TM*, representing plasmids containing *E* and *M* genes respectively (7).

Sensitivity and Limit of Detection (LOD)

To determine the reaction sensitivity, the minimum copy number of the target gene to be able to show a visible bond in the PCR, was calculated. For this reason, 10- fold serial dilutions (10^{-1}-10^{-7}) of the *pTZ57R/TE* and *pTZ57R/TM* plasmids with certain concentration were prepared. After PCR on the serial dilutions of plasmids, the last dilution that showed a visible bond was determined as the limit of detection (LOD) of the method. Finally, the plasmid concentrations of the last dilutions were calculated and converted as the copy number of the respective gene (8).

The PCR product for *E* and *M* genes was sequencing in some samples for confirmation of *SARS-CoV-2* and received an accession number from Gene bank.

Phylogenetic analysis

The PCR Products were purified from the gel by Sinaclon Gel recovery kit (Sinaclon Co., Iran) and PCR purified template sequencing. Sequencing data assembling, editing and analyses were made using CLC main work bench version 6.8.1 homology analysis of 156 and 547bp region was done to identify reference sequence and alignment multiple sequence using Mega X version 10.0.05. The phylogenetic tree was constructed with neighbor- joining method (9).

Results

RT-PCR reaction for E and M genes

The extracted samples were reading the OD rate and concentration examined by nanodrop. Then, after RT-PCR reaction, the results of RT-PCR reaction on *E* and *M* genes were positive and was observed 156 and 547 bp on the gel. Figure 1 shows the bonds associated of these genes amplification.

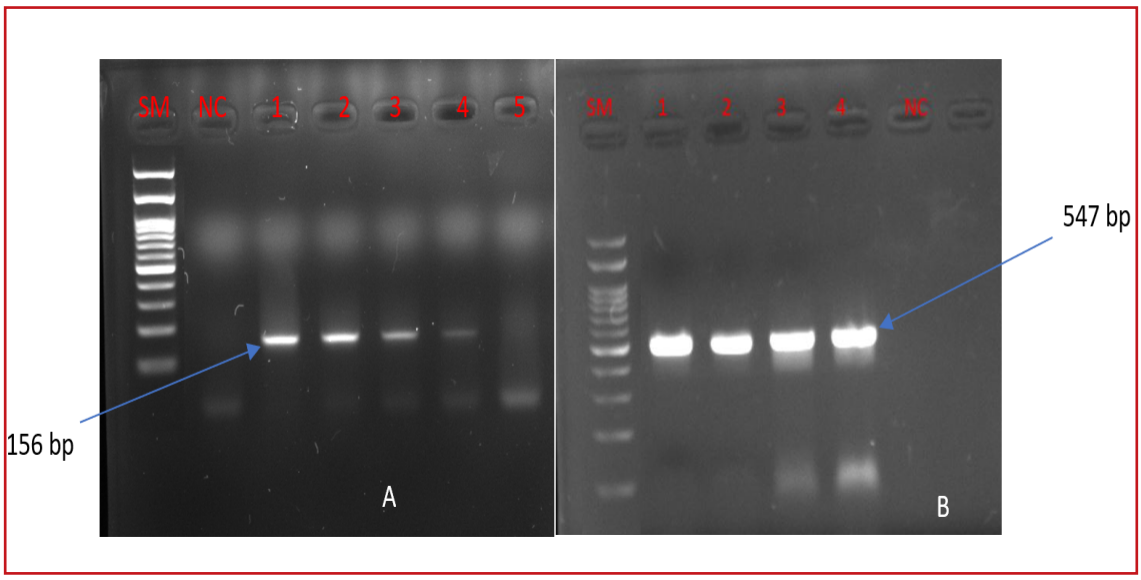


Figure 1. RT-PCR Amplification of *E gene* 156 bp band (A) and *M* gene product 547 bp band (B)

Sensitivity determination results

In sensitivity determination assays, the last dilutions of the *pTZ57R/T-E* and *pTZ57R/T-M,* recombinant plasmid that showed clear bond on agarose gel were10^{-6} and 10^{-5} respectively. The copy number of last detectable for *E* and *M* equals 60 and 82 copy (figure 2).

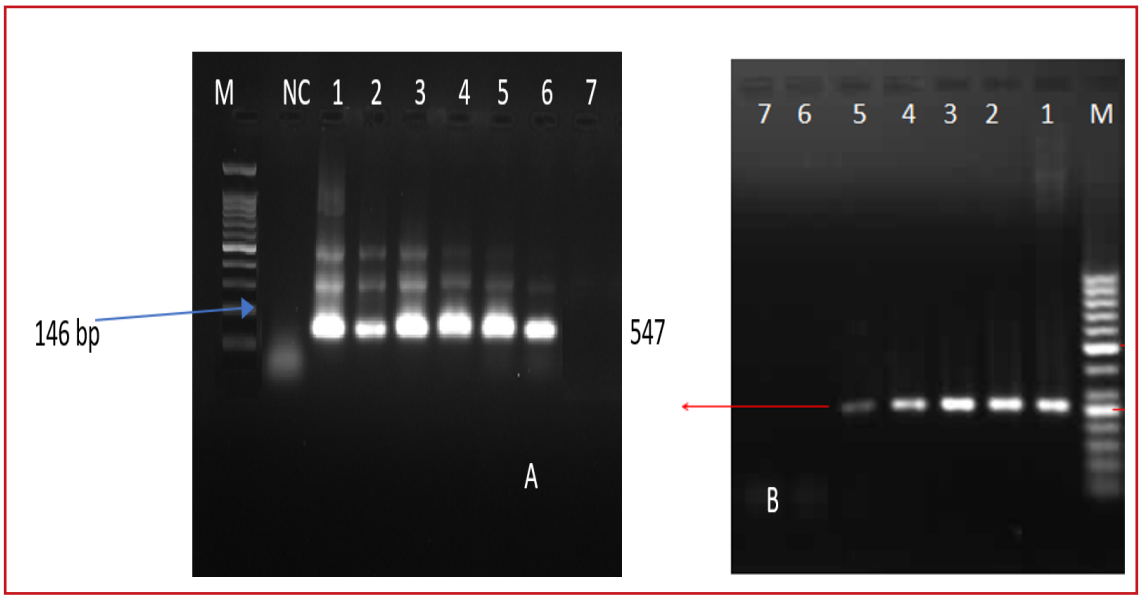


Figure 2. PCR Amplification for E,100bp DNA Ladder,2 PCR product 146 bp Limit of Detction for *E* in detrmined 10^{-6} and PCR Amplification for *M* PCR product 547 bp and Limit of Detction for *M* in detrmined 10^{-5}
NCBI Blast was used to detemine the sequence similarty and homology of the *E* and *M* genes

Results indicated that nucleotide homology among these isolates with these genes were 100%. The more detailed results of phylogenetic finding will be discussed (figure 3, 4).

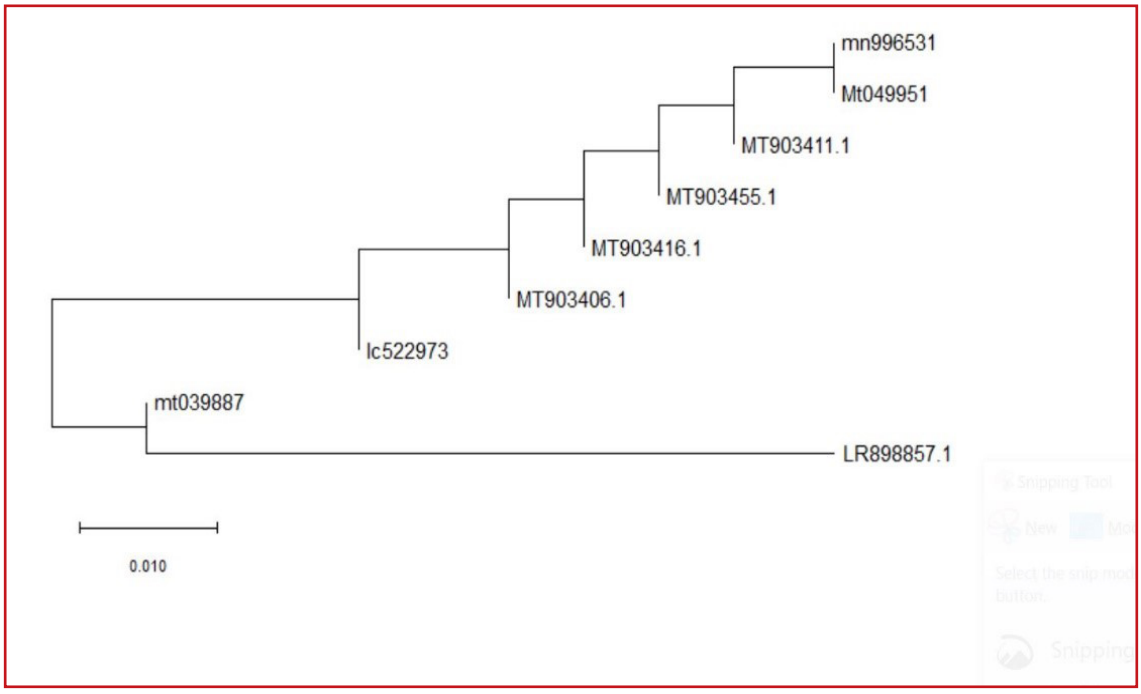


Figure 3. phylogenetic analysis of *E* gene

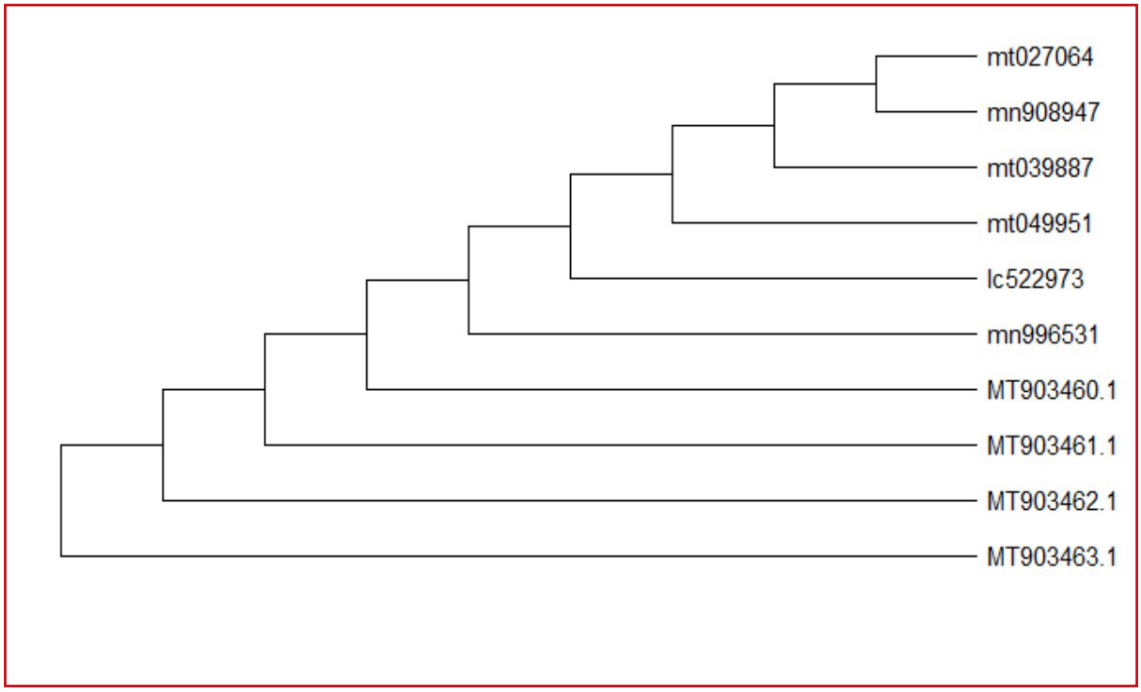


Figure 4. phylogenetic analysis of *M* gene

Database accession number

The sequences are deposited in the Gene Bank with the accession numbers MT903455-MT903411-MT903416-MT903406 for *E* gene and MT903460-MT903461-MT903462-MT903463 for *M* gene.

Discussion

The outbreak of *SARS-CoV-2* globally has emphasized the importance of the laboratory detection of people with coronavirus infection in order to stop the spread as well as to appropriately treat those patients who have a serious infection (9). The purpose of this study to design and optimize rapid detection of *SARS-CoV-2* by molecular method. RT-PCR technique is very simple, appropriate, fast with high sensitivity, high specificity and suitable repeatability for diagnosis of this virus and it can be easily prevented (10). The main concern in this study is designing primers 15 and 18 sequences for *E* and *M* genes which were used from the NCBI database, and then through alignment, the designed primers were used for conserved regions. The main point in designing a multiple RT-PCR method is the possibility of nonspecific products and primer dimers. It is essential to design primers of the same Tm values. In this study compared with other same studies all the declared points are put into action (11) which usually were the cause of the same diseases and did not show any reaction (12). After amplification and optimization of the reaction, the PCR products were cloned in *pTZ57R/T* plasmid and in this study the confirmed plasmids were used as positive controls. The cloned gene in the vector had a stable positive control and possible to take the desired number of plasmids. The sensitivity of the test was determined by limit of detection (LOD) (13). The target genes had been cloned in plasmid and serial dilution was prepared from it to infer the LOD for each gene. Consequently, in a 25 µl RT-PCR assay the LOD was 60 and 82 copies for *E* and *M* genes. 20 swab samples from patient were positive by this homemade set up method. RT-PCR assay could detect fewer genome of virus than other convention technique and diagnosis DNA of Virus from swab sample in a rapid method (14). To our knowledge, this diagnostic assay is set up Hommade diagnostic kit based on RT-PCR for *SARS-CoV-2* infection among pateint. Molecular method based on PCR proved to be an effective detection method for *SARS-CoV-2* with analytic specificity determination and analytic method sensitivity for designing primer (15). Sequences of *E* and *M* genes had a closer relationship with *SARS-CoV-2*. Additional complete genome sequence from *SARS-CoV-2* strains isolated in different parts of the Iran will be helpful in elucidation of epidemiology of the virus, the exact phylogenetic *SARS-CoV-2* strains and subsequently the genetic evolution of the virus. Design and optimization of In-house RT-PCR assay for rapid detection of *SARS-CoV-2* in Iran is a significant step towards success in this field and is thought of as improvement in diagnostic laboratories for precise diagnosis of these viruses.

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Conflict of Interest

The authors declare that no conflict of interests lies among them.

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