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Original Article

Lack of Human Coronaviruses Detection in Samples Collected from Patients with Respiratory Tract Infections during 2014-2015

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Abstract

Background and Aims: Coronaviridae family cause respiratory diseases ranging from common cold to severe Respiratory diseases such as SARS, MERS and new emerging coronavirus disease COVID-19. In addition, the family including four other human coronaviruses (HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43) with flu likes symptoms. Coronaviruses cannot be distinguished clinically from other respiratory infectious agents. Based on the health importance and widespread distribution of respiratory infections, the current study was designed for diagnosis of Pancoronaviruses.

Materials and Methods: Nasopharyngeal swabs from 200 patient suspected viral upper respiratory tract infection analyzed using optimized RT-PCR assay. The constructive specific degenerate primers were used for amplification of rep1b ORF from coronaviruses genome. The 354bp DNA fragment related to 229E coronavirus polymerase gene was amplified from Amplirun Total Respiratory Viral Panel Control (Vircell) template by RT-PCR. Amplified product ligated into T-easy vector (Promega). Plasmid then transformed to Top 10 F' strain by chemical method and Positive colonies were selected using colony PCR with gene specific primers. Diagnostic restriction enzyme digestion was done with EcoRI restriction enzyme. Vector was linearized by SacI restriction enzyme and In-vitro transcription was performed using TranscriptAid T7 High Yield Transcription Kit. DNA was removed with DNase I treatment. Then the detection limit of the specific Rep1b primers was determined by Two-Step RT-PCR with synthetic RNA concentration gradient. All of samples were negative for Pancoronavirus.

Results and Conclusion: All of these samples were negative for Pancoronavirus. Larger sample sizes and proper sampling procedure may improve the chances of viral RNA detection.

Keywords: Coronavirus, human coronaviruses, RT-PCR Optimization, respiratory tract infections

Introduction

The majority of upper respiratory tract infections caused by viruses and (1) often are self-limiting, but common cold is one of the main causes of absenteeism (2). However acute respiratory infections (ARIs) are the leading cause of morbidity and mortality worldwide (3). Coronaviruses are the second most prevalent agents that can cause ARIs (4). Coronaviruses belong to Coronaviridiae family and Nidovirales order which have the largest known RNA genomes (from 26 to 32 kb) (5).

They can infect man, mammals and birds (6). Because of the large genome size and high rates of recombination, Coronaviruses exhibit remarkable genetic diversity and this features allows them to cross species barriers and emerge in new hosts (7). There are seven species of coronavirus known to infect humans including (HCoV)-OC43, HCoV-229E, HCoV-HKU1, and HCoV-NL63 which cause upper respiratory tract infection (URI) with mild symptoms and SARS coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) which can lead to severe acute respiratory diseases with atypical pneumonia and high mortality rate (8, 9). The newest member of human coronavirus emerged in December 2019 in Wuhan, China and spread all over the world. The virus has been named as SARS-CoV-2, and the disease it causes has been named coronavirus disease 2019 (COVID-19) (10). The 5′ two-thirds of the genome contains non-structural protein coding regions that encode polyproteins (pp1a) and pp1ab with polymerase and helicase activity. The 3′-end of genome creating subgenomic RNAs by discontinuous transcription process coding structural and nonessential accessory protein (11). Human coronaviruses are difficult to isolate in cell culture. Common laboratory and diagnostic testing for coronavirus infections are serologic tests and molecular diagnostics methods.

Serologic testing has little clinical value in diagnosis of infection (12) and PCR-based molecular tests such as reverse-transcriptase polymerase chain reaction (RT-PCR), nested RT-PCR, and real-time RT-PCR are preferred. These tests can be easily performed on RNA extracted from respiratory tract samples such as nasopharyngeal swab, sputum, tracheal aspirate or bronchoalveolar lavage (13).

These nucleic acid amplification techniques are very common due to rapid and accurate detection of respiratory viruses (14) and several studies have been done in this context previously.

Leen Vijgen and colleagues (2008) proposed a degenerate primer for amplification a 251-bp fragment of coronaviruses polymerase gene that provides detection of all known and may be unknown human coronaviruses (15). Iran geographically is located near the Middle East respiratory syndrome coronavirus outbreak in Asia, which increases the risk of virus transmission in the country. In this study the mentioned method was used to investigate the possible presence of previous Coronavirus infection during the years of 2014-2015.

Methods

Clinical specimens. Nasopharyngeal swabs from 250 patient suspected viral upper respiratory tract infection were obtained from Department of Influenza and other respiratory viruses, Pasteur Institute of Iran from spring 2014 to fall 2015.

Clinical specimens were influenza negative confirmed by WHO approved Real-Time Reverse Transcription-PCR. Samples were transported in viral transport medium containing DMEM, PenStrep and Amphotericine B and stored at -70 º C before analysis.

Viral RNA extraction and cDNA synthesis. RNA was extracted from the collected specimens using High Pure viral nucleic acid kit (Roche) according to the manufacture protocol.

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Amplification of the target gene was carried out reverse transcriptase-polymerase chain reactions (RT-PCRs) in two steps; cDNA synthesis and PCR.

cDNA synthesis process was performed according to Sinaclon First-strand cDNA synthesis kit in a 20-µL reaction volume containing 4 µL of RNA template, 1 µL random hexamers 100 µM, 2 µL 10× RT buffer, 2 µL dNTP 10Mm, 1 µL M-MuLV Reverse transcriptase (10 U/ml), 0.5 µL Ribolock and 8.5 µL deionized water.

The Reactions were incubated at 25°C for 10 minutes, 37 °C for 60 minutes and 95°C for 2 minutes.

Primers. Degenerate primers for detecting Pancoronaviruses were: forward 5′-ACWCA-RHTVAAYYTNAARTAYGC-3′, reverse 5′-TCRCAYTTDGGRTARTCCCA-3′ (15).

Specific primer sequences for Rep1b HCoV-229E gene were: forward 5′- TATTAGT-TATGAGGAACAAGATGC-3′ and reverse 5′- ATAGCCGACAACATACGAATC-3′.

Internal RNA extraction control primer (RNase P) were: forward 5′-AGATTTGGACCTGCG-AGCG-3′ and Revers 5′-TTCTGACCTGA-AGGCTCTG-3′

PCR conditions. The PCR was performed in 25-µl reaction volume containing 12.5 µl PCR Ampliqon 2x master mix, 1 µl Forward primer 100µM , 1 µl Reverse primer 100µM, 0.3 µl Taq DNA Polymerase, 100 ng of cDNA in a 5 µl, 5.5 µl DW, applying the following Amplification cycles: 95°C for 5 min, followed by 35 cycles of amplification (95°C for 5 s, 57°C for 30 s and 72°C for 30 s) and terminated with an extension step at 72˚C for another 10 min. The RT-PCR products were detected by electrophoresis besides the positive and negative controls in a 2% agarose gel electrophoresis. Amplirun Total Respiratory Viral Panel Control (SWAB) Vircell was used as the positive controls. RNase P gene primer were used as an internal positive control to monitor inhibitors of the PCR reaction. Samples that amplify 251-bp fragment of coronaviruses polymerase gene and the internal control were should be considered positive.

In vitro transcription of 229E Rep1b-354 was performed using TranscriptAid T7 High Yield Transcription kit and continued with DNase I treatment to remove the template. RNA transcripts were purified and quantified before used in standardization.

In-vitro transcribed RNA controls. Because of the low copy number of corona virus copies in the control panel, pGEM-T Easy Vector (Promega) recombinant plasmid containing 354 bp fragment of Rep1b HCoV-229E gene were constructed as standardized positive control. The desired fragments multiplied by primers previously mentioned which the product includes the target sequence for Pan-coronavirus diagnostic primers using QIAGEN One-Step RT-PCR Kit and then extracted from agarose gel by Bioneer's AccuPrep® Gel Purification Kit. Then 354 bp fragment were cloned into T Easy Vector using the Fermentas cloning kit. E. coli TOP10 (ThermoFisher Scientific) used as a bacterial host for plasmid multiplication and purification of recombinant was performed using FavorPrep™ Plasmid Extraction Kit. Clones were screened by PCR using 229E-354 F and 229E-354 R-primers, and enzyme digestion. Serial dilutions of linearized recombinant plasmid (1 ng/µl) were prepared by 10-fold increment dilutions up to 10–7 ng/µL. Invitro transcrip-tion and RT-PCR was performed on serial dilution of synthetic RNA.

Result

In the present study, the detection limit of the specific Rep1b primers was determined by Two-Step RT-PCR with synthetic RNA concentration gradient. All PCR reactions were negative which indicates complete removal of the plasmid from the RNA synthesis reaction and Two Step RT-PCR reactions were positive (Figure 1). As the same way, determining detection limit of the Pancoronavirus diag-nostic degenerate primers was determined 10-3 ng/µL (Figure 2).

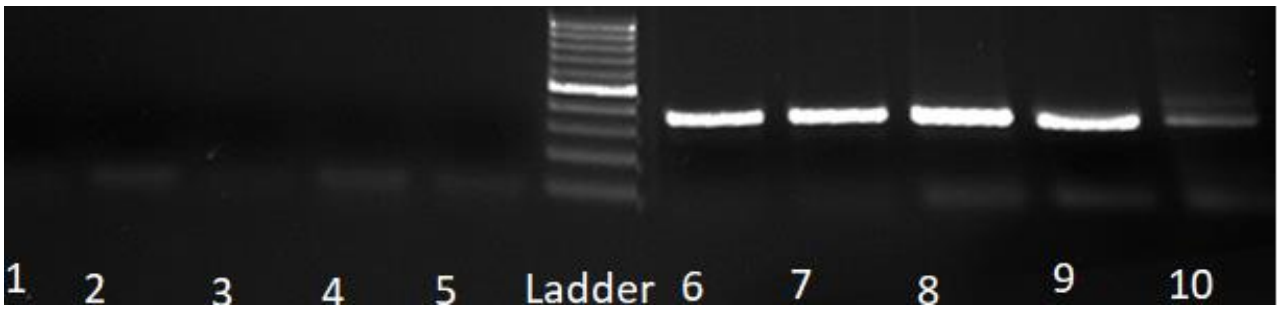

Fig. 1. PCR and RT-PCR results on serial dilution of synthetic RNA. Lane 1-5: PCR results on serial dilution of synthetic RNA (10^{-1} -10^{-5}), Lane 6-10: Two step RT-PCR results on serial dilution of synthetic RNA (10^{-1} -10^{-5}).

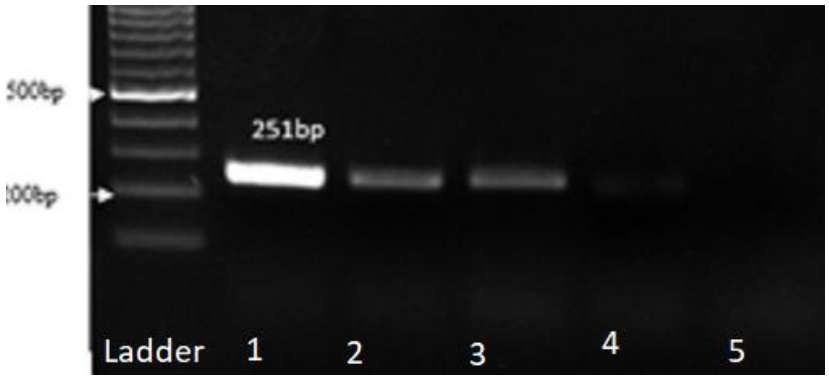

Fig. 2. RT-PCR results serial dilution of synthetic RNA using degenerate primers. Lane 1-4: serial dilution of synthetic RNA (10^{-1} -10^{-4}), Lane 5: Negative control.

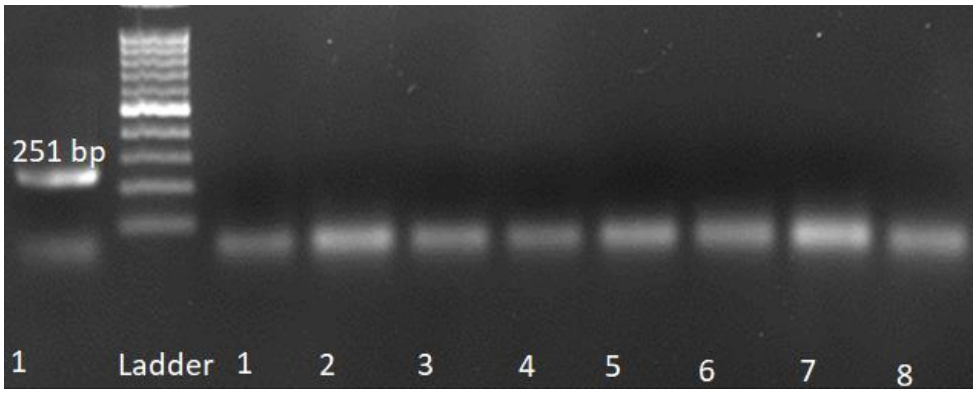

Fig. 3. Gel electrophoresis RT-PCR amplification pattern of clinical samples. Lane 1 positive control, Lanes 1 to 8 clinical samples.

Then we investigated 250 nasopharyngeal swabs from 250 patients with the diagnosis of acute upper respiratory tract infection. All procedure from RNA extraction and positive controls were performed carefully and confirm with amplification of RNase P gene. Then we investigate all 250 samples using degenerate primers for detecting Pancoronaviruses and specific primer sequence for Rep1b HCoV-229E gene, all examined samples were negative for human coronaviruses (Figure 3).

Discussion

Human coronaviruses can cause acute respiratory tract infection in children and adults. The prevalence of HCoVs has been reported 3-16% which tend to rise in the winter months in temperate climates (16, 17) and this may be affected by seasonal variation and region and also population sampled in various studies (17). Previous studies show that most

coronaviruses don't circulate continuously every year and some types of virus outbakes occur every 2-4 years (18). HCoV-229E infection is usually found in immunocompromised persons and negative result are more expected in general population which have been investigated in this study (19).

Generally, lower respiratory tract samples such as sputum or bronchoalveolar lavage fluid samples compared to upper respiratory tract specimens including nasopharyngeal or throat swabs have higher viral load for molecular virus detection methods such as PCR (20).

In this study, failure to detect the virus was probably due to low level circulation of coronaviruses during the study period or relatively small sample size considering the overall variable prevalence of HCoVs. It can be concluded that larger studies may be producing more reliable results which increase the likelihood of detecting virus.

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Authors' Contribution

Hoorieh Soleimanjahi and Fatemeh Fotouhi have designed the present study. Somayeh Alipour has done the experiments and written the article draft, and Hoorieh Soleimanjahi has edited the article. Somayeh Alipour and Zohreh Farahmand have been responsible for collecting the data and laboratory work.

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