Research Article

Development of Dual TaqMan Based One-Step rRT-PCR Assay Panel for Rapid and Accurate Diagnostic Test of MERS-CoV: A Novel Human Coronavirus, Ahead of Hajj Pilgrimage

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Abstract

Background: Coronaviruses (CoVs) are large ribonucleic acid (RNA) viruses causing primarily respiratory disease in humans. A novel human coronavirus, subsequently named middle east respiratory syndrome coronavirus (MERS-CoV), was first reported in Saudi Arabia in September of 2012. With increasing numbers of infections and deaths from MERS-CoV, development of a rapid and reliable kit was crucial to prevent further spread of MERS-CoV.

Objectives: In this study, we present two real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays for in-house rapid and sensitive diagnostic testing of MERS-CoV, detecting the regions upstream of the envelope gene (upE) and open reading frame (ORF)1b, respectively, for initial screening and final confirmation of MERS-CoV infection, as recommended by the world health organization (WHO).

Materials and Methods: In this experimental study, acquiring patient samples was difficult; thus, according to WHO recommendations and standard protocols, we synthesized RNA sequences of upE and ORF1b genes as the template signatures and TaqMan based-diagnostic rRT-PCR assays were carried out using these synthetic genes for detection of MERS-CoV. In this research, we also inaugurated a cell-free system to transcribe these RNA sequences using the DNA templates synthesized.

Results: The upE and ORF1b based one-step rRT-PCR assays were optimized by testing several times via different synthetic RNAs, and validation results were highly successful. The sensitivity obtained for upE was fewer than ten copies of RNA template per reaction and for ORF1b was 50 or fewer copies per reaction.

Conclusions: This study showed that the developed rRT-PCR assays are rapid, reliable, reproducible, specific, sensitive, and simple tools for detection of MERS-CoV. Finally, a kit consisting of two assay signatures and controls was assembled, which can be distributed to public health laboratories in Iran to support international MERS-CoV surveillance and public health response.

Keywords: Hajj Pilgrimage, MERS-CoV, Diagnosis, Real-Time RT-PCR, upE, ORF1b

1. Background

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense ribonucleic acid (RNA) viruses (1). They are widespread and can be found in many species of mice, horses, whales, birds, cats, dogs, pigs, and humans (1). Development of the infection, can lead to additional complications, including respiratory tract disease and organ dysfunction, particularly renal failure and immune suppression, enteric, neurologic or hepatic diseases (2, 3). The majority of patients have typical symptoms, such as fever with or without cough, and breathing difficulties (4). Middle east respiratory syndrome coronavirus (MERS-CoV) is a new and unknown origin respiratory virus with genotypic and phenotypic diversity; thus, this virus can mutate, increasing its virulence and even causing tissue tropism. There is a high-frequency mortality rate around > 50% and median age of the majority of identified cases affected with this virus is 56 years. Very little is known about its behavior (5, 6). Humans are known to maintain circulation of four different human coronaviruses (hCoVs) at a global population level. These are a part of the spectrum of agents that cause the common cold. The severe acute respiratory syndrome (SARS) CoV constitutes the fifth hCoV, which was

in circulation for a limited time during 2002 and 2003, when a virus appeared in humans and caused an outbreak affecting at least 8,000 people. Symptoms matched the clinical picture of acute primary viral pneumonia. MERS-CoV, a novel coronavirus, was detected for the first time in September of 2012 in Saudi Arabia, an area heavily impacted by this virus at present. Between April and June 2013, 81 cases of infection by this hCoV were reported in Saudi Arabia. Of those, 49 patients died, so fatality rate is high (2). This virus (MERS-CoV) causes severe acute respiratory infection in humans (7). Subsequently MERS-CoV has been reported in other countries, including Tunisia, United Arab, Emirates, Italy, United Kingdom, Germany, France, and Qatar (2, 8-11). The first cases of MERS-CoV infections reported in Iran were two cases in Kerman, a city in southeast Iran. The virus killed one of them, a 53-year-old woman. With the appearance of new cases, in view of the risk of MERS-CoV transmission to humans (6), and because of severe infections that have been observed among the elderly, there is considerable concern about this virus (5). Although few cases have been reported annually (around 34 case as of 12 May 2013), the morbidity and mortality rate of this infection is alarming (7). Unfortunately, at present, there are no specific treatments or effective drugs for this deadly disease, and no vaccine. In the absence of an effective treatment, the appropriate infection controls include rapid diagnostics and isolation of patients, useful strategies for preventing further transmission and spread of this infectious agent (5). Currently, real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays are used for detection of MERS-CoV in respiratory, blood, and stool samples of patients. Real-time RT-PCR assay is highly sensitive and is able to detect viruses even in low copy numbers (12, 13).

2. Objectives

Our aims in this research were to present two rRT-PCR assays for in-house rapid and sensitive diagnostic testing of MERS-CoV, detecting the regions upstream of the envelope gene (upE) and open reading frame (ORF) 1b, respectively, for initial screening and final confirmation of MERS-CoV infection (according to world health organization (WHO) recommendations).

3. Materials and Methods

3.1. Templates for Design of Assays

In this experimental study, the sequences, obtained from the National center for biotechnology information (NCBI; GenBank accession number: JX869059 for the Rotterdam virus isolate, termed hCoV-EMC), were synthesized (Eurofins Genomics, Germany) and served as templates for assay design. The DNA templates from upE and ORF1b of novel human coronavirus were amplified and confirmed using the corresponding real-time PCR specific probes and primers (forward and reverse) of each signature.

3.2. Inauguration of a Cell-Free System for Transcription of Synthetic DNA Templates and Preparation of RNA Transcripts as Viral Templates

Target regions upE and ORF1b, synthesized by Eurofins Genomics (Germany) in the same vector (pGH cloning vector) were subcloned into the pSPT18 (Roche) expression vector using the HindIII restriction site (results not presented) and subsequently were transcribed into RNA for preparation of viral templates using the SP6/T7 Transcription kit (Roche) to synthesize both single-stranded RNAs. At all steps, contamination with RNase must be avoided through the usual diethylpyrocarbonate (DEPC) treatments, the use of disposable plasticware, wearing gloves, etc. After DNase I digestion, both RNA transcripts were purified using Roche High Pure Viral RNA Kit and quantified by ultraviolet (UV) light spectroscopy. No DNA content remained at this stage due to its removal, and results of common real-time PCR assays were negative (results not presented), whereas the results of real-time reverse-transcription polymerase chain reaction (rRT-PCR) assays were positive.

3.3. Real-Time RT-PCR Assay Panel Step 1: Suitable for Initial Screening of MERS-CoV Infection by Detection of Upstream Region of the Envelope (upE) Gene

The novel in-house one-step rRT-PCR assay was developed using the QuantiFast RT-PCR kit (Qiagen, Hilden, Germany) on Corbet (Rotor-Gene) 6000 (Qiagen, Germany) real-time PCR instrument. Each 25 μL reaction mixture contained 12.5 μL of 2 $\times$ Master Mix, 1 μL of reverse transcriptase/Taq DNA polymerase mixture, 0.4 mM of each dNTP and 3.2 mM magnesium sulfate, 5 μL of RNA, 400 nM concentrations of upE forward primer (GCAACGCGC-GATTCAGTT) and upE reverse primer (GCCTCTACACGGGAC-CCATA), and 200 nM of upE probe (6-carboxyfluorescein [FAM]-CTCTTCACATAATCGCCCCGAGCTCG-6-carboxy-N, N, N, N'-tetramethylrhodamine [TAMRA]). Thermal cycling included 55°C for 20 minutes, followed by 95°C for 3 minutes and then 45 cycles of 95°C for 15 s and 58°C for 30 seconds. It should be mentioned that common one-step real-time RT-PCR kits formulated for application with probes should all provide satisfactory results with default reaction mix compositions as suggested by their manufacturers.

3.4. Real-Time RT-PCR Assay Panel Step 2: Suitable for Final Confirmation of MERS-CoV Infection by Detection of Open Reading Frame (ORF) 1b Gene

This confirmatory assay had the same conditions as described above for the upE rRT-PCR, albeit the primer and probe sequences were different. This assay was performed with ORF1b forward primer (TTCGATGTTGAGGGT-GCTCAT) and reverse primer (TCACACCAGTTGAAAATCC-TAATTG), as well as its specific probe (6-carboxyfluorescein [FAM]-CCCGTAATGCATGTGGCACCAATGT-6-carboxy-N, N, N, N' tetramethylrhodamine [TAMRA]). This target gene did not overlap with those of known pan-CoV assays (2, 6, 7).

3.5. In Vitro Synthesis of RNA Controls (Standard RNAs)

As noted above, two transcribed candidate signatures (target regions) upE and ORF1b, were used in both assays. Because of the difficulties in acquiring patient samples, according to WHO recommendation, these two RNA sequences were also synthesized (Eurofins Genomics, Germany) and used as RNA controls (standard RNAs) in optimization of the two assays and for preparation of serial dilutions for the evaluation of sensitivity. All RNA dilutions were prepared in RNase-free water.

3.6. Evaluation of Analytical Sensitivity and Specificity of Assays

3.6.1. Analytical Sensitivity

A) Limit of detection - MERS-CoV upE gene: serial dilutions of quantified standard upE RNAs (10, 100, 1000, 10000) were prepared in 10 mM TE buffer and tested by upE assay signature in 25 replicates. The highest dilution of upE RNAs at which all replicates were positive was defined as the limit of detection (LoD) for the upE assay.

B) Limit of detection - MERS-CoV ORF1b gene: sensitivity evaluation was optimized by testing candidate probes and primers in the ORF1b signature assay with serial dilutions (5, 50, 500, 5000) of MERS-CoV quantified standard ORF1b RNAs in separate reactions and comparing them. Ten-fold serial dilutions of MERS-CoV ORF1b RNAs were prepared in buffer as above and tested in 25 replicates. The highest dilution of ORF1b RNAs at which all replicates were positive was defined as the limit of detection (LoD) for the ORF1b assay. A volume of 5 μL was used in two real-time RT-PCR assays of upE and ORF1b.

3.6.2. Analytical Specificity

Reactivity with different MERS-CoV strains (in silico prediction): In addition to demonstrating reactivity of the rRT-PCR assay with the MERS-CoV strain Jordan-N3/NCV, primer/probe sequences were evaluated against an additional eight recently published genome sequences from seven patients collected from June 2012 to May 2013: GenBank accession numbers JX869059, KC776174, KC164505, KC667074, HPA Website (http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_-C/1317138176202), KF192507, KF186564-KF186567. Primer/probe sequences for all signatures were 100% identical to all published virus strains (results not presented).

4. Results

4.1. Confirmation of Synthesized DNA Template of upE and ORF1b

The DNA templates synthesized from upE and ORF1b of novel human coronavirus were amplified and confirmed using 2% gel-based traditional PCR (Figure 1) and common real-time PCR with the corresponding specific probes and primers (forward and reverse) of each signature (Figures 2 and 3).

4.2. Real-Time RT-PCR Assay Panel Step 1: Suitable for Initial Screening of MERS-CoV Infection by Detection of Upstream Region of the Envelope (upE) RNA

This confirmatory one-step rRT-PCR assay was performed for synthetic standard RNA containing upE signature using specific probe and primers (Figure 4). This test can initially screen for MERS-CoV infection bebecause it is a highly sensitive assay. The results of RNA transcripts (containing upE signature) were confirmatory, and were also similar to those of synthetic standard RNAs (results not presented here).

4.3. Real-Time RT-PCR Assay Panel Step 2: Suitable for Final Confirmation of MERS-CoV Infection by Detection of Open Reading Frame (ORF) 1b RNA

This confirmatory one-step rRT-PCR assay was performed for synthetic standard RNA containing ORF1b signature using specific probe and primers (Figure 5). This test can conclusively detect MERS-CoV infection bebecause it is a highly specific assay. The results of RNA transcripts (containing ORF1b signature) were confirmatory, and were also similar to those of synthetic standard RNAs (results not presented here).

4.4. Evaluation of Analytical Sensitivity of Assays

We prepared serial dilutions of standard RNAs for evaluation of analytical sensitivity of assays (Figure 6). As shown in Tables 1 and 2, the stages of sensitivity (minimum detection limit) determination for each of these genes were done separately and the real-time RT-PCR assays showed different sensitivities for upE and ORF1b target regions.

Figure 1. Electrophoresis Analysis of upE and ORF1b Regions Amplified by 2% Gel-Based Traditional PCR
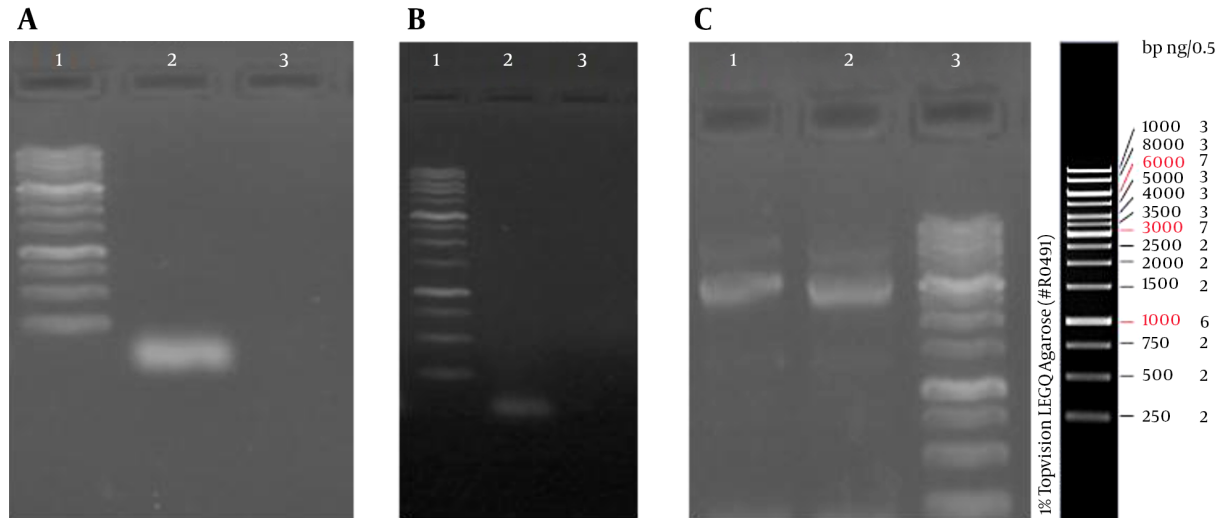
A, lane 1, 1 Kb DNA ladder (Fermentas); lane 2, upE amplicon with a length of ~ 90 bp; lane 3, negative control; B, Lane 1, 1 Kb DNA ladder (Fermentas); lane 2, ORF1b amplicon with a length of ~ 80 bp; lane 3, negative control; C, lanes 1 and 2, synthesized DNA template (plasmid) containing upE and ORF1b regions; lane 3, 1 Kb DNA Ladder (Fermentas).
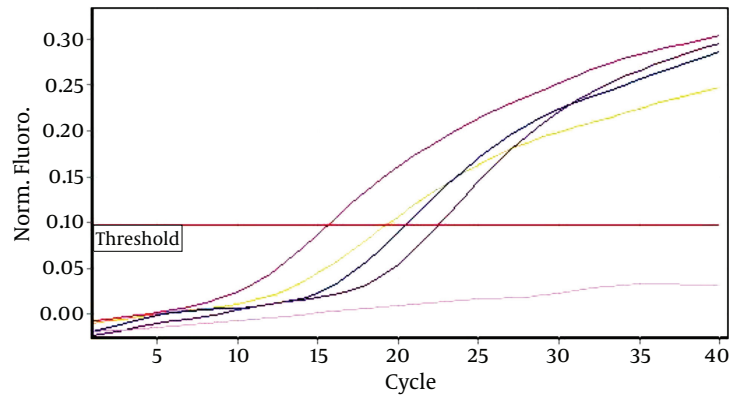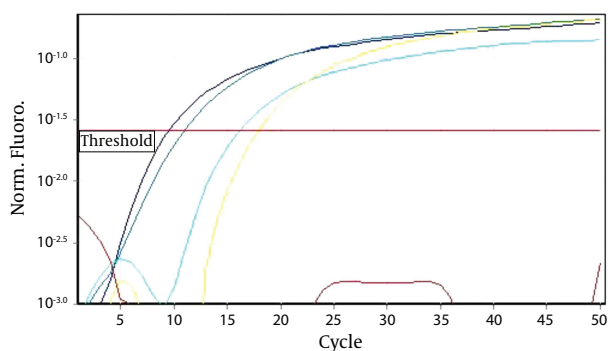

Figure 2. Confirmatory Common Real-Time PCR Using the Corresponding Specific Probe and Primers (Forward and Reverse) of the upE Signature Assay for Several DNA Template Samples
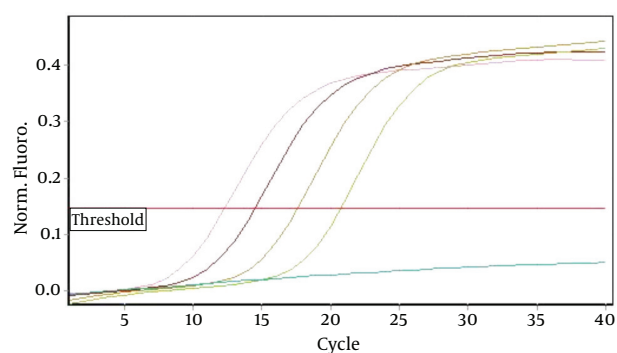
Table 1. MERS-CoV rRT-PCR Assay Limit of Detection for upE Gene[a]

Serial Dilution (Copies/Reaction)	Positive Tests upE Region, %
10	100
100	100
1000	100
10000	100

[a]The highest dilution at which 100% of rRT-PCR replicates were positive is underlined.

Table 2. MERS-CoV rRT-PCR Assay Limit of Detection for ORF1b Gene[a]

Serial Dilution (Copies/Reaction)	Positive Tests upE Region, %
5	10
50	100
500	100
5000	100

[a]The highest dilution at which 100% of rRT-PCR replicates were positive is underlined.

Figure 3. Confirmatory Common Real-Time PCR Using the Corresponding Specific Probe and Primers (Forward and Reverse) of the ORF1b Signature Assay for Several DNA Template Samples
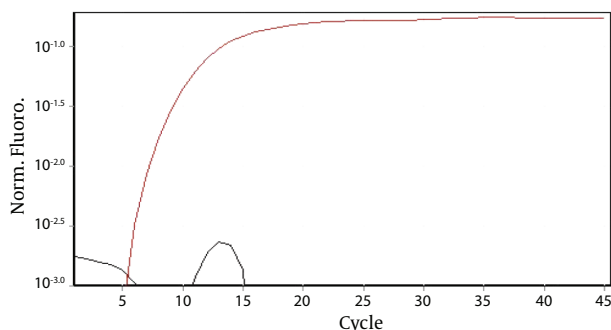

Figure 4. Confirmatory One-Step Real-Time RT-PCR Assay for One Standard RNA Sample Containing upE Signature Using Specific Probe and Primers
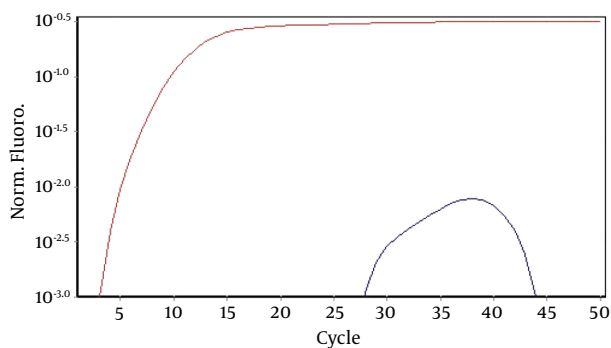

Figure 5. Confirmatory One-Step Real-Time RT-PCR Assay for One Standard RNA Sample Containing ORF1b Signature Using Specific Probe and Primers

Figure 6. Representative Amplification Plot of Developed rRT-PCR Assay, Showing Serial Dilutions of Standard RNAs for Evaluation of Analytical Sensitivity of Assays

A) Limit of detection (LoD) of the MERS-CoV upE gene: The serial dilutions containing 10, 100, 1000, and 10000 copies of standard RNAs per reaction were analyzed using the real-time RT-PCR assay. The highest dilution of upE RNAs at which all replicates were positive was defined as the limit of detection (LoD) for the upE assay. The minimum detection limit of the assay signature was fewer than ten copies of standard RNAs per reaction (Table 1), as the higher dilutions were not evaluated.

B) Limit of detection (LoD) of the MERS-CoV ORF1b gene: The serial dilutions containing 5, 50, 500, and 5000 copies of standard RNAs per reaction were analyzed using the real-time RT-PCR assay. The highest dilution of ORF1b RNAs at which all replicates were positive was defined as the limit of detection (LoD) for the ORF1b assay. The minimum detection limit of the assay signature was 50 or fewer copies of standard RNAs per reaction (Table 2), as the dilutions between 5 and 50 copies of standard RNAs per reaction were not evaluated.

5. Discussion

Since the human coronavirus (hCoV) termed SARS-CoV caused fatalities in Southeast Asia during 2002 and 2003, spread of another CoV, termed MERS-CoV, has caused considerable concern in the international community. As Saudi Arabia is the main center for the spread of MERS, it has a further importance in Islamic countries, where large numbers of people travel to Saudi Arabia for the Hajj pilgrimage. Annually, millions of world Muslims travel to Saudi Arabia in order to perform Hajj rituals and amassing of people can result in the spread of infection and disease transmission into different countries. Unfortunately, at present there is no specific treatment, such as an effective drug, and no vaccine for this deadly infection. In

absence of an effective treatment, the appropriate infection controls include rapid diagnostics and isolation of patients, useful strategies for preventing further transmission and the spread of this infectious agent (5). Development of a rapid and accurate diagnostic kit can facilitate early-stage detection of infected individuals with respiratory symptoms who have returned from the Hajj and are re-entering the country, preventing the spread of infection. The best methods for detection of MERS-CoV, recommended by WHO, are real-time RT-PCR assays, for upE gene as the initial screening of disease and for ORF1b (or ORF1a) gene as the final confirmation of MERS-CoV infection. It is notable that traditional (gel-based) RT-PCR methods require amplification in a thermo cycler (after cDNA preparation via RT process) and product analysis by electrophoresis gel, both of which are time-consuming and laborious (14). By eliminating the need for post-amplification product processing, the real-time RT-PCR method enables shortened turn-around times for reporting results, which is critical for determining isolation for suspected case patients and contact tracing. Real-time RT-PCR assays are sensitive, rapid, and can be automated. They also have high reproducibility and since the need for post-PCR processing is eliminated, they can prevent carry-over contamination (14). TaqMan probe-based real-time RT-PCR assays not only provide confidence in identification of target genes due to specific probing, but also reduce the risk of laboratory product contamination, because the amplification reaction and detection of PCR products are performed in a single tube (15). Despite these advantages of rRT-PCR assays, these techniques are two-step. Step 1 is reverse transcription (RT) of RNA for cDNA preparation, and step 2 is the real-time PCR. In this study, we developed a novel one-step rRT-PCR assay, based on specific TaqMan probes, instead of traditional two-step rRT-PCR assays, enabling both steps to be accomplished in a single step. This increases the sensitivity of the assays and is affordable economically, in addition to the other advantages mentioned above.

Since, acquiring patient samples and the complete viral genome is difficult, according to WHO recommendations and standard protocols, we instead synthesized RNA sequences of upE and ORF1b genes as the template signatures, and the TaqMan-based diagnostic rRT-PCR assays were carried out using these synthetic genes for detection of MERS-CoV. In this research, we also inaugurated a cell-free system to transcribe these RNAs using the synthesized DNA templates. As previously shown, the stages of sensitivity determination for each of these genes were done separately and the real-time RT-PCR assays showed different sensitivities for upE and ORF1b target regions. It is notable that the ORF1b assay has lower sensitivity than the upE assay.

5.1. Conclusion

This study showed that both the developed novel one-step rRT-PCR assays based on specific TaqMan probes are rapid, reliable, reproducible, specific, sensitive, and simple molecular methods for detection of MERS-CoV. Finally, the kit consisting of two assay signatures and controls was assembled, and it can be distributed to public health laboratories in Iran to support international MERS-CoV surveillance and public health response.

Acknowledgments

Footnotes

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