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ORIGINAL ARTICLES

Development of quantitative real-time RT-PCR assay for detection and viral load determination of Crimean-Congo Hemorrhagic Fever (CCHF) virus

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Abstract: *Background and Aim: The CCHF (Crimean-Congo hemorrhagic fever) virus causes a severe disease in human with a case fatality rate of up to 50%. Since, there is no specific treatment or approved vaccine against CCHF viral infections, an accurate and early detection as well as a reliable surveillance and quantitative determination of viral load is necessary for patient improvement and case management. In this research, our aim was to develop a probe based one-step real-time reverse-transcription polymerase chain reaction (rRT-PCR) assay for in-house quantitative detection of CCHF virus.*

Methods: At first, the highly conserved S-fragment sequence of CCHF virus genome was adapted from GenBank and the specific probe and primers targeting this region were designed. Then, viral RNAs were extracted from 37 blood samples of different patients from east of Iran (Zahedan). The specificity and sensitivity of the probe and primers were also evaluated in positive blood samples, confirmed to have CCHF virus. A standard (PTG19-T vector containing S-fragment) for quantization was also constructed and the viral load was determined in some of positive samples.

Results: From a total of 37 suspicious blood samples, 15 samples were confirmed to be positive for CCHF virus by this probe based one-step rRT-PCR assay and no false-positive result was detected according to sequencing data. The predicted fragment of 176 bp was also confirmed in all positive samples by gel-based electrophoresis analysis. The assay was linear between 10 to 103 copy numbers per each microliter of extracted plasmid for this technique and the viral load determined in one of patient blood samples was 55,000 viral particles per each milliliter, for example. Bioinformatics and experimental evaluations approved the specificity of this assay. The LOD of the assay was 10 (or fewer) copy numbers of viral genome per each microliter of the extracted genome.

Conclusions: This research showed that the developed probe based one-step rRT-PCR assay is a specific, rapid, sensitive and the simple tool for detection and viral load determination of the CCHF virus.

Keywords: *CCHF Virus, Real-time RT-PCR, Quantitative, viral load, S-region*

INTRODUCTION

The CCHF (Crimean-Congo hemorrhagic fever) is a widespread disease caused by Nairovirus (a tick-borne virus) of the Bunyaviridae family. The CCHF virus causes a severe disease (a zoonotic viral hemorrhagic fever) in human and is endemic in Africa, the Balkans, Asia, and Europe [1-6]. In Iran, this virus has also caused serious challenges in the Sistan-va-Baluchestan province [7-15]. The fatality rate is about 10-50% [16, 17]. Since, there is no specific treatment or approved vaccine against CCHF viral infections, an accurate and early detection as well as a reliable surveillance and quantitative determination of viral load is necessary for case management, patient improvement, and protection of medical staffs.

Several different laboratory assays can be used for CCHFV diagnosis, including enzyme-linked immune sorbent assay (ELISA), serum neutralization, antigen detection, virus

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isolation by cell culture and reverse-transcriptase polymerase chain reaction (RT-PCR) assay [4, 18-21]. CCHFV RNA from different clinical samples is a proper diagnosis target during the acute phase of the infection or even before the beginning of disease when detection of antibody is untrustable or impossible [22-24].

Unlike the traditional two-step RT-PCR technique, the one-step real-time RT-PCR (rRT-PCR) assay does not need to pre-PCR (post-RT) sample handling, avoiding the PCR product dependent probable contamination transmission and resulting in more sensitive, rapid and also higher efficient assays. On the other hand, the probe based rRT-PCR assays have a very high specificity in addition to the mentioned features. Therefore, it has become an accepted recognition method for detecting many microbes [25]. However, in this research we developed a quantitative probe based one-step rRT-PCR assay for detection and viral load determination of the CCHF virus in suspected blood samples of the Sistan-va-Baluchestan prov¬ince of Iran. The aim of this experimental study was to develop a probe based one-step rRT-PCR assay for in-house quantitative detection of the CCHF virus.

METHODS

Collection of sample

To perform this study, we provided 37 blood samples of different CCHF suspicious patients from the Zahedan city in the southeast of Iran. A few samples had already been confirmed to be positive for CCHF viral RNA existence by rRT-PCR analysis (Pasteur Institute, Tehran, Iran) that were also used as the positive control and they had been stored at -70°C.

Extraction of viral genome

Viral genomes were extracted from 0.2 ml of blood samples using the High Pure Viral RNA Kit (Roche-Germany) in 30 minutes, according to manufacturer's instructions. Then, each one of the extracted genomes were dissolved in 50 µl of RNase free water and stored at -70°C until rRT-PCR analysis (of which only 5 µl was required in each assay). It is noteworthy that all of manipulations were performed in a biological hood with class II biosafety.

Designing and synthesizing the specific probe and primers

The nucleotide sequence of S-region of the available CCHFV strains was adapted from the NCBI database (GenBank) and the highly conserved sequences in this region were determinated and specified from the alignment data analysis obtained from MEGA 7 software. Then the specific probe and primers targeting this region were designed using the Oligo software, version 7.0 (Molecular Biology Insights, Cascade, CO, USA) and GeneRunner (Hastings Software, Hastings, NY, USA).

The probe sequence designed for S-region internal sequence was FAM-5'-CAA AAC AGG ATC TAC ATG CAC CCT GCC-3'-BHQ as well as the forward and reverse oligonucleotide primers for S-region internal sequence were 5'-TGG GGK AAG AAR CTY TAT GAG CT-3' and 5'-ATG GAC TTG GTR TGY CCA GAT CC-3', respectively. K, R and Y are the wobble bases. Eventually, the probe and primers were synthesized by the Bioneer Company (Korea).

One-step real-time RT-PCR assay

The probe based one-step rRT-PCR assays were developed by use of QuantiTect® Probe RT-PCR Kit (Qiagen, Hilden, Germany) using both the Applied Biosystem (ABI) 7500 (USA) and Corbet (Rotor-Gene) 6000 (Germany) real-time PCR instruments. The amplification process was performed in 60 minutes within 20 µl reaction mixtures including 5 µl of the viral RNA template, 1 µl (10 pmol) of both primers (0.5 µM in the reaction) and 0.5 µl of the probe (0.25 µM in the reaction). The conditions of real-time cycler were as follows: two single steps of 30 minutes at 50°C (required for reverse transcriptase activity) and 15 minutes at 95°C (required for HotStarTaq DNA polymerase activation); followed by 50 cycles of 15 seconds at 94°C; and 60 seconds at 60°C (collecting the fluorescence data was accomplished at the end of each 60°C step). So, the real-time PCR products were detected trough an increase in the fluorescence intensity from cycle to cycle. Cycling curves were evaluated with respect to negative and positive controls (as the quality controls of the process) in the real-time analysis. The rRT-PCR products were then approved by gel-based electrophoresis.

Evaluation of sensitivity and specificity of the assay

Analytical sensitivity

To determine the LoD (limit of detection) of the assay, serial dilutions containing 1, 10, 100 and 1000 copies of the positive control sample (plasmid) per each microliter of the extracted sample were analyzed. The highest dilution of sample at which 100% of rRT-PCR replicates were positive was considered as the LoD of the assay. In this assay, the LOD was 10 or fewer copy numbers of viral genome per each microliter of the extracted genome (2500 or fewer copy numbers of viral genome per each milliliter of patient blood sample).

Analytical specificity via in silico prediction and rRT-PCR assy

As previously mentioned, none of the probe and primer sequences demonstrated genomic cross-reactivity with

other viruses, human genome and other likely interfering genomes in a BLAST search analysis and only detected a 176 bp fragment of the CCHFV S-region in bioinformatics analysis (data not shown). On the other hand, in the experimental rRT-PCR assay, some of the accessible RNA viruses such as norovirus, flu A, HCV, WNV and CCHF (as the positive control) were also evaluated in terms of specificity of the assay.

Linearity range and repeatability of the assay

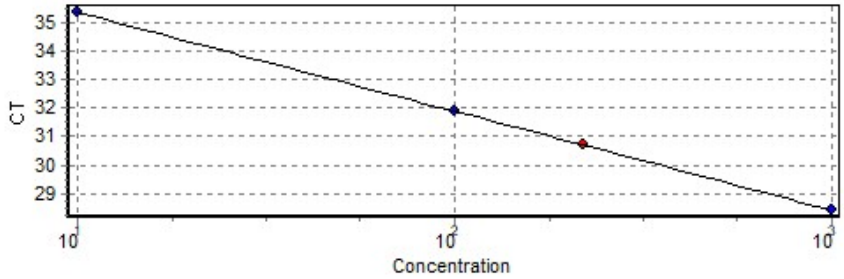
Linearity of the assay was evaluated by analysis of serial dilutions containing 10 to 105 copy numbers of positive control sample per each microliter of extracted plasmid and the assay was linear between 10 to 103 copy numbers per each microliter of extracted plasmid for this technique (Figure 1 – *Ct was plotted against the quantity of plasmid DNA (common logarithmic scale). Measurement of the copy number of CCHF virus was estimated from Ct by quantitative rRT-PCR< ■ [in Ct = 30.69] represents unknown patient sample and ■ [in Cts = 35.36, 31.87 and 28.42] represents the standard dilution*). These assays were evaluated and repeated by at least three different users and three times by each one of them on different days and the repeatability of the assays was approved. Also the assays were developed using both the Applied Biosystem (ABI) 7500 (USA) and Corbet (Rotor-Gene) 6000 (Germany) real-time PCR instruments.

Quantitative determination of viral load in positive samples

Construction of a standard for quantization: The fragment of 176 bp within the S-region sequence of CCHF virus genome was amplified by rRT-PCR and the amplicon was cloned into the PTG19-T vector using a T/A cloning Kit (Vivantis, Malaysia). The plasmid was then purified using a plasmid purification kit (Qiagen, Hilden, Germany). After the performance confirmation of cloning process by PCR, the accuracy of this process and the sequence of cloned fragment was confirmed by sequencing. Determination of recombinant plasmid concentration was then performed by a NanoDrop.

For preparation of a standard curve, the plasmid was diluted serially from 103 to 10 copy numbers per microliter as a master of standard positive control. As soon as optimizing the assay conditions, we obtained a standard curve with a linear range across at least three logs of DNA (recombinant plasmid) concentration, from which the Ct values can be referred to the virus copy number (Figure 1).

Figure 1. The standard curve with a linear range across three logs of different concentrations of the recombinant plasmid DNA containing the S-region sequence of CCHFV genome.



Determination of the Ct values and construction of the standard curve were performed using the Rotor-Gene 6000 software. In all real-time PCR assays, the correlation coefficient of the standard curve was greater than 0.980. Ct was plotted against the quantity of plasmid DNA (common logarithmic scale).

Measurement of the copy number of CCHF virus in unknown blood samples from different patients was estimated from Ct by quantitative rRT-PCR. Since, the viral genome was extracted from 200 µl of a patient blood sample, on the other hand, the extracted genome was dissolved in 50 µl of RNase-free water, as well as, 5 µl was only used in each quantitative assay, the final viral load in 1 ml of blood sample would be 50 folds.

RESULTS

Diagnostic assay of CCHF viral RNA in suspicious blood samples

From a total of 37 suspicious blood samples, 15 cases were approved to be positive and 22 were negative for CCHF viral RNA by use of this technique and no false positive was detected in the results of sequencing. The predicted length of 176 bp resulted by rRT-PCR amplification was approved in all the positive samples by electrophoresis analysis (Figure 2A). The amplification curve related to some of the suspicious blood samples evaluated is shown in Figure 2B.

Evaluation of analytical sensitivity of the assay

The serial dilutions prepared containing 1, 10, 100 and 1000

copies of the positive control sample (plasmid) per each microliter of the extracted genome were analyzed in rRT-PCR for the analytical sensitivity evaluation of the assay. As shown in table 1, the highest dilution at which 100% of rRT-PCR assays were positive (underlined) is considered as the LOD, which in this assay was 10 (or fewer) copy numbers of viral genome per each microliter of the extracted genome or 2500 (or fewer) viral particles per each milliliter of patient blood sample.

Figure 2. A) The predicted fragment with the length of 176 bp, resulted by rRT-PCR amplification. Lad is a 50 bp DNA Ladder (SinaClon, Iran).

B) The rRT-PCR amplification curve related to some of the suspicious blood samples considered (■ represents NTC [Not template control] or negative control sample, ■ [in Ct = 23.29] represents positive control sample, ■ and ■ represent negative patient sample, ■ [in Ct = 28.12] and ■ [in Ct = 26.85] represent positive patient sample)

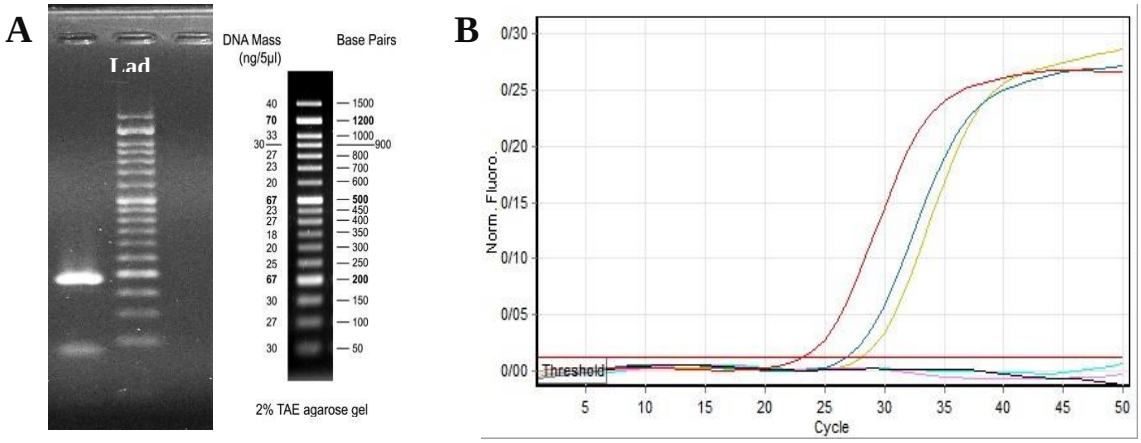


Table 1. CCHFV rRT-PCR assay limit of detection with positive control sample

Serial dilution *(copies/microliter)*	Positive tests (%) s – region
1	variable
10	100
100	100
1,000	100

Evaluation of analytical specificity via in silico prediction and rRT-PCR assay

As shown in Figures 3A and 3B, the highly conserved sequences (yellow) in the S-region were determinated from the alignment data analysis obtained from the MEGA 7 software which were quite specific for CCHF virus, as previously mentioned and the specific primers targeting this region were designed using the mentioned softwares (bioinformatics data for the probe is not shown).

Figure 3. The alignment data resulted from the MEGA 7 software related to the S-region of CCHFV. A) The Yellow zone is the highly conserved sequence used for designing the specific forward primer. B) The Yellow zone is the highly conserved sequence used for designing the specific reverse primer.

The results of experimental rRT-PCR assay on the accessible RNA viruses such as Norovirus, Flu A, HCV, WNV and CCHFV (as the positive control) showed that this assay was quite specific for CCHFV and other viruses were not detected by the designed probe and primers (Figure 4 - ■ represents NTC [Not template control] or negative control sample, ■ [in Ct = 21.44] represents the positive control [CCHFV] sample, ■ represents the negative result from Norovirus sample assay, ■ represents the negative result from Flu A sample assay, ■ represents the negative result from HCV sample assay and ■ represents the negative result from WNV sample assay).

Figure 4. The rRT-PCR amplification curve related to some of the accessible RNA virus samples

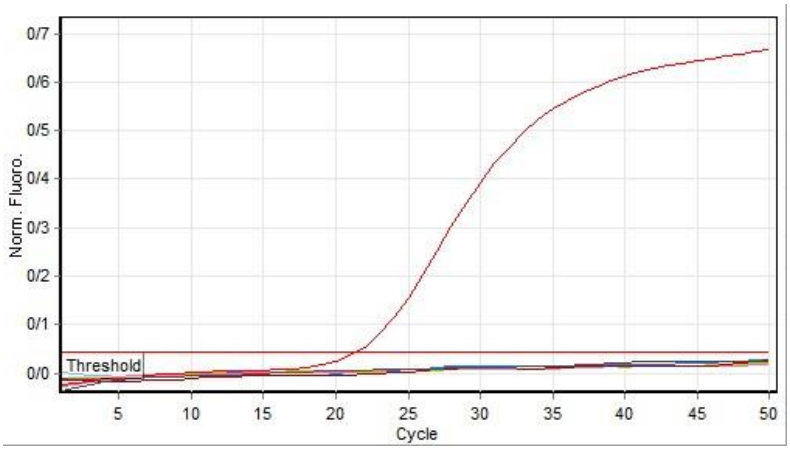
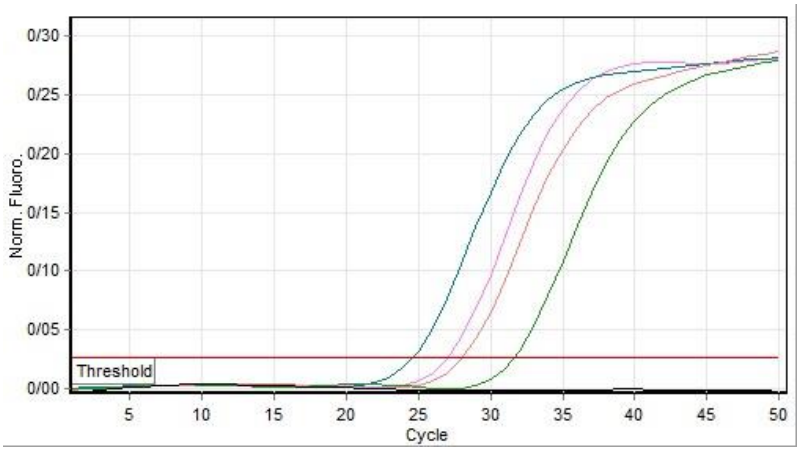


Figure 5. Representative amplification plot of the developed CCHFV quantitative rRT-PCR assay (quantitative curve) showing three ten-fold dilutions of the standards (Cts = 35.36, 31.87 and 28.42) and one unknown sample (Ct = 30.69).



Quantitative determination of viral load in positive samples

The copy number of CCHF virus in different patient blood samples was estimated from Ct by the rRT-PCR assay (Table 2), based on the quantitative curve (Figure 5). For example, the viral load determined in patient blood sample-1 was 220 copy numbers of viral genome per each microliter of the extracted genome or 55,000 (250 folds) viral particles per each milliliter of patient blood sample.

It is notable that viral load decreased slowly in most of cases in subsequent samplings (severe form of disease) and in others (moderate form of disease) decreased very faster. The results of subsequent evaluations is not reported in this article.

Table 2. The calculated viral load of CCHF virus in several patient blood samples based on the quantitative curve and Ct, NTC is "not template control" or "negative control" sample

No.	Color	Name	Type	Ct	Given Conc. *(copy number/µl)*	Calc. Conc. *(copy number/µl)*	Calc. Conc. *(viral particle/ml)*
1		Sample 1	Unknown	30/69	------	220	55,000
2		Sample 2	Standard	28/42	1,000	994	248,500
3		Sample 3	Standard	31/87	100	101	25,250
4		Sample 4	Standard	35/36	10	10	2,500
5		Sample 5	NTC	------	------	------	------

DISCUSSION

The CCHF (Crimean-Congo hemorrhagic fever) is an acute widespread zoonotic illness, caused by Nairovirus (a tick-borne virus), which is characterized by a severe and often hemorrhagic course of disease, with a fatality rate of about 10-50% [16, 17].

The CCHF viral RNA from various clinical samples is a proper tracing target during the acute phase of the infection, or even before the beginning of the disease when detection of antibody is impossible or unreliable [22-24]. As there is no specific treatment or approved vaccine against CCHF virus, an accurate and early detection as well as a reliable surveillance and quantitative determination of viral load is necessary for case management, patient improvement, and protection of medical staffs.

Several different laboratory assays can be used for CCHFV diagnosis, including enzyme-linked immune sorbent assay (ELISA), serum neutralization, antigen detection, virus isolation by cell culture and reverse-transcriptase polymerase chain reaction (RT-PCR) assay [4, 18-21]. RT-PCR is the best molecular diagnostic techniques in clinical laboratories owing to its simplicity, high specificity and sensitivity [24]. Unlike the traditional two-step RT-PCR technique, the one-step real-time RT-PCR (rRT-PCR) assay does not need to pre-PCR (post-RT) sample handling, avoiding the PCR product dependent probable contamination transmission and resulting in more sensitive, rapid and also higher efficient assays. On the other hand, the probe based rRT-PCR assay has a very high specificity in addition to the mentioned features, compared with the SYBR Green based rRT-PCR assay that was also developed in our previous study [22]. Therefore, it has become an accepted diagnosis method for detecting many microbes [25] and also has greater repeatability when the need for post-amplification processing (analysis by agarose gel electrophoresis) is eliminated, thus avoiding the transfer contamination, difficulty and time-consuming [26, 27].

The assay was linear between 10 to 10^3 viral genomes per each microliter of the extracted genome for this technique and the bioinformatics and experimental evaluations approved high specificity of this assay. On the other hand, the LOD of the assay was 10 (or fewer) copy numbers of viral genome per each microliter of the extracted genome. Measurement of the CCHF virus copy number and quantitative determination of viral load in positive samples was estimated from Ct via the rRT-PCR on the basis of the quantitative curve. It is notable that construction of a standard for quantization of this assay is necessary and very difficult, which was devoted a lot of time in this study.

CONCLUSIONS

This research showed that the developed probe based one-step rRT-PCR assay is a specific, reliable, rapid, sensitive, repeatable and simple tool for detection as well as quantitative determination of viral load of the CCHF virus in various suspicious patient samples.

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