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Original Article

Rapid and Quantitative Detection of Crimean-Congo Hemorrhagic Fever Virus by One-Step Real-Time Reverse Transcriptase-PCR

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SUMMARY: In this article, the development of a new TaqMan-based one-step real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay for detection and quantification of Crimean-Congo hemorrhagic fever virus (CCHFV) RNA is described. Selected oligos targeting the highly conserved S region of CCHFV were designed by using our oligo design and analysis software, Oligoware 1.0. None of the primer sequences showed genomic cross-reactivity with other viruses or cells in a BLAST (NCBI) search analysis. The sensitivity and specificity of the primers and the probe were tested using 18 serum samples from patients from East Anatolian who were suspected of having CCHFV, including 2 samples that had already been confirmed to be positive for CCHFV. Among the 16 previously unconfirmed samples, 5 were positive by TaqMan-based one-step real-time RT-PCR and 1 was positive by non-nested RT-PCR, and these results were confirmed with DNA sequencing analysis. The 2 previously confirmed CCHFV RNA samples were also positive by both TaqMan-based one-step real-time RT-PCR and non-nested RT-PCR tests. To ensure the quantitative reproducibility of TaqMan-based one-step real-time RT-PCR, the procedure was repeated several times and the same results were obtained (SD = 0.84 [maximum value]). The developed assay was able to sensitively quantify the concentration of CCHFV RNA, which ranged from 10^2 to 10^7 copies/ml per reaction, using plasmid standards generated from the CCHFV RNA (correlation coefficiency = 0.989). The results of the one-step real-time RT-PCR assay were more sensitive than those of the non-nested RT-PCR assay. It can be concluded that our one-step real-time RT-PCR assay is a reliable, reproducible, specific, sensitive and simple tool for the detection and quantification of CCHFV.

INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV) is the causative agent of a serious human hemorrhagic fever known as Crimean-Congo hemorrhagic fever (CCHF) with a fatality rate ranging from 10 to 60%. CCHFV has been reported in Africa and the western part of China, and in Eastern European and Middle Eastern countries (1-3). Humans are often infected by a bite from or contact with infected ticks or contact with bodily fluids or tissues of infected livestock. Most infected humans live or work in close contact with livestock in areas where CCHFV is endemic (4,5). Health care workers can also become infected following treatment of, or surgery on a patient with unsuspected CCHFV (6,7). Because of the mode of transmission, high fatality rates, and the need to institute proper barrier nursing precautions and public health measures during outbreaks, it is essential to establish an early diagnosis in the course of the disease. During a CCHFV outbreak, the ability to establish a rapid diagnosis may help save the lives of patients presenting with treatable diseases, such as other infections. On these occasions, proper triage of patients depends heavily on a rapid and accurate diagnosis.

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Conventional assays currently available for the diagnosis of CCHFV infections include virus culture, electron microscopy (EM), and antigen and antibody detection (8-11). Each method has both advantages and disadvantages. Virus culture is sensitive but must be performed under a biosafety level 4-laboratory condition. EM is a rapid technique (3 - 5 h), but it is usually available only in well-equipped research facilities. Antibody detection with enzyme immunoassay (EIA) for CCHFV infections is often reliable but not in patients in an early stage of infection. To date, the most rapid and sensitive assay for CCHFV has been based on reverse transcriptase-polymerase chain reaction (RT-PCR) for early stage of infection (1,12,14). However, most of the published RT-PCR assays are time consuming (4-8 h), as they include a separate cDNA synthesis step, followed by the RT-PCR, agarose gel analysis of PCR products, and in some instances, a second round of nested amplification or Southern hybridization (1,12). These assays require multiple manipulations of the samples, increasing the risk of carry-over contamination and providing no quantification of the viral target copy. In contrast, real-time PCR is relatively easy and has a high throughput capacity, and it has therefore become the most widely used gene detection and quantification method.

The purpose of the present study was to demonstrate a highly sensitive, specific, and rapid detection and quantitative assay for CCHFV. Oligos for the TaqMan-based one-step real-time RT-PCR assay were designed from the S RNA

segment of the CCHFV genome on the basis of maximal consensus sequences. The design of these sequences was made possible by the ability of our oligo design and analysis program (Oligoware 1.0) to select and analyze maximally conserved regions of the viral genome for the unique primers and the probe.

MATERIALS AND METHODS

Serum samples: Serum samples were gathered from 18 patients in East Anatolian who were suspected of having CCHF, including 2 samples that had already been confirmed to be positive for CCHFV by ELISA and RT-PCR analyses (Institute Pasteur, Lyon, France), were tested retrospectively by one-step real-time and classical non-nested RT-PCR assays after they had been stored at −80°C for up to 8 months.

Viral RNA preparation: The stored human sera were thawed and cleared by centrifugation on a benchtop centrifuge at 12,000 × g for 3 min. All of the manipulations were performed in a biosafety class II cabinet. Viral RNA was prepared from 100 μl of a cell-free serum sample using an EZ-RNA total RNA isolation kit (Biological Industries, Kibbutz Beit Haemek, Israel) according to the manufacturer's instructions. Extracted viral RNA was dissolved in 100 μl DNase- and RNase-free distilled water.

Oligonucleotide design: The oligos, primers and probe used in this study were designed with the recently developed program Oligoware 1.0 (15) and synthesized by MWG Biotech (Munchen, Germany). Oligoware 1.0 can accurately detect one of the highly conserved regions of any nucleic material. The software is able to select the oligos based on its own gene database of the sequences generated from the EMBL, Genbank and DDBJ databases. We chose primers and a probe from the S genomic region, which is a partly conserved region in CCHFV (1) (GenBank accession numbers: AF415236, AF527810, AF358784, AF362745, AF481802, AF481801, AF481800, AF527810, AY297689, AY277677, AY277672, AY049081, AY049080, AY049078, AY049079, U88414, U88411, U04958, U88412). Using the program, we analyzed oligos to determine whether they met the optimum real-time RT-PCR assay conditions. In addition, Oligoware 1.0 gave us the opportunity to determine whether or not there were any degenerative bases (Wobble) on the primers or the probe. The TaqMan probe was labeled with 6-carboxyfluorescein at the 5′ end (FAM) as a reporter and with 6-carboxy-N,N,N',N'-tetramethylrhodamine at the 3′ end (TAMRA) as a quencher.

TaqMan based one-step real-time RT-PCR assay: The assay was performed in a Perkin-Elmer 7700 Sequence Detection System by using the combination of reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) and hot start Taq DNA polymerase (Bioron GmBH, Munchen, Germany) enzymes. Amplifications were carried out in 25 μl reaction mixtures containing 5 μl of the target virus RNA, 5 pmol of each primer, 4 pmol of TaqMan probe, 0.2 mM of each dNTP (containing doubled dUTP) and 6 mM $MgCl_2$. Cycling conditions were as follows: a single cycle of 30 min at 42°C, 5 min at 95°C; followed by 40 cycles of 15 s at 95°C; and a final cycle of 1 min at 60°C. TaqMan PCR products were detected as an increase in fluorescence from cycle to cycle. The software generates two detection responses, i.e., the fluorescence intensity by a normalized reporter value (ARn) and the threshold cycle (Q). The ARn value is obtained by subtracting the value for the reporter signal R^+n (emission intensity of the reporter) from the value for the background signal, R^-n (emission intensity of the reporter [no template]). The C_t value is the cycle number at which the reporter fluorescence is greater than the fixed threshold fluorescence, a parameter defined as 10 times the standard deviation of the background fluorescence intensity, which is measured between cycle 3 and 15. The log 10 of the number of targets initially presented is proportional with the Q and can be measured on a standard curve (16).

Quantification: A special segment (the S genomic region) of CCHFV RNA was amplified with our designed primers, CCReal P1 and CCReal P2, and then the amplicon (103 bp) was cloned with the pGEM-T Easy Vector System I (Promega, Madison, Wis., USA). This plasmid, called CCQ, was used for the CCHFV quantification assay. The serial dilutions of CCQ plasmid were also used to determine the dynamic range of quantification. The calculation of standards was performed by using a spectrophotometrically and the Oligoware 1.0 software.

Test evaluation: We performed conventional non-nested RT-PCR (12) and TaqMan-based one-step real-time RT-PCR with the designed primers to compare the sensitivity for 18 serum samples from patients suspected to have CCHFV, including two serum samples already determined to be positive for CCHFV. The variability of the complete RT-PCR procedures, including the TaqMan-based one-step real-time RT-PCR, was determined by undertaking three RT reactions in parallel on aliquots of standards of CCHFV RNA. All final products (amplicons) of non-nested and one-step real-time RT-PCR tests - i.e., 10 samples - were sequenced on an ABI PRISM 310 sequencer using a Big Dye Terminator Sequencing Kit (PE Biosystems Co., Foster City, Calif., USA).

RESULTS

Design of primers and the 5′ nuclease detection probe: Novel primers and a novel probe were also designed for CCHFV. Real-time detection of PCR products by 5′-nuclease

Table 1. Oligonucleotide sequences used in this study

Primers[1)] and probe[2)]	Amplicon length (bp)	Reference
5′-ATGCAGGAACCATTAA**R**TCTTGGGA-3′ 5′-CTAATCATATCTGACAACATTTC-3′	228	12
CCReal P1 5′-TCTT**Y**GC**H**GATGA**Y**TC**H**TT**Y**C-3′ CCReal P2 5′-GGGAT**K**GT**Y**CC**R**AAGCA-3′ PROBE 5′-FAM ACA**SR**ATCTA**Y**ATGCA**Y**CCTGC TAMRA-3′	103	This study

[1)]: H, K, R, S and Y are designated for degenerative bases (Wobble bases), where H=A/C/T, K=G/T, R=A/G, S=G/C, Y=C/T.
[2)]: FAM and TAMRA are fluorescent labels in the probe.

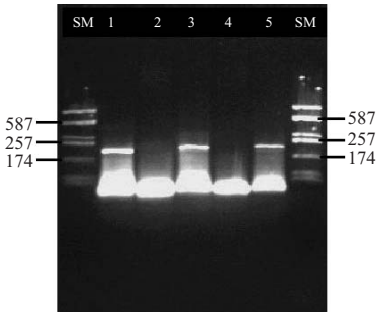

Fig. 1. A 228-bp region of the S segment of the CCHFV genome was amplified using CCHFV-specific oligonucleotide primers, directly from human serum samples by non-nested RT-PCR and detected by agarose gel stained with ethidium bromide. Lanes 1 and 5, confirmed patient's sera; lane 3, one of the suspected CCHF patient's sera; lane 2, negative control (distilled water); and lane 4, negative patient's serum. SM, Size Marker (DNA MicroMake; Amresco Inc., Cleaveland, Ohio, USA).

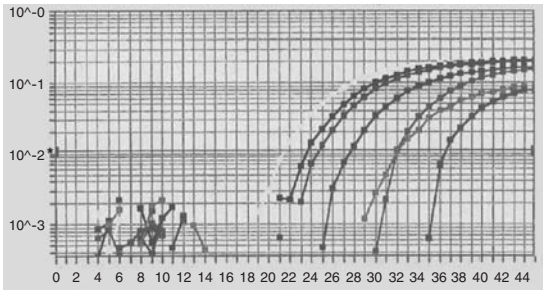

Fig. 2. TaqMan-based one-step real-time RT-PCR test results of CCHFV-suspected sera, including two confirmed patients' sera. The X axis shows the cycle numbers of the one-step real-time PCR and the Y axis shows the C_t values. The C_t values are calculated automatically by the PE 7700 real-time PCR device. The asterisk indicates the baseline for C_t value.

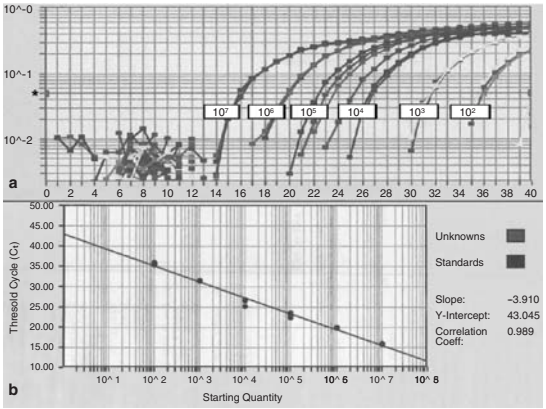

Fig. 3. a) C_t values and linear detection range between 10^7 and 10^2 copies/ml in triplicate sampling. The asterisk indicates the baseline for C_t value. b) The calibration curves were determined in triplicate sampling. The correlation coefficiency of the test was 0.99. The X axis reveals quantitation standards, and diluted recombinant plasmids prepared with spectrophotometric analyses. The Y axis shows C_t values for each triplicate standards.

technology (TaqMan) is highly specific for CCHFV detection. The oligos were chosen from one of the partly conserved regions of the viral genome. We detected some different bases based on bioinformatic analysis using Oligoware 1.0, and replaced the mutant bases with Wobble bases (Table 1). None of the primer sequences showed homology with other database sequences by a BLAST (NCBI) search.

Table 2. Intraassay variability and reproducibility

Genome equivalents	Proportion positive	Cycle threshold (mean)	CV%	SD
10^7	3/3	16.03	1.32	0.21
10^6	3/3	19.95	0.73	0.14
10^5	3/3	23.11	2.81	0.65
10^4	3/3	26.32	3.21	0.84
10^3	3/3	31.74	0.25	0.07
10^2	3/3	35.93	1.01	0.36

Detection of viral RNA in 18 suspected sera: DNA bands corresponding to the predicted 228 bp were detected in 3/18 stored serum samples with ethidium bromide-stained agarose gels (Figure 1). Seven of 18 suspected serum samples were demonstrated to be positive with one-step real-time RT-PCR (Figure 2). Two confirmed CCHFV RNA were also positive by non-nested RT-PCR and TaqMan-based one-step real-time RT-PCR tests performed in triplicate. One-step real-time RT-PCR using the novel designed primers and probe for detected extra four positive CCHFV RNA by comparison to the non-nested PCR test in stored 18 suspected sera. The sequences of all positive PCR products were confirmed to be those of CCHFV by an ABI 310 DNA sequencer (data not shown).

Reproducibility and analytic sensitivity of TaqMan-based one-step real-time RT-PCR assay: A one-step real-time RT-PCR assay for the detection and quantification of CCHFV was designed and evaluated. To allow quantification of CCHFV RNA, calibration curves were generated by amplification of serial dilutions of the CCQ plasmid, and Q values were determined in triplicate and plotted against the plasmid copy number. Figure 3b presents the resulting calibration curves from the assay, indicating a linear detection range between 10^7 and 10^2 copies/ml and a correlation coefficiency (R) of 0.99. Although we repeated the one-step real-time RT-PCR assay three times, we did not detect any variability in the analytic sensitivity with plasmid standards. The intraassay variability (standard deviation of C_t values) was low - e.g., 0.21 - for 10^7 copies/ml and 0.36 for 10^2 copies/ml. In addition, the intraassay reproducibility was high: CV %<3.21 (Table 2).

DISCUSSION

Although the clinical and biological features of CCHFV infection are known, the diagnosis of human CCHFV infection is currently based on the detection of specific antibodies, virus isolation and RT-PCR. Because virus isolation should be done under biosafety level 4-laboratory conditions and the virus culture requires viable viruses, most diagnostic laboratories are unable to culture samples that have been gathered directly from the patient. Serological tests are often reliable methods as diagnostic tools for CCHFV, but they are not suitable for patients who die before humoral immune response (1,11-14). In the acute stage of the illness, nested RT-PCR has been repeatedly shown to be more sensitive than the culture techniques and the antibody assays for the detection of CCHFV (1,12,14). However, analysis of the discrepant results and assessment of the clinical performance of PCR have been carried out less frequently. The use of only RT-PCR with ethidium bromide-stained gels permits a presumptive diagnosis of CCHFV to be reported within approximately

Table 3. Comparison of non-nested RT-PCR and real-time RT-PCR (TaqMan) tests

Assay features	Non-nested RT-PCR	Real time RT-PCR (TaqMan)
Run time	Approximately 3.5 h	Approximately 2.5 h
No. of primers	2 oligonucleotides	2 oligonucleotides plus 1 probe
Primer matching	Selected oligonucleotides for non-nested RT-PCR assay may not match well with target genome sequences of CCHFV	Selected oligonucleotides and probe via Oligoware 1.0 in one-step real-time RT-PCR assay match well with target genome sequences of CCHFV
Detection method	The use of ethidium bromide-stained gels may cause sensitivity problem	The use of a probe with fluorophore dye in a special equipped machine has a better sensivity and specificity
Quantification	Impossible	Possible. It has a quantitative linearity and a correlation coefficiency = 0.99

8 h after receiving a specimen. Sensitivity can be improved by the use of Southern blots and labeled probes (1).

For the diagnosis of many virus infections, the real-time PCR assays have excellent analytical sensitivity, as shown previously for other PCR methods (1,12,15-17). In the present study, we have described for the first time a method of one-step real-time RT-PCR that allows the identification and quantification of CCHFV RNA in a short time.

When any PCR method is used in a diagnostic setting, a thorough validation of the analytical performance, especially the sensitivity and specificity, is mandatory. Standardized virus stocks with defined numbers or units of genomes per milliliter are the test material of choice. For less common viruses such as CCHFV, the PCR sensitivity has been determined by making a comparison between classical virological quantification methods (1). Because of setbacks in the management of our facilities, especially in regard to biosafety level 4-laboratory conditions, we currently have no source for standardized virus stocks with defined numbers or units of genomes per milliliter or classical virological quantification methods. We have used cloning and DNA sequencing methodology to overcome these problems. We have found significant results, in terms of sensitivity, specificity and quantitative linearity, in our TaqMan-based one-step real-time RT-PCR assay. In quantitative analysis' 10^2 to 10^7 copies/ml, the correlation coefficiency was found to be 0.99 in plasmid derived standards. The sensitivity of the one-step real-time RT-PCR assay was evaluated to be good with respect to another ethidium bromide-stained gel method. Positive test results for CCHFV RNA in stored serum samples were higher (7/18) in one-step real-time RT-PCR. On the other hand, in terms of specificity, we have analyzed the sequences of the positive amplicons, which have been detected in classical and real-time RT-PCR. The sequences analysis revealed that all of the amplified products are related to CCHFV RNA.

As a result, among the 18 serum samples suspicious for CCHFV, 3 (16%) tested positive by non-nested RT-PCR and 7 (38%) tested positive by one-step real-time RT-PCR assays. The results of this study showed that the use of ethidium bromide-stained gels might cause a sensitivity problem for the non-nested RT-PCR test.

Since the numbers of the tested samples were inadequate for statistical analysis, we thought that we could describe the observed differences between non-nested RT-PCR and one-step real-time RT-PCR assays as shown in Table 3. Rapid and accurate diagnosis of virus infection is essential for the appropriate clinical management of patients. These assays must be simple to perform, accurate, and reproducible. Therefore, use of the newly designed fluorogenic 5′ nuclease assay (TaqMan) described in this paper would be the first step for diagnosis and quantification of CCHFV. On the other hand, in order to analyze potent antiviral drugs in cell culture and then use them for patients, it is necessary to appreciate their ability to inhibit virus replication and then to determine their concentration-dependent activity. As a reference method, the titration of infectious viral particles is currently used to evaluate antiviral activity. However, this method requires a time-consuming cell culture analysis and special facilities with biosafety level 4-laboratory conditions. In this study, we obtained a good correlation when plasmid standards derived from CCHFV RNA were quantified by TaqMan-based one- step real-time RT-PCR.

In conclusion, our laboratory has developed and extensively evaluated a rapid, reproducible TaqMan-based one-step real-time RT-PCR assay for the detection and quantification of CCHFV RNA. We have carefully selected primers and a probe for CCHFV based on the sequences of CCHFV in the GenBank using Oligoware 1.0 software. We conclude that our TaqMan-based one-step real-time RT-PCR assay is a reliable, specific, sensitive and simple tool for the diagnosis and quantification of CCHFV RNA. We anticipate that this technique will be important in future studies to validate the selected primers-probe set against the CCHFV.

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