

# Development and Evaluation of a Real-Time RT-qPCR for Detection of Crimean-Congo Hemorrhagic Fever Virus Representing Different Genotypes

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## Abstract

Crimean–Congo hemorrhagic fever (CCHF) is a zoonotic disease caused by a nairovirus belonging to family Bunyaviridae. The CCHF virus (CCHFV) can be transmitted to humans by *Hyalomma* ticks as well as by direct contact with infected body fluids or tissues from viremic livestock or humans. Our aim was to set up a fast RT-qPCR for detection of the different CCHFV genotypes in clinical samples, including an inactivation step to make the sample handling possible in lower biosafety levels (BSL) than BSL-4. This method was evaluated against commercial reference assays and international External Quality Assessment (EQA) samples. The analytical limit of detection for the developed CCHFV-S RT-qPCR was 11 CCHFV genomes per reaction. After exclusion of four dubious samples, we studied 38 CCHFV-positive samples (using reference tests) of which 38 were found positive by CCHFV-S RT-qPCR, suggesting a sensitivity of 100%. CCHFV-S RT-qPCR detected all eight different CCHFV strains representing five different CCHFV genotypes. In conclusion, the CCHFV-S RT-qPCR described in this study was evaluated using various sources of CCHFV samples and shown to be an accurate tool to detect human CCHFV infection caused by different genotypes of the virus.

**Key Words:** Crimean–Congo hemorrhagic fever—In-house RT-qPCR—CCHF genotypes.

## Introduction

**C**RIMEAN-CONGO HEMORRHAGIC FEVER (CCHF) is a zoonotic arbovirus disease caused by a nairovirus belonging to family Bunyaviridae. This zoonosis can be transmitted to humans by *Hyalomma* ticks as well by direct contact with CCHF virus (CCHFV)-infected body fluids or tissues from viremic livestock or humans. CCHFV infection can be asymptomatic in livestock such as cattle, sheep, goats, hares, and pigs (Whitehouse et al. 2004). Clinical symptoms in human CCHF cases include onset of high fever, severe myalgia, malaise, and gastrointestinal symptoms, in addition to extensive hemorrhages

occurring at later stages of the disease leading up to a 50% fatality rate (Ergonul et al. 2006, Vorou et al. 2007). However, symptoms of CCHFV infection seem to be milder in pediatric patients (Kızılgün et al. 2013, Oflaz et al. 2013). According to the World Health Organization (WHO), the majority of CCHF cases occur in Africa, the Middle East, Asia, the Balkans, and Russia. Outbreaks have been encountered in Turkey, and an endemic case was reported in Greece in June of 2008, whereas only few imported cases have occurred in Europe, e.g., in France and the United Kingdom (Jaureguiberry et al. 2005, Midilli et al. 2007, Papa et al. 2010, Chamberlain et al. 2013). People traveling to CCHF-endemic areas generate the need for worldwide

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diagnostic preparedness. Our aim was to develop a fast real-time RT-qPCR for detection of CCHFV RNAs from chemically inactivated patient samples and to evaluate the method with reference data from two commercial CCHFV RNA assays and international External Quality Assessment (EQA) samples obtained from the European Network of Imported Viral Disease (ENIVD) and CCHFV strains Swedish Institute for Communicable Disease Control (SICDC, Sweden).

**Materials and Methods**

*RT-qPCRs*

The CCHFV-S RT-qPCR was set up and carried out using primers and probes targeting the nucleoprotein gene region (S segment). The primers were: CCHFV-forw (5'-GGACATA GGTTCCTGTCAG-3'), CCHFV-rev1 (5'-TCCTTCTAA TCATGTCTGACAGC-3'), and CCHFV-rev2 (5'-TCTGAC AGCATCTCTTTGACAGAC-3') with final concentrations of 400 nM and CCHFV MBG probe1 (6'FAM 5'-TGGCG AAATTGTGATGTCTG-3'MGB), probe2 (6'FAM 5'-CTT GCAGAGTACAAGGTT- 3'MGB), and probe3 (6'FAM 5'-TRAGCAACAAAGTCCT- 3'MGB) 200 nM using SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen™, Life Technologies Europe BV, Finland). The test was performed using 7 µL of RNA template in a final volume of 25 µL using cycling conditions of 15 min at 48°C (reverse transcription step) followed by 2 min at 95°C, and 45 cycles of 15 s at 95°C followed by 50 s at 60°C using a MX3005P QPCR System (Agilent Technologies Finland Oy, Espoo, Finland).

*Virus strains, sample panels, and RNA extraction*

To determine the limit of detection (LOD) and sensitivity of the developed CCHFV-S RT-qPCR, CCHFV strains were obtained from the ENIVD and SICDC. EQA samples comprised of four different CCHFV strains representing different genotypes: Europe 1 (strain Hoti, isolated in Kosovo), Europe 2 (strain AP-92, isolated in Greece), Asia 1 (strain Afg09-2990, isolated from a human CCHFV infection acquired in Afghanistan), and Africa 3 (strain ArD 39554, isolated in Mauritania) (ENIVD; Escadafal et al. 2012). In addition, four CCHFV strains representing different genotypes of Africa 3 (strain IbaR 10200, accession no. U88410), Asia 1 (strain Iran Zahedan, accession no. KC867274), Africa 1 (strain Senegal), and Europe 1 (strain Turkey Samsun) (SICDC, Sweden) were also included.

Gamma-irradiated CCHFV strain Hoti quantified previously using a commercial RealStar® CCHFV RT-PCR kit (Altona Diagnostics GmbH, Hamburg, Germany) was used to determine the LOD. Two different CCHFV-positive serum panels were tested in parallel by developed and commercial methods. Thirty-two samples obtained from Ankara Numune Education and Research Hospital and Ankara University that were previously tested as CCHFV RNA-positive in Turkey using the method described by Yapar et al. (2005) were screened using a commercial CCHFV Real Time RT-PCR kit (in vitro diagnostics, Liferiver Shanghai ZJ Bio-Tech Co., Ltd. Shanghai, China). Ten serum samples (originating from Turkey) were obtained from SICDC, and tested using CCHFV-S RT-qPCR and a RealStar® CCHFV RT-PCR kit (Altona Diagnostics GmbH, Germany).

TABLE 1. LIST OF RESULTS AND CCHFV STRAINS TESTED USING DEVELOPED CCHFV-S RT-qPCR

Origin of the inactivated virus strains or clinical samples	Other reference Escadafal et al. 2012 (number, genotype) <sup>a</sup> ; Ankara, Turkey (number) <sup>b</sup>		CCHFV Real Time RT-PCR kit (Liferiver) (number) <sup>c</sup>		RealStar® CCHFV RT-PCR kit (Altona) (number, genotype) <sup>d</sup>		In-house CCHFV-S RT-qPCR results (number)	
	POS	NEG	POS	NEG	POS	NEG	POS	NEG
	EQA panel (ENIVD) <sup>a</sup>	6, gt Europe 1 2, gt Europe 2 1, gt Asia 1 1, gt Africa 3 1, <sup>c</sup> Africa 3	1	—	—	—	—	6 2 1 1 1
Total SICDC (Sweden), viral culture	—	1	—	—	1, gt Africa 3 1, gt Europe 1 1, gt Asia 1 1, gt Africa 1	0	1 1 1 1	0
Total SICDC (Sweden), positive patient panel originated from Turkey	—	—	—	—	4	0	4	0
Ankara (Turkey), positive patient panel <sup>b</sup>	32	0	28	4	—	—	30	2
HUSLAB (Finland), negative panel	—	60	—	—	—	—	—	60

<sup>a</sup>EQA panel, External Quality Assessment panel.

<sup>b</sup>Detected previously positive in Ankara Hospital and Ankara University (Turkey).

<sup>c</sup>CCHFV Real Time RT-PCR kit (Liferiver Shanghai ZJ Bio-Tech).

<sup>d</sup>RealStar® CCHFV RT-PCR kit (Altona Diagnostics GmbH).

<sup>e</sup>CCHFV Ibar 10200 nucleoprotein cloned in pGEM-4Z.

CCHFV, Crimean–Congo hemorrhagic fever virus; POS, positive; NEG, negative; EQA, External Quality Assessment; ENIVD, European Network of Imported Viral Disease; gt, genotype; SICDC, Swedish Institute for Communicable Disease Control, Sweden.

To determine the specificity, a negative panel of 60 human serum samples sent for antibody detection of dengue virus and tick-borne encephalitis virus in HUSLAB (Finland) during the years 2011–2012 were analyzed. The viral RNA was extracted from CCHFV strains and patient sera were stored in TRIzol reagent (Life Technologies Europe BV, Finland) or in QIAamp viral lysis buffer (Qiagen, Germany) using Viral RNA kit (Qiagen, Germany).

## Results

A new CCHFV-S RT-qPCR was set up based on the S-segment, and the performance was evaluated using different CCHFV strains prior testing the clinical samples. Strains representing five genotypes tested were shown to be detected (Table 1). The LOD was determined using six parallel tests of dilution series of extracted CCHFV strain Hoti RNA and found to be 11 CCHFV genomes per reaction.

Thirty-two Turkish CCHFV patient samples (Ankara Numune Education and Research Hospital and Ankara University, Turkey) detected earlier as being CCHV positive were tested using both CCHFV-S RT-qPCR and a commercial CCHFV kit (Liferiver). Using CCHFV-S RT-qPCR, 30/32 samples were found positive for CCHFV RNA (Table 1). The commercial method (Liferiver) detected 28 samples as positive, whereas four remained negative. These four samples were referred as negatives, even though these samples were previously detected as positive in Turkey (Table 1). Both developed and commercial assays found the same two samples negative. All 10 CCHFV patient samples from another patient panel (originating from Turkey) provided by SICDC were positive using both developed and commercial assays (Altona). When compared to the reference commercial tests, the performance characteristics of the CCHFV-S RT-qPCR were as follows: Sensitivity was 100% ( $n=38$ ; 95% confidence interval [CI] 91–100%), and specificity 97% ( $n=64$ ; 95% CI 89–100%). These values were calculated using the diagnostic test evaluation program (MedCalc Software).

## Discussion

CCHF is an emerging disease with a high fatality rate, thus requiring rapid diagnosis and a high biosafety level. We developed a novel rapid RT-qPCR for detection of the various genotypes of CCHFV from chemically inactivated patient samples and evaluated the test using different CCHFV sample panels. The LOD was 11 genomic copies per reaction. All eight different strains representing five out of seven recognized CCHFV genotypes were detected with this assay. The results from EQA and patient sample panels were in good concordance with reference results. The CCHFV-S RT-qPCR was more sensitive (30/32) for CCHFV RNA detection than a commercial assay (28/32, Liferiver). There have been several published RT-qPCR tests for CCHFV; *e.g.*, the RT-qPCR described by Yapar et al. (2005) has a run time of approximately 2.5 h whereas the method described here is about 68 min. Overall, the results showed that CCHFV-S RT-qPCR is an accurate and rapid tool for diagnosis of CCHF.

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## Author Disclosure Statement

No competing financial interests exist.

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