Virus Detection and Monitoring of Viral Load in Crimean-Congo Hemorrhagic Fever Virus Patients

Roman Wölfel,*† Janusz T. Paweska,‡ Nadine Petersen,† Antoinette A. Grobbelaar,‡ Patricia A. Leman,† Roger Hewson,§ Marie-Claude Georges-Courbot,¶ Anna Papa,# Stephan Günther,† and Christian Drosten†

We developed a real-time reverse transcription–PCR that detected 1,164 copies/mL of Crimean-Congo hemorrhagic fever virus per milliliter of serum at 95% probability (probit analysis) and was 100% concordant with nested PCR on 63 samples from 31 patients with confirmed infection. Infected patients who died appeared to have higher viral loads; low viral loads correlated with IgG detection.

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne viral zoonosis that occurs widely in Africa, Asia, and Eastern Europe. It is caused by CCHF virus (CCHFV), a segmented, negative-stranded RNA virus belonging to the family Bunyaviridae, genus Nairovirus. CCHF has a fatality rate of ≈30% and a potential for nosocomial spread (1). Early diagnosis of CCHF is important for case management and protection of medical staff.

Diagnostic assays for CCHF include virus culture, antigen-detection enzyme immunoassay (EIA), antibody-detection EIA, and reverse transcription–PCR (RT-PCR) (2). Virus detection is the main diagnostic method in the acute stage of disease, and RT-PCR is most sensitive method of detection. However, because of the remarkable genetic variability among CCHFV strains, all current RT-PCRs either lack sensitivity or focus on the detection of local CCHFV variants only (3–6).

We describe the first real-time RT-PCR that rapidly and reliably detects the global spectrum of clinically relevant virus strains. An extended strategy of probe design was implemented to cover such high variability. Sensitivity was demonstrated by testing virus strain collections from several different Biosafety Level 4 laboratories, essentially covering the full range of global diversity of CCHFV (Figure 1). A comprehensive panel of original clinical samples from persons with confirmed cases of CCHF was used for clinical evaluation; the samples were collected by World Health Organization reference facilities.

The Study

Primers and probes were selected on the basis of an alignment of S segment sequences of 61 CCHFV isolates from all known CCHF-endemic regions worldwide (7) (representative sequences shown in expanded online version of Figure 2, available from www.cdc.gov/EID/content/13/7/1097-G2.htm). Oligonucleotide melting points, folding characteristics, and cross-hybridization properties were determined by using the Primer Express software package (Applied Biosystems; Foster City, CA, USA). Primers were selected to amplify a 181-bp region near the 5’-end of the S segment. The capability of these primers to amplify 12 representative CCHFV strains from distinct CCHF-endemic regions was confirmed initially by gel detection RT-PCR (data not shown) (Figure 2, expanded online version, panel B).

For real-time PCR, identifying a simple detection probe compatible with all known CCHFV strains was not possible. Therefore, a broad-range probe was formulated...
on the basis of the observation that the non–Watson/Crick base pair G:T is almost as thermodynamically stable as regular Watson/Crick base pairs, whereas A:C is very unstable (8). Thus, the probe was placed on the DNA strand that provided more G:T mismatches than complementary A:C mismatches, and the resulting G:T mismatches were not compensated for. As shown in the left panel of Figure 2B (see expanded online version), this probe, designated SE01, detected all 12 representative strains. Because signal intensity varied according to the strain detected, a strain that provided low signal (BT956, Figure 2B expanded online version, left panel) was chosen for evaluation of sensitivity. Its full S segment RNA was cloned and transcribed in vitro to obtain a quantitative RNA standard (9). Cloning, in vitro transcription, purification, and quantification were performed as previously described (10). End-point dilution showed that single copies of RNA could be detected despite low overall fluorescence (data not shown). Nevertheless, variation in signal intensity between strains was adjusted by the following 2 modifications. First, an additional oligonucleotide (SE03) was introduced at the same binding site as SE01. This probe had 2 effects: first, a pyrimidine base (IUB-code “Y,” 50% C and 50% T) was generated at 2 positions of balanced C/T polymorphisms. Second, a “keto” base (IUB-code “K,” 50% G and 50% T) resulted at 1 position of total variability (A, C, G, T). RNA from the 12 representative strains was tested, and those strains that still provided low signal were realigned separately. On the basis of the second alignment, an additional probe was selected at an alternative binding site to prevent interference with probes at the first binding site. It was placed on the minus strand to obtain more G:T mismatches than complementary A:C mismatches (see above). The improvement obtained by the additional probes on the set of representative strains is shown in Figure 2B, middle panel (expanded online version). The final assay protocol is summarized in the Table.

For precise evaluation of analytical sensitivity, a series of human plasma samples was spiked with the RNA standard from strain BT-958 in concentrations ranging from 100,000 to 10 copies per mL. Testing was done on 5 replicate reactions per concentration, and probit analysis was conducted as shown in the expanded online version of Figure 2, panel B, right graph (11). The calculated limit of detection, defined as the concentration down to which >95% of conducted tests can be expected to be positive, was 13.6 copies per reaction (p = 0.05). This corresponded to 1,164 copies per mL of plasma (95% confidence interval, 780–2990 copies/mL).

Cross-reactivity was excluded by testing DNA or RNA from cultures or high-titered clinical samples containing Dugbe virus, Rift Valley fever virus, Sudan Ebolavirus Gulu, Lassa virus AV, yellow fever virus, dengue virus types 1–4, Japanese encephalitis virus, West Nile virus Uganda, Venezuelan equine encephalitis virus, Sindbis virus, Ross River virus, Epstein-Barr virus, hepatitis C virus, human cytomegalovirus, monkeypox virus, poliomyelitis virus types 1–3, rabies virus RSDD, Bacillus anthracis, Leptospira interrogans, Listeria monocytogenes, Neisseria meningitidis, Coxiella burnetii, Rickettsia prowazekii, R. rickettsii, and Plasmodium falciparum. An additional
128 blood specimens collected during the course of the study from 128 patients with conditions other than CCHF all tested negative for CCHF virus.

The real-time RT-PCR was used to test and quantify viral loads for CCHFV infection; the samples were obtained 1–18 days after symptom onset. All samples had nested RT-PCR results positive for CCHFV (3), and all were also positive by the new real-time RT-PCR. For 21 patients with confirmed CCHF (17 from South Africa, 3 from Iran, and 1 from Pakistan), viral load was quantified and compared with other standard diagnostic methods for CCHFV detection (online Appendix Table, available from www.cdc.gov/EID/content/13/7/1097-appT.htm). Again, sensitivity of the new assay was at least as high as that of nested PCR. As shown in Figure 2, there was a clear correlation between viral load and duration of symptoms in these patients. Clinical outcome could not be correlated clearly with viral load, although patients who died of the disease seemed, in general, to have higher viral loads (Figure 2, filled squares). The appearance of antibodies correlated clearly with lower viral loads (Figure 2).

Conclusions

To our knowledge, this is the first PCR validated with representative CCHFV strains from nearly all regions worldwide where the virus is endemic. High sensitivity enables reliable detection of virus in early stages of the infection, when antibody detection is unreliable or impossible. By eliminating the need for postamplification product processing, real-time RT-PCR enables shortened turnaround times for reporting results, which is critical for deciding on isolation and contact-tracing for suspected case-patients. Quantification of viral load may assist in estimating patient’s infectivity. It may also assist in predicting the clinical outcome and could be used to monitor viral load in patients receiving ribavirin treatment (12). Our study provides baseline data on CCHF viral load throughout the acute stage of the illness. High viral load tended to indicate fatal outcome, and lower viral load was generally associated with detectable antibodies. Because detectable antibody response correlates with good outcome (13), viral load will probably be a useful predictor of clinical progress. These preliminary data are highly encouraging for further studies on larger patient cohorts.

Acknowledgments

We are grateful to Britta Liedigk, Beate Becker-Ziaja, and John Chamberlain for excellent technical assistance.

This work was supported by the European Commission (contracts SSPE-CT-2003-502567 and SSPE-CT-2005-022639), the Bundeswehr Medical Service (contract E/B41G/1G309/1A403), and the Federal Office of Civil Protection and Disaster Assistance (contract BBK-F-440-00-1).

Dr Wölfel is a medical microbiologist at the Department of Virology and Rickettsiology of the Bundeswehr Institute of Microbiology in Munich, Germany. He is working in the area of medical defense against biological warfare and terrorism, and his interests include viral hemorrhagic fevers and rickettsial diseases.

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Address for correspondence: Christian Drosten, Bernhard Nocht Institute for Tropical Medicine, Bernhard Nocht Str 74, 20359 Hamburg, Germany; email: drosten@bni-hamburg.de
**Appendix Table.** Comparison of 4 standard diagnostic methods with the novel quantitative real-time reverse transcriptase–PCR (qPCR) assay, using primary specimens and quantification of Crimean-Congo hemorrhagic fever viral load*  

| Patient, age in years (year sample collected, patient outcome)† | No. days after disease onset | Mouse brain inoculation‡ | Vero cell culture‡ | Antibodies§ | Conventional PCR¶ | Novel qPCR viral copies (log/mL) |
|---|---|---|---|---|---|---|
| JN, 47 (2001, survived) | 4 | Pos | Pos | Neg | Pos | 6.6 |
| | 7 | Pos | Pos | Pos (IgM only) | Pos | 6.1 |
| | 9 | Pos | ND | Pos | ND | 5.0 |
| | 10 | Neg | ND | Pos | ND | 4.7 |
| | 13 | ND | ND | Pos | ND | Neg |
| | 17 | ND | ND | Pos | ND | Neg |
| WK, 33 (2001, survived) | 5 | Pos | ND | Neg | Pos | 6.8 |
| | 7 | Neg | ND | Pos | ND | 4.1 |
| | 8 | Neg | ND | Pos | ND | 4.0 |
| | 10 | Neg | ND | Pos | ND | 3.9 |
| | 11 | Neg | ND | Pos | ND | 2.9 |
| | 12 | Neg | ND | Pos | ND | Neg |
| EH, 46 (2001, died) | 5 | Pos | ND | Pos | ND | Neg |
| LK, 54 (2001, died) | 3 | Pos | Pos | Neg | Pos | 7.7 |
| JNM, 33 (2002, survived) | 1 | Pos | Pos | Neg | Pos | 7.0 |
| | 5 | Pos | ND | Pos | ND | 6.0 |
| | 7 | Pos | ND | Pos | ND | 4.5 |
| | 8 | Neg | ND | Pos | ND | 4.2 |
| | 9 | Neg | ND | Pos | ND | 3.7 |
| | 13 | ND | ND | Pos | ND | Neg |
| FF, 67 (2004, died) | 3 | Neg | ND | ND | ND | >8.7 |
| ME, 51 (2006, died) | 10 | Pos | ND | Pos | ND | Neg |
| | 12 | ND | ND | Pos | ND | Neg |
| NVDM, 53 (2001, died) | 6 | Pos | Pos | Pos | Pos | 5.9 |
| RDT, 44 (2001, survived) | 7 | Pos | Pos | Pos | Pos | 5.4 |
| PK, 65 (2002, died) | 3 | Pos | Pos | Neg | Pos | 7.4 |
| PJM, 49 (2002, died) | 4 | Pos | Pos | Neg | Pos | 7.7 |
| WZ, 69 (2004, died) | 4 | Pos | Pos | Pos | ND | 6.4 |
| PTK, 35 (2004, died) | 11 | Neg | ND | Pos | ND | Neg |
| AM, 25 (2006, died) | 10 | Pos | ND | Pos | Pos# | 3.2 |
| | 12 | ND | ND | Pos | ND | Neg |
| | 18 | ND | ND | Pos | ND | Neg |
| NVDM, 53 (2001, died) | 6 | Pos | Pos | Pos | Pos | 5.9 |
| RDT, 44 (2001, survived) | 7 | Pos | Pos | Pos | Pos | 5.4 |
| PK, 65 (2002, died) | 3 | Pos | Pos | Neg | Pos | 7.4 |
| PJM, 49 (2002, died) | 4 | Pos | Pos | Neg | Pos | 7.7 |
| WZ, 69 (2004, died) | 4 | Pos | Pos | Pos | ND | 6.4 |
| PTK, 35 (2004, died) | 11 | Neg | ND | Pos | ND | Neg |
| AM, 25 (2006, died) | 10 | Pos | ND | Pos | Pos# | 3.2 |
| | 12 | ND | ND | Pos | ND | Neg |
| | 18 | ND | ND | Pos | ND | Neg |
| NVDM, 53 (2001, died) | 6 | Pos | Pos | Pos | Pos | 5.9 |
| RDT, 44 (2001, survived) | 7 | Pos | Pos | Pos | Pos | 5.4 |
| PK, 65 (2002, died) | 3 | Pos | Pos | Neg | Pos | 7.4 |
| PJM, 49 (2002, died) | 4 | Pos | Pos | Neg | Pos | 7.7 |
| WZ, 69 (2004, died) | 4 | Pos | Pos | Pos | ND | 6.4 |
| PTK, 35 (2004, died) | 11 | Neg | ND | Pos | ND | Neg |
| AM, 25 (2006, died) | 10 | Pos | ND | Pos | Pos# | 3.2 |
| | 12 | ND | ND | Pos | ND | Neg |
| | 18 | ND | ND | Pos | ND | Neg |
| NVDM, 53 (2001, died) | 6 | Pos | Pos | Pos | Pos | 5.9 |
| RDT, 44 (2001, survived) | 7 | Pos | Pos | Pos | Pos | 5.4 |
| PK, 65 (2002, died) | 3 | Pos | Pos | Neg | Pos | 7.4 |
| PJM, 49 (2002, died) | 4 | Pos | Pos | Neg | Pos | 7.7 |
| WZ, 69 (2004, died) | 4 | Pos | Pos | Pos | ND | 6.4 |
| PTK, 35 (2004, died) | 11 | Neg | ND | Pos | ND | Neg |
| AM, 25 (2006, died) | 10 | Pos | ND | Pos | Pos# | 3.2 |
| | 12 | ND | ND | Pos | ND | Neg |
| | 18 | ND | ND | Pos | ND | Neg |
| NVDM, 53 (2001, died) | 6 | Pos | Pos | Pos | Pos | 5.9 |
| RDT, 44 (2001, survived) | 7 | Pos | Pos | Pos | Pos | 5.4 |
| PK, 65 (2002, died) | 3 | Pos | Pos | Neg | Pos | 7.4 |
| PJM, 49 (2002, died) | 4 | Pos | Pos | Neg | Pos | 7.7 |
| WZ, 69 (2004, died) | 4 | Pos | Pos | Pos | ND | 6.4 |
| PTK, 35 (2004, died) | 11 | Neg | ND | Pos | ND | Neg |
| AM, 25 (2006, died) | 10 | Pos | ND | Pos | Pos# | 3.2 |
| | 12 | ND | ND | Pos | ND | Neg |
| | 18 | ND | ND | Pos | ND | Neg |

*Neg = negative test result; Pos = positive test result; ND = not done.
†All patients except ME were male.
‡As described in Shepherd et al. (1).
Indirect immunofluorescence assay; >1:80 for immunoglobulin G (IgG) and IgM is positive, as described in Swanepoel et al. (2).

Burt et al. (3).

#Only in nested PCR.