

## A Patient with Crimean-Congo Hemorrhagic Fever Serologically Diagnosed by Recombinant Nucleoprotein-Based Antibody Detection Systems

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**We treated a male patient with Crimean-Congo hemorrhagic fever (CCHF). The diagnosis of CCHF was confirmed by reverse transcription-PCR and recombinant nucleoprotein (rNP)-based immunoglobulin G (IgG) and IgM capture enzyme-linked immunosorbent assays of serially collected serum samples. The patient was treated with intravenous ribavirin and recovered with no consequences. The study indicates that rNP-based CCHF virus antibody detection systems are useful for confirming CCHF virus infections. This case also suggests that intravenous ribavirin therapy may be promising for the treatment of CCHF patients.**

**Case report.** We treated a patient with Crimean-Congo hemorrhagic fever (CCHF). The diagnosis of CCHF was confirmed by reverse transcription-PCR (RT-PCR) and a recombinant CCHF virus (CCHFV) nucleoprotein (rNP)-based enzyme-linked immunosorbent assay (ELISA) for detection of immunoglobulin G (IgG) to CCHFV (6). Furthermore, an IgM capture ELISA using the CCHFV rNP was used as a serological tool for the diagnosis of this patient. This patient was successfully treated by intravenous administration of ribavirin.

The patient was a 28-year-old male shepherd who lived in an area of the western part of the Xinjiang Uygur Autonomous Region, People's Republic of China, where CCHF is endemic. Taking the day on which the fever first appeared as day 1, he visited a local clinic on day 1. He spent 3 nights at home but deteriorated abruptly. He was transferred to our hospital and hospitalized on day 4. He did not know whether he had been bitten by a tick, one of the main reservoirs of CCHFV. He presented on admission with low-grade fever, unconsciousness, and severe hemorrhage from the nostrils, gingiva, skin, and gastrointestinal tract. He had anemia, and the erythrocyte count and hemoglobin level were  $3.41 \times 10^{12}$  cells/liter (normal range,  $4.5 \times 10^{12}$  to  $5.9 \times 10^{12}$  cells/liter) and 10.0 g/dl (normal range, 13.5 to 17.5 g/dl), respectively. Thrombocytopenia was noticed, with a platelet count of  $84 \times 10^9$ /liter (normal range,  $150 \times 10^9$  to  $400 \times 10^9$ /liter). The alanine transaminase, aspartate aminotransferase, and lactate dehydrogenase levels were 173 U/liter (normal range, 5 to 40 U/liter), 216 U/liter (normal range, 5 to 40 U/liter), and 268 U/liter

(normal range, 114 to 240 U/liter), respectively, suggesting liver dysfunction. Mild hyperbilirubinemia, hypoproteinemia, and hypoalbuminemia were also present. Renal function was preserved. The patient was intravenously administered ribavirin (0.6 g/dose twice a day, 2-h drip infusion) from day 4 to 11. The symptoms improved gradually, and the patient recovered with no consequences.

Blood samples were drawn for diagnostic tests on days 1, 5, and 9. The blood sample drawn on day 1 was collected at the initial clinic visit and was brought to our hospital. Serum was separated and kept at  $-20^\circ\text{C}$  until use. A nested RT-PCR was performed for amplification of the viral genome. Viral RNA was extracted from 200  $\mu\text{l}$  of serum with a High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. The primers were modified from those reported by other investigators (4) in accordance with the nucleotide sequence of Chinese CCHFV isolate 8402 (GenBank accession no. AJ010649). Five microliters of purified RNA was added to the Ready to Go RT-PCR mixture (0.5-ml tubes; Amersham Pharmacia Biotech Inc., Piscataway, N.J.) as a template, and then the primer set of 50 pmol each of CCHF/F2C (5'-TGGATACTTTCACAACTC-3') and CCHF/R3 (5'-GACAAATTCCTGCACCA-3') and an appropriate amount of water were added to the tube. The RT-PCR was performed in accordance with the manufacturer's instructions. The tube was kept at  $42^\circ\text{C}$  for 30 min for the RT reaction. The reverse transcriptase was then heat inactivated at  $95^\circ\text{C}$  for 5 min. The PCR conditions were as follows: 35 cycles of denaturation at  $95^\circ\text{C}$  for 30 s, annealing at  $52^\circ\text{C}$  for 30 s, and elongation at  $72^\circ\text{C}$  for 30 s, followed by an additional elongation at  $72^\circ\text{C}$  for 5 min. For the nested PCR, 1  $\mu\text{l}$  of the first-round PCR product was added to a 0.5-ml Ready to Go PCR tube (Amersham Pharmacia Biotech Inc.) as a template and then 50 pmol each of primers CCHF/F3C (5'-GAGTGTGCCTGGGTTAGCTC-3') and CCHF/R2C (5'-GACATTAC

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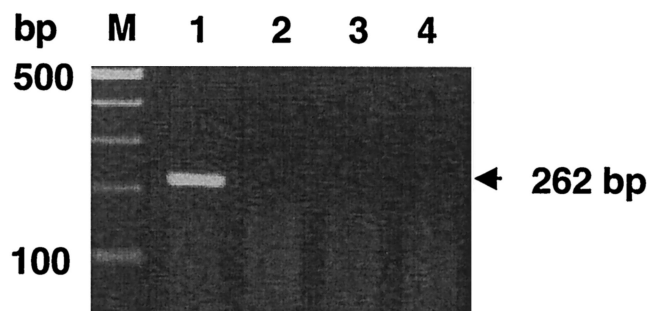


FIG. 1. Results of the nested RT-PCR assay of serum samples serially collected from the patient. Lanes: M, 100-bp markers; 1, 2, 3, and 4, day 1, 5, and 9 serum and negative control samples, respectively. A PCR product of the expected size (262 bp) was amplified only from the day 1 serum sample.

AATTCGCCAGG-3') and an appropriate amount of water were added to the PCR tube. The second-round PCR was performed under the same conditions as described above. The PCR product was separated by electrophoresis in a 2% agarose gel and visualized by staining with ethidium bromide. The expected size of the PCR product was 262 bp.

IgG antibodies to CCHFV were detected by a CCHFV rNP-based IgG ELISA (6). IgM antibodies to CCHFV were detected by an IgM capture ELISA with the same antigen. The ELISA plate was coated with goat anti-human IgM antibody ( $\mu$  chain specific; Zymed Laboratories Inc., South San Francisco, Calif.) at an approximate concentration of 100 ng/well at 4°C overnight. After the plate was washed with phosphate-buffered saline solution containing 0.05% Tween 20 (T-PBS), it was treated with the blocking reagent, 200  $\mu$ l of T-PBS containing 5% skim milk (T-PBS-M) per well, at 37°C for 1 h. The plate was washed with T-PBS, and the top four and bottom four wells of the plate were inoculated with heat-inactivated test serum samples (100  $\mu$ l/well), which were diluted twofold from 1:50 to 1:400 with T-PBS-M. The plate was incubated at 37°C for 1 h. After being washed with T-PBS, the top four wells of the plate were inoculated with the purified CCHFV rNP in T-PBS-M (100  $\mu$ l/well) at a concentration of 1  $\mu$ g/ml while the bottom four wells were also inoculated with T-PBS-M as a negative control. After being washed with T-PBS, all of the wells were inoculated with anti-CCHFV rNP rabbit serum at a dilution of 1:1,000 (6). After being washed with T-PBS, the plate was inoculated with goat anti-rabbit IgG antibody labeled with horseradish peroxidase (Zymed Laboratories Inc.) at a dilution of 1:1,000. The plate was washed with T-PBS, 2,2'-azino-di[3-ethylbenzthiazolin sulfate (6)] (ABTS) solution (Roche Diagnostics GmbH) was added to each well, and the plate was incubated at 37°C for 30 min. The optical density at 405 nm ( $OD_{405}$ ) was measured with a reference of 490 nm. The  $OD_{405}$  was adjusted by subtracting the  $OD_{405}$  of the non-antigen-inoculated well from that of the corresponding antigen-inoculated well. The cutoff value for the IgG ELISA at a dilution of 1:400 was defined as reported previously (6). The cutoff value that determined IgM positivity or negativity was calculated as the average plus 3 standard deviations of serum samples collected from 48 subjects with no history of CCHFV infection.

The viral genome was detected by nested RT-PCR in the day

TABLE 1. IgG and IgM antibodies to CCHFV detected by ELISAs in serum samples drawn sequentially

Class of antibodies and dilution	$OD_{405}$ of samples and antibody <sup>a</sup>			Cutoff <sup>b</sup>
	Day 1	Day 5	Day 9	
IgM				
1:50	0.000	2.692	2.709	ND <sup>c</sup>
1:100	0.020	2.672	2.711	0.205
1:200	0.044	2.528	2.767	ND
1:400	0.040	1.606	2.001	ND
IgG				
1:100	0.075	0.924	1.882	ND
1:400	0.031	0.486	0.972	0.213
1:1,600	0.000	0.152	0.384	ND
1:6,400	0.000	0.045	0.132	ND

<sup>a</sup> The  $OD_{405}$  values shown here were adjusted as described in the text.

<sup>b</sup> Cutoff values for IgM and IgG responses were defined at dilutions of 1:100 and 1:400, respectively, as described in the text. According to these cutoff values, both the IgM and IgG responses were demonstrated in the serum samples drawn on days 5 and 9 but not in the day 1 sample.

<sup>c</sup> ND, not determined.

1 serum sample but not in those from days 5 and 9 (Fig. 1). Neither IgM nor IgG antibodies to CCHFV were detected in the day 1 serum sample. On the other hand, IgM and IgG were both detected in the serum samples collected on days 5 and 9 (Table 1). The titers were higher on day 9 than on day 5.

The virological and immunological status of CCHFV infection was closely followed by nested RT-PCR and the ELISAs. We previously reported the CCHFV rNP-based antibody detection systems (5, 6). In the present study, we used a CCHFV rNP-based IgM capture ELISA along with an IgG ELISA. We applied these assays to the serum samples serially collected from the patient. The results suggest that both the CCHFV rNP-based IgG ELISA and the IgM capture ELISA are useful for the diagnosis of CCHF. We believe that this is the first case of CCHF serologically diagnosed by CCHFV rNP-based antibody detection systems.

Ribavirin, an anti-RNA virus agent, has been reported to have an inhibitory effect on the replication of CCHFV in vitro and in vivo (2, 7, 9). There have been several reports on ribavirin therapy for CCHF (1, 3, 8). However, the efficacy of ribavirin for CCHF has not yet been proved. Oral ribavirin was used for postexposure prophylaxis of CCHFV infection with promising results, but its efficacy was not formally assessed (8). The patient in the present study was treated by intravenous administration of ribavirin. To our knowledge, this is the first case report of a CCHF patient treated with intravenous ribavirin therapy. The previous reports, along with the present study, suggest that ribavirin is a promising drug for the treatment of CCHF.

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