

Investigation of the Presence of Crimean-Congo Hemorrhagic Fever Virus RNA in Tears of Eleven Infected Patients

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Abstract

Purpose: To investigate the presence of viral RNA in tears of patients with Crimean-Congo hemorrhagic fever (CCHF) disease.

Methods: This study was conducted on eleven patients with CCHF cases. The diagnosis of CCHF was based on clinical and epidemiological findings and the detection of CCHF virus (CCHFV)-specific IgM and of CCHFV RNA in the serum samples of the patients by ELISA and a real time reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. CCHFV RNA positive patients tear samples were then subjected for investigation of viral RNA. Blood and tears of patients were collected at the same time within 24 hours of hospitalization of patients. A TaqMan-based one-step real time RT-PCR was applied to detect CCHFV RNA in sera and tears of patients.

Results: Eight patients were male and three patients were female. One of 11 patients had only CCHF virus-specific IgM antibodies and four of 11 patients' blood sera had only positive for viral RNA. Both viral RNA and virus specific IgM were detected in six out of eleven patients' sera. Although the presence of CCHFV RNA was confirmed in 10 (90.9%) patients' sera, none of the patients' tear had viral RNA.

Conclusions: The preliminary results obtained in the present study showed that the presence of CCHFV RNA was not detected in tear fluids in patients having CCHF disease. This suggested that tears of CCHF patients may not have a potential for CCHFV virus transmission.

Keywords: Crimean-Congo hemorrhagic fever; Reverse transcriptase-polymerase chain reaction; Tears

Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the genus *Nairovirus*, a tick borne RNA virus in the family *Bunyaviridae* [1]. CCHFV is a contagious viral zoonosis and associated with a severe acute hemorrhagic disease known as *Crimean-Congo hemorrhagic fever* (CCHF) in humans with mortality reaching up to 30% [2]. Human individuals acquire the infection either through tick bites of the genus *Hyalomma*, especially *Hyalomma marginatum marginatum* or direct contact with blood or tissues of infected humans or viremic livestock. Thus, the healthcare workers dealing with infected patients, livestock breeders contacting infected animals are under risk groups. The infection of CCHFV in humans often results in sudden onset of the disease with fever, and other nonspecific flu-like symptoms. The severity of the disease correlates with the amount of the viruses in blood of patients (up to 10⁹ genome equivalents/ml of blood) [3]. The first case of CCHF disease was confirmed in Tokat province in the Kelkit Valley of Turkey located in northern Turkey in 2002 [3,4]. Between 2002 and 2009 4453 CCHF confirmed cases were reported by the *Turkish Ministry of Health* [6].

The reverse transcriptase polymerase chain reaction (RT-PCR) used for the detection of viral RNA in sera of infected patients is a highly specific, sensitive and rapid method for the diagnosis of CCHFV disease [2]. Alternatively, the viral antigens can also be detected by immunofluorescence assay or by ELISA using recombinant nucleocapsid protein (NP) [3,7,8]. In general, detection of viral RNA by RT-PCR and/or detection of CCHF virus-specific IgM by ELISA are used in the laboratory diagnosis of the Crimean-Congo hemorrhagic fever. Both IgG and IgM antibodies are detectable by IFA or ELISA about 7 days after the onset of illness. The IgM antibodies declines to undetectable levels from the fourth month after infection, and IgG

titres may also begin to decline gradually at this time, but remain demonstrable for at least 5 years [1]. Viral RNA could be detected in samples up to day 16 of illness, whereas, infective virus was progressively cleared from the serum after the first week of illness [9].

Body fluids are important in transmission of several diseases including CCHF [10-12]. Several nosocomial outbreaks of CCHF, mainly focused on blood-borne transmission, have been reported [13,14]. Bodur et al. [15] reported the presence of the viral RNA genome of CCHFV in saliva and urine samples of infected patients. It is likely that other body fluids including tears may have a potential in transmitting diseases between humans. For instance, there is a risk of transferring several viral infections such as hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) by tears [11,12,16-18]. Thus, the potential of tears of CCHF disease patients in transmitting the CCHF disease need to be searched.

The aim of the present study was to investigate the presence of CCHFV in tears of patients.

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Methods

The physical examination revealed 1–2 cm ecchymosis in 3 patients on their legs. Two of the 11 patients developed epistaxis during follow-up. One female patient developed early vaginal bleeding. However, none of these patients had active bleeding at the time of the samples collection. All patients had fever and thrombocytopenia. This study included the patients coming from endemic region for CCHF, tick bites and / or ticks, and the previously known healthy patients who developed fever and thrombocytopenia. Patients who have serologically negative results for CCHF IgM and/or RT-PCR were excluded from the study. None of the patients were treated with ribavirin. Supportive therapies were applied to all our patients.

Collection of study sample

The present study was undertaken in Cumhuriyet University School of Medicine Hospital, Sivas, Turkey between May and September in 2010. The study protocol was approved by the Human Ethics Committee of Cumhuriyet University School of Medicine. A total of 21 blood serum samples and tears were collected aseptically upon admission.

Tears were collected aseptically from 21 patients by two experienced ophthalmologists (HE and MKA). Both blood sera and tear fluids were collected at the same time within 24 hours of hospitalization. In addition, the presence of serum IgM of patients was also tested by ELISA in acute and convalescent phase sera. A convalescent phase serum sample was obtained from each patient after at least 7 days when possible. All patients were followed up at the Department of Infectious Diseases and Clinical Microbiology at Cumhuriyet University Hospital.

Reflex tear stimulation was carried out nontraumatically by holding open the upper lid to prevent blinking, a procedure that eventually stimulated a heavy production of reflex tears. Tear samples were collected from inferior fornix using sterile disposable blunted glass microcapillary tubes. Precautions were taken to avoid damaging and bleeding the lid margins, the conjunctiva or the corneal surface. Collected tear fluids were transferred immediately to a sterile eppendorf tubes and they were stored at -40 °C until testing. Following collection of serum samples and tears were immediately sent to the Virology Laboratory of Refik Saydam National Public Health Agency (RSNPHA) Ankara, Turkey.

Patients	Serum ELISA IgM	Blood sera RT-PCR	Blood viral load (copies/ml)	Tear fluids RT-PCR	Duration of symptoms* (days)
Case 1	+	+	10.243	-	6
Case 2	+	+	672.650	-	4
Case 3	+	+	35.443	-	4
Case 4	+	+	169.514	-	4
Case 5	+	+	107.747	-	10
Case 6	+	+	86.110	-	2
Case 7	-	+	399.977	-	4
Case 8	-	+	146.786	-	10
Case 9	-	+	3.160	-	3
Case 10	-	+	1.740	-	4
Case 11	+	NT		-	4

CCHF = Crimean-Congo hemorrhagic fever

RT-PCR = Reverse transcriptase polymerase chain reaction

NT = None-tested

+ = Positive

- = Negative

* = Time between the beginning of symptoms and collection of tear fluids

Table 1: Results of ELISA, CCHF real-time RT-PCR tests and viral loads in blood and tear fluids.

Diagnosis of CCHF disease and testing the presence of viral RNA in tear fluids of patients

The presence of CCHFV RNA was tested in both blood sera and tear fluids using a TaqMan-based real time RT-PCR assay as described by Yapar et al. [19]. Briefly, 5 µl of CCHFV RNA was added to 20 µl of mixture containing 5 pmol of primers, 4 pmol of TaqMan probe, 0.2mM of each dNTP (containing dUTP), and 6mM magnesium chloride. The cycle conditions were as follows: 42°C for 40 min, 95°C for 10 min and then 40 cycles as follows: 15 s at 95°C, 1 min at 60°C. The assays were run on a Perkin-Elmer 7700 Sequence Detection System by using a combination of reverse transcriptase (MBI Fermentas) and Hot Start Taq Polymerase (Bioron GmbH, Germany). In addition, ELISA was also used to detect CCHFV specific IgM antibodies.

Results

The presence of CCHFV RNA was confirmed in 10 out of 21 individuals that were suspected to have CCHF disease according to epidemiological and clinical findings using a TaqMan-based real time RT-PCR assay as described previously [19]. In addition, the virus specific IgM antibodies were also detected in 11 out of 21 patients by ELISA. Among 11 patients, 8 (72.7%) were male and 3 (27.3%) were female and the median age of the patients was 51 (min. and max: 22-73). One patient died due to the disease complications during the hospitalization period. One out of 11 patients sera (9.1%) was positive only for CCHF virus-specific IgM antibodies by ELISA and four out of 11 patients' sera (36.4%) were positive only for viral RNA by a TaqMan-based RT-PCR. Both viral RNA and virus specific IgM were detected in 6 out of 11 (54.5%) patients' sera. Thus, the presence of CCHFV RNA was determined in 10 out of 11 patients' sera (90.9%). Although one patient no 11's convalescent sera was positive for virus specific IgM, acute phase sera was not obtained for viral RNA detection. The 11 CCHFV blood sera positive patients tears were also tested by a TaqMan based one-step RT-PCR and viral RNA was not detected in any of the patient's tear samples (Table 1).

Discussion

CCHF is one of the most important fatal zoonosis and emerging disease causing infections and fatalities in humans [2,20]. It has been endemic in about 30 countries in Africa, Asia, Europe, the Balkan Peninsula, the Middle East and has become a serious threat to public health in Eurasia [20,21]. Since 2002, CCHF is an endemic disease in Turkey and there has been substantial increase in the number of cases especially in the provinces of the Kelkit Valley [5].

According to informations from the Turkish Ministry of Health, 60% of the patients acquire the CCHF disease through the tick bite [15] whereas; the source of infection in the remaining 40% of patients is usually unknown. It has been suggested by seroepidemiological studies that CCHFV IgG specific antibodies were present in humans in endemic areas despite they did not present symptoms of the disease [22,23].

Since human infections can lead to nosocomial outbreaks and very infectious nature of the CCHFV, it is essential to determine the potentials of various blood fluids including tears for transmitting CCHFV. To date, there have not been any reports investigating CCHFV RNA in tears of infected patients with CCHFV. In the present study, we investigated the presence of CCHFV in tears of patients. The test of 11 patient's 10 of whose blood sera was positive for viral RNA by RT-PCR did not show any evidence of viral RNA in tears.

Several body fluids including saliva, urine, and tears have been investigated for the presence of viral RNA in several viral infections such as dengue, Ebola hemorrhagic fever (Ebola HF), HIV, HBV and HCV infections [11,17,18,24,25]. Chakravarti et al. [24] detected dengue (DEN)-specific IgG and IgM antibodies in saliva by ELISA and they concluded that the detection of DEN-specific salivary IgG and IgM antibodies are useful markers for dengue infection. Various clinical specimens from patients as well as from environmental surfaces have been investigated for the presence of Ebola HF virus [25]. They found that Ebola HF virus can be shed through various body fluids including saliva, breast milk, stool and tears during the acute phase of illness. In their study, sixteen clinical specimens from 12 patients were positive by virus culture (4 specimens) and/or RT-PCR (16 specimens), including saliva (8 of 16), skin swab (1 of 11), stool (2 of 4), semen (1 of 2) breast milk (2 of 2), tears (1 of 1), and nasal blood (1 of 1). They concluded that EBOV is shed in a wide variety of bodily fluids during the acute period of illness. Kibadi et al. [26] have reported that twenty-two days after the onset of disease, Ebola viral antigens were no longer detectable in blood or plasma by an antigen-capture ELISA test, but the Ebola virus remained detectable by polymerase chain reaction of a conjunctival swab.

Bodur et al. [13] previously investigated the viral RNA of CCHFV in saliva and urine samples of infected patients. In their study, the genome of CCHFV virus was detected in the saliva in five out of six patients and in the urine samples in two out of three patients. Interestingly, in their study, the levels of viral load in the saliva and urine samples were similar to those in the blood samples in all but one patient, in whom higher levels were detected in blood compared to saliva or urine.

In the present study, the potential role of tears for transmission of CCHFV in tears of 11 laboratory-confirmed CCHFV patients were investigated. Although CCHFV RNA was detected in 10 out of 11 patients' sera, CCHFV RNA was not detected in these patients' tears. The failure of the demonstration of CCHFV RNA in tears could be attributed to several reasons. The number of cases subjected for this study was not enough to make a sufficient conclusion. The larger numbers of cases are indeed necessary and the collection of tear samples should be started as early as possible from the suspected and confirmed cases of CCHF disease. In this study, our limitations were not collecting the sample in consecutive days and the low number of subjects.

In conclusion, CCHFV RNA was not detected in tears of infected patients although the viral RNA was detected in almost all tested patients' sera. From our preliminary results, it seems that tears of CCHFV infected patients may not likely to have potential for virus transmission and a possible nosocomial outbreak. However, our present study should be extended to larger numbers of patients to make a sufficient conclusion.

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