GENOTYPIC ANALYSIS OF S SEGMENT OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS IN TURKEY

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The Crimean-Congo hemorrhagic fever (CCHF) virus (CCHFV) is a tick-borne virus, whose viral RNA consists of S, L, and M segments. The virus is migrating through the ticks with animals and migratory birds and the geographical distribution can be investigated based on genetic analysis. To better understand the connection between the seropositivity and the mortality rate, the key factor could be the temporal and spatial analysis of the different strains. In this study, serum samples ($n = 26$) were selected from CCHFV RNA-positive patients and subjected to sequence analysis of the gene regions encoding the S segments. According to the neighbor-joining analysis, the obtained partial sequences were linked to the European strain. The strains were closely related to Turkey-Kelkit06, Turkey 200310849 viruses, and viruses from Russia and Kosovo. The comparison with previously analyzed isolates from the GenBank showed 95%–99% sequence similarity. The isolates in phylogenetic branches were divided into two groups. AST, platelet, and APTT levels were found significantly higher in Group 2 compared to Group 1. Nucleotide differences can be prognostic factor in CCHF disease. Increasing CCHF cases not originating from local isolates were circulating strains imported from different neighboring countries of Turkey. The results show new evidence to the emerging threat of the CCHF disease.

Keywords: Crimean-Congo hemorrhagic fever virus, S segment, genotypic analysis

Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a zoonotic disease caused by the CCHF virus (CCHFV). The virus is primarily transmitted to humans by a bite of *Hyalomma marginatum*. Birds are playing an important role in the transmission of the virus, since they are resistant to it [1]. CCHF is mainly found

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in Africa, Southeast Europe, and Asia. Tokat, a province of Turkey, is known for the CCHF endemic [2, 3]. In Turkey, 900 new CCHF cases are currently found yearly, and a total of 9,787 cases have been reported to the Minister of Health from 2002 through December 2015, of whom 469 (4.79%) died [4]. CCHF in humans starts with non-specific symptoms, such as general muscle aches, headache, nausea, vomiting, and diarrhea, and progresses to hemorrhagic syndrome [5]. Death is mostly caused by the development of hepatorenal insufficiency, acute respiratory distress syndrome, and disseminated intravascular coagulopathy [3]. The mortality rate of CCHF is between 3% and 30% [4]. In Turkey, it is between 2% and 12% [6].

The CCHFV is a single-stranded negative-polarity RNA tick-borne virus of the genus *Nairovirus* in the new order of *Bunyavirales* [7]. Viral RNA consists of three segments, namely S, L, and M segments. The small (S) segment of the genome encodes the major protein of nucleocapsid, the medium (M) segment encodes a glycoprotein precursor, resulting in the two envelope glycoproteins, G1 and G2, and the large (L) segment encodes an RNA-dependent RNA polymerase [8, 9]. The recombination in S segment of CCHFV was eventually demonstrated by Lukashev [10] in 2005. The results of other studies depicted evidence of potential recombination among S segment in CCHFV [2]. Genetic alterations in RNA viruses are often caused by mutations, but reassortment can also result in a different genetic structure. Recently, it has been emphasized that global warming might affect the migration patterns of birds and maturation processes of ticks creating new habitats for ticks [2]. Furthermore, it has also been suggested that the increased tick populations might play a role in the increased incidence of tick-borne infectious diseases [11]. Nucleotide sequencing has shown a recombination in the S segment of the CCHFV [2]. The aim of this study is to investigate genotypic relationship in the S segment of CCHFV obtained from patients in endemic region of Turkey.

Materials and Methods

This project was approved by the ethics committee of Gaziosmanpasa University (number: 15/KAEK/048). In this study, serum samples of 26 patients diagnosed with CCHF were analyzed. All serum samples were stored at −80 °C until processed.

RNA isolation

RNA was collected with the Bosphore CCHFV Quantification Kit (Anatolia Geneworks, Turkey). Lyophilized proteinase K and carrier RNA were liquefied

with buffers and RNase-free water. In total, 400 µl of serum was added to 10 µl carrier RNA (1 mg/ml) and 20 µl proteinase K (10 mg/ml), and RNA isolation was performed on the Magnesia 16 isolation device (Anatolia Geneworks), in accordance with the manufacturer's recommendation. All collected RNAs were stored at +4 °C, if used within a short period of time or at −20 °C.

Polymerase chain reaction (PCR) stage

An amount of 10 µl of RNA was added to 15 µl PCR Master Mix (Bosphore® CCHFV Quantification Kit v1) and PCR was performed with Montania 4896 Real-Time PCR (Anatolia Geneworks). The thermal protocol was as follows: initial denaturation at 95 °C for 14.5 min followed by 50 cycles of denaturation at 95 °C for 0.5 min, and binding and synthesis at 52 °C for 1.5 min. After a total of 50 cycles, the PCR was completed by incubating at 22 °C for 5 min. The primer sequences used in the PCR are proprietary to the manufacturer.

Sequencing stage

Post-PCR purification was performed with the Spin Column Method Bosphore® PCR Product Purification Spin Kit (Anatolia Geneworks). The DNA concentrations were measured by nanodrop. The concentration of >25 ng/µl was determined as the study's acceptance criteria. A total of 27 samples with DNA concentrations >25 ng/µl were sequenced. DNA samples were prepared to be about 250 ng with dH_2O with a total volume of 20 µl. The relevant primer and sequencing mix (Bosphore® CCHFV Sequencing Kit; Anatolia Geneworks) prepared by the manufacturer were added to the samples. After the PCR, 2 µl of 3 M sodium acetate (pH = 5.2), 2 µl of 100 mM Na_2EDTA (pH = 8), and 1 µl of 20 mg/ml glycogen were added to the tubes and mixed for 20–30 s. An amount of 120 µl of cold 95% ethanol (−20 °C) was added followed by a centrifugation at $12,500 \times g$ for 15 min. The supernatant was removed and the pellet was mixed with 240 µl of cold 70% ethanol (−20 °C). After centrifugation ($12,500 \times g$ for 5 min), the pellet was incubated at 37 °C for 1 h. DNA was reconstituted with 40 µl sample loading solution and incubated at 37 °C for 5 min. The sequencing was performed by a CEQ 8000 Genetic Analyzer (Beckman Coulter, USA).

A total of 450 base-pair section of the S segment was amplified and 360 base-pair section was sequenced. The Clustal W program [12] was used to analyze viral nucleotide and amino acid sequences, while BLAST analysis of sequencing results was performed according to the National Center for Biotechnology Information

GenBank (http://blast.ncbi.nlm.nih.gov). All isolates were compared with previously genetically analyzed isolates.

Phylogenetic analyses

Phylogenetic analyses were conducted by MEGA version 6 [13]. Nucleotide sequences of strains already described were obtained from GenBank and used to construct alignments and phylogeny. The accession numbers of these strains were: complete S segment of Senegal DQ211639, Nigeria KY484036, S Africa DQ211646, S Africa DQ211647, China DQ211642, Russia Drosdov DQ211643, Russia DQ206447, Turkey 200310849-DQ211636, Turkey-Kelkit06 GQ337053, and Kosovo AF428144 and partial S segments. Neighbor-joining tree was constructed using Kimura 2 Parameter substitution model [14]. The branching pattern was statistically evaluated by bootstrap analysis of 1,000 replicates.

Statistical analysis

The difference between levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), white blood cell (WBC), platelet, prothrombin time (PT), and activated partial thromboplastin time (APTT) in Groups 1 and 2 were investigated with unpaired t test. The statistical significance level of p was 0.05. Statistical analysis was performed using commercial software (IBM SPSS Statistics 20, SPSS Inc., an IBM Co., Somers, NY, USA). Data are presented as mean $\pm$ SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

Sixteen (61.5%) of the patients were male and 10 (38.5%) were female; 25 (96.2%) of them were living in Tokat, 1 (3.8%) of them in Amasya. Twenty patients (77%) were living in the village and six patients (13%) were living in the city. Evaluation of the patients' occupation showed that most of them were farmers ($n = 14$, 53.8%). The mean age was 50 ± 6.8 year. Twenty-one patients (80.8%) had tick bite, whereas five (19.2%) were not fully aware of the tick bite. The neighboring joining analysis of the partial sequence obtained from the patients suggested that ticks are related to the European strain. We also found that our viral isolates were closely related to Turkey-Kelkit06 and Turkey 200310849 viruses, as well as viruses from Russia and Kosovo. Their amino acid sequences were also similar. The isolates designated as KRM are shown in the phylogenetic tree in

Figure 1. The comparison with previously analyzed isolates from the GenBank showed 95%–99% sequence similarity. The names of the compared isolates and their GenBank access numbers are shown in Table I. The isolates were separated in two distinct subbranches. According to the distinct subbranch, strains were separated into two groups. Group 1 consisted of KRM 17, KRM 28, KRM 2, KRM 13, KRM 7, KRM 35, KRM 37, KRM 16, KRM 4, KRM 22, KRM 5, KRM 30, KRM 3, KRM 5, KRM 24, KRM 29, KRM 21, and KRM 15 and Group 2 consisted of KMR 10, KRM 19, KRM 26, KRM 27, KRM 32, KRM 38, KRM 14,

Figure 1. Demonstration of CCHF-diagnosed isolates in the phylogenetic tree

Table I. Names and GenBank accession numbers of the compared isolates

No.	The name of the compared isolates	Accession numbers in the GenBank	No.	The name of the compared isolates	Accession numbers in the GenBank
1	Tokat 555-2010	HQ685839	18	Bingol-2008	FJ601862
2	Tokat 92-2008	FJ601882	19	Samsun-2008	FJ60186
3	Tokat 89-2008	FJ601881	20	Sivas 167-2008	FJ601859
4	Turkey/Yozgat 20-2012	KR059974	21	Samsun-2008	FJ601861
5	Yozgat 19-2012	KR092375	22	Tokat 3944-2009	HQ188914
6	Turkey/Corum 22-2012	KR059958.1	23	Yozgat 714-2010	HQ188920
7	Sivas 561-2010	HQ188917	24	Sivas 515-2009	HQ173898
8	GOU-OT 07	EU727456	25	Kastamonu 627-2010	HQ188913
9	Tokat 1728-2009	HQ173902	26	Turkey_812955	KY362517
10	Yozgat 207-2011	KR092377	27	Turkey/Corum 434-2011	KR059970
11	Tokat 1731-2009	HQ659777	28	Kastamonu 627-2010	HQ188913
12	Yozgat 8-2007	FJ601871	29	Tokat 1731-2009	HQ659777
13	Yozgat 19-2012	KR092375	30	Corum 6-2007	FJ601876
14	Turkey_812955	KY362517	31	Yozgat 207-2011	KR092377
15	Iran-Isfahan 78	KJ027522	32	Kastamonu 229-2010	HQ675009
16	Corum 618-2009	HQ675004	33	Turkey/Çorum 22-2012	HQ188914.1
17	Tokat 1728-2009	HQ173902			

and KRM 40. AST and APTT levels were significantly increased in Group 2 compared to Group 1 (**$p < 0.01$). Platelet level was significantly increased in Group 2 compared to Group 1 (***$p < 0.001$). There were no significant difference in ALT, PT, and WBC levels in Group 2 compared to Group 1 ($p > 0.05$). The level of the laboratory parameters and statistical data of Groups 1 and 2 were shown in Table II. The statistical data of the laboratory parameters of Groups 1 and 2 was shown in Figure 2.

Discussion

The CCHFV was first isolated in 2002 in the province of Tokat, Turkey [6]. The number of CCHFV-associated cases has increased over the years. Most CCHF cases occur in Kelkit valley and surrounding areas including Tokat, Sivas, Yozgat, and Çorum [4].

People who work in farms and slaughterhouses, live in rural areas, and are in contact with blood and tissue of infected animals are at higher risk of CCHF infection [4]. Seropositivity was reported in 79% of the sera collected from 400 cattle in Tokat region [15]. Moreover, Kırbaş et al. [16] examined 100 cattle and 100 sheeps that belonged to the households in the endemic regions, which

Table II. The statistical data of the laboratory parameters of CCHF strains

	Group 1 (mean $\pm$ SEM)	Group 2 (mean $\pm$ SEM)	p value
AST	72.79 $\pm$ 14.32	143.9 $\pm$ 16.89**	0.0031
ALT	43.71 $\pm$ 8.037	62.53 $\pm$ 6.315	0.0742
Platelet	123,778 $\pm$ 9,191	68,059 $\pm$ 5,951***	<0.0001
WBC	2,523 $\pm$ 224.9	2,132 $\pm$ 142.9	0.1509
PT	13.36 $\pm$ 0.3475	14.21 $\pm$ 0.3619	0.0987
APTT	33.78 $\pm$ 2.695	33.78 $\pm$ 2.695**	0.0020

Note: The table shows the blood parameters of Groups 1 and 2. Data are presented as mean $\pm$ SEM. Unpaired t test was used (**$p < 0.01$, ***$p < 0.001$), Group 1 compared to Group 2. CCHF: Crimean-Congo hemorrhagic fever; SEM: standard error of measurement; AST: aspartate aminotransferase; ALT: alanine aminotransferase; WBC: white blood cell; PT: prothrombin time; APTT: activated partial thromboplastin time.
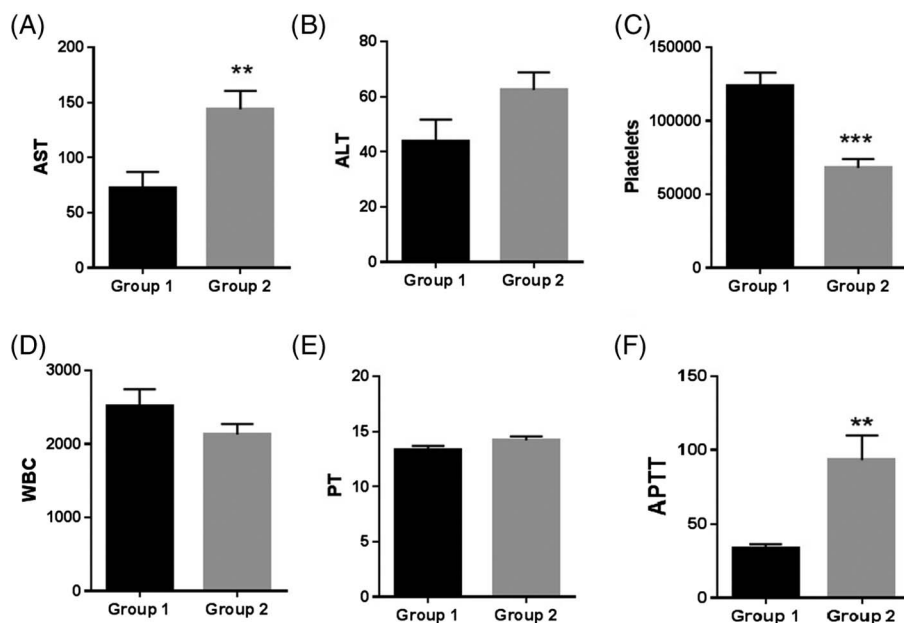

Figure 2. The difference between laboratory levels in Groups 1 and 2. (A) AST levels were significantly increased in Group 2 compared to Group 1 (**$p < 0.01$). (B) There were no significant difference in ALT level in Group 2 compared to Group 1 ($p > 0.05$). (C) Platelet level was significantly increased in Group 2 compared to Group 1 (***$p < 0.001$). (D) There were no significant difference in WBC level in Group 2 compared to Group 1 ($p > 0.05$). (E) There were no significant difference in PT level in Group 2 compared to Group 1 ($p > 0.05$). (F) APTT levels were significantly increased in Group 2 compared to Group 1 (**$p < 0.01$)

included Elazığ, Samsun, Sivas, Tokat, and Yozgat, and found that 17% of cattle and 37% of sheeps were positive with CCHF viral IgG. The CCHF positivity rates in this region may be the result of contamination with blood and tissues of these animals.

In this study, most of the patients were living in the endemic region, which supports the notion that living in the rural areas and being in contact with animals are the risk factors for CCHF. Reports have shown that infectious transmission may occur not only through tick bites, but also through nosocomial outbreaks, direct contact with blood or bloody secretions of the patient with severe manifestations, and through airway [17]. In this study, the presence of tick bite in 21 of the patients (80.8%) suggests that the most important source of infection in endemic areas is still tick bites. CCHF poses a great risk of farmers, slaughterhouse workers, veterinarians, animal caregivers, and healthcare workers [18]. In addition, a significant proportion of the patients were farmers (53.8%) and there were no healthcare workers among the patients. These results are in agreement with Yilmaz et al. (51.8%) [19]. Based on this, we suggest that it is important to inform the population in endemic regions to take precautions against tick bites.

Over the years, a genetic variability between the viruses in different geographical regions has been noticed. The sequence of the S segment of the CCHFV was used in phylogenetic analyses. The S segment plays a role in the encapsulation of viral RNA by forming ribonucleoprotein complexes [20] and is important in assessing the topology of viruses [2]. Based on the CCHF viral S segment sequences, there are seven genetic groups showing different geographical distribution characteristics [21]. In the CCHFV, the genetic variability rates between the segments are diverse, as it is up to 30% at the nucleotide level; however, variability is much lower at the amino acid level [22].

In 2004, Hewson et al. [23] performed nucleotide sequencing of S and M segments and reported genetic differences between isolates and suggested that these differences are due to reassortment. The study from Iran found a recombination in the S segment of CCHFV and emphasized that this could contribute to epidemiological and vaccination studies [24].

A number of studies showed that the CCHFV responsible for the infections in Turkey belongs to the European strain I, which is also widespread in southwestern Russia and the Balkan peninsula [25, 26], whereas others reported that virus belongs to European strain II, which includes Greece AP92 [27, 28].

In 2010, Ozkaya et al. [11] showed no genetic variability in the S segment in Turkish isolates and found that they were closely related to the European strain. Moreover, in 2012, Kalaycıoglu et al. sequenced the S segment and found that it was genetically homogeneous. It has been reported that the CCHFVs that cause

infections in Turkey are closely related and are associated with the European strain I class that includes Eastern Europe-Russia (Drosdov, Kashmanov and Balkan peninsula, Kosovo, and Bulgaria) [29]. In 2016, Chinikar et al. [24] indicated that their study provides evidence of recombination in the S segment of CCHFV and switching of different genomic regions between different strains by recombination could contribute to CCHFV diversification and evolution.

Consistent with previous studies, this study showed that the majority of isolates were clustered in the genetic group V, which represents the geographical area of Europe/Turkey. The increase in CCHF cases in Turkey was thought to be related to the increased number of local viral isolates rather than the introduction of new strains from neighboring countries [27]. Furthermore, climate change has also been suggested to be responsible for the increase in tick-borne infectious diseases by facilitating the growth of the tick population [21].

Cevik et al. [30] reported that laboratory abnormalities, which include leukopenia and thrombocytopenia, high levels of ALT and AST, prolonged bleeding time, PT and APTT, were independent predictors of fatality in CCHF disease. Based on the nucleotide differences, the strains were separated by the two subbranches in the phylogenetic tree. The strains in these branches were divided into two groups and were statistically analyzed. AST, platelet, and APTT levels were found significantly higher in Group 2 compared to Group 1. Nucleotide differences can be a prognostic factor in CCHF disease.

The results of this study suggest that CCHFV's genetic variations may be transported through migrating birds, as well as through viremic animals from countries to the east of Russia and Turkey [2, 11]. Therefore, the proliferation of tick populations and the increased incidence of tick-borne infectious diseases will have vital importance, especially for endemic regions.

Along with the prevalence studies in endemic areas, it is vital to conduct genotypic research in order to establish a countrywide data bank. This information will enable a better understanding of this virus, help to prevent the spread of CCHF, increase the number of treatment options, and help to develop vaccines to prevent the disease.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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