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RESEARCH ARTICLE

Genetic Analysis of the Partial M RNA Segment of Crimean-Congo Hemorrhagic Fever Viruses in Turkey

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Summary

Crimean-Congo hemorrhagic fever (CCHF) is a fatal tick-borne zoonosis extensively common in Africa, Asia, Eastern Europe and the Balkan Peninsula. CCHF has been reported in Turkey with high frequency since 2002. Genetic diversity of CCHF virus (CCHFV) isolates circulating in Turkey were studied by two recent studies from 2006 to the end of 2010. Since CCHFV disease has been an important public health concern in Turkey, it is necessary to continue genetic analysis of CCHFV viruses for the assessment of future patterns of disease. The aim of the present study was to genetic analysis of CCHFV isolates derived from infected patients over a two-year period (2011 and 2012) in several provinces of Turkey. Serum samples (n=10) were selected from CCHFV RNA positive patients and subjected to sequence analysis of the gene region encoding partial M segment. The nucleotide sequence alignments of the 10 partial M segments of CCHFV isolates showed that the nucleic acid relatedness of CCHFV isolates ranged from 94.4% to 100%. Phylogenetic analysis of M segment sequences revealed that CCHFV isolates circulating in Turkey belonged to the European lineage I and were closely related to the viruses previously found in Turkey and in the Eastern European-Russian and Balkan Peninsula. The results of the present study indicated the genetic stability and the lack of the genetic diversity of CCHFV isolates circulating in Turkey.

Keywords: *Crimean-Congo hemorrhagic fever, Genetic diversity, M segment, Glycoprotein precursor, Reassortment, Recombination, Turkey*

Türkiye'deki Kırm Kongo Kanamalı Ateşi Virüslerinin Kısmi M-segmentlerinin Genetik Analizi

Özet

Kırım-Kongo kanamalı ateşi (KKKA), kene kaynaklı, ölümcül bir zoonotik hastalık olup, Doğu Avrupa, Asya, Afrika ve Balkan yarımadasında yaygın olarak görülmektedir. Türkiye'de 2002 yılından itibaren artan bir sıklıkta görülmektedir. KKKAV'leri genetik analizi, yakın zamanda yapılan iki çalışmada 2006 ile 2010 yıllarını kapsayan süreçte araştırılmıştır. Kırım Kongo hastalığının ülkemizde önemli sağlık sorunu oluşturması, gelecek için hastalık sürecinin değerlendirilmesi ihtiyacı; virüsün genetik yapısının sürekli izlenmesini gerekli kılmaktadır. Bu çalışmada 2011 ve 2012 yılları arasındaki iki yıllık süreçte hastalardan elde edilen KKKA virüs (KKKAV) izolatlarının genetik analizi amaçlanmıştır. KKKAV pozitif 10 hastanın serumlarından elde edilen RNA örnekleri kullanılarak kısmı M segment sekansları elde edilmiştir. Elde edilen sekansların eşleştirilmesi sonucu izolatların sekans benzerliği %94.4 ile %100 arasında bulunmuştur. Filogenetik analiz sonucu sekans analizi yapılan izolatların Avrupa I kümesinde yer aldığı, Türkiye, Doğu Avrupa-Rusya ve Balkan Yarımadasında daha önce analiz edilen virüslerle yakın ilişkili olduğu tesbit edilmiştir. Bu çalışmada Türkiye'de dolaşımda olan KKKAV'lerinin genetik olarak sabit ve çeşitlilik yönünden de kısıtlı olduğu sonucu gözlenmiştir.

Anahtar sözcükler: *Kırım-Kongo kanamalı ateşi, Genetik çeşitlilik, M segment, Glykoprotein precursor, Reassortment, Recombination, Türkiye*

INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV) is a member of the genus *Nairovirus,* a tick borne RNA virus in the family *Bunyaviridae* [1,2]. In humans, CCHFV is highly contagious and causes a severe acute hemorrhagic disease

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known as Crimean-Congo hemorrhagic fever (CCHF) with mortality rates reaching 30%. Disease is transmitted either through tick bites (primarily of the genus *Hyalomma*) or direct contact with infected blood or tissues of viremic hosts [3,4]. Since human infections can lead to nosocomial outbreaks, CCHF cases are required to be reported to the public health authorities [5].

CCHF has now been reported in more than 30 countries in Africa, Asia, Eastern Europe, the Middle East and Balkan Peninsula. Disease distribution correlates well with the geographical distribution of the tick vector *Hyalomma marginatum marginatum* [4,6]. In addition, it is possible that migratory birds may also play a part in viral dissemination by carrying infected ticks over great distances [7].

In Turkey, the first case of CCHF disease was confirmed in the Tokat province in the Kelkit Valley located in northern Turkey in 2002. The majority of cases were reported from the middle and eastern part of Anatolia, particularly from the provinces of Tokat, Sivas, Corum, Yozgat and Erzurum [8-10]. Between 2002 and 2010, 5,317 CCHF confirmed cases, and 267 deaths (average fatality rate of 5%) were reported by the Turkish Ministry of Health (http://www.saglik.gov.tr). Potential reasons for the emergence and increase in the number of CCHF cases in Turkey include climate change that may have a significant impact on *Hyalomma* tick reproduction rates as well as anthropogenic factors such as changes in agricultural and hunting habits [5,11].

CCHFV possesses a negative sense, single stranded, three segmented RNA genome comprised of small (S), medium (M) and large (L) segments [12]. The S segment codes for the nucleoprotein (NP) [13], the M segment encodes for a glycoprotein precursor which gives rise to two structural glycoproteins Gn (37 kDa) and Gc (75 kDa) and also encodes for a non-structural protein (NS_m) [13-15]. The glycoproteins, Gn and Gc, encoded by M-RNA segment, are responsible for virus attachment and induction of virus neutralizing antibodies [16]. Therefore, M segment is considered critical to the elicitation of immunopathologic responses in humans [17,18]. The large L segment encodes the RNA-dependent RNA polymerase enzyme (L protein) [1].

Genetic analysis studies based on S, M and L RNA segment sequences of CCHFV isolates have been used to define genetic groups or lineages [2,7,17,19-22]. These studies have indicated the natural occurrence of recombination and reassortment events resulting in worldwide genetic diversity of CCHFV [2,21,23]. Several studies have showed that the majority of CCHFV isolates from infected humans and ticks in Turkey belonged to the European lineage 1 that includes south-western Russia and Balkan Peninsula [8,17,22,24-26]. In addition, some viruses isolated in Turkey were grouped within the European lineage II that originally included CCHFV strain AP92 isolated in Greece [22,27-29].

As a result of frequent reassortment event(s) associated with M RNA segments, phylogenetic analysis based on M segment RNA sequences differ from those based on S and L RNA segment analyses [2]. It is likely that reassortment events associated with M RNA segments may result in enhanced virulence [17,18,30]. In addition, M-segment variability may also result in affecting antigenic and immunogenic epitopes of CCHFV. Therefore, investigations of M segment variability are of a great importance to the identification of new isolates and a better understanding of virulence mechanisms associated with respective CCHFV isolates.

Genetic analysis studies of CCHFV isolates involved in disease seasons from 2006 to the end of 2010 were conducted by two recent previous studies [22,26]. The results of these studies indicated that CCHFV viruses circulating in Turkey were closely related viruses and belonged to European lineage I. Since CCHF associated disease is an important health problem in Turkey, and the possible risk that new CCHFV isolates from other countries could be introduced into this region, a continuous investigation designed to define the genetic analysis of the circulating CCHFV isolates needs to be carried out.

The aims of the present study were i) to carry out phylogenetic analyses of partial 10 M RNA segments sequences from CCHFV isolates obtained from selected human cases from 2011 through 2012, ii) to investigate M segment based CCHFV genetic heterogeneity between isolates in Turkey and to define their relationship to sequences isolated from neighbouring countries.

MATERIAL and METHODS

Study Samples: The present study examined CCHFV positive samples of patients (n=10) from the provinces of the Kelkit Valley (situated in the middle Black Sea region) and other provinces of Turkey during CCHFV disease season in 2011 and 2012. The presence of CCHFV RNA was confirmed using a TaqMan-based real time RT-PCR assay as previously described by Yapar et al.[31]. A total of 10 CCHFV RNA positive samples (5 from 2011 and 5 from 2012) were selected from the Virology Reference and Research Laboratory Turkish National Public Health Agency (TNPHA), Ankara, Turkey.

Viral RNA Extraction and RT-PCR: CCHFV RNA was extracted from respective samples using a viral RNAeasy Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A one step RT-PCR kit was used to amplify a 890 bp partial M segment of 10 CCHFV isolates using primers F (5'- ACAGGCTTTAGGAAACTAC-3') and R (5'-CAC CTGCAATAGCTTTCT-3'). The one step RT-PCR was carried out in a 50 µl reaction volume containing 10 µl of template RNA, 10 µl 5x reaction buffer, 3 µl of each primer (10 pmol), 2 µl enzyme mix, 2 µl dNTP mix (10 nm each) and 20 µl nuclease free water. The reaction mixture was amplified in a thermocycler at the following conditions: 50°C for 30 min and an initial denaturation of 15 min at 95°C followed by 33 cycles at 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min followed by a final step at 72°C for10 min [26].

DNA Sequencing: Following amplification of the 890 bp M CCHFV segments, amplicons were purified using Agencourt Ampure (Beckman Coulter, Brea, CA) and sequencing reactions were set up. Briefly, sequence reaction mixtures consisted of 3.5-5 ml of purified amplicon, 5 pmol primer and 4 ml of Dye terminator cycle sequencing Quick Start Kit (Beckman Coulter, Brea, CA). The sequencing reaction was then carried out as follows: initial denaturation at 94°C for 3 min followed by 30 cycles at 96°C for 20 s, 55°C for 20 s and 60°C for 4 min. PCR products were purified using a DyeTerminator removal kit (Agencourt Cleanseq, Beckman Coulter) and 20 ml of purified product was sequenced using a CEQ 8000 Genetic Analyser (Beckman Coulter Brea, CA).

Phylogenetic Analysis: Clustal W was used to align viral nucleotide and amino acid sequences using representative M nucleotide and amino acid sequences downloaded from GenBank [32]. The CCHFV sequences used for comparison and phylogenetic analysis were given in the phylogenetic tree and their GenBank accession numbers were indicated in brackets.

The evolutionary history of the Turkish isolates based on regional sequence comparisons was inferred using the neighbour-joining (NJ) method. The evolutionary distances were computed using the Maximum Composite Likelihood method [33] and are reflected as units of the number of nucleotide substitutions per site. Phylogenetic analyses were conducted using MEGA4 [34].

RESULTS

Amplification of CCHFV Partial M Segments: Single PCR bands corresponding to the 890 bp of partial M segments (envelope glycoprotein precursor Gn) from the 10 selected CCHFV isolates were successfully amplified and sequenced. The assigned accession numbers for Turkish CCHFV isolates and their provincial origin are described in *Table 1*.

Phylogenetic Analysis of CCHFV M-RNA Segment Sequences: The nucleotide sequence alignments of the 10 M segments of CCHFV isolates derived from patients in 2011 and 2012 showed sequence similarities ranging from 94.4 to 100%. The M segment nucleotide sequence similarity of these isolates with those derived from patients in 2009 and 2010 analysed in the previous study [26] was ranged from 94.9 to 99.7%. In addition, the sequence similarity between isolates subjected for the present and the previous study and Bulgarian vaccine strain V24/81 was ranged from 94.9 to 95.9%. The NJ-based phylogenetic analysis of 10 partial M segment sequences revealed that the CCHFV isolates analysed in the present study belonged to the European lineage I. These isolates were closely related to viruses characterized from Eastern Europe, Russia and the Balkan Peninsula including Hoti, Kosovo, Kashmanov and Drosdov *(Fig. 1)*.

Based on the partial M segment based phylogenetic analysis, the 10 CCHFVs were classified in two groups (I and II) together with representative viruses from Eastern Europe and the Balkan Peninsula *(Fig. 1)*. Group I included eight isolates that were closely related to Turkish representative viruses identified previously (Turkey200310848 and Turkey-Kelkit06) and isolates derived from patients in 2009 and 2010 (KYSR1761/09, AYDN193/10 and KSTM229/10). Two isolates from 2011 (KYSR207/11 YZGT327/11) comprised group II along with Russian viruses ROS/HUVL-100 and Kashmanov *(Fig. 1)*.

Alignment of the amino acid sequences deduced from the translation of the partial M segment nucleotide sequences coding for the envelope glycoprotein precursor identified several amino acid variations in 10/10 isolates primarily located between amino acids 674 and 760 *(Fig. 2)*. Similarity indices of amino acid sequences of the 10 isolates varied between 95.4 and 100%. Compared to the

Table 1. *Provincial origin of CCHFV isolates and their assigned accession number by GeneBank*
Tablo 1. *KKHA virüslerinin illere gore dağılımı ve GeneBank tarafından verilen resmi erişim numaraları*

Province	Isolation Year of Viruses				Total
	2011		2012		
	Virus Code	Accession No.	Virus Code	Accession No.	
Kayseri	KYSR207/11	JX308613			1
Yozgat	YZGT327/11	KC150005			1
Erzurum	ERZRM633/11 ERZRM634/11	JX308615 JX308614			2
Çorum	CRM2020/11	JX308616			1
Kastamonu			KSTM169/12 KSTM241/12 KSTM170/12	KC150000 KC150001 KC150002	3
Edirne			EDRN622/12	KC150003	1
Kırklareli			KRKLAREL707/12	KC150004	1
Total	5		5		10

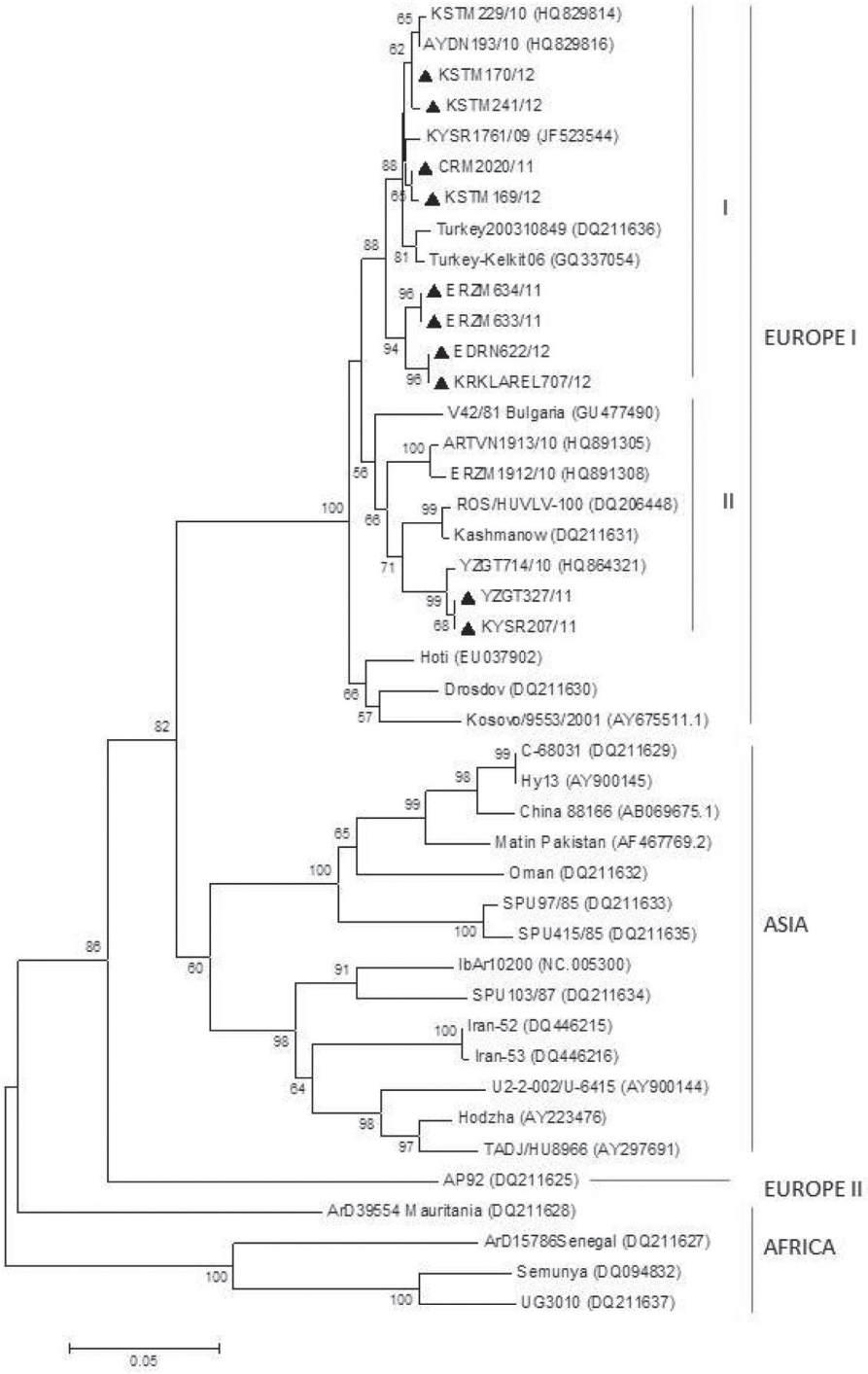

Fig 1. Phylogenetic analysis

A 890 bp of partial M segment amplified from 10 RNA samples obtained from CCHFV isolates between 2011 and 2012 were compared to representative viruses using the neighbour-joining method with Kimura two-parameter distances using MEGA 4 software. Isolates subjected for the present study are indicated by a triangle

Şekil 1. Filogenetik analiz

2011 ve 2012 yıllarına ait KKHAV virüs izolatlarından elde edilen 10 adet RNA örneklerinden 890 bç boyutunda M segmentler çoğaltılmıştır. M segment sekansları referans sekanslarla MEGA 4 yazılımı (neighbour-joining metot ve Kimura two-parameter distances) yöntemi kullanılarak karşılaştırılmıştır. Bu çalışmada kullanılan izolatlar üçgen ile belirtilmiştir

Turkish representative TR200310849 virus sequence, the most striking variation was observed in the replacement of an S to F at amino acid position 711 in all tested isolates in this study and in representative viruses, including Turkey-Kelkit06, Kashmanov, ROS/HULV-100, Drosdov, and Hoti. Isolates YZGT327/11 and KYSR207/11 that displayed identical nucleotide sequence and derived from patients with fatal outcome presented with four amino acid variations, including I674V, K679R, A681V, and I729F. An amino acid variation, A746T, was shared in the isolates of ERZM633/11 and ERZM634/11 that had identical nucleotide sequence and derived from patients with fatal outcome. Isolates EDRN622/12 and KRKLAREL707/12 that had also identical nucleotide sequence and derived from patients from fatal outcome displayed two amino acid variations, including K728R and A741T. An amino acid variation, I710L, was observed in isolate KSTM241/12 derived from another patient with fatal outcome. There was no amino acid variation in isolates CRM2020/11 and KSTM169/12 derived from recovered patients from the disease. Bulgarian vaccine strain V42/82 did not display any common amino acid variation with any of the tested isolates *(Fig. 2)*.

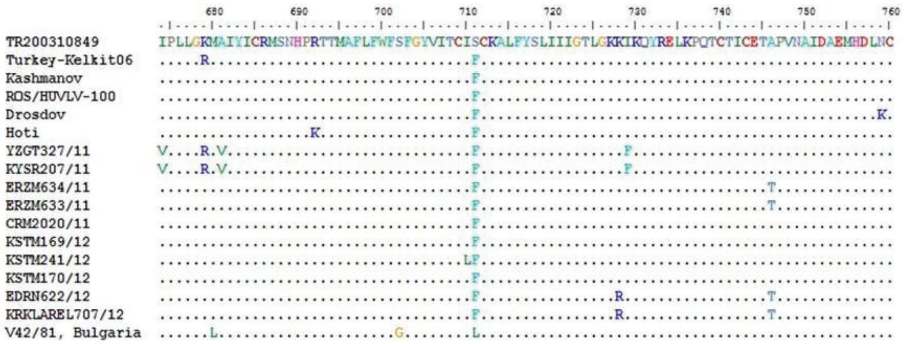

Fig 2. Alignment of the deduced partial CCHFV M segment amino acid sequence corresponding to the variable region between amino acids 674 and 760

Representative CCHFV isolates from Turkey (TR200310849, Turkey-Kelkit06) and from neighbouring countries (Kashmanov, ROS/HUVL-100, Drosdov, Hoti and V42/82 Bulgaria) were included in the alignment

Şekil 2. KKHAV kısmi M segmentinin 674 ve 760 amino asit dizisine karşılık gelen değişken bölgelerinin karşılaştırılması

Türkiye kökenli (TR0310849, Turkey-Kelkit06) ve komşu ülke kökenli izolatlar (Kashmanov, ROS/HUVL-100, Drosdov, Hoti ve V42/82 Bulgaria) karşılaştırmaya dahil edilmiştir

DISCUSSION

Since the first case of CCHF disease was emerged in 2002, an increasing number of CCHF disease cases resulting in fatal outcomes in humans have been reported in Turkey with majority of cases reported in the provinces of Kelkit Valley.

The recombination and reassortment events associated with the segmented RNA genome have inevitably resulted in worldwide genetic diversity of CCHFVs. In particular, more frequent reassortment event(s) occurring in M-RNA segment compared to reassortment events occurring in S and L segments make it more variable than S and L segments [2,21]. Thus, the genetic analysis of M segment is more beneficial for investigating CCHFV diversity.

In the present study, the genetic analysis of Turkish CCHFV isolates obtained from 10 confirmed human clinical cases from 2011 to 2012 was investigated by comparing the partial M segment sequences in phylogenetic analysis.

The NJ-based phylogenetic analysis of the M-RNA segments showed that all tested CCHFV isolates belonged to the European lineage I, including viruses from Eastern Europe-Russia (Drosdov, Kashmanov) and the Balkan Peninsula (Kosovo, Bulgaria) *(Fig. 1)*. The distribution of 10 CCHFVs in two groups on phylogenetic analysis was due to M-segment variability as expected.

The results obtained in our present study have been compared to two recent studies performed by Ozkaya et al.[22] and Kalaycioglu et al.[26] who described the molecular epidemiology of CCHFVs from patients between 2006 and 2008 and investigated genetic diversity of CCHFVs from patients between 2009 and 2010 in Turkey, respectively. The present study extended the phylogenetic analysis of viruses circulating in this region by including viruses collected from 2011 to 2012. In particular, comparison of the partial M-segment sequences involving in viruses studied between 2009 and 2010 in Kalaycioglu's previous study [26] and the present study confirmed the existence of a close relationship between the CCHFV isolates circulating in Turkey *(Fig. 1)*. In addition, a close relationship between the Turkish CCHFVs and Bulgarian vaccine strain V42/82 Bulgaria, the only available inactivated vaccine, may be beneficial for focusing protective efficacy of this vaccine or development of a novel vaccine *(Fig. 1)*.

Amino acid variations between amino acids 674 and 760 of the partial M segment glycoprotein precursor (Gn) protein region was showed the replacement of an S to F at amino acid position 71 in all tested isolates in the present study *(Fig. 2)*. Except for two isolates (CRM2020/11 and KSTM169/12) derived from patients recovered from the disease, the remaining viruses which isolated from individuals with fatal outcome displayed some amino acid variations *(Fig. 2)*. Whether or not these variations important for determining the virulence of viruses remain to be investigated. In addition, other parts and/or the full length M-RNA segment (s) of CCHFV isolated need to be sequenced in future studies for determining nucleotide and amino acid variability that may be critical for virulence mechanism of CCHFV.

The results of our study showed that CCHFV isolates circulating in Turkey were closely related and phylogenetically belonged to the European lineage I as described in previous studies. Our findings were in agreement with the Ozkaya's suggestion [22] that local topotype viruses were circulating and responsible for infections in Turkey. In addition, our results were also in agreement with the description of lack of the genetic diversity of Crimean-Congo haemorrhagic fever viruses in Turkey as described by Kalaycioglu et al.[26]

Since CCHFV is known to be a migrating pathogen, Turkey may not only serve as a 'donor' country for Europe as suggested by Mild et al.[7] but may also be a 'recipient' of

new CCHFV isolates from other parts of the world where the disease is endemic. Generally, CCHFV strains tended to be region or continent specific with certain lineages predominating and circulating in particular areas of the world, including Turkey unless new viruses are introduced either by viremic animals or animals carrying infected ticks. The indroduction of new viruses belonged to other lineages may result in reassortment event(s) between viruses that may lead to appearance of new isolates with enhanced virulence. Therefore, Turkish CCHFV cases need to be monitored for such incursions.

In conclusion, the results based on the genetic analysis of M segment of CCHFV isolates confirmed genetic similarity and stability between viruses circulating in Turkey. This information could be beneficial in future studies focusing on identifying the most potentially effective vaccine strain(s). It should also be noted that there is a potential risk for importing novel viruses from neighbouring and other countries. The more and continuous genetic analysis studies involving in full length M segments are essential for future studies in Turkey.

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