



Spatial and Phylodynamic Survey on Crimean-Congo Hemorrhagic Fever Virus Strains in Northeast of Iran

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Abstract

Background: Crimean-Congo hemorrhagic fever (CCHF) is asymptomatic in infected animals, yet the virus poses a serious threat to humans causing a symptomatic, hemorrhagic disease with a high case-fatality rate. Numerous genera of ticks serve as both vectors and reservoirs of the Crimean-Congo hemorrhagic fever virus (CCHFV).

Objectives: The aim of the present study is to determine the CCHFV prevalence in ticks from northeast Iran to establish a phylogenetic relationship of the tick-derived CCHFV strains circulating in Iran.

Methods: During April to June 2015, a total of 93 hard ticks were collected from different animals in the Damghan district. The S-segment of positive samples was fully sequenced using the Sanger technique. A total of 142 CCHFV sequences comprised full-length of CCHFV sequences obtained in this study were aligned using the MAFFT algorithm, then phylogenetic tree was constructed using Geneious v 7.1.8.

Results: The identified tick species included *Hyalomma marginatum* (6.5%), *Hy. dromedarii* (21.5%), *Hy. anatolicum* (15.1%), *Hy. asiaticum* (3.2%) and *Hy. schulzei* (2.2%), as well as *Rhipicephalus sanguineus* (47.3%). The CCHFV RNA was detected in 4 samples of 93 tick samples (4.3%) by RT-PCR. A total of 4 CCHFV sequences were obtained in this study clustered within clade IV (Asia-1 and Asia-2).

Conclusions: We demonstrated that 4 species of hard ticks could be a vector for CCHFV in Iran. In addition, our findings indicate the circulation of CCHFV clade IV strain in the northeast of Iran and provide a solid base for more targeted surveillance and prevention programs in Iran.

Keywords: Crimean-Congo Hemorrhagic Fever, Arbovirus, Tick, Molecular Epidemiology, Iran

1. Background

Crimean-Congo hemorrhagic fever (CCHF) is an acute, viral, zoonotic disease with hemorrhagic manifestations and considerable mortality in humans. The virus is widely distributed around the world and reports of recent outbreaks have increased. Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the Nairovirus genus of the Bunyaviridae family and has a three-segmented genome: the small (S) segment encodes the nucleoprotein that encapsidates the genome and forms ribonucleoprotein (RNP) complexes (1), the medium (M) segment encodes 2 glycoproteins, and the large (L) segment encodes an RNA-

dependent RNA polymerase (2). The virus is transmitted to humans either by a tick bite, by direct contact with fresh meat or blood of infected animals, or via nosocomial transmission (3-6).

Crimean-Congo hemorrhagic fever disease has been reported in some Asian, European, and African countries. Sporadic outbreaks of CCHF have been reported from Kosovo, Senegal, Turkey, Bulgaria, Iran, Pakistan, and Mauritania (2, 7-9). The predominant hosts of CCHFV include wild and domestic mammals and birds. Sheep, goats, and cattle develop high virus titers in their blood, without notable clinical signs (10). The genus *Hyalomma* is the main vector and reservoir of CCHFV (11, 12).

Iran is a known CCHF-endemic country, from where the disease was first reported in 1970 and the virus isolated in 1978 from ticks. Crimean-Congo hemorrhagic fever virus is detected in 27 of 31 provinces of Iran (13). Data, with respect to the gender, acquired infection in Iran have shown that CCHF infection in males is more frequent than in females, which seems to be due to male dominance in high risk professions. In this regard, farmers and their families, abattoirs, as well as healthcare workers have been considered high risk professions for acquiring CCHF (14). Although the infection rate fluctuates annually and geographically, Sistan va Baluchistan (Southeast of Iran), Khorasan (east of Iran), and Isfahan (central of Iran) provinces have the highest rates of CCHF in humans (15).

An unknown divergence time exists between 2 CCHFV species and can be estimated through the calculation of the gene evolution rate by comparing changes at the nucleotide or amino acid level of the viral genome (16). Crimean-Congo hemorrhagic fever virus has a high degree of genetic diversity, leading to 7 phylogeographic clades: clade I comprising CCHFV strains mainly isolated from West-Africa, clade II comprising CCHFV strains mainly isolated from Central Africa, clade III comprising CCHFV strains mainly isolated from South Africa and West Africa, clade IV comprising CCHFV strains mainly isolated from Middle East and Asia (this clade are divided into 2 distinct clades, Asia-1 and Asia-2), and clade V comprising CCHFV strains mainly isolated from Europe and clade VI comprising AP29 strain isolated from Greece (17, 18). In addition, the different strains of CCHFV are probably correlated with different degrees of pathogenicity (19). Consequently, understanding the molecular epidemiology of CCHFV is essential to enable the development and implementation of surveillance strategies for its control.

2. Objectives

The aim of the present study is to determine the CCHFV prevalence in ticks from Damghan (northeast of Iran), using real-time RT-PCR, and to establish a phylogenetic relationship of the tick-derived CCHFV strains circulating in Iran.

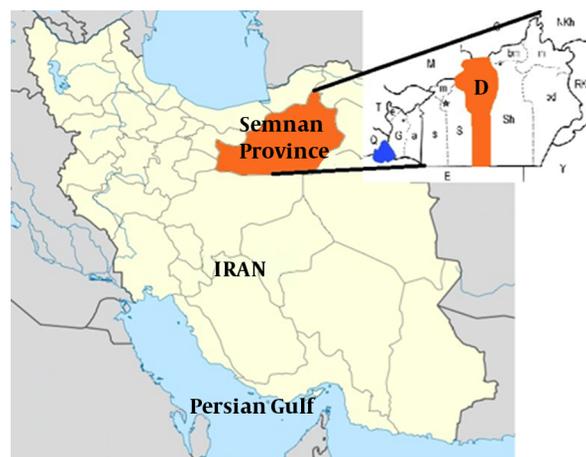
3. Methods

3.1. Study Area and Sampling

Samples were collected from Damghan county (35°30'N 54°20'E), the center of Semnan province, in Iran. All samples were collected in 2015 during the spring in 4 regions including Reza Abad, Hassan Abad, Jazan, and Damghan corresponding to 3 different climates in

Damghan county (Figure 1). Ticks were subsequently collected from sheep, cow, camel and goats. In each region, animals were checked randomly for tick collection. The entire body of each animal was checked for the presence of ticks. Ticks were collected and kept alive in separate labeled vials and then transferred to the Medical entomology laboratory, school of public health, Tehran University for morphological discrimination and sex determination (20).

Figure 1. Semnan Province is Located in North of the Country



D indicates Damghan County.

3.2. RNA Extraction, RT-PCR and Sequencing

All identified ticks had been kept in micro tubes and were transferred to the Arboviruses Laboratory at Pasteur institute of Iran for further analysis. Ticks were individually washed with PBS and crushed with a mortar and pestle in 200 - 300 μ L of PBS. Total RNA was extracted from the samples using the RNA easy kit (QIAGEN, Viral RNA mini kit, Germany) based on the manufacturers protocol. The extracted RNAs from tick samples were screened for CCHFV RNA by RT-PCR. A master mix was prepared with QIAGEN one step RT-PCR kit (QIAGEN, Germany). In an initial screening for CCHFV RNA, a partial segment of S-gene (536-bp) were amplified using specific forward primer (5'-TGGACACCTTCACAAACTC-3') and reverse primer (5'-GACAAATTCCTACACCA-3') (21, 22). Subsequently, the full length of S-gene for positive samples was amplified and completely sequenced using Sanger technique according to the previously described methods with specific primers (13, 23-25).

3.3. Phylogenetic Analysis Based on Complete S-Gene

The 4 complete S-gene sequences derived from tick samples have been deposited in GenBank (assigned accession numbers: KU707898-KU707901). The data set used for this analyses consisted of a total of 142 sequences comprised of the complete S-gene of the 4 Iranian sequences obtained in this study and 138 globally representative isolates retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Only GenBank submissions that included both a date (between 1956 and 2015) and location of origin were considered. The alignment was performed using the MAFFT algorithm and the initial phylogenetic tree was developed by Geneious v7.1.8. Similar sequences with the same isolation year and country were omitted from the original set of 142 sequences to provide a representative dataset without duplicate sequences, which resulted in a dataset consisting of 55 sequences. A phylogenetic tree was constructed by the neighbor-joining algorithm (NJ) with HKY genetic distance model using a 1000 bootstrap replicate in Geneious v7.1.8 (26).

3.4. Nucleocapsid Protein Structure and Pairwise Identity Score

The nucleocapsid protein (N-protein) structure of 4 CCHFV S-gene sequences obtained from ticks in Iran was predicted with approximately 65% accuracy by using EMBOSS v 6.5.7 (27). In order to tentatively classify the S-gene of CCHFV sequences obtained in this study with other CCHFV sequences, pairwise comparison and identity calculation was performed using MUSCLE, ClustalW, and MAFFT methods implemented in Sequence Demarcation Tool (SDT) v 1.2 (www.cbio.ucl.ac.za/SDT/) (28).

4. Results

Among 95 investigated animals, 26 (27.4%) were infested by ticks. The most infestation rate belonged to camels (60%) from the Hassan Abad region. In contrast, the lowest infestation rate belonged to sheep (17.5%) from Reza Abad (Table 1). The dispersion of different tick species collected from different districts of Damghan County located in Semnan province is shown in Table 2.

4.1. Tick Species

A total of 93 ticks belonging to 6 species in 2 genera (*Rhipicephalus* and *Hyalomma*) were collected in 4 sampling areas (Reza Abad, Hassan Abad, Jazan, and Eqbalyeh), *Rhipicephalus* County (Table 2). The tick species were *Hy. marginatum* (6.45%), *Hy. dromedarii* (21.5%), *Hy. anatolicum* (15.1%), *Hy. asiaticum* (3.2%), *Hy. schulzei* (2.2%), and *Rh. Sanguineus* (47.3%). Meanwhile, 4.3% of ticks were female *Hyalomma* sp. (Table 3).

Table 1. Detection of Infection of Tick Ectoparasites on Livestock, Studied in Damqan County, Semnan Province, Iran^a

Livestock	No.	Infestation No.	District
Cow	8	3	E
Goat	8	3	E
Cow	12	2	J
Camel	10	6	H
Sheep	40	7	R
Goat	17	5	R
Total	95	26	N A

Abbreviations: E, Eqbalyeh; J, Jazan; H, Hasan Abad; R, Reza Abad.

^aIn total, 95 female livestock were studied to check if they were infected with ticks. In total, 26 of 95 livestock (27.4%) were infected.

4.2. Crimean-Congo Hemorrhagic Fever Virus Prevalence in the Tick Populations

The CCHFV RNA was identified in 4 tick samples (4.3%). The infected samples belonged to a female *Rh. sanguineus*, a female *Hy. dromedarii*, a female *Hy. Anatolicum*, and a male *Hy. dromedarii* collected from sheep, camel, cow, and goat, respectively. A total of 3 positive tick samples were female ticks (75%) and 1 male tick (25%). It is notable that 52 of 93 tick samples (55.9%) were female. The infection rate of female and male ticks is proportional with the female and male ectoparasite samples that were collected (Table 3).

4.3. Phylogenetic Analysis of Full-Length S-gene Crimean-Congo hemorrhagic Fever Virus Strains

The phylogenetic tree was deduced from 51 previously published CCHFV strains and 4 CCHFV sequences in this study. The phylogenetic tree inferred from the complete coding region (ORF) of CCHFV S-gene sequences revealed 7 distinct clades (Figure 2). All 7 main clades were confirmed by the maximum likelihood inference and supported by bootstrap values. The phylogenetic analyses indicated that all 4 recent CCHFV sequences obtained from tick samples fell into clade IV. Meanwhile, CCHFV sequences obtained from 2 *Hy. dromedarii* (KU707898 and KU707900) and 1 *Hy. anatolicum* (KU707899) grouped within clade IV (Asia-1), whereas the remaining CCHFV sequence obtained from *Rh. sanguineus* (KU707901) clustered with other CCHFV strains within clade IV (Asia-2).

4.4. Structural Comparison of Iranian Crimean-Congo Hemorrhagic Fever Virus N-Proteins and Identity Matrix

The S-gene of CCHFV encodes 482 amino acids (102 alpha helices, 89 beta strands, 146 coils, and 147 turns), forming the monomeric N protein, which when combined

Table 2. Dispersion of Tick Species Collected From Different Districts of Damqan County, Located in Semnan Province, Iran

Districts/Tick Species	<i>Rh. sanguineus</i>	<i>Hy. marginatum</i>	<i>Hy. anatolicum</i>	<i>Hy. asiaticum</i>	<i>Hy. schulzei</i>	<i>Hy. dromedarii</i>	<i>Hy. sp.</i>	Total	Percentage (%)
Reza Abad	43	1	-	2	-	-	3	49	52.69
Hasan Abad	-	-	3	-	2	20	-	25	26.88
Jazan	-	-	11	-	-	-	-	11	11.83
Egbalyeh	1	5	-	1	-	-	1	8	8.60
Total	44	6	14	3	2	20	4	93	100

Table 3. Details of Collected Ticks and Their Infection to CCHFV Studied in Damqan County, Semnan Province, Iran^a

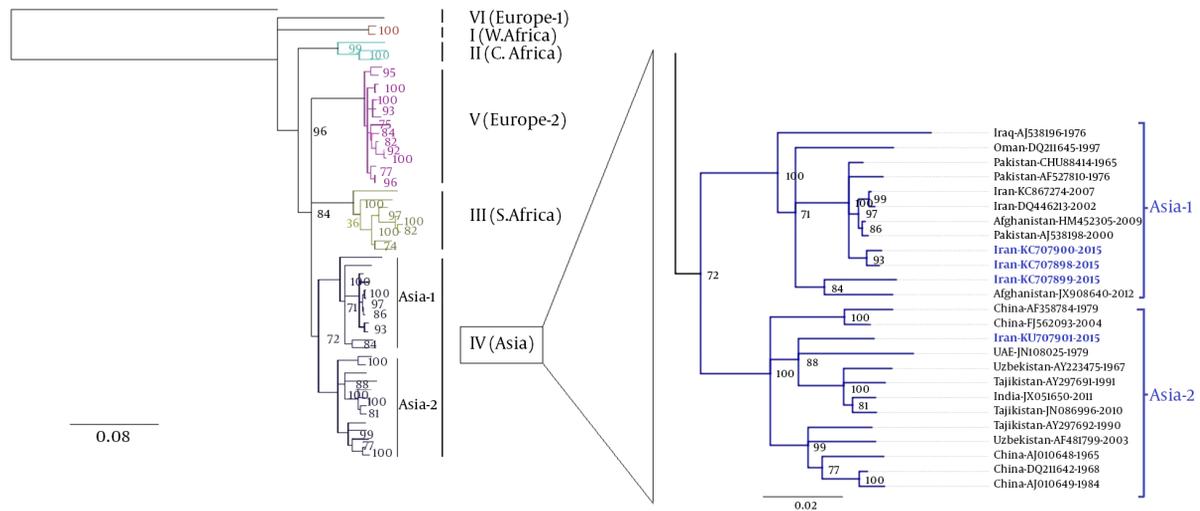
No.	Host	Sample Location	Genus/Species	No. Collected Ticks			No. Infection to CCHFV			GenBank Accession No
				F	M	Total	F	M	Total	
1	Sheep	R	<i>Hy. asiaticum</i>	1	1	2	-	-	-	-
			<i>Rh. sanguineus</i>	2	-	2	-	-	-	-
2	Camel	H	<i>Hy. dromedarii</i>	2	3	5	1	-	1	KU707898
			<i>Hy. schulzei</i>	1	-	1	-	-	-	-
3	Camel	H	<i>Hy. schulzei</i>	1	-	1	-	-	-	-
4	Camel	H	<i>Hy. dromedarii</i>	4	5	9	-	-	-	-
5	Sheep	R	<i>Rh. sanguineus</i>	4	-	4	-	-	-	-
6	Goat	R	<i>Hy. dromedarii</i>	1	-	1	-	1	1	KU707900
7	Camel	H	<i>Hy. anatolicum</i>	-	3	3	-	-	-	-
8	Sheep	R	<i>Rh. sanguineus</i>	3	2	5	1	-	1	KU707901
9	Camel	H	<i>Hy. dromedarii</i>	2	1	3	-	-	-	-
10	Sheep	R	<i>Hy. sp.</i>	1	-	1	-	-	-	-
11	Sheep	R	<i>Rh. sanguineus</i>	6	3	9	-	-	-	-
12	Sheep	R	<i>Rh. sanguineus</i>	4	2	6	-	-	-	-
13	Goat	R	<i>Rh. sanguineus</i>	-	2	2	-	-	-	-
14	Sheep	R	<i>Hy. marginatum</i>	-	1	1	-	-	-	-
			<i>Rh. sanguineus</i>	5	1	6	-	-	-	-
15	Goat	R	<i>Rh. sanguineus</i>	1	3	4	-	-	-	-
16	Cow	J	<i>Hy. anatolicum</i>	3	3	6	1	-	1	KU707899
17	Goat	R	<i>Hy. sp.</i>	1	-	1	-	-	-	-
			<i>Rh. sanguineus</i>	3	-	3	-	-	-	-
18	Goat	R	<i>Rh. sanguineus</i>	2	-	2	-	-	-	-
19	Cow	J	<i>Hy. anatolicum</i>	2	3	5	-	-	-	-
20	Cow	E	<i>Hy. marginatum</i>	-	1	1	-	-	-	-
21	Cow	E	<i>Rh. sanguineus</i>	-	1	1	-	-	-	-
22	Camel	H	<i>Hy. dromedarii</i>	2	1	3	-	-	-	-
23	Cow	E	<i>Hy. marginatum</i>	-	2	2	-	-	-	-
			<i>Hy. asiaticum</i>	-	1	1	-	-	-	-
24	Goat	E	<i>Hy. sp.</i>	1	-	1	-	-	-	-
25	Goat	E	<i>Hy. marginatum</i>	-	1	1	-	-	-	-
26	Goat	E	<i>Hy. marginatum</i>	-	1	1	-	-	-	-
Total	26 livestock	4 districts	6 species	52	41	93	3	1	4	-

Abbreviations: E, Egbalyeh, H, Hasan Abad; J, Jazan; R, Reza Abad.

^aIn total, 52 and 41 female and male ticks respectively were collected from infected livestock. PCR was carried out on all of tick samples and finally the RT-PCR results revealed that 32 female and 24 male collected ticks were infected with CCHFV.

with CCHFV RNA forms the viral nucleocapsid structure (29). The overall protein structural topology from 4 CCHFV strains obtained in this study was similar. Interestingly, comparison of the primary sequences of 4 CCHFV strains

indicated 16 a.a. differences among 482 positions, however, only 8 a.a. changes caused secondary structure alternation. The main secondary structural difference was the 5 divisions of long alpha helices in strains KU707899

Figure 2. CCHFV Phylogenetic Tree of Complete S-Gene Sequences Using Genious v7.1.8 (Left)

Enlargement of the section of tree containing CCHFV sequences obtained from Iran in bold (right). Dugbe virus is considered as the out-group. The numbers indicate percent bootstrap replicates. Sequences in the right tree are indicated as country-accession number-isolation year.

and KU707901 at a.a. positions 48 to 60, strain KU707901 at a.a. positions 78 to 85, strains KU707899, KU707900 and KU707898 at a.a. positions 110 to 134, strains KU707900 and KU707898 at a.a. positions 328 to 333, and strain KU707901 at a.a. positions 338 to 355, into 2 shorter alpha helices. One beta sheet was shortened and a turn in secondary structure occurred at a.a. positions 137 to 141 in strain KU707899 and an extended alpha helix at a.a. position 208 after turn in strain KU707900. An alpha helix changes to coil at a.a. positions 357 to 359 in strain KU707901. One alpha helix was shortened and a turn in secondary structure occurred at a.a. position 406 in strain KU707901 (Figure 3).

The SDT analysis showed pairwise identity scores using a color-coded matrix and provided an insight into the overall relationship between sequences in the dataset. All 3 alignments resulted in similar demarcation patterns (data not shown). Only the MAFFT alignment identity matrix is shown. Obvious demarcation between different clades of CCHFV is distinguishable (Figure 4).

5. Discussion

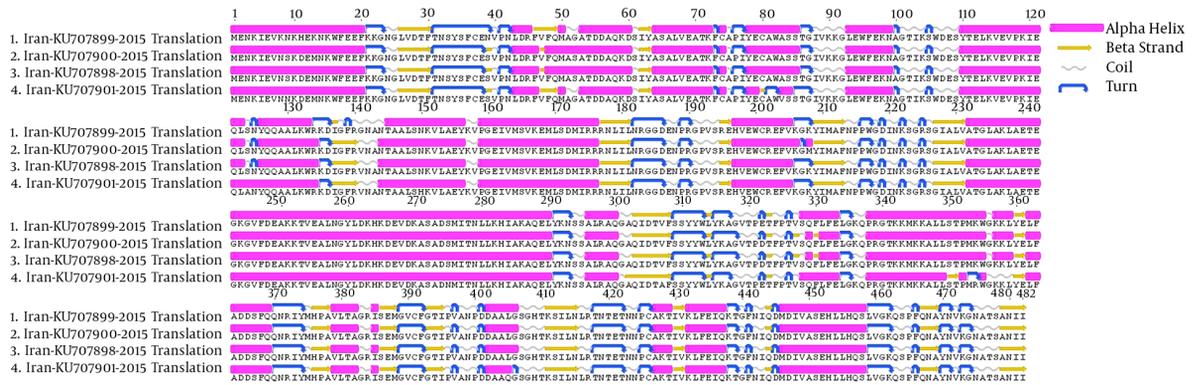
Several arboviruses are circulating in Iran (30-35), however, CCHF human causes are considered as the most severe arbovirus in Iran with considerable mortality rate each year (36, 37). The seroprevalence of CCHFV in humans and animals in Iran was investigated for several years (38). Approximately, 100 human CCHF cases per year have been reported in Iran since 2000 (39). Given the essential role of

ticks as the vectors and reservoirs of human and animal diseases, they are the subjects of many studies in Iran (40). Our results showed that 6 tick species are infesting livestock in the investigated areas. In addition, we demonstrated the presence of CCHFV in 2 *Hy. dromedarii*, 1 *Rh. sanguineus*, and 1 *Hy. anatolicum* from Damghan county in Semnan province. The detection of CCHFV in ticks is important, because different tick species are responsible for transmission of CCHFV and are available in Iran, which might increase the risk of CCHFV transmission to humans (41).

In a recent study, tick species infesting livestock in a neighboring province of Semnan province, Mazandaran province, was screened for CCHFV by RT-PCT. As a result, CCHFV RNA was detected in *Rh. sanguineus*, *Rh. bursa*, *Ixodes ricinus*, *Boophilus annulatus*, *Haemaphysalis punctate*, and *H. numidiana* (42). In our report, *Rh. sanguineus* was also identified as a positive tick reservoir for CCHFV. Both the phylogenetic analysis and identity matrix analysis, based on the full length of S-gene, demonstrated the clustering of CCHFV strains within 7 distinct clades, and revealed the close genetic relationship of 4 CCHFV strains obtained in the current study with other CCHFV strains in clade IV, including Asia-1 and Aisa-2.

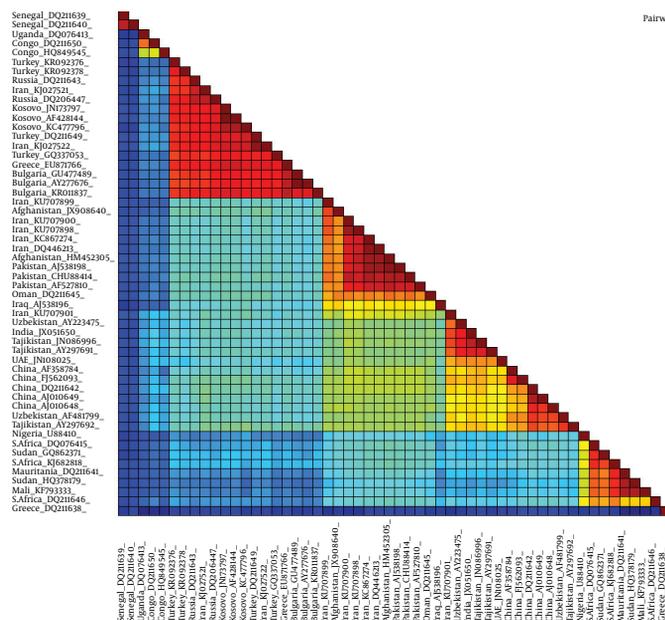
The variations of CCHFV genome sequences combined with sample collection dates and locations can be used to identify the source and the evolutionary history of circulating strains. The results of our genome-based epidemiology revealed that 2 CCHFV variants are circulating in this part

Figure 3. Primary Amino Acid Sequences of N-Protein of Four CCHFV Strains Obtained in This Study



The secondary structures are shown schematically. The figure was made using EMBOSS v 6.5.7.

Figure 4. Color-Coded Pairwise Identity Matrix Generated from Complete Sequence of S-Gene of CCHFV Representing All 7 Clades



Each colored cell represents the percentage identity score between 2 sequences (one indicated horizontally to the left and the other vertically at the bottom). A color key indicates the correspondence between pairwise identities and the colors displayed in the matrix. High genetic identity (100%) is indicated (dark red color). In contrast, genetically distant sequences are also coded (blue color)

of Iran, which is in accordance with previous phylogenetic data (30). Grouping of the complete CCHFV S-segment sequences obtained in the current study are in conformity with the previous reports (36, 37).

Understanding of N protein is noteworthy, as the N-protein interacts with the cellular antiviral defense factor MxA and was recently shown to have a role as a substrate for the apoptosis mediator caspase-3 (43, 44). Thus, amino

acid substitutions may lead to protein structure modification that would consequently alter its ability to combat cellular defense mechanisms against CCHFV and also influence its functions such as RNA binding and oligomerization, which are required for virus replication. Additional studies are required to further understand how the host antiviral defense against circulating CCHFV strains in Iran is activated, which will enable therapeutic molecules to be

designed and used for human use (45). In addition, the identification of pathogen transmission mechanisms in ticks may provide the opportunity to disrupt transmission routes and may contribute to the development of vaccines against tick-borne diseases (46) including strains of CCHFV that circulate in Iran.

6. Conclusion

To conclude, the continuous emergence of CCHFV is a public health concern in Iran (47). Crimean-Congo hemorrhagic fever virus is circulating in several countries surrounding Iran e.g Turkey (48), Pakistan (49), and Afghanistan (50) with sporadic outbreaks, which demands collaboration between involved countries to cope with CCHFV re-emergence. Accordingly, public health authorities in the mentioned countries should pay more attention to vector control measures to reduce the density of CCHFV vectors.

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Footnotes

Authors' Contribution: All persons designated as authors are contributed in this study and qualified for authorship.

Financial Disclosure: Authors declare that no competing financial interests exist.

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