

Original Article**Molecular Epidemiology and Phylogeny of Crimean-Congo Haemorrhagic Fever (CCHF) Virus of Ixodid Ticks in Khorasan Razavi Province of Iran**

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(Received 14 June 2017; accepted 21 Dec 2020)

Abstract

Background: Crimean–Congo hemorrhagic fever (CCHF) is a fatal disease caused by Nairovirus classified within the Bunyaviridae family. The virus is transmitted to humans through the bites of infected ticks or direct contact with viremic animals or humans. The current study aimed to detect the virus genome in ticks from Khorasan Razavi Province.

Methods: One hundred hard ticks were collected randomly from 100 sheep in four different areas of the province. Collected ticks were kept alive and identified. All the ticks were analyzed for the presence of CCHF virus genome using reverse transcriptase polymerase chain reactions (RT-PCR).

Results: The identified ticks were belonging to *Hyalomma marginatum* (16% female and 6% male), *Rhipicephalus turanicus* (52% female and 25% male), and *Dermacentor raskemensis* (1%). The CCHF virus genome was found in *Hyalomma marginatum* (5% male from Taibad and Sabzevar region and 1% female from Taibad). Genetic analysis of the virus genome isolated from two regions (Sabzevar and Taibad) showed 100% identity.

Conclusion: This study indicated that CCHF should be regarded as a risk-borne infection in this province. Therefore, special health management is needed to control this disease.

Keywords: Crimean-Congo haemorrhagic fever; Ixodid ticks; Khorasan Razavi province

Introduction

Crimean-Congo Hemorrhagic Fever (CCHF) is a serious zoonotic viral disease which can be fatal.

Crimean-Congo Haemorrhagic Fever has been reported in Asian, African and Eastern European countries (1) and can be transmitted to humans via a number of routes including ticks bites, direct contact with infected animal blood or meat, direct contact with infected individuals, and through nosocomial and community outbreaks (2). The virus causing the disease has a single stranded RNA genome and is

known as Nairovirus in the family of Bunyaviridae (3-5). Although the disease is usually asymptomatic in infected animals, it causes a severe haemorrhagic syndrome in humans, with a fatality rate up to 50% (6).

Crimean-Congo Hemorrhagic Fever has the highest rate of distribution amongst those areas of the world with global distribution of ticks, especially *Hyalomma* (7).

Diagnosis of viral infection is achieved using three methods, ELISA, isolation of the CCHF virus and molecular methods to detect

the viral genome (8). Jorjani, first reported the disease in 1100 AD in his book and pointed out that ticks were associated with the haemorrhagic syndrome (9). In 1975, it was discovered that the virus existed around the Caspian Sea and the East Azerbaijan Province of Iran. Three years later, in Iran, the virus was reported from infected ticks (10, 11). Chinikar et al. analyzed the CCHF virus genetically in Iran and deposited the sequence of partial fragments in GenBank (AY366373 and AY-366979). Furthermore, their discovery was recognized globally as they had reported two genetic branches for of the virus in Iran (12). So far, CCHF virus has been reported in more than 31 different types of ticks including Ixodidae and Argasidae ticks in the world (13, 14). There had been numerous reports on CCHF in Khorasan Razavi Province (8, 12, 15), but not on its vectors. Therefore, the purpose of this study was to determine molecular evidence of CCHF viruses in ixodid ticks from Khorasan Razavi, Iran

Materials and Methods

Study area

Khorasan Razavi Province (Fig. 1) is located in north-east of Iran (33°52' and 37° 42') (Fig. 1). Regarding its massive area, this province consists of various climates and natural environments but in terms of the temperature averages is 13.5 °C/56.2 °F and also rainfall categorized as nearly an average receiver of rain (the annual rainfall is 251mm/9.9 inch) (<https://www.worlddata.info/asia/iran/climate-razavi-khorasan.php>).

Tick collection and molecular procedure

The ticks were collected from 17 regions of 4 cities namely Mashhad, Sabzevar, Kalat and Taibad (Fig. 1). One hundred ticks were collected from entire body of each 100 sheep and kept in proper humid conditions in the capped tubes and transferred to the Department of Parasitology, Tehran faculty of veterinary medicine. The ticks were identified using iden-

tification keys (16). After identification of the ticks, they were analyzed using RT-PCR in the Arboviruses and Viral Hemorrhagic Fevers laboratory (National Reference Laboratory) at the Pasteur Institute of Iran.

Ticks were individually washed twice with PBS (PBS1×, pH= 7.4) and crushed with mortar and pestle in 200–300µL of PBS 1×. Then, viral RNA was extracted from each tick by using the QIAamp Viral RNA Kit according to the manufacturer's instructions (QIAgen GmbH, Hilden, Germany). The extracted viral RNA was subsequently analyzed by RT-PCR with the One-Step RT-PCR Kit (QIAgen GmbH, Hilden, Germany) using specific published primers: F2 5'TGGACACCTTCACAAA CTC3' and R35'GACAATTCCTACACC3' (17). These primers were selected to amplify a 536bp fragment of a highly conserved region inside the S-segment of the CCHFV genome. The PCR was carried out in a total volume of 50µl for 30min at 50 °C, 15min at 95 °C, and 40 cycles including 30s at 95 °C, 30s at 50 °C, 45s at 72 °C, and, finally 10min at 72 °C as a final extension. Positive and negative controls were used in each PCR reaction (18). Then, PCR products were analyzed by performing 1.5% agarose gel electrophoresis in TBE buffer with a 100bp DNA Ladder. DNA bands were visualized with ethidium bromide staining under ultraviolet transilluminator (Genius Co., USA).

Sequence analysis

For verification of positive samples, amplified fragments were sent for sequencing to Takapouzist Co. Then, Finch TV software was used for quality control of sequenced data. Subsequently, the Sequenced data were compared with the corresponding sequence data registered in Data bank by BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) after verification of similarity, closest hits were selected and all similarity blast results retrieved.

For constructing the phylogenetic tree, ClustalW and Bioedit software version 7.7.9 were used. After alignment of Iranian S gene

sequences of CCHF with other sequences, redundant sequences or very short sequences and areas with ambiguous alignment or containing poly-N stretches were excluded from the analyses. For probable similarity and cluster analysis, the MEGA 6 software package was used for construction of phylogenetic trees using the neighbor joining method with bootstrap 1000 (19).

Results

The ticks collected from the four different regions (Mashhad, Taibad, Sabzevar and Kalat) and were classified in 3 different species as: female *Hyalomma marginatum* (Fig. 2) (16% female and 6% male), *Rhipicephalus turanicus* (Fig. 3) (52% female and 25% male) and *Dermacentor raskemensis* (Fig. 4) (1%) (Table 1).

CCHF virus infection was found in *Hyalomma marginatum* (5% male ticks (from Taibad and Sabzevar and 1% female ticks from Taibad) (Table 2).

Genetic analysis of the virus genome isolated from the two different regions (Sabzevar and Mashhad) showed 100% identity with some registered isolates in GenBank. The obtained nucleotide sequences of CCHF virus, showed 100% similarity with the CCHF virus from several registered sequences from the Hamedan Province of Iran (AY366378.1, AY 366379.1, GU456725.1). Moreover, blast data of the ss RNA segment of CCHF virus with sequences from GenBank showed 100% similarity with sequences from Pakistan and Afghanistan (AY

905662.1, AJ538198.1, HM 452305.1) and 99% similarity with three other sequences from Iran (GU456728, DQ446212.1 and DQ446213.1). Furthermore, the obtained sequences showed similarity between 85–95% with the registered sequences from various other states as follows: Iraq (AJ 538196.1), China (DQ211642.1), South Africa (AY 905664.1, DQ211648.1), Sudan (GQ862372.1), Bulgaria (JF807428.1), Turkey (HQ664913.1, FJ601847.1) and with some sequences from Iran (U15022, AY 905653.1).

In order to construct phylogenetic trees based on the partial nucleotide sequence of the S segment, sequences belonging to neighboring countries to Iran and some very similar sequences from a BLAST search (Africa and China) were used. The phylogenetic tree in Fig. 5 showed the close relationships between Taibad and Sabzevar sequences with previous reports of Iranian CCHF sequences (S segment) and also with isolates from Afghanistan and Pakistan. They are all located in group 1. Sequences from Oman (DQ211645) and Iraq (AJ538196) and Afghanistan (JX908640) had lower similarity to Iranian sequences. Also, the nearest relatives to Iranian sequences are Tajikistan, China and India sequences that are clustered together in group 2. Furthermore, isolates from different provinces of Turkey grouped together in a separate clade (group 3). Interestingly one of the Iranian isolates from Rasht was located in this group.

Table 1. The variety of identified ticks in four cities of Khorasan Razavi Province, Iran

Location	Ticks	Identified ticks (%)					
		Number of collected ticks	<i>Hyalomma marginatum</i> Female	<i>Hyalomma marginatum</i> Male	<i>Rhipicephalus turanicus</i> Female	<i>Rhipicephalus turanicus</i> Male	<i>Dermacentor raskemensis</i> Female
Sabzevar		30	43/3	10	30	16/6	0
Taibad		38	7/8	7/8	57/8	26/3	0
Mashhad		14	0	0	35/7	64/2	0
Kalat		18	0	0	88/8	5/5	5/5
Total		100	-	-	-	-	-



Fig. 1. Map of Iran shows the location of Khorasan Razavi Province (the black area)



Fig. 2. *Hyalomma marginatum* (Male, above and Female, below)

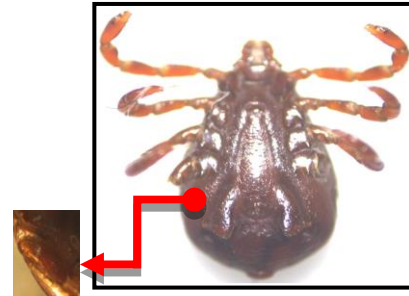


Fig. 3. *Rhipicephalus turanicus* (Male, dorsal and ventral surfaces)



Fig. 4. *Dermacentor raskemensis* (Female)

Table 2. The Results of tick infection by Crimean-Congo Haemorrhagic Fever virus using Reverse Transcription Polymerase Chain Reaction

Ticks	<i>Hyalomma marginatum</i>	<i>Hyalomma marginatum</i>	<i>Rhipicephalus turanicus</i>	<i>Rhipicephalus turanicus</i>	<i>Dermacentor raskemensis</i>
Location	♀	♂	♀	♂	♀
Kalat	0	0	0	0	0
Mashhad	0	0	0	0	0
Taibad	+1	+3	0	0	0
Sabzevar	0	+2	0	0	0
Total	1	5	0	0	0

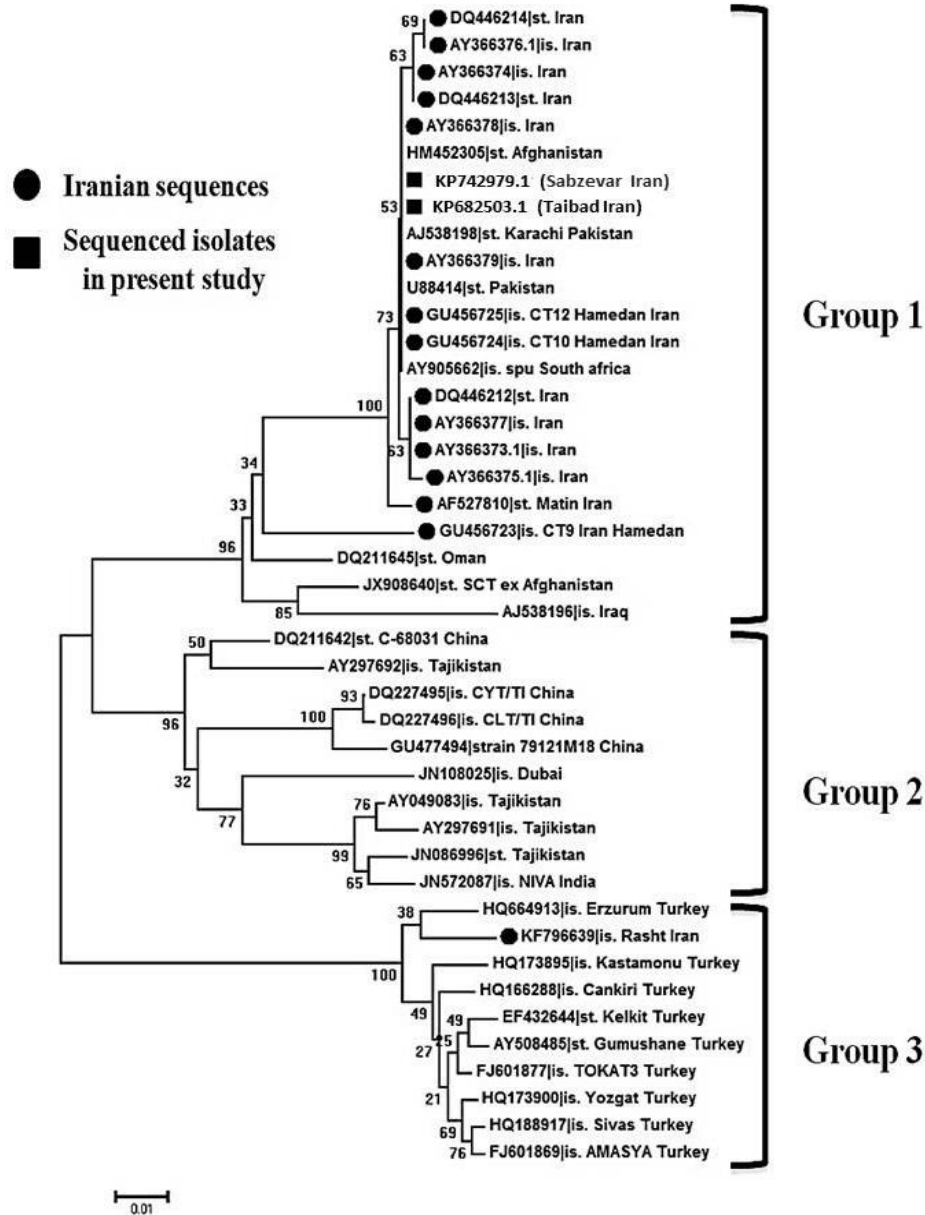


Fig. 5. The tree of Iranian partial S segment nucleotide sequences constructed by MEGA 6. The tree was constructed by using the neighbor-joining (NJ) algorithm based on differences in S sequences of different isolates. Units at the bottom of the tree indicate the number of substitution events. The length of each pair of branches represents the distance between sequence pairs. The dataset was re-sampled 10,000 times using the bootstrap method. The sequence information at the tips of the branches includes the accession number of the sequence, name of the isolate and strain

Discussion

Crimean-Congo hemorrhagic fever (CCHF) is a severe and often fatal viral disease in human caused by a Bunyavirus and is transmitted by infected body fluids, secretions or persons with ticks (18, 20, 21). It is considered as

one of the zoonosis diseases. This disease is reported from Asian, African and Eastern and central European states. There are some studies about diagnosis of its agents and distribution of this tick borne diseases (12, 18, 20-23).

The global warming and climatic changes in Iran, cause changes in tick fauna and tick borne diseases including CCHF throughout the different regions. Therefore, it seems that conducting more periodic studies on virus distribution and its genetic variation in order to prevent a wider distribution of the virus is needed (12, 18).

Amongst the different regions in Iran, the Khorasan Province has crucial importance because of neighboring with Afghanistan and Pakistan where the ticks and CCHF exist. Reports of CCHF in Iran were related to sporadic cases between 1970–1978; while there was no evidence of CCHF investigations between 1978–1999 (10, 11, 24, 25). Virus reported from different provinces (Esfahan, Sistan and Baluchestan, Fars, Tehran, Khorasan and Khuzestan) which resulted some death cases in years between 2000–2009 (15). Another study which was carried out in Yazd, proved that *Hyalomma* species is the best transmitter of the virus. None of the ticks genus *Rhipicephalus* and *Dermacentor* harbored the virus genome (27). Telmadarraiy et al detected IgG against CCHF in examined sheep (27.8%) of Hamedan Province and showed 16.4% of the ticks were certain transmitters of CCHF especially those belonging to the *Hyalomma*, *Rhipicephalus* and *Haemaphysalis* species (23). Chini-kar et al. also analyzed the CCHF virus genetically to show that nucleotide sequences of the S and M segments of viruses from 9 Iranian patients had 98% similarity. Furthermore, their physiological analysis proved that they were similar to the branches of the same virus found in Madagascar and Pakistan. They could also establish that there have been at least 2 genetic lineages in circulation in Iran (12). Analysis of S segment nucleotide sequences of CCHF between Taibad and Sabzevar showed 100% identity. Also, comparison of these results with another sequence in GeneBank showed 100% identity with Pakistan (AJ 538198.1, AY905662.1) and Afghanistan viruses (HM425305.1) related sequences. These

similarities can be attributed to the transportation of livestock between neighbor provinces in Iran or transmitting the ticks by migrating birds between neighbor countries.

The present study points out that *H. marginatum* ticks in the regions of Taibad and Sabzevar are certain transmitters of CCHF. The above claim can be proven by the help of a comparison between the results obtained by Ghorbani who reported numerous similarities in the nucleotide sequences extracted from Iranian patients in different regions as well as Pakistan's (28). Tahmasebi et al. showed genetic diversity in isolated virus from ticks of different parts of Hamedan province, which attributed to virus replication and recombination phenomena in vector ticks (21).

Conclusion

Regarding to significance of ticks in CCHF transmission and similarity of obtained CCHF virus sequences in this study with Afghanistan and Pakistan sequences, suitable strategy is that infected ticks to virus and illegal transactions of infected livestock in these borders might be considered to control CCHF disease in Khorasan Razavi Province.

Acknowledgements

The authors would like to thank the faculty of Veterinary Medicine, University of Tehran for funding this project and staff of the Arbovirus Laboratory, Pasteur Institute of Iran (National Laboratory of Iran) for their technical support and veterinary Office of Sabzevar and Dr Joghataei.

The authors declare that there is no conflict of interests.

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