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Molecular epidemiology of Crimean-Congo hemorrhagic fever virus genome isolated from ticks of Hamadan province of Iran

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Abstract

Background & objectives: Crimean-Congo hemorrhagic fever (CCHF) virus is a tick-borne member of the genus *Nairovirus*, family *Bunyaviridae*. CCHFV has been isolated from at least 31 different tick species. The virus is transmitted through the bite of an infected tick, or by direct contact with CCHFV-infected patients or the products of infected livestock. This study was undertaken to study the genetic relationship and distribution of CCHFV in the tick population of Hamadan province of Iran.

Method: In this study, RT-PCR has been used for detection of the CCHFV genome.

Results: This genome was detected in 19.2% of the ticks collected from livestock of different regions of the Hamadan province in western Iran. The infected species belonged to *Hyalomma detritum, H. anatolicum, Rhipicephalus sanguineus* and *Argas reflexus*. With one exception, genetic analysis of the virus genome isolates showed high sequence identity to each other. Even though they clustered in the same group with the strain circulating in Iran, they had a closer relationship to the Matin strain.

Interpretation & conclusion: Vector control programs should be applied for reducing population density of potential tick vectors in this province. Further surveys are indicated in this region to provide a better view of the distribution and epidemiology of the virus.

Key words *Argas reflexus;* CCHF; RT-PCR

Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne virus with a negative-sense, single-stranded (ss) RNA genome containing S (small), M (medium), and L (large) segments. It belongs to the genus *Nairovirus* and the family *Bunyaviridae*. The virus causes a severe hemorrhagic syndrome in humans, with a fatality rate of up to 50%, while in animals, the virus causes subclinical infection[1–6]. The CCHFV is transmitted to humans through the bite of *Ixodid* ticks (mostly *Hyalomma* genus) or by contact with blood or tissues from infected livestock. Moreover, it has been demonstrated that most statistics about transmission routes in Iran relate to people in close contact with tissues and blood from CCHF-affected livestock[7–15]. In addition to zoonotic transmission, CCHFV can spread from person to person and is one of the rare hemorrhagic fever viruses able to cause nosocomial outbreak in hospitals[16–17]. The virus is distributed across Africa, Eastern Europe, the Middle East, and Asia. The dis-

tribution of CCHFV coincides precisely with the distribution of *Hyalomma* ticks[3,5,16, 18,19].

Although sporadic surveys of CCHF in livestock and humans have been undertaken since 1970, it wasn't until the 1999 outbreak that CCHF was recognized as one of the country's major public health concerns. Since 2000, CCHF infection has been demonstrated in 23 out of the 30 provinces of Iran; with Sistan-va-Baluchistan, Isfahan, Fars, and Khuzestan being the most heavily infected provinces. The first confirmed case of human CCHF in the Hamadan province in western Iran, was reported in Hamadan city in 2004. Recent surveys have shown circulation of the virus in livestock existing in the province[16,20], but no further data on the genetic variability of the CCHFV strain from the Hamadan province have been available. However, several partial and complete S-genome segment sequences have become available through GenBank (Accession Nos. AY366373–AY366379 for partial sequences and DQ446212–DQ446215 for complete sequences). This study describes the genetic relationship and distribution of CCHFV in the tick populations from Hamadan province.

Material & Methods

Study area: Hamadan province is one of the highest and coldest regions in Iran. This district is located between 45° 32´ and 48° E and 34° 47´ and 35° 1´ N and consists of eight townships. Hamadan is among the major sheep and goat raising provinces in Iran. The economy is mainly based on agriculture and livestock husbandry, and so a large proportion of the population comes into close contact with livestock. This provides a high risk of exposure to CCHFV. In this study the province was divided into three regions, with two townships selected randomly from each region. Therefore, six townships were studied—Razan and Kabotar-Ahang in the north; Hamadan and Bahar in the centre; and Malayer and Toyserkan in the south of the province (Fig. 1).

Sample collection and preparation: In this study, carried out during 2007, 70 villages were randomly selected as the study area. In each village, 20–30 sheeps were checked for tick collection. The entire body of each sheep, but mainly: the ears, nape of the neck, perineum, scrotum and the tail base, were inspected for the presence of ticks. Collected ticks were

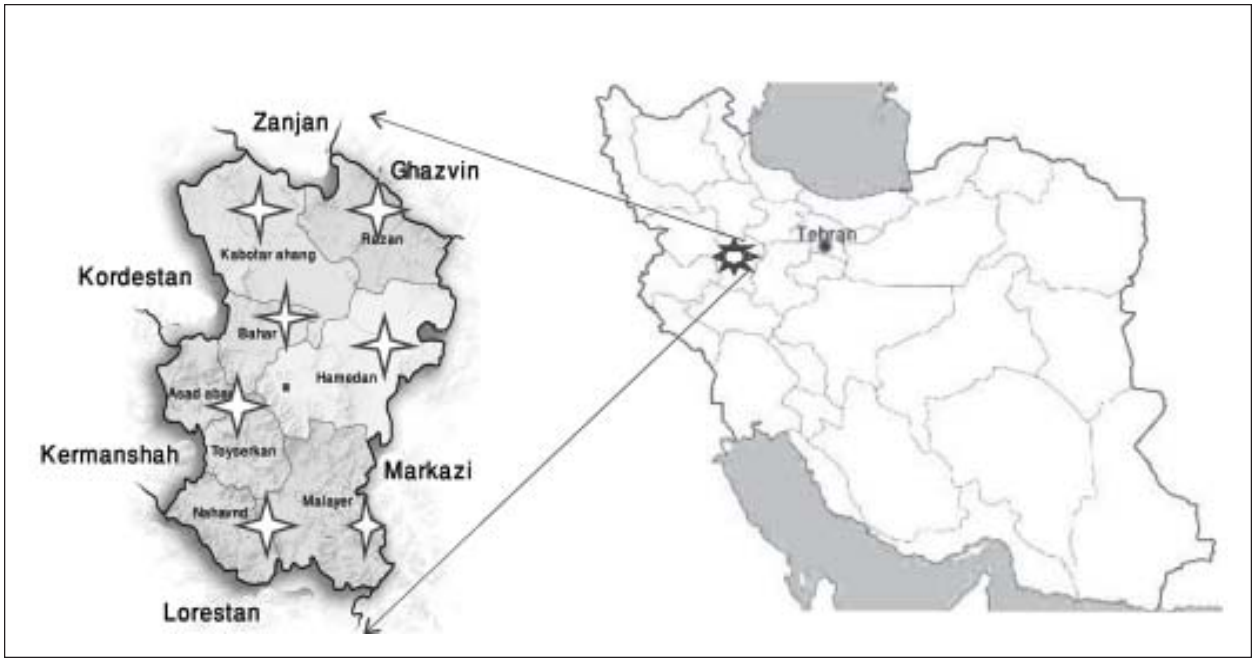

Fig. 1: Locations of collecting the ticks to characterize the situation of CCHFV infection in Hamadan province, the sampled townships are shown with stars

kept alive in separate labeled vials and sent to the Pasteur Institute of Iran, for species identification and molecular detection of CCHFV.

RT-PCR: Ticks were individually washed twice by PBS (PBS, pH 7.4) and crushed with a mortar and pestle in 200–300 µl of PBS. Total RNA was extracted using an RNeasy mini kit (QIAGEN, Cat No. 2215716) according to the manufacturer's instructions. The extracted total RNA was stored at –70°C until use. For the RT-PCR, a master mix was prepared as follows: 28 µl of RNase free water, 10 µl of buffer (5 × conc.), 2 µl of dNTP mixture, 2 µl of enzyme mixture containing reverse transcriptase and Taq DNA polymerase enzymes, 1 µl of primer F2 (5′ µ-TGGACACCTTCACAAACTC-3µ), 1 µl of Primer R3 (5′ µ-GACAATTCCCTACACC-3′ µ) and 1 µl of RNase inhibitor. The F2 and R3 primers amplify a 536 bp fragment inside the S-segment of the CCHFV genome. For qualitative analysis in real-time RT-PCR, 1 µl of SYBR Green dye with 1/10000 dilution was also used in the master mix. The thermal cycling program for the RT-PCR, for both techniques, included 30 min at 50°C for reverse transcription reaction (cDNA synthesis); followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec, and a final extension at 72°C/5 min. For gel-based RT-PCR product analysis, 5 µl of the PCR products was mixed with 1 µl loading buffer (6 × conc.) and then analyzed by gel electrophoresis. In real-time analysis, both amplification and dissociation curves were evaluated with respect to negative and positive controls, as well as in the gel-based analysis[1,8,16,21].

Sequencing and genetic analysis: PCR products were purified using quick-spin PCR purification kit (Intron) and directly underwent sequencing by specific primers. All sequenced amplicons were placed in the GenBank with Accession Nos. GU456723 to GU456728 related to CT9, CT10, CT12, CT13, CT14 and CT15, respectively. For phylogenetic analysis, a 499 bp long fragment of the S-segment was used. The sequences of CCHF viruses were analyzed by a neighbor-joining method with Kimura two parameter distances by using Mega 4 software. Bootstrap confidence limits were based on 500 replicates. Evolutionary divergence, distance matrix and subsequently sets of phylogenetic trees were calculated by the software[22].

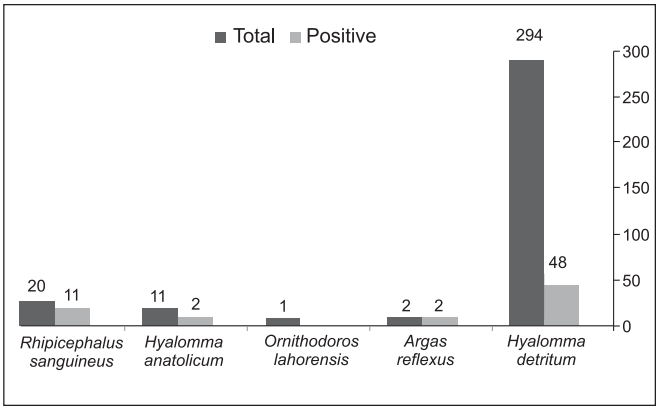

Fig. 2: The proportion of tick species infected with CCHFV

Results

A total of 328 adult ticks (199 males and 129 females) were collected from the north, center and south of Hamadan province. These ticks were grouped into five species, including: *H. detritum* (89.6%) (Found at all locations), *Hyalomma anatolicum* (3.4%), and *Rhipicephalus sanguineus* (6.1%) [both species found in central and southern regions, *Argas reflexus* (0.4%) collected from northern parts], and *Ornithodoros lahorensis* (0.3%). The two latter species, *A. reflexus* and *O. lahorensis*, showed narrow distributions (Fig. 2).

The presence of CCHFV was confirmed by RT-PCR technique in which a 536 bp amplicon is amplified inside the S segment of the CCHFV genome isolates from 63 collected ticks. Among positive ones, 42.9 and 57.1% were males and females, respectively. The infected tick species belonged to *H. detritum* (16.32%), *H. anatolicum* (18.18%), *R. sanguineus* (55%) and *A. reflexus* (100%). *Ornithodoros lahorensis* was RT-PCR negative.

The genome isolates belonging to different parts of the study area were named as: CT9 (isolates of *R.*

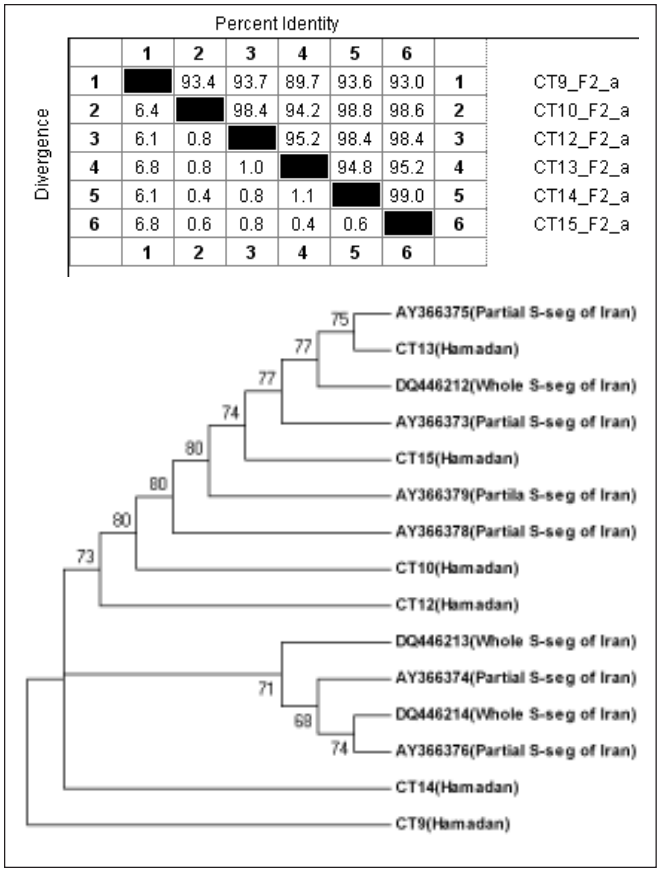

Fig. 3: Phylogenetic tree (neighbour-joining method) calculated for CCHFV S-segment sequences. New sequences in this paper (CT9, CT10, CT12, CT13, CT14 and CT15) and previously described sequences from Iran (AY 366373–8, DQ446212–14) with exceeding of 70% are shown.

sanguineus); CT10, CT12 and CT15 (all isolates of *H. detritum*); CT13 (isolated from *A. reflexus*); and CT14 (isolated from *H. anatolicum*). By using various methods for reconstructing phylogeny, a CCHFV phylogenetic tree was obtained based on individual sequences of the 536 bp fragment of the nucleoprotein gene. The genetic analysis revealed some nucleotide substitutions. Notably, the isolates (with the exception of CT9) were all clustered with Matin strain (Pakistani strain) and showed a closer relationship to this strain as the previously published Iranian isolates of CCHFV genome had been clustered in one group close to the Matin strain. The CT9 isolate showed lower identity (89.7%) and more divergence (6.8) compared to the other isolates (Fig. 3).

Discussion

The data obtained provide evidence for the presence of CCHFV in different regions of Hamadan province. CCHFV infection is shown in *H. detritum, H. anatolicum, R. sanguineus* and *A. reflexus*. These findings are in concordance with previous studies in other parts of the country. *Hyalomma* ticks are the primary vectors for the widespread transmission of CCHFV throughout Europe, Asia, the Middle East, and Africa[3,5,19]. Although in the epidemiology of CCHF virus, *Hyalomma* ticks are considered to be the most important vector and reservoir, the virus has also been reported from other genera of ticks. In 1979, CCHFV was first isolated in Iran from *O. lahorensis*[23]. In a study done in Ardabil province, north-west of Hamadan, 33.3% of the ticks, including *Hyalomma* sp, *Rhipicephalus* sp and *Ornithodoros* sp, were infected with CCHFV. In another study in Bahar (central part of Hamadan Province) 11.3% of ticks, including: *H. marginatum, H. dromedarii, H. anatolicum, R. bursa, R. sanguineus* and *H. punctata,* were CCHFV positive[24]. Current research shows CCHF infection in 19.3% of ticks collected from Hamadan province. Therefore, people in this province, involved with livestock, should protect themselves from direct contact with CCHFV contaminated tissue and blood.

In the present study, CCHFV contamination was confirmed in *A. reflexus*; an argasid (soft) tick which mainly infests pigeons, but occasionally infests mammals. This suggests that more consideration should be given to the detection of the CCHF virus genome in soft ticks. Soft ticks have a long life-span and can preserve the CCHF virus for long periods, potentially providing a persistent virus reservoir, for infection of the corresponding livestock and hard ticks in a region, and consequently, possible outbreaks of CCHF.

This report presents data on the CCHF situation in ticks, and the genetic variability of the CCHFV isolates obtained from different parts of Hamadan province. The relatively high divergence of the CT9 iso-

late, compared to the other isolates, may correlate with high geographical distance. CT9 was isolated from the southern part of the province, whereas the other isolates were mostly found in the central and northern parts. The remarkable genetic variability of this isolate could also be attributed to amplification of the virus and recombination events which may have occurred in the species of host tick from which it was isolated: *R. sanguineus*. This uncertainty shows that further molecular epidemiology surveys are required in this region to identify the exact phylogenetic relationship between the circulating strains[6,25]. Comparison of the data produced from three regions in the province suggested that ticks in the northern parts are infected more than the central and southern regions. Persons with high risk occupations such as shepherds, farmers, veterinarians, and other people who are in close contact with ticks should take precautions to avoid exposure to virus-infected ticks or virus-contaminated animal blood or tissues. Applying commercially available insect repellents, such as diethyl tolumidean DEET, to exposed skin, and the use of clothing impregnated with permethrin, can give some protection against tick bites. This study indicates that CCHF should be regarded as a serious health problem, worthy of consideration by the health centers in this province, and neighbouring regions, in order to develop and implement strategies to decrease the tick population, and alert high risk professionals. Further surveys on human and animal populations in these regions should be recommended to provide a better view of the distribution and epidemiology of the virus in this province.

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