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# Molecular epidemiology of Crimean-Congo hemorrhagic fever virus in ticks in northwest of Iran

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**Abstract**
Crimean-Congo hemorrhagic fever (CCHF) is a fatal viral disease that occurs in different provinces of Iran. The causative agent of the disease is a tick-borne virus of the family Bunyaviridae, genus Nairovirus. The virus is transmitted to humans through infected bite of ticks, squashed ticks or by contact with blood or tissues of infected livestock or human. Ticks are important vectors and reservoirs of Crimean Congo Hemorrhagic Fever (CCHF) virus. This study was conducted to determine the rate of CCHFV infection in ticks in Eastern Azerbaijan Province of Iran. Reverse transcription– polymerase chain reaction (RT-PCR) method was used to detect the CCHFV genome based on S segment in 177 ticks. RT-PCR technique showed the occurrence of CCHFV in 9 out of 177 tested hard tick samples (5.08%). All positive ticks belonged to *Hyalomma* and *Dermacentor* genera. Infected species were *Dermacentor marginatus* (66.6%), *Hyalomma marginatum* (22.2%) and *Hyalomma sp* (11.1%). All the infected ticks were isolated from sheep. Our Results exhibited that *D. marginatus*, *H. marginatum* and *Hyalomma sp* were the main vectors of CCHFV in the study area.

**Keywords:** CCHF, RT-PCR, hard ticks, arbovirus, tick-borne disease, Ixodidae, Iran

## 1. Introduction
Crimean-Congo haemorrhagic fever (CCHF) is a zoonotic disease caused by tick-borne virus that in humans can result in a severe haemorrhagic fever with a fatality rate of 13–50%. Being a public health problem in many countries including Iran, the disease has been reported from different parts of Eastern Europe, Africa, the Balkans, Russia, the Central Asian Republics, Turkey, China and the Middle East [1-8]. CCHF was first described as a viral human disease amongst soldiers of former Soviet Union in the Crimean region during world war II (1944) and subsequently reported from different countries including a number of southern Soviet republics, Bulgaria and South Africa [1]. The viral agent of CCHF is a member of the genus Nairovirus from family Bunyaviridae [9-13]. The agent is a negative-sense, single-stranded enveloped RNA virus, which possesses a tripartite genome [16, 17, 19]. Hosts of CCHF virus are predominantly wild and domestic mammals and birds. Although, sheep, goats and cattle develop high viremia titres, they do not develop the illness and show no symptoms except of very mild and unnoticed fever. Humans are usually infected with CCHF virus through a tick bite or close contact with viral-contaminated tissues, blood or body fluids of both infected human and domestic animals [1, 18]. Nosocomial outbreaks among hospital staff due to CCHF with sever manifestation and high mortality rate are also another route of the disease contamination [19, 5, 20]. The Ixodid (hard) ticks are able to pass the infection of CCHF virus through vertical and horizontal transmissions to a variety of wild and domestic mammals upon contact with blood or tissues of infected livestock [21, 17]. The geographical distribution of CCHFV cases corresponds most closely with the distribution of *Hyalomma* ticks, suggesting their principal role as a vector of CCHF. Some species of *Hyalomma*, *Dermacentor* and *Rhipicephalus* genera have been shown to be capable of trans-stadial transmission (i.e. passing the virus from larva to nymph to adult) of CCHFV after feeding on a viremic host. Once infected, the virus can persist in ticks and take its route to vertebrates that are needed to provide blood meals for ticks' development [22-25].
CCHF was first reported from Iran in 1970, when 45 out of 100 sheep sera collected from Tehran abattoir was shown to react positively for CCHF virus infection by Institute of Poliomyelitis and Viral Encephalitides in Moscow [1].

In 1974, a typhoid-like illness in Eastern Azerbaijan was described in 60 cases having hemorrhagic syndromes, and this was suspected to be CCHF disease [26]. In the period between 1974 to 1975, more similar clinical cases were reported from the same province [27].

The aim of this study was to determine CCHF infection in hard ticks (Ixodidae) in Heris County of Eastern Azerbaijan Province, Northwest of Iran using RT-PCR technique.

## 2. Materials and Methods

### 2.1 Study area

Heris County (38°14′N 46°50′E) is located in Eastern Azerbaijan Province, Northwest of Iran (Fig 1). As per 2006 census, the county's population was recorded as being 70,000 inhabitants. The county possesses 4 towns and 100 villages, 30 percent of which are located in mountainous area (north-east and north) and the rest in plain area (south and southwest). The county has cold winters with freezing temperatures and mild summers. The average temperature may drop to -25 °C during winter and rise to a maximum of 35 °C during summer. Comprising 70% of the county, the plain areas provide suitable environment for ongoing agricultural activities and livestock farming hence the occurrence of zoonotic diseases. In total 6 villages were randomly selected for hard tick collection, 4 villages in mountainous area and 2 in plains. The selected villages were visited on a seasonal basis for sample collection from January to December 2014.


**Fig 1:** Location of Heris County in East Azerbaijan Province, Northwest of Iran.

### 2.2 Tick collection

Tick sampling was carried out in the study area during 4 seasons thought the year 2014. A total of 649 sheep and goats in 6 villages were examined for tick infestation. Various tick attachment sites on animals such as ears, limbs, dewlaps, neck, tail, axial, groin and abdomen and chest were scrutinized and ticks were gently removed using forceps. The samples were preserved in glass vials blocked with wet cotton. The vials were numbered and full profile of the samples including animal type, age and sex as well as the name of the animal owner, place and date of collection were recorded in registration forms. Tick samples were then transferred in cold boxes to the laboratory for further studies. After identification, the tick specimens were stored at −20°C. Tick identification was done using the recommended comprehensive keys including Hoogstraal (1956) and Pomerantsev (1950) [1, 28]. The tick specimens were then sent to the National Arbovirus Laboratory of Pasteur Institute in Tehran (Iran) for CCHFV detection.

### 2.3 RNA Extraction and RT-PCR on ticks

A total of 177 tick samples were randomly selected and analyzed for CCHF virus in the biosafety level 4 laboratory of Pasteur Institute of Iran. Ticks were individually washed twice with PBS 1X and crushed with a mortar and pestle in 200-300 µl of PBS 1X. The total RNA of specimens was extracted using the RNA easy kit (QIAGEN, Viral RNA mini kit, GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA was dissolved in 50 mL of RNase-free water and stored at -70°C until used. A master mix was prepared with QIAGEN one step RT-PCR kit (QIAGEN GmbH, Hilden, Germany) as follow: 28µl of RNase free water (RFW), 10µl buffer 5X, 2µl dNTP mixed, 2µl Reverse Transcriptase Enzyme and Taq Polymerase, 1µl of Primer A (Forward) (5'TGGACACCTTCACAAACTC-3') and 1µl of Primer B (Reverse) (5'GACAAATTCCCTACAC CA-3') and 1 µl RNase inhibitor. A volume of 45 µl of master mix was added to PCR tubes and 5 µl of extracted RNA was added to the individual PCR tubes (Total volume 50 µl). The master mix typically contains all the components required for RT-PCR except the template RNA. After amplification, samples were stored either overnight at 2 to 8 °C, or at -20 °C for longer term storage. PCR product was separated by electrophoresis in a 1.5% agarose gel, stained with Safe Stain and visualized by UV trans-illumination.

## 3. Results

This study was carried out in 6 villages, in which a total of 423 sheep (65.1%) and 226 goats (34.9%) were inspected for tick infestation during four seasons. During the study period, a total of 780 ticks were collected and identified using standard identification keys. The occurrence of ticks on sheep and goat were 28.6% and 30.9% respectively. Table 1 outlines the tick species encountered during the collection seasons, all belonged to the family Ixodidae. The collected ticks included 4 *Haemaphysalis* species, 1 *Dermacentor* species and 6 *Hyalomma* species as described (Table 1). The species, *Dermacentor marginatus,* was the most abundant species infesting animals of both sexes, whereas *Haemaphysalis punctate* and *Haemaphysalis inermis* were the least frequently encountered ticks. Out of 780 collected ticks, female and male ticks comprised 51.66% and 48.33% respectively. About 94 percent of the ticks were caught in villages located in mountainous regions, and the rest, 6% from rural plain areas. RT-PCR analysis confirmed the presence of CCHFV infection in 9 specimens out of 177 ticks tested indicating a rate of infection of 5.08% (Table2). The highest infection rate was

detected in *Dermacentor marginatus* (3.38%), followed by *Hy. marginatum* and *Hyalomma sp* with infection rates of 1.12% and 0.56% respectively. In this study, all infected ticks were isolated from sheep. We found that 88.9% of infected ticks were isolated from mountainous villages and 11.1% from the plain areas. The result of RT-PCR analysis for detection of S segment of CCHFV genome extracted from tick samples is depicted in Fig 1.

**Table 1:** Classification of Ticks found in sheep and goat

| Phylum | Class | Order | Family | Species |
|---|---|---|---|---|
| Arthropoda | Arachnida | Ixodidae | Ixodidae | *Haemaphysalis erinacei* |
| | | | | *Haemaphysalis inermis* |
| | | | | *Haemaphysalis punctate* |
| | | | | *Haemaphysalis sulcata* |
| | | | | *Hyalomma sp* |
| | | | | *Hyalomma* nymph |
| | | | | *Hyalomma marginatum* |
| | | | | *Hyalomma anatolicum* |
| | | | | *Hyalomma asiaticum* |
| | | | | *Dermacentor marginatus* |

**Table 2:** Prevalence of CCHF in 177 Ticks found in sheep and goat

| Species | Total | Infected x/177 | % Infected |
|---|---|---|---|
| *Haemaphysalis erinacei* | 64 | 0 | 0 |
| *H. inermis* | 1 | 0 | 0 |
| *H. punctate* | 1 | 0 | 0 |
| *H. sulcata* | 16 | 0 | 0 |
| *Hyalomma sp* | 14 | 1 | 0.56 |
| *Hyalomma* nymph | 13 | 0 | 0 |
| *Hy. marginatum* | 49 | 2 | 1.12 |
| *Hy. anatolicum* | 8 | 0 | 0 |
| *Hy. asiaticum* | 14 | 0 | 0 |
| *Dermacentor marginatus* | 600 | 6 | 3.38 |

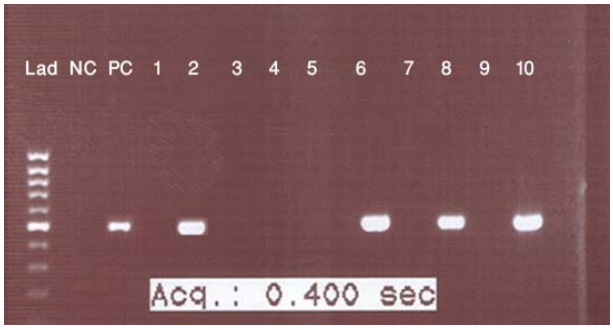


**Fig 1:** Result of RT–PCR analysis indicating S segment of CCHF genome of 536 bp from positive tick samples; Lad: Marker (100 bp DNA ladder), NC: negative control; PC: CCHF positive control; lanes 2, 6, 8 and 10: positive samples; other lanes: negative samples.

## 4. Discussion

Crimean-Congo Hemorrhagic Fever (CCHF) is a zoonotic viral infection that may result in a mortality rate of up to 50% in human. This fatal disease is transmitted by ticks of various genera in more than 30 countries worldwide. Most of neighboring countries of Iran including Pakistan and Afghanistan on the eastern border and Turkey on the western border are known to be endemic for human CCHF, posing serious threat to already complicated scene of disease outbreaks in the country where various CCHF genotypes are circulating [16]. CCHF is a well-known infectious disease in Iran since its first report in 1975. The disease has been reported from 23 out of 30 provinces of Iran of which Sistan-va-Baluchistan, Isfahan, Fars, Tehran, Khorasan, and Khuzestan provinces were the most infected respectively [17].

In the present study, the collected ticks from sheep and goat herds were identified to be members of three hard tick genera namely, *Hyalomma*, *Dermacentor* and *Haemaphysalis* as reported to occur on domestic ruminants by other studies in Iran [30-32]. Out of six *Haemaphysalis* species found in Iran, we encountered four species in the study area including *H. erinacei*, *H. inermis*, *H. punctate* and *H. sulcata*. However, only one *Dermacentor* species, viz. *D. marginatus* and six out of eight reported *Hyalomma* species from Iran, including two *Hyalomma sp*, *Hy. anatolicum*, *Hy. asiaticum* and *Hy. Marginatum* were identified [36]. We found *Dermacentor marginatus* to be the dominant species of hard ticks in Heris County, whereas *Hy. anatolicum* was reported to be the most abundant hard ticks in neighboring province of Western Azerbaijan [31].

Ticks are considered to be the most important in the epidemiology of CCHF for their role as vector and reservoir of the virus. So far, CCHFV infection of 88 ticks belonging to 4 genera of hard ticks including *Rhipicephalus*, *Haemaphysalis*, *Dermacentor* and *Ixodid* as well as 2 species of soft ticks namely *Argas persicus* and *Ornithodoros lahorensis* were confirmed by laboratory examination [33, 34]. However, *Hyalomma* spp, have been incriminated to be the most important vector and reservoir of CCHF virus [33]. Our study confirmed the infection of only 3 species of collected hard ticks, including *Dermacentor marginatus*, *Hyalomma marginatum* and *Hyalomma sp*. Other ticks including *Haemaphysalis* species including *H. erinacei*, *H. inermis*, *H. punctate* and *H. sulcata*, and *Hyalomma spp*, *H. anatolicum* and *H. asiaticum* were negative for CCHFV infection. A molecular survey undertaken during 2008–2009 in Yazd province (Iran) revealed that 5.71% of hard ticks (Ixodidae) were contaminated with CCHFV genome. All positive ticks belonged to *Hyalomma* genus and included: *Hy. dromedarii*, *Hy. marginatum*, *Hy. anatolicum*, *Hy. detritum*, *Hy. asiaticum* [32]. In Zahedan (Southeast of Iran), the genus *Hyalomma* was also tested positive [17]. Likewise, in studies conducted in Kurdistan province (West of Iran), the CCHFV test was positive solely for *Hyalomma* spp. but not for other genera such as *Haemaphysalis*, *Rhipicephalus*, and *Dermacentor* [35]. However, this was not the case for *Rhipicephalus sp.* and *Haemaphysalis sp.* in Hamadan province (Centre of Iran) where these were first reported positive for CCHFV infection in the country [33]. The infection rate of 5.08% of hard ticks in Heris County mainly from mountainous areas is high enough to alarm about the creeping risk of CCHF to cold and moderate regions such as Eastern Azerbaijan province. It is, therefore, recommended that further molecular surveys be undertaken to elucidate the enzootic and endemic status of CCHF disease in other counties of Eastern Azerbaijan province, should a comprehensive provincial plan be devised to prevent CCHF outbreaks.

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