Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3 (2): 156-159

Received: 23-01-2015
Accepted: 12-02-2015

Faezeh Faghihi
Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran

Sadegh Chinikar
National Reference Laboratory of Arboviruses and Hemorrhagic Fevers, Pasteur Institute, Tehran, Iran

Zakkyeh Telmadarraiy
Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Hasan Bakhshi
Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran

Sahar Khakifirouz
National Reference Laboratory of Arboviruses and Hemorrhagic Fevers, Pasteur Institute, Tehran, Iran

Tahmineh Jalali
National Reference Laboratory of Arboviruses and Hemorrhagic Fevers, Pasteur Institute, Tehran, Iran

Gidiglo Godwin Nutifafa
Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Correspondence:
Zakkyeh Telmadarraiy
Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

Crimean-congo hemorrhagic fever: A seroepidemiological and molecular survey in north of Iran

Faezeh Faghihi, Sadegh Chinikar, Zakkyeh Telmadarraiy, Hasan Bakhshi, Sahar Khakifirouz, Tahmineh Jalali, Gidiglo Godwin Nutifafa

Abstract
Crimean Congo Hemorrhagic Fever (CCHF) is an arboviral zoonotic disease. Numerous genera of ticks serve as both vector and reservoir of CCHF virus. CCHF is an endemic disease in different provinces of Iran.
Having used the ELISA method, IgG antibodies against CCHF virus were detected in 12 (38.7%) out of 31 examined sheep. RT-PCR showed CCHFV in 9.52% of hard tick samples. The infected species included *Haemaphysalis punctata*, *Haemaphysalis punctata*, *Rhipicephalus bursa* and *Rhipicephalus sanguineus*. We revealed presence of *Rhipicephalus, Dermacentor, Haemaphysalis* and *Hyalomma* genera of ticks in the study area with 9 species. The most frequent ticks were *Rhipicephalus bursa* and *Hyalomma marginatum* with a frequency of 40.9% and 11.5% respectively. We also found *Haemaphysalis numidiana*, *Rh. sanguineus*, *Dermacentor marginatus*, *H. sulcata*, *H. punctata*, *H. concinna* and *H. inermis*.
This study confirms the circulation of the virus in the afore-mentioned region.

Keywords: CCHF, RT-PCR, ELISA, Mazandaran Province, Iran

1. Introduction
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 outbreaks have recently increased [1]. Crimean-Congo Hemorrhagic Fever Virus (CCHFV) is a tick-borne disease [2]. The virus is transmitted to humans by several methods including: tick bites, direct contact with fresh meat or blood of infected animals [3]. Nosocomial outbreaks among hospital staff due to CCHF with high mortality are also another aspect of the disease [4-6]. The disease has a worldwide distribution and it is considered as an endemic disease in many countries of Asia, Europe, and Africa. New outbreak of this disease was recorded in Kosovo, Senegal, Turkey, Bulgaria, Iran, Pakistan and Mauritania [2, 7-9]. CCHF is caused by an RNA virus as a *Nairovirus* genus from Bunyaviridae family. Predominant hosts of CCHF virus are wild and domestic mammals and birds. Sheep, goats and cattle develop high titres of virus in blood, but tend not to fall ill. Humans are usually infected with CCHF virus through a tick bite or close contact with viral-contaminated tissues or blood of the domestic animals. Blood and secretions from the infected patients could be distributed by way of infection so, medical laboratory staff and health-care workers are included in high-risk groups [10]. In Iran, CCHFV was reported in 1970 and first isolated in 1978 from ticks [11-12], Afterwards, the case of disease was not reported properly. In 1999, an outbreak was reported from Chaharmahal and Bakhtiari provinces, South-west of Iran [13-15]. According to the latest records, CCHFV exists in 24 out of 31 provinces of Iran [9, 13]. The aim of this study was to determine CCHFV infection in hard ticks (Ixodidae) by RT-PCR method and to determine CCHFV infection in sheep by the use of ELISA method, IgG antibodies against CCHF virus in Southeastern region of Mazandaran province, North of Iran.

2. Materials and Methods
2.1 Study area
Mazandaran province (Figure 1) (36.5656°N 53.0588°E) is located in the North of Iran. This province has an area of 23,842 km², and is one of the most densely populated provinces in Iran. The province's four largest counties are Sari, Amol, Nur, and Tonekabon. The diverse nature of the province features plains, prairies, forests and rainforest stretching from the sandy

beaches of the Caspian Sea to the rugged and snowcapped Alborz sierra, including Mount Damavand, one of the highest peaks and volcanos in Asia. In this study we selected the south-east region of the province for investigations on infection of sheep due to the fact that the most livestock in this part of the province is sheep. Also, we selected this region for investigation on infection of ticks to CCHFV. We caught ticks and also studied livestock of Kiasar, Chaloo, Manzel Darreh, Tooseh, Terkam, Sanoor, Ivel, Telmadarreh, Kordmir, Era, Malkhast, South Mazandaran boarder region and Kwat districts. This study was carried out during year 2006-2007.

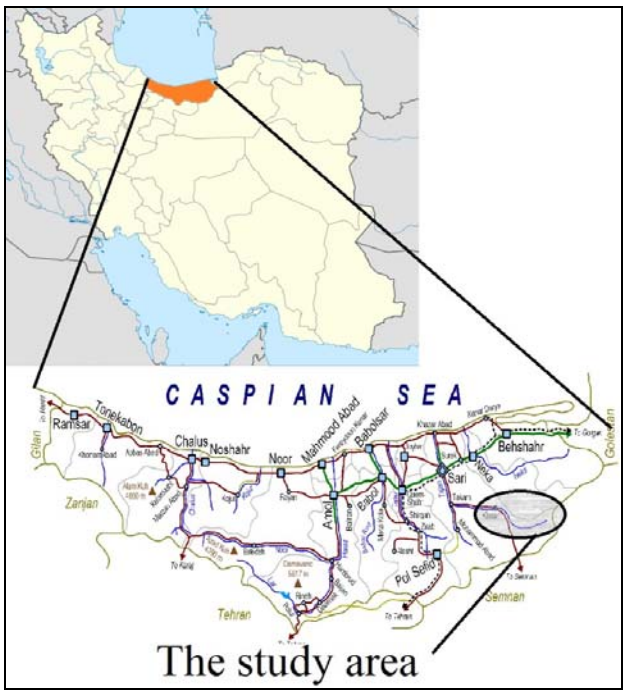

Fig 1: Mazandaran Province is located in north of Iran. Our study districts are located in north-east region of Mazandaran province

2.2 Sample collection

A cross-sectional study on sheep and tick population which had infected the sheep, was conducted in the summer, fall and winter of 2006 and spring of 2007 in 12 districts of the south-east region of Mazandaran province, including Kiasar, Chaloo, Manzel Darreh, Tooseh, Terkam, Sanoor, Ivel, Telmadarreh, Kordmir, Era, Malkhast, South Mazandaran boarder region and Kwat districts. A total of 337 hard ticks were collected from sheep.

Blood samples (10mL each) were collected from the total of 31 enrolled sheep subjects and immediately centrifuged at the local laboratory in a veterinary organization for serum taking. The sera were kept in – 20 °C and transmitted on ice to Arboviruses and Viral Hemorrhagic Fevers laboratory (National Ref. Lab) in Pasteur Institute of Iran. All laboratory tests including molecular and serological analyses were performed in biosafety level 2 under negative pressure [16]. The RT-PCR master mix solution was provided in a filtered clean chamber with positive pressure to avoid a false-positive result.

Collected ticks from each host were kept alive in separate labeled tubes, then were transferred to the laboratory of Medical Entomology, School of Public Health, Tehran University of Medical Sciences and were identified by morphological characteristics using a stereo- microscope based on valid identification keys [3, 17]. All identified ticks were kept in micro tubes and were transferred to the Arboviruses Laboratory, Pasteur institute of Iran (National Reference Laboratory of Iran) for determination of presence of CCHFV by reverse transcription-polymerase chain reaction (RTPCR) method.

2.3 RNA Extraction and RT-PCR

In the molecular laboratory, ticks were individually washed twice with PBS 1X and crushed with a mortar and pestle in 200-300 µl of PBS 1X. Total RNA was extracted from the samples using the RNA easy kit (QIAGEN, Viral RNA mini kit, GmbH, Hilden, Germany) according to the recommendations of the supplier. The RNA was dissolved in 50 mL of RNase-free water and stored at -70°C until use. 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'GACAAATTCCCTACACCA-3') and 1 µl RNase inhibitor. 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) [18]. 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. Five µl of the PCR products were mixed with 1 µl loading buffer and then electrophoresis on 1.5% agarose gels in Tris-borate EDTA buffer (TBE) was carried out. DNA bands were stained with ethidium bromide and were visualized on a UV transilluminator [13-15, 18].

2.4 Serological assay

For IgG detection, ELISA plates were coated with mouse hyperimmune ascetic fluid diluted in 1X PBS and incubated overnight at 4 °C. The native (produced in biosafety level 4 containment) or recombinant antigen (a recombinant nucleoprotein expressed in mammalian cells via the recombinant Semliki Forest alphavirus replicon), diluted in PBSTM (PBS containing 0.05% Tween 20 and 3% skim milk), was added to the plates, and the plates were incubated for 3 h at 37°C and extensively washed. Serum samples diluted in PBSTM were added, and the plates were incubated for 1 h at 37°C. After washing, the peroxidase-labeled anti-animal immunoglobulin diluted in PBSTM was added to each well, and the plates were incubated for 1 h at 37°C. The plates were then washed thrice with phosphate-buffered saline containing 0.5% Tween (PBST), which had also been used for washing the plates after each of the incubation periods. Finally, hydrogen peroxide and 3,3',5,5'-tetramethylbenzedine were added, and the plates were incubated for 15 min at room temperature. The enzymatic reaction was stopped by the addition of sulfuric acid (4N). The plates were read by an ELISA reader at 450 nm. Positive and negative control sera were included in each test [14, 19]. The sample with an OD higher than 0.15 was considered positive and this is deducted from the OD of negative controls.

3. Results

In this study, we tried to catch ticks from sheep in south-east region of Mazandaran province due to this fact that the most livestock in the region are sheep. Totally, 337 ticks were caught from Kiasar, Chaloo, Manzel Darreh, Tooseh, Terkam, Sanoor, Ivel, Telmadarreh, Kordmir, Era, Malkhast, Kwat and South Mazandaran border districts. We revealed presence of *Rhipicephalus, Dermacentor, Haemaphysalis* and *Hyalomma* genus of ticks in these districts with 9 species (Table 1). The most frequent tick was *Rhipicephalus bursa* with a frequency of 40.9%. The second frequent tick was *Dermacentor*

marginatus with a frequency of 18.6%. We could find also *Haemaphysalis numidiana* (9.7%), *Rh. sanguineus* (9.1%), *Hyalomma marginatum* (11.5%), *H. sulcata* (5.3%), *H. punctata* (3.5%), *H. concinna* (0.5%) and *H. inermis* (0.3%). A total number of 31 sheep were examined for presence of CCHFV genome by the use of ELISA method, IgG antibodies against CCHF virus. Sheep sera were collected from different parts of high-risk regions in the south-east region of Mazandaran province including Kiasar, Chaloo, Manzel Darreh, Tooseh, Terkam, Sanoor, Ivel, Telmadarreh, Kordmir, Era, Malkhast, South Mazandaran boarder region and Kwat districts. Our examination revealed that 12(38.7%) out of 31 sheep examined from south-east region of Mazandaran province, North of Iran were positive. Reverse transcription-polymerase chain reaction (RT-PCR) showed CCHFV in 9.52% of hard tick samples, including two of 16(12.5%) *Haemaphysalis* and two of 14 (14.4%) *Rhipicephalus* genera of ticks were infected. Infected species were *Haemaphysalis punctata*, *Haemaphysalis punctata*, *Rhipicephalus bursa* and *Rhipicephalus sanguineus* which were collected from Manzel Darreh, Tooseh, Telmadarreh and Kiasar respectively. Result of RT-PCR amplification of S segment of CCHFV genome using RNA extracted from each ticks showed a 536 bp PCR band.

Table 1: Seasonal activity and ticks caught from south-east region of Mazandaran province, North of Iran

Seasons	***D. marginatus***	***Hy. marginatum***	***Rh. bursa***	***H. numidiana***	***H. punctata***	***Rh. sanguineus***	***H. sulcata***	***H. concinna***	***H. inermis***	***Total***
Winter 2007	0	0	0	9	11	0	17	0	0	37
Spring 2007	0	0	115	0	0	16	0	0	0	131
Summer 2007	14	39	23	4	1	15	0	0	0	96
Autumn 2007	49	0	0	20	0	0	1	2	1	73
Total	63	39	138	33	12	31	18	2	1	337

4. Discussion

As ticks are very important vectors of human and animal diseases, they are subject of many studies in Iran. There is very limited information about geographical distribution of tick infestation in certain geographical areas, especially in Iran [20]. In a recent study, Hosseini Vasoukolaei *et al.* investigated on tick species parasiting domestic ruminants in Ghaemshahr county, Mazandaran province [21]. The mean number of ticks on each animal in that study was low. This is in agreement with our study. Hosseini Vasoukolaei *et al.* found *Rhipicephalus sanguineus, Rhipicephalus bursa, Ixodes ricinus, Boophilus annulatus, Haemaphysalis punctata* and *Haemaphysalis numidiana* in their investigation [21]. We found *Rh. Bursa, H. numidiana, H. punctata* and *Rh. Sanguineus* too but could not find *Ixodes ricinus and Boofilus annulatus* in our investigation; although we found *H. sulcata, H. concinna* and *H. inermins* that Hosseini Vasoukolaei *et al.* could not find. *Rhipicephalus sanguineus* was the most abundant species in Ghaemshahr; although our most abundant species was *Rh. bursa*.

Serological and molecular epidemiology survey will certainly help in determining CCHFV in our study area. An increasing number of human CCHFV infections have been reported from various regions of Iran [22]. An early seroepidemiological survey in northern/central parts of the country was carried out in 1974, thus showing the presence of virus in local livestock [23].

During 2008–2009, a molecular survey on hard ticks *(Ixodidae)* was conducted in Yazd province, Iran. The results revealed that CCHFV genome was found in 5.71% of hard ticks. All positive ticks were from *Hyalomma* genus. Positive ticks included: *Hyalomma dromedarii*, *Hyalomma marginatum, Hyalomma anatolicum, Hyalomma detritum, Hyalomma asiaticum* [24]. During the years 2003 to 2005, of the 448 livestock sera collected from the Khorasan province, northeast part of Iran, 77.5% of 298 sheep samples and 46% of 150 goat samples were positive [16].

In a similar investigation which was carried out to clarify the epidemiological aspects of CCHF infection among sheep in various geographical regions of Mazandaran province, 270 blood samples were collected from sheep in eastern, central, and western Mazandaran between 2010 and 2011, and the specific ELISA test for CCHF virus was carried out on the blood samples in the National Reference Laboratory in the Pasteur Institute, Tehran, Iran. The CCHF infection rate according to this study was 3.7% [25]. A weak statistical relationship (p=0.063) was seen between the different geographical regions, with a gradual decrease in the infection rate noted, stretching from the eastern to the western portions of the province (eastern 6.8%, central 2.8%, and western 0%) [25]. Our investigation showed an infection of 38.7% of sheep in south-east region of Mazandaran province; so the infection rate of our study is higher than that from the Mostafavi el al., study. Mostafavi *et al*., demonstrated that the eastern portion of the province had a 6.8% infection rate [25]. This reveals that the infection rate increases when we stretch from the eastern to the western portions of the province. As the infection rate in Mazandaran is high among sheep due to this study, CCHF disease is considered a serious health problem in Mazandaran. This is contrary to the Mostafavi *et al*., statement. Although *Hyalomma* ticks are considered the most important vector and reservoir in the epidemiology of CCHFV, the virus has also been reported from other genera of ticks. We did not detect any virus from *Hyalomma* ticks but our study revealed the infection of 4 hard tick samples, including two of 16(12.5%) *Haemaphysalis* and two of 14 (14.4%) *Rhipicephalus* genera of ticks were infected. Infected species were *Haemaphysalis punctata*, *Haemaphysalis punctata*, *Rhipicephalus bursa* and *Rhipicephalus sanguineus*.

It is recommended that further studies be carried out on additional livestock, high-risk groups of humans, and ticks, to determine the CCHF disease status in Mazandaran especially at the Eastern part of the province. It is also suggested that a similar study be carried out on other northern neighboring provinces (Golestan and Gilan), to clarify the epidemiological features of CCHF disease in the provinces bordering the Caspian Sea.

5. Acknowledgments

We appreciate the financial support from Tehran University of Medical Sciences, Iran (Grant No. 3765-63-02).

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