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Crimean Congo Hemorrhagic Fever in Northeast of Iran

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Abstract: Crimean-Congo hemorrhagic fever (CCHF) is an arboviral zoonotic disease that is asymptomatic in infected animals, but a serious threat to humans. Humans become infected with Crimean-Congo hemorrhagic fever virus (CCHFV) either through bites of infected ticks, or by direct contact with virus contaminated tissues or blood of animals. CCHF now occurs sporadically throughout much of Africa, Asia, Europe and recent outbreaks of CCHF have occurred in Afghanistan and Pakistan. Since 1999, human clinical cases of CCHF have been reported from different parts of Iran. Although, most of human reports came from the Southeast of Iran, we analyzed samples from the Northeast provinces to check the geographical extension of CCHF. From October 2001 to the end of 2007, 10(15.9%) of 63 probable human patients confirmed to be infected with CCHFV using ELISA and RT-PCR tests in Khorasan region, Northeast of Iran, close to Afghanistan and Pakistan. No specific antibodies against other viral fevers such as Yellow fever, Rift Valley fever, Dengue 2 and Chikungunya were detected in the sera of these probable cases. During the years 2003 to 2005, of 448 livestock sera collected form different townships of this region, IgG antibodies were noted in 77.5% (95% confidence interval (CI): 72.5-82%) of 298 sheep samples and 46% (95% CI: 38.1-54.0%) of 150 goat samples. The frequency of infection was more or less equal in both sexes and was age dependent. Regardless whether CCHF is newly enzootic or has long established enzooticity, the potential exists for sporadic or clustered outbreaks of CCHF in humans, so persons in close contact with animals and also health care workers should be alarmed.

Key words: Crimean-Congo hemorrhagic fever, RT-PCR, ELISA, Khorasan region, Iran

INTRODUCTION

Crimean-Congo hemorrhagic fever virus is a tick-transmitted member of the *Bunyaviridae* family (*Nairovirus* genus) that causes severe hemorrhagic diseases in humans. It is also classified as one of the potential agents of bioterrorism. CCHF outbreaks constitute a threat to public health services because of its epidemic potential, high case fatality rate (30-50%), potential for nosocomial outbreaks and the difficulties in treatment and prevention (Ergonul, 2006; Whitehouse, 2004).

CCHF was first observed in the Crimea of Russia in 1944 and isolated in Congo of Africa in 1956. There are reports of viral isolation and/or disease from more than 30 countries in Africa, Asia and Europe; and recent outbreaks have occurred in Afghanistan and Pakistan (Whitehouse, 2004; Wallace *et al*., 2002; Altaf *et al*., 1998).

Chumakov *et al*. (1970) first suggested the presence of CCHFV in Iran (Chumakov *et al*., 1970). There was no report of human clinical CCHF in Iran until 1999, when an outbreak reported form Shahr-e-Kord city (central part of Iran) and subsequently other outbreaks were recorded in

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different provinces of Iran. Up to October 2004, 169 (68%) of 248 Iranian CCHF cases reported from Sistan Baloochestan province, Southeast of Iran, bordered with Afghanistan and Pakistan (Chinikar *et al*., 2005; Izadi *et al*., 2004).

Although, many domestic and wild vertebrates are infected with CCHFV, birds, in general, appear to be refractory to infection with CCHFV (Whitehouse, 2004). In mammals the infection is usually subclinical and asymptomatic. Domestic ungulates, especially sheep and goats appear to play a central role as virus hosts in the maintenance cycle of CCHFV in endemic areas and also because of their role as principal hosts of CCHFV tick vector (Gonzalez *et al*., 1998). The most efficient and common ticks are the members of the genus *Hyalomma*. The biological role of ticks is important not only as virus vectors, but also as reservoirs of the virus in nature (Ergonul, 2006; Whitehouse, 2004). Shepherds, farmers, veterinarians, abattoir workers and other persons in close contact with animals and ticks are high risk for infection. Although, the focus of epidemiological interest is on the pattern of human cases, explanations for the described distribution and abundance of infections come from understanding the extent of animal infection (Ergonul and Whitehouse, 2007).

The present study is a report of CCHF in humans and livestock of Northeast of Iran, neighboring Sistan Baloochestan province and Afghanistan and close Pakistan, in the recent outbreaks of the disease.

MATERIALS AND METHODS

Study area: Northeastern part of Iran containing Northern Khorasan (pop. 811,572), Razavi Khorasan (pop. 5, 593, 079) and Southern Khorasan (pop. 636, 420) provinces, covering an area of 313.335 sq km and includes one fifth of the Iran's total area.

Iran is among the major sheep and goat raising countries of the world, with a sheep population of 53.9 million heads and a goat population of 25.8 million. Of these, Khorasan region have approximately 8.6 million sheep and 3.7 million goats (Ministry of Jihad-e-Keshavarzi Report, 2002).

Khorasan region geographically has long boarders with high risk areas. It is bounded northward by Turkmenistan, eastward by Turkmenistan and Afghanistan, westward by Yazd, Semnan and Golestan provinces and southward by Sistan Baloochestan and Kerman provinces (Fig. 1).

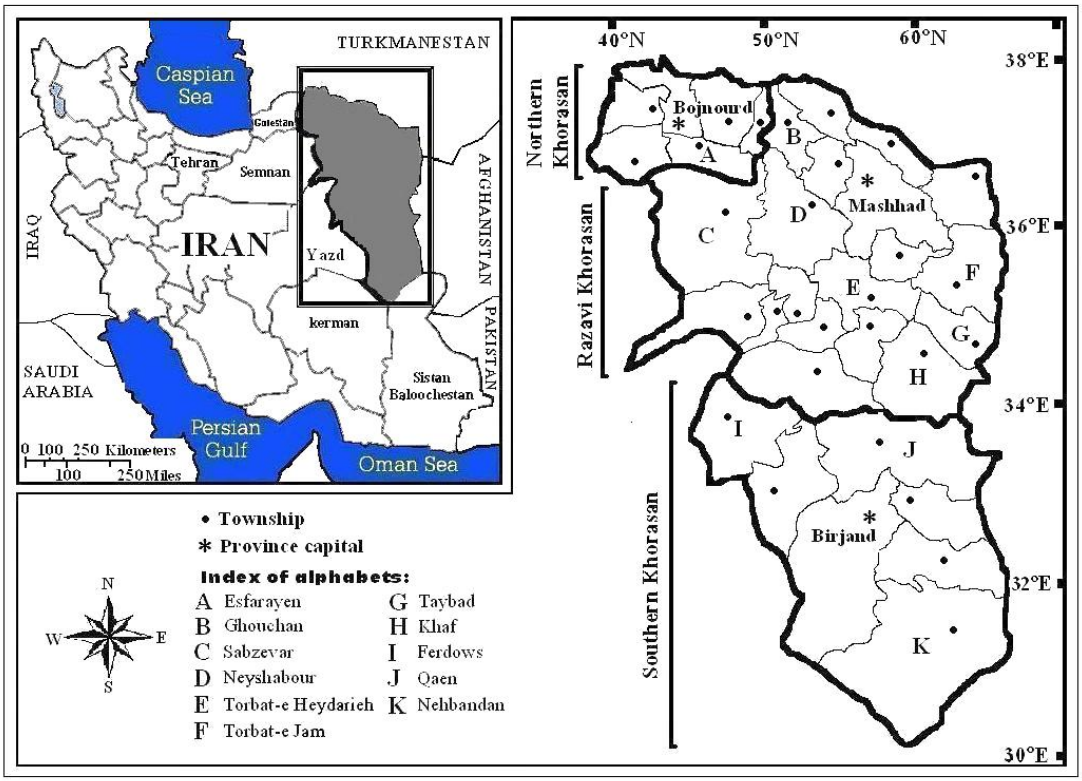

Fig. 1: Locations visited in order to characterize the prevalence of CCHF virus infection in sampled sheep and goats and sera of individuals probable to be infected with CCHFV collected in Khorasan region, Northeast of Iran

The area is mainly mountainous and arid. People's economy is mainly based on agriculture and livestock husbandry and so the people are in close contact with animals. There is also some nomadic population in sparse and scattered villages and their usual occupation is trading livestock. There is a sizeable Afghan community in this area due to the influx of refugees from Afghanistan and Pakistan in recent years. Moreover, this emigrant community for many reasons crosses the boarder frequently.

Sample collection: As part of the National surveillance system, sera of probable human cases are collecting by the health system and transferring to the national reference laboratory of research and diagnosis on arboviruses and viral hemorrhagic fevers of Pasteur Institute of Iran for serological and molecular assessment. In this study, we analyzed the results of all confirmed patients between October 1st, 2001 and the end of 2007. A probable human case was defined as an acutely ill person with clinically observed signs and symptoms of acute onset of fever, myalgia and bleeding; epidemiological risk factors and/or laboratory data such as thrombocytopenia and leucopenia.

Northeast of Iran contains 32 townships in 3 provinces. We randomly chose 13 townships; in each one we collected serum samples from 15 sheep and/or goats using convenience sampling. Also, we recorded the sex and the estimated age of animals by dental examination. The sera transferred to a local laboratory, extracted, froze and transmitted to the national reference laboratory of Pasteur Institute of Iran.

Laboratory tests: Immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies are detectable by specific enzyme-linked immunosorbent assay (ELISA) from about 7 days after the onset of the CCHFV. IgM antibodies can be detected in sheep and goats for only 3-7 weeks, whereas in human they remain for three to 5 months. IgG remains detectable for at least 5 years (Ergonul, 2006; Gonzalez, 1990).

Probable human sera were referred for CCHF specific antibody (IgG, IgM) detection on the 1st, 5 and 10th day of admission. Reverse transcription-polymerase chain reaction (RT-PCR) technique is done on the first samples of the patients. Presence of IgM, IgG antibody by ELISA and/or presence of CCHFV by RT-PCR method were used for confirming a definite case. Human sera were also tested for other viral fevers such as Yellow fever, Rift Valley fever, Dengue 2 and Chikungunya.

IgG ELISA tests done on livestock sere for detection of CCHF infection.

IgG detection: The ELISA plates were coated with the mouse hyperimmune ascetic fluid and incubated overnight at 4°C. The native or the recombinant antigen was added and the plates incubated for 3 h at 37°C. Diluted Serum was added and the plates were incubated for 1 h at 37°C. After adding the diluted Peroxydase-labeled anti-human or anti-animal immunoglobulin, the plates were incubated for 1 h at 37°C. The plates then were washed 3 times with phosphate-buffered saline (PBS) containing 0.5% Tween after doing each of the incubations. Finally, hydrogen peroxide (H2O2) and 3, 3', 5, 5' tetramethyl benzidine (TMB) was added and the plates were incubated for 15 min at room temperature. The enzymatic reaction was stopped by the addition of 4N H2SO4. The plates were read by ELISA reader at 450 nm (Garcia *et al*., 2006; Chinikar *et al*., 2005).

IgM detection: The ELISA plates were coated with goat IgG fraction to human IgM (anti μ chain) diluted in PBS 1x and incubated overnight at 4°C. Then, the sera sample diluted in PBS containing 0.5% Tween (PBST) and 3% skim milk (PBSTM) and the plates were incubated for 1 h at 37°C. After dilution of the Antigen in PBSTM, it was added and the plates were incubated for 3 h at 37°C. Diluted immunoascite then was added and the plates were incubated for 1 h at 37°C. Peroxydase-labeled anti mouse immunoglobulin was added and the plates were incubated for 1 h at 37°C. After each of the incubations, the plates were washed 3 times with PBST. Finally, H2O2 and 3, 3', 5, 5' TMB was added and the plates were incubated for 15 min at room temperature. The enzymatic reaction was stopped by the addition of 4N H_2SO_4. ELISA reader read the plates at 450 nm (Garcia *et al*., 2006; Chinikar *et al*., 2005).

RNA extraction and RT-PCR on human samples: Total RNA was extracted from the samples using the RNA easy kit (Qiagen, Viral RNA mini kit, GmbH, Hilden, Germany).

A master mix was prepared with Qiagen, one-step RT-PCR kit, GmbH, Hilden, Germany as fallowing: 28 μL of RNase free water(RFW), 10 μL buffer 5x, 2μL dNTP mixed, 2μL reverse transcription enzyme and taq polymerase, 1μL of primer A (Forward) (5' TGGACACCTTCACAAACTC-3'), 1μL of primer B (Reverse) (5' GACAAATTCCCTAC ACCA-3') and 1 μL RNase inhibitor. A 45 μL of master mix was added to PCR tubes and 5 μL of extracted RNA was added to the individual PCR tubes.

Thermal program for RT-PCR included:

RT reaction	30'	50°C (cDNA synthesis)	
Denaturation	15'	95°C	
Denaturation	30"	95°C	40 cycles
Annealing	30"	50°C	
Extension	45"	72°C	
Final Extension	10'	72°C	

After amplification, samples were stored either overnight at 2-8°C, or at -20°C for longer-term storage. 5 μL of the PCR products were mixed with 1 μL loading buffer and then were electrophoresed on 1.5% agarose gels in Tris-borate EDTA buffer (TBE). DNA bands was stained with ethidium bromide and were visualized on a UV transilluminator (Burt *et al*., 1998; Chinikar *et al*., 2004).

Statistical analysis: Data were analyzed using SPSS software version 15.0. To compare qualitative variables, the chi-square test was used. p-values <0.05 were considered significant. Descriptive statistics (i.e., frequencies and percentages) were used to summarize the quantitative variables.

RESULTS AND DISCUSSION

In this research CCHF is reported in human and livestock of Northeastern part of Iran. It demonstrates that more attention should be made to the disease in this region.

During 2000-2004, an increasing number of human CCHFV infections have been reported from different provinces in Iran. Most of the human cases have been reported in Sistan Baloochestan province, in Southeastern Iran (Chinikar *et al*., 2005).

Different surveys have shown confirmed patients and circulating virus in livestock living in Turkmenistan, Pakistan and Afghanistan, countries neighbouring East and Northeast of Iran (Wallace *et al*., 2002; Altaf *et al*., 1998; Smirnova *et al*., 1978). Livestock trading from these countries to Iran is reported to have increased during recent years. In Sistan Baloochestan province, shepherds raising sheep and goats move their herds for grazing to Khorasan pastures. So, the occurrence of human cases and livestock infection in Khorasan could associate with infectivity of recently imported tick infested and viremic domestic animals. The recent phylogenic analysis done on CCHF viruses isolated from eastern region of Iran showed that new Iranian CCHFV was similar to the strain in Pakistan (Chinikar *et al*., 2004).

In our study, sera of 63 probable patients of different townships of Khorasan region, referred to the national reference laboratory of Pasteur Institute. Among them, 10 (15.9%) cases confirmed to be infected with CCHFV using specific ELISA and RT-PCR tests. No specific antibodies against Yellow fever, Rift Valley fever, Dengue 2 and Chikungunya were detected in these sera.

Most of the human confirmed patients (30%) reported in 2001, there was no report in 2007. Nine (90%) of the confirmed cases where from Razavi Khorasan including Mashhad (4 cases), Sabzevar (2 cases) and Torbat-e Heydarieh and Chenaran (one case) and one of the cases was from Bojnourd in Northern Khorasan. There was no report from Southern Khorasan. One of the cases was Afghan refugees hospitalized in Mashhad; whether he is infected in Iran or Afghanistan is not known.

The mean age was 32.5±22.05 (range: 20-53) years. All the confirmed patients were males and 2 (20%) ones were from rural areas. All of 10 cases were IgM and IgG positive and between them 5 (50%) cases were RT-PCR positive too (Table 1).

Eight (80%) of the patients recovered and unfortunately two (20%) ones died. Fever less than 2 weeks (90%), haemorrhage (petechiae, hematuria, epistaxis, melena, ecchymosis) (80%), thrombocytopenia (platelet count less then 150000/cc) (80%), leucopenia (WBC count less then 3000 cells mm^{-3}) (70%), myalgia (60%) and Serum glutamic oxaloacetic aminotransferase (SGOT) (above 100 IU dL^{-1}) (50%) were the most frequent symptoms.

Among these confirmed patients the fallowing epidemiological risk factors have been seen: 90% (nine) handled livestock, 40% (4) had a high risk profession (butchers and farmer), 20% (2) had a history of tick bite and 10% (1) had a history of contact with a recognized CCHF case. Total 20% (2) of these patients had none of these risk factors.

Most of the risk factors for CCHF such as close contact with livestock (animal husbandry and slaughtering livestock), a history of tick bite and contact with a recognized CCHF case (Izadi *et al*., 2004; Ozkurt *et al*., 2006; Fisher-Hoch *et al*., 1992) have been seen in confirmed patients. Male risk factors primarily associated with herding activities and contact with sick animals. All of the patients were male and it shows that their job plays an important role as a risk factor.

Most of the human cases reported from Mashhad, the center of Razavi Khorasan. This is the second largest city in Iran containing 2 million populations and a sizable Afghan community with an appropriate surveillance system and developed primary health care (PHC) systems for diagnosing the disease.

In 1978, Sureau *et al*. (1980) isolated the CCHFV from an *Alveonasus lahorensis* tick in Khorasan province. The occurrence of human CCHFV infections in this region may be newly recognized but virus circulation in ticks and local livestock may have been enzootic and unrecognized for many years.

In endemic areas, sheep and goat antibodies appear to be one of the best indicators of risk to humans. Moreover, sheep are the most representative domestic animals due to their abundance and proximity to humans (Gonzalez *et al*., 1998). In Iran, in different surveys on CCHF antibody, sheep and goats have been 25-80% seropositive. In 1970, the presence of CCHFV demonstrated when antibodies to the virus in sera of 45(45%) of 100 sheep from the Tehran (North of Iran) abattoir were detected (Chumakov *et al*., 1970). In 1970-1971, CCHF antibody was detected in 54% of sheep and goat sera from northern area (Chumakov and Smirnova, 1972). Saidi *et al*. (1975) reported positive reactions in sera of sheep (38%), goats (36%) and cows (18%) mostly from near the Caspian Sea in Northern provinces. Afterward the recent outbreak in Iran, Chinikar *et al*. (2002) reported specific CCHF IgG antibodies in 32.95% of 607 sheep and 12.64% of 356 goats of different regions of Iran during 2000-2002. Total 78.9% of 372 local sheep in Isfahan province in central part of Iran were seropositive for CCHF in 2002 (Ataei *et al*., 2006).

In our survey, samples from 448 heads of sheep and goats collected respectively from 13 and 9 townships. Serological evidence for CCHFV infection was present in 77.5% (95% CI: 72.5-82%) of 298 sheep samples and 46% (95% CI: 38.1-54.0%) of 150 goat samples and it shows that Northeastern part of Iran is a hyper enzootic region for CCHF. The high infection rates among these animals suggest that they are an important part of the ecology of CCHF, if only to provide a source of blood meal to infected ticks.

In sheep samples, the minimum seroprevalence (40%) was seen in Nehbandan and the maximum (100%) in

Table 1: Characteristics of confirmed CCHF patients in Khorasan region, Iran, 2001-2007

Province	City resident	Year report	Age	Profession	Test result
Razavi Khorasan	Mashhad	2001	53	Butchers	IgM+, IgG+
		2001	35	Butchers	IgM+, IgG+
		2001	40	Framer	IgM+, IgG+
		2002	25	Mason*	IgM+, IgG+, RT-PCR+
	Sabzevar	2003	20	Student	IgM+, IgG+, RT-PCR+
	Torbat-e Heydarieh	2003	44	Farmer	IgM+, IgG+
	Mashhad	2004	32	Employee	IgM+, IgG+, RT-PCR+
	Sabzevar	2005	34	Tailor	IgM+, IgG+, RT-PCR+
	Chenaran	2006	21	Soldier	IgM+, IgG+, RT-PCR+
Northern Khorasan	Bojnourd	2006	21	Soldier	IgM+, IgG+

* An afghan refugees hospitalized in Mashhad

Table 2: Characteristics of animals tested in Khorasan region, Iran, 2003- 2005 (Infected ones are all ELISA IgG positives)

Species	Province	City	No. Tested	No. infected (% per group)
Sheep	Northern Khorasan	Esfarayen	21	21(100)
	Razavi Khorasan	Ghouchan	15	15(100)
		Sabzevar	17	16(94.12)
		Neyshabour	15	15(100)
		Mashhad	25	25(100)
		Torbat-e Heydarieh	22	15(68.18)
		Torbat-e Jam	17	16(94.12)
		Taybad	18	10(55.56)
		Khaf	16	7(43.75)
	Southern Khorasan	Ferdows	20	14(70)
		Qaen	72	53(73.61)
		Birjand	25	18(72)
		Nehbandan	15	6(40)
		Total	298	231(77.52)
Goat	Northern Khorasan	Esfarayen	17	9(52.94)
	Razavi Khorasan	Ghouchan	17	12(70.59)
		Sabzevar	15	7(46.67)
		Neyshabour	17	4(23.53)
		Torbat-e Heydarieh	16	8(50)
		Torbat-e Jam	15	4(26.67)
	Southern Khorasan	Ferdows	16	4(25)
		Birjand	17	10(58.82)
		Nehbandan	20	11(55)
		Total	150	69(46)

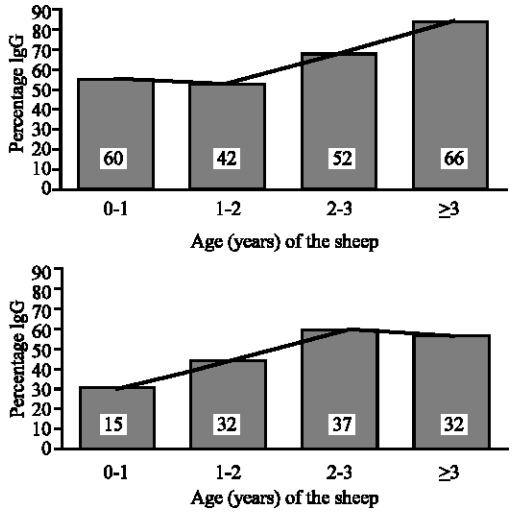

Fig. 2: Prevalence of IgG antibodies to CCHF virus by age of sheep and goats from Northeast of Iran between October 2003 and June 2005. Sheep and goats which their age was recorded are included. Number of sheep and goats in samples are shown at the base of each bar

Ghouchan, Mashhad, Neyshabour and Esfarayen. In goat samples, the minimum seroprevalence (25%) was seen in Ferdows and the maximum (70.59%) in Ghouchan. The details are shown in Table 2.

The difference in seropositivity for CCHF between males and females was not significant. Total 169 (75.4%) of 224 ewe sera and 62(83.8%) of 74 ram sera were IgG positive ($p = 0.60$). In goat samples, 51(47.7%) of 107 nanny goat sera and 18(41.9%) of 43 billy goat sera were IgG positive ($p = 0.69$).

Sheep and goats were categorized into 4 groups: 0-1 years, 1-2 years, 2 -3 years and = 3 years. Compatible with our expectation, we observed an increasing trend in the prevalence of Antibody by age. Fifty five and thirty percents of sheep and goats with less than one year old showed antibody, while the corresponding figures in more than 3 year olds were 83.3 and 56.2% (Fig. 2).

As IgG remains detectable for at least 5 years, we anticipated and observed an age-related increase in seroprevalence of sheep and goats. Sexes were infected equally. That IgG was found in sheep and goats of all ages suggests enzootic transmission throughout this region. These observations were such as findings in Senegal (Wilson *et al*., 1990).

Regardless whether CCHF is newly enzootic or has long established enzooticity, the potential exists for sporadic or clustered outbreaks of CCHF in humans, so persons in close contact with livestock and also health care workers should be alarmed.

Whereas our, study showed a high prevalence of livestock infection but it revealed few reports of human clinical cases in Khorasan region. The ratio of inapparent-to-apparent infections has been estimated to be 5:1 (Goldfarb *et al*., 1980). It can be assumed that the number of asymptomatic cases were high such as in Russia which only 20% of those seropositive for CCHF virus reported significant illness (Mertz *et al*., 2002).

Certain treatment and control measures such as using acaricidal treatment and dipping or spraying livestock upon entry into the region, enforcing environmental tick control by cleaning and treating market places, trucks, ships, slaughterhouses and quarantine facilities with suitable short-term and residual acaricides and perhaps most importantly, use precautions such as gloving and booting to reduce skin contact of humans and being alarm of nosocomial transmission of disease, can decrease the potential for distribution of vectors, host animals and clinical disease in humans.

Continued surveillance and strictly enforced importation and quarantine practices will be required to prevent human exposure and ongoing dissemination of infected ticks and animals in this region.

The known occurrence of CCHF coincides with the global distribution of *Hyalomma* ticks (Hoogstraal, 1979; Ergonul, 2006). *Hyalomma* is reported as the most abundant and dominant tick in different provinces of Iran (Rahbari *et al*., 2007; Mazlum, 1971). Further studies are needed to assess the extent of tick CCHFV infection, also it would be very interesting to perform a serological survey on the human populations in this region in order to have data on the presence and spread of antibodies to CCHF virus.

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