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Crimean-Congo Hemorrhagic Fever: A Seroepidemiological and Molecular Survey in Bahar, Hamadan Province 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. Numerous genera of ticks serve both as vector and reservoir for CCHF virus. CCHF is an endemic disease in different provinces of Iran. In this study by the use of ELISA method, IgG antibodies against CCHF virus were detected in 15(27.8%) out of 54 sheep examined whereas none of the high risk human samples were positive in Bahar Township, a western region of Iran. Reverse transcription-polymerase chain reaction (RT-PCR) showed CCHFV in 16.4% of hard tick samples. Six of 43(13.9%) *Hyalomma* ticks were infected. CCHFV was detected in 3 of 23 (13%) *Rhipicephalus* sp. and 1 of 5 (20%) of *Haemaphysalis* sp. Up to now there have been few confirmed human cases in this region. This study confirms the circulation of the virus in this region and so persons in close contact with livestock and also health care workers should be alarmed.

Key words: CCHF, RT-PCR, ELISA, Bahar, Hamadan

INTRODUCTION

Crimean-Congo hemorrhagic fever virus is a tick-transmitted member of the Bunyaviridae family (*Nairovirus* genus) that causes severe hemorrhagic diseases in humans. The virus is able to have nosocomial transmission (Elliott *et al*., 2000; Whitehouse, 2004). Many vertebrates are able to replicate CCHF virus. In mammals the infection is usually subclinical and asymptomatic. Domestic ungulates, especially cattle, sheep and goats, were suspected to be the source of the virus during epidemic manifestations. Shepherds, farmers, veterinarians, abattoir workers and other persons in close contact with livestock and ticks are at risk of infection. The typical route of transmission in nature is through tick bite. CCHFV has been isolated from at least 31 species of ticks in Ixodidae (hard ticks) and Argasidae family (soft ticks) (Whitehouse, 2004).

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Although the focus of epidemiological interest is on the pattern of human cases, explanations for the described distribution and abundance of infections must come from understanding the underlying ecological processes. Epidemiologic studies suggest that ecological systems play an important role in CCHFV circulation and maintenance of the virus. In general, CCHF outbreaks have developed against a background of favorable climatic factors and enviroumental changes beneficial for the survival of large numbers of Hyalomma ticks (Despommier *et al*., 2007; Ergonul and Whitehouse, 2007).

CCHF was first observed in the Crimea of Russia in 1944 and isolated in Congo in Africa in 1956. It has a worldwide distribution and is reported in about 30 countries of Africa, Asia and Europe. The disease propagation in tropical and subtropical areas occurs more easily than in other locations (Drosten *et al*., 2003).

CCHFV was reported in Iran in 1970 (Chumakov and Smirnova, 1972). There was no report of Clinical CCHF until 1999, when an outbreak reported form Shahr-e-Kord Township and subsequently other outbreaks were recorded in different provinces of Iran. In 2000, CCHF was recognized as a major public health problem necessitating implementation of reliable methods for antibody detection (Chinikar *et al*., 2002).

A confirmed human case was reported in 2004 in Hamadan city and recent surveys has reported confirmed human cases and already circulating virus in animals living in provinces neighboring Hamadan (Chinikar *et al*., 2002, 2005) but no study has been done yet to assess the presence and extend of host and vectors of the CCHFV in this province.

This study describes the situation of CCHFV in Bahar Township, one of the highest and coldest cities of Iran.

MATERIALS AND METHODS

Study Area

Hamadan province is in the Western part of Iran. Bahar Township is located in the north west of Hamadan (Fig. 1). The highest altitude of the Township is 2964 m and the lowest is 1700. It has a cold mountainous climate, with snowy winters and short mild summers. Bahar has an area of 1334 km^2. It is an important sheep-raising area. This Township comprises two regions differing in topography; the mountainous region and plateau region. In this study the sheep and tick samples were collected from 17 mountainous and 14 plateau villages.

Sample Collection

This survey has been performed in 2006. Fifty four indigenous sheep serum samples were collected using convenience sampling and the history of their sex, age and location was recorded. Twenty one serum samples were obtained from high risk humans including Shepherds (5), farmers (5) and abattoir workers (11). Sheep and human blood samples were transferred to a local laboratory; sera were separated by centrifugation at 1500 rpm for 10 min and frozen until analyses. The frozen sera under cold chain regulations were transferred to the Arbovirus laboratory, Pasteur Institute of Iran for IgG capture Enzyme-Linked Immunosorbent Assay (ELISA) tests.

Ticks collected from the mentioned sheep were kept alive in separate vials and labeled, collection points were noted. The ticks were sent to the Entomology laboratory, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences for species identification. The identified ticks were pooled into microtubes and transferred to the Arbovirus laboratory, Pasteur Institute of Iran. Eighty eight ticks (11 species) were tested for determination of the presence of CCHF virus by reverse transcription-polymerase chain reaction (RT-PCR) method.

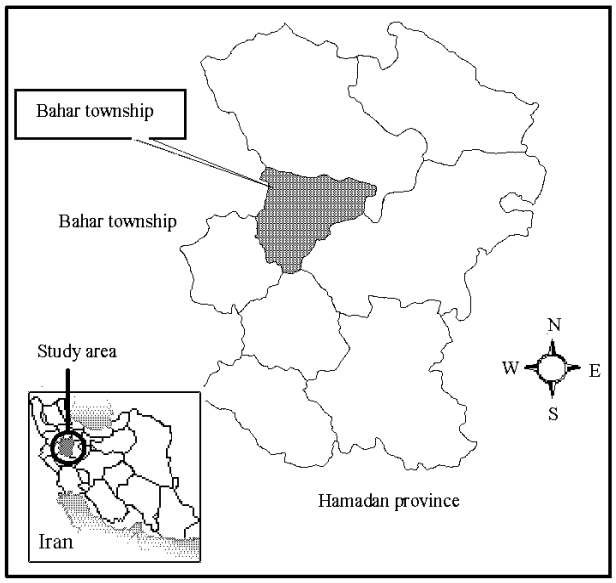

Fig. 1: Locations visited in order to characterize the prevalence of CCHF virus infection in sampled sheep, ticks and individuals involved in animal-contact occupations in Bahar Township, Hamadan province, 2006

IgG Detection

The ELISA plates were coated with the mouse hyper immune ascetic fluid (diluted at 1:1000 in phosphate-buffered saline (PBS 1x) and incubated overnight at 4°C. Following washing step, the native or the recombinant antigen diluted at 1:500 in PBS containing 0.5% Tween (PBST) and 3% skim milk (PBSTM), were added and the plates were incubated for 3 h at 37°C. Serum diluted at 1:100 in PBSTM was added and the plates were incubated for 1 h at 37°C. Peroxidase-labeled anti-human or anti-animal immunoglobulin diluted at 1:1000 in PBSTM was added and the plates were incubated for 1 h at 37°C. The plates were washed 3 times with PBST after each incubation. Finally, hydrogen peroxide (H_2O_2) 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 4 N H_2SO_4. The plates were read by ELISA reader at 450 nm (Garcia *et al*., 2006).

RNA Extraction and RT-PCR on Ticks

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 fallow: 28 µL of RNase Free Water (RFW), 10 µL buffer 5x, 2 µL dNTP mixed, 2 µL Reverse Transcriptase Enzyme and Taq Polymerase, 1 µL of Primer A (Forward) (5'TGGACACCTTCACAAACTC-3') and 1 µL of Primer B (Reverse) (5'GACAAATT-CCCTACACCA-3') and 1 µL RNase inhibitor. Forty five microliter 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) (Burt *et al*., 1998). (The master mix typically contains all the components required for reverse transcription-polymerase chain reaction (RT-PCR) except the template RNA.

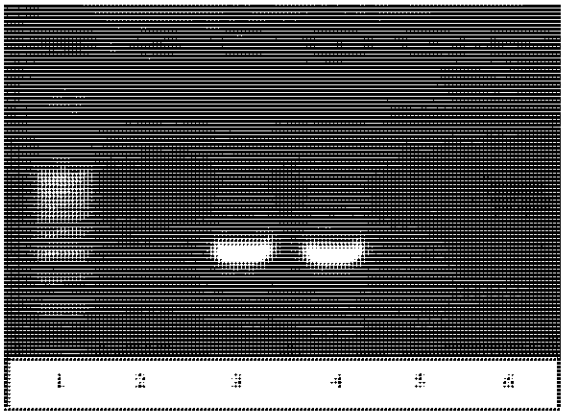

Fig. 2: A 536 bp region of the S segment of CCHF genome was amplified, directly from tick samples by RT-PCR. Lane 1: Marker, Lane 2: negative control, Lane 3: positive control, Lane 4: RT-PCR positive samples from infected ticks and Lane 5-6: RT-PCR negative samples

Thermal program for RT-PCR includes:

RT reaction	30′	50°C	(cDNA synthesis)
Denaturation	15′	95°C	
Denaturation	30″	95°C	
Annealing	30″	50°C	40 cycles
Extension	45″	72°C	
Final extension	5′	72°C	

After amplification, samples were stored either overnight at 2 to 8°C, or at -20°C for longer-term storage. Five microliter 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 were stained with ethidium bromide and were visualized on a UV transilluminator (Burt *et al*., 1998) (Fig. 2).

Statistical Analysis

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

RESULTS AND DISCUSSION

CCHFV was detected in 10(11.3%) of 88 ticks tested. 10(16.4%) of 61 Ixodidae family samples were RT-PCR positive whereas none of 17 Argasidae family (*Ornithodoros lahorensis*) showed signs of virus contamination. Six of 43(13.9%) *Hyalomma* sp., 3 of 23 (13%) *Rhipicephalus* sp. and 1 of 5(20%) of *Haemaphysalis* sp. were infected. 8(16%) of 50 ticks from mountainous region and 2(5.26%) of 38 ticks from plateau regions were RT-PCR positive (Table 1).

Serological evidence for CCHF virus infection was present in 15(27.8%) of 54 sheep samples. 9(29%) of 31 female sheep sera and 6 (26.1%) of 23 males were IgG positive. Most of the seropositive sheep were ≥ 4 years old which 5 (31.2%) of 16 sheep were infected. Seven (26.9%) of 26 and 8 (28.6)

Table 1: Characteristics of ticks tested in Bahar Township, 2006 (Infected ones are all RT-PCR positives) (n = 88)

Parameters	No. tested (% of total)	No. infected (% per group)
Genus and species		
Hyalomma dromedarii	14(15.9)	2(14.2)
Hyalomma margintum	14(15.9)	3(21.4)
Hyalomma anatolicum	10(11.36)	1(10)
Hyalomma detritum	5(5.68)	0(0)
Rhipicephalus bursa	13(14.77)	1(7.6)
Rhipicephalus sanguineus	9(10.23)	2(22.2)
Rhipicephalus turanicus	1(1.14)	0(0)
Haemaphysalis concina	2(2.27)	0(0)
Haemaphysalis pncntata	1(1.14)	1(100)
Haemaphysalis sulcata	2(2.27)	0(0)
Ornithodoros lahoreusis	17(19.32)	0(0)
Living environment		
Mountainous regions	50(56.8)	8(16)
Plateau regions	38(43.2)	2(5.3)

Table 2: Characteristics of sheep tested in Bahar Township, 2006 (Infected ones are all ELISA IgG positives) (n = 54)

Parameters	No. tested (% of total)	No. infected (% per group)
Age groups		
0-1 years	4(7.4)	1(25)
1-2 years	15(27.8)	4(26.7)
2 -3 years	19(35.2)	5(26.3)
≥3 years	16(29.6)	5(31.2)
Living environment		
Mountainous regions	26(48.2)	7(26.9)
Plateau regions	28(81.8)	8(28.6)
Sex		
Male	23(42.6)	6(26.1)
Female	31(57.4)	9(29)

of 28 sheep samples collected from mountainous and plateau regions were positive, respectively. But the difference was not statistically significant between different age groups (Table 2).

The human study group included 21 high risk men in close contact with animals. The mean age was 37.38 ± 19.41 years (median = 35 years, range = 14-80 years). None of these human samples were positive for IgG CCHF virus antibodies.

Findings in this study for sheep seroprevalence and RT-PCR positive ticks provide ample evidence for the presence of CCHF virus in Bahar Township.

Although *Hyalomma* sp. ticks are considered the most important in the epidemiology of CCHF as main vectors and reservoir of the virus, the virus has been reported in other 7 genera of hard ticks (i.e., *Rhipicephalus*, *Haemaphysalis*, *Dermacentor* and *Ixodes* sp.) and also in 2 species of soft ticks (*Argas persicus* and *Ornithodoros lahorensis*) (Logan *et al*., 1989). Sureau *et al*. (1980) isolated the CCHF virus for the first time from *Ornithodoros lahorensis* in Khorasan province in northeast of Iran. Chinikar *et al*. (2004) showed the presence of CCHFV by RT-PCR in one pool of collected *Hyalomma* tick in Zahedan city, in southeast of Iran. Shirani *et al*. (2004) in a survey on soft ticks in Chaharmahal Bakhtiari province detected 22.8% of *Ornithodoros lahorensis Neumann* to be RT-PCR positive. This survey is the first report of *Rhipicephalus* sp. and *Haemaphysalis* sp. infection with CCHFV in Iran.

In endemic areas, sheep and cattle 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*., 1990). In Iran, sheep have been 25-80% seropositive to CCHFV antibodies. In 1970, the presence of CCHFV in Iran demonstrated when antibodies to the virus in sera of 45 (45%) of 100 sheep from the Tehran abattoir detected (Chumakov and Smirnova, 1972). Chumakov and Smirnova (1972) detected CCHF antibody in 62% of sheep sera from northern area and 28% from northeastern area. Saidi *et al*. (1975) reported positive reactions in sera of 38% of sheep

mostly from near the Caspian Sea. After the new outbreaks in Iran, Chinikar *et al*. (2002) examined the sera of 607 sheep of 15 provinces during Jun 2000 to Jun 2002 and reported specific IgG antibodies to CCHFV in 32.9% of them. Of 372 local sheep in Isfahan province 78.9% were seropositive for CCHF (Darvishi *et al*., 2005). As mentioned in the results section, the seroprevalence of anti-CCHF antibodies was 27.8% and this shows that Bahar Township has a prevalence similar to other enzootic regions in Iran.

Although in different surveys in Iran and other endemic countries CCHF infection is recorded in different animals and ticks, yet in some reports human infection has not been detected in the same region (Chumakov and Smirnova, 1972; Saidi *et al*., 1975; Ergonul *et al*., 2007). This result could be related to the high rate of compliance to universal precautions, which seem to have been snfficient to give protection against CCHFV infection. In this study, since the number of human samples was small, additional and wider survey is needed.

Regardless whether CCHF virus is newly endemic or has long been established in this region, the potential exists for sporadic or clustered outbreaks of CCHF 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 livestock in this region.

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