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Serological and molecular epidemiology of Crimean-Congo hemorrhagic fever in Ghaemshahr county in Mazandaran province; Iran

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Abstract. Crimean-Congo Hemorrhagic Fever (CCHF) is a tick-borne viral hemorrhagic fever disease which is known as an endemic disease within some provinces of Iran. Ticks play an important role in transmission of the disease. As vector and reservoir, ticks transmit CCHF virus from livestock to human. The current study reports the presence of CCHFV in Ghaemshahr county of Mazandaran province, in north of Iran based on the evidences obtained from ELISA and RT- PCR. Based on our results, IgG antibodies against CCHFV were detected in 4(4.8%) out of 84 sheep sera samples. Forty sera were obtained from people who were in close contact with the examined sheep, none of which had IgG antibodies against CCHFV. Using RT-PCR, we confirmed the existence of CCHFV genome in 1.7% of hard tick samples. Sequence analysis demonstrated that CCHFV genomes isolated from ticks were 100% identical to those isolated from the corresponding livestock. This study confirms the presence of the virus in this region; so people in close contact with livestock and health care workers should be alerted.

INTRODUCTION

Crimean-Congo Hemorrhagic Fever (CCHF) is a viral hemorrhagic fever with up to 50% fatality rate in human beings (Morikawa *et al*., 2007). Known as an endemic disease in many countries in Africa, Europe, and Asia, the outbreak of CCHF was reported in Kosovo, Albania, Iran, Pakistan, and South Africa, so far (Gear *et al*., 1982; Van Eeden *et al*., 1985; Swanepoel *et al*., 1987; Ergonul, 2006; Chinikar *et al*., 2008).The CCHF virus (CCHFV), belongs to the Bunyaviridae family and *Nairovirus* genus (Donets *et al*., 1977) which transmits to human beings through the bite of ixodid and argasid ticks (Telmadarraiy *et al*., 2010) or by direct contact with blood or tissues from infected livestock (Camicas *et al*., 1993). Statistically, the highest infection rate in Iran occurs in people who are in close contact with the blood and tissues of livestock infected with CCHFV (Chinikar *et al*., 2004). In addition to zoonotic transmission cycle, CCHFV can be spread from a person to person and is one of the hemorrhagic fever viruses causing nosocomial outbreaks in hospitals (Van Eeden *et al*., 1985; Garcia *et al*., 2006). The virus-harboring ticks require a vertebrate host to provide blood meals (Logan *et al*., 1989; Shepherd *et al*., 1991; Gonzalez *et al*., 1992), including a variety of livestock (e.g.,

sheep, goat, cattle, and ostriches), large wild herbivores, hares, and hedgehogs that all can be infected with CCHFV. In contrast to human, infection in animal hosts generally result in unapparent or sub-clinical symptoms (Swanepoel *et al*., 1987), however, during the viremic period the livestock will be able for transmitting CCHFV to humans (Hoogstraal, 1979).

Due to an outbreak in Chaharmahal-va-Bakhtiari in 1999, CCHF was recognized as a major public health problem in Iran (Chinikar *et al*., 2008). Mazandaran province, the location of the present study, is neighbored by Semnan, Golestan and Gilan provinces where some human CCHF cases have been reported (Chinikar *et al*., 2008). To control and prevent the disease in high risk areas, the transmission and spread of CCHFV in these areas should be monitored through epidemiological surveys. The aim of this study was to determine the presence of CCHFV in ticks and their host animals as well as human sera in Ghaemshahr county of Mazandaran province in Iran.

MATERIALS AND METHODS

Study Area: Mazandaran is located in the northern part of Iran. Ghaemshahr is one of five largest counties of the province, with stockbreeding profession in rural area seriously participating in the economy of country. Ghaemshahr experience Hyrcanian climate as a moderate, subtropical weather with an average temperature of 25°C in summer and about 8°C in winter, which is suitable for farming activities and animal husbandry. The study area is comprised of two regions differing in topography: the mountainous and plateau regions. The former is an important sheep-raising area.

Sample collection: The survey was carried out from the summer of 2008 to the winter of 2009. The livestock, tick and human samples were collected from 4 villages in Ghaemshahr; most of the villages in our study were located in the mountainous region. Eighty four indigenous sheep and 40 human serum samples were collected using convenience sampling. Sheep and human blood samples were transferred to a local laboratory, and sera were separated by centrifugation at 1500 rpm for 10 min then frozen until use. The frozen sera were transferred to the arboviruses and viral hemorrhagic fevers laboratory (National Reference Laboratory) at the Pasteur Institute of Iran for IgG ELISA tests. The collected ticks were identified using taxonomical key (Walker *et al*., 2003). The identified ticks were then stored at -70°C until molecular investigation.

Serological Assay: For IgG detection, ELISA plates were coated with mouse hyper immune ascitic fluid (HMAF) diluted in 1X PBS and incubated overnight at 4°C. The corresponding antigen produced at arboviruses and viral hemorrhagic fevers laboratory (National Ref. Lab) was diluted in PBSTM and added to the plates. The plates were incubated for 3 hours at 37°C. Then the serum samples diluted in PBSTM were added, and the plates were incubated for 1 hour at 37°C. After adding the Peroxydase-labeled anti-human or animal immunoglobulin diluted in PBSTM, the plates were incubated for 1 hour at 37°C. The plates were then washed 3 times with phosphate-buffered saline containing 0.5% Tween (PBST), which also had been used for washing the plates after each of the incubation periods. Finally, hydrogen peroxide and 3, 3', 5, 5' tetra methyl Benzedrine (TMB) were added, and the plates were incubated for 15 minutes at room temperature. The enzymatic reaction was stopped by the addition of sulfuric acid (4N). The plates were read by an ELISA reader at 450nm. Taken together, an IgG-positive serum sample was considered a positive control and a negative serum was considered a negative control in the IgG ELISA (Swanepoel *et al*., 1987; Garcia *et al*., 2006).

Molecular Assay: Viral RNA was extracted from 140 µl of serum or phenol-extracted tick suspensions using the QIAamp Viral RNA Kit according to the manufacturer's instructions (QIAgen GmbH, Hilden, Germany). The extracted viral RNA was subsequently

analyzed by gel-based and qualitative real-time RT-PCR with the one-step RT-PCR kit (QIAgen GmbH, Hilden, Germany) and the use of specific primers: F2 5'–TGGACACCTTCACAAACTC–3' and R3 5'–GACAATTCCCTACACC–3'. These primers amplified a 536-bp fragment of the S segment of the CCHFV genome. The PCR reaction was done in a total volume of 50µl for 30 minutes at 50ºC, 15 minutes at 95ºC, and 40 cycles including 30 seconds at 95ºC, 30 seconds at 50ºC, 45 seconds at 72ºC, and finally, 10 minutes at 72ºC as a final extension. The CCHFV genome was used as the positive control and no template control (NTC) were used as the negative controls in both RT-PCR techniques (Chinikar *et al*., 2004; Chinikar *et al*., 2008).

RESULTS

At RT-PCR, the used primers amplified a 536-bp fragment of the S segment of CCHFV genome detected in the examined tick (Figure 1). The CCHFV was detected in 1(1.7%) of 58 examined ticks. All ticks were belonged to *Rhipicephalus sanguineus* species, most prevalent tick species in Ghaemshahr county. One (4.3%) out of 23 ticks were RT-PCR positive in plateau regions, whereas none of the ticks collected from the mountainous regions were RT-PCR positive (Table 1). Based on the serological evidence, 4(4.8%) out of 84 sheep samples were affected to the disease; while no CCHFV was detected in forty human serum samples. Totally, 3 (6.7%) out of 43 sheep sera samples collected from mountainous area and 1 (2.4%) out of 41 samples in plateau regions were IgG positive for the disease (Table 2). The sequence analysis of the isolated virus genome from tick *R. sanguineus* and sheep serum showed 100% homogeneity with each other. The data of this study imply the presence of CCHFV in Ghaemshar county of Mazandaran province.

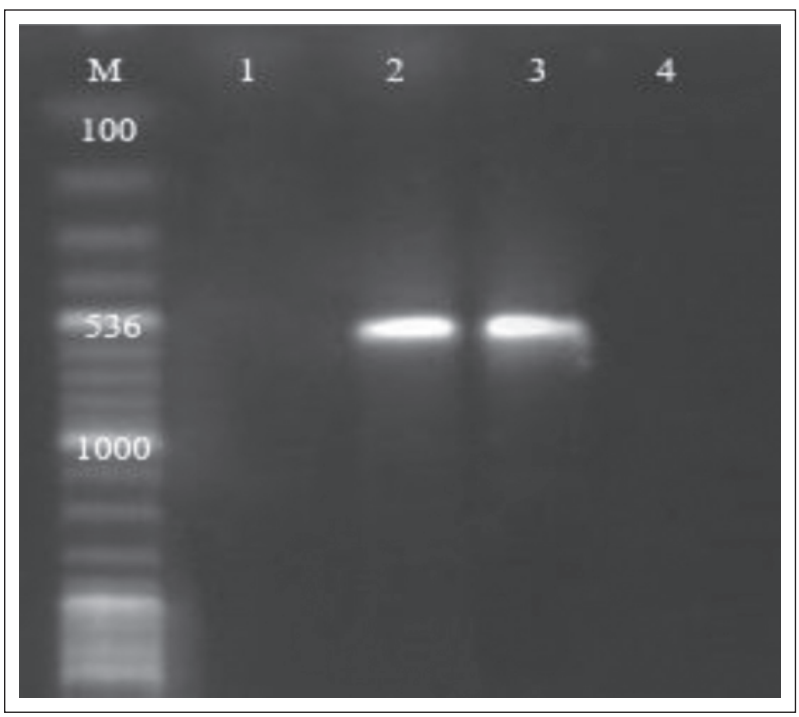

Figure 1. Amplification of S segment of CCHF virus genome in ticks sample using RT-PCR in Mazandaran province. Lane 1: negative control, lane 2: positive control (536bp), lane 3: RT-PCR positive sample from infected tick (536bp) and lane 4: negative sample. M:100 bp size marker.

Table 1. Characteristics of molecular detection of CCHFV in ticks by RT-PCR in Ghaemshahr county, 2008-2009

	Positive / total (%)		
	Female	Male	Total
Tick species			
Rhipicephalus sanguineus	1/37 (2.7)	0/21(0)	1/58 (1.7)
Living environment			
Mountainous region	0/23 (0)	0/12 (0)	0/35 (0)
Plateau regions	1/14 (7.1)	0/9 (0)	1/23 (4.3)
Host age groups			
<2	0/13 (0)	0/12 (0)	0/25 (0)
2-3	0/8 (0)	0/2 (0)	0/10 (0)
3-4	1/5 (20)	0/4 (0)	1/9 (11.1)
> 4	0/11 (0)	0/3 (0)	0/14 (0)

Table 2. Characteristics of livestock and human tested in Ghaemshahr county, 2008-2009 (Infected ones are all IgG ELISA positive)

	Positive / total (%)		
	Female	Male	Total
Sheep age groups			
<2	0/16 (0)	1/13 (7.7)	1/29 (3.4)
2-3	1/15 (6.7)	0/0 (0)	1/15 (6.7)
3-4	0/15 (0)	0/3 (0)	0/18 (0)
> 4	2/21 (9.5)	0/1 (0)	2/22 (9.5)
Living environment			
Mountainous region	2/30 (6.7)	1/13 (7.7)	3/43 (6.7)
Plateau regions	1/37 (2.7)	0/4 (0)	1/41 (2.4)
Human			
< 20 years	0/2 (0)	0/3 (0)	0/5 (0)
20-30 years	0/3 (0)	0/4 (0)	0/7 (0)
30-40 years	0/5 (0)	0/3 (0)	0/8 (0)
40-50 years	0/4 (0)	0/0 (20)	0/9 (0)
50-60 years	0/4 (0)	0/0 (50)	0/6 (0)
> 60 years	0/4 (0)	0/1 (0)	0/5 (0)

DISCUSSIONS

Our molecular assays using RT-PCR detected CCHFV in 1.7% of *Rhipicephalus sanguineus*, as the most prevalent tick species in Ghaemshahr. In our previous faunistic study we determined the prevalence of different tick species; including six species belonging to four genera as follows: *Rhipicephalus sanguineus*, *R. bursa*, *Ixodes ricinus*, *Boophilus annulatus*, *Haemaphysalis punctata*, and *Ha. erinacei* with *R. sanguineus* 82.35% of all as the most prevalent tick species in Ghaemshahr (Hosseini-Vasoukolaei *et al.*, 2010).

The comparison between sequences of the isolated virus genome from tick *R. sanguineus* and sheep showed 100%

homogeneity. This verifies the transmission cycle of the virus between ticks and livestock, so understanding of this cycle is crucial for controlling the transmission of the virus to humans in the county. Similar to our previous study in Bahar township of Hamadan province in west of Iran, *R. sanguineus* infected with CCHFV was also found in Ghaemshahr county (Telmadarraiy *et al*., 2008).

It appears that in the endemic and high risk areas of CCHFV diseases, sheep and cattle antibodies against CCHFV maybe one of the best indicators detecting the rate of risk for humans. Moreover, sheep are the most domesticated animals due to their abundance and proximity to humans (Gonzalez *et al*., 1992). In Iran, 25-80% of sheep have IgG antibodies against CCHFV. In 1970s, the presence of CCHFV in Iran was confirmed when antibodies against the virus were detected in the sera of 45% of 100% examined sheep (Chumakov, 1972). In addition, CCHFV-specific antibodies were detected in 62% and 28% of sheep sera collected from the northern and northeastern areas of Iran, respectively (Chumakov, 1972). A study reported positive reactions in the sera of 38% of the sheep found mostly near the Caspian Sea (Saidi *et al*., 1975). After the new outbreaks in Iran, 607 sheep sera from 15 provinces were examined and reported that 32.9% of the samples were IgG positive against CCHFV (Chinikar *et al*., 2002). Of the 372 sheep sera collected from Isfahan province, 78.9% were seropositive for CCHFV (Darvishi *et al*., 2005). A similar study detected CCHFV-specific antibodies in 27.8% of the sheep sera from Bahar township in Hamadan province (Telmadarraiy *et al*., 2008). As mentioned in the results, high prevalence of seropositive infected livestock and ticks point to high risk to public health in this province. Therefore, a prevention and control program should be planned and executed for this province. According to these findings, a sporadic or clustered outbreak potential of CCHF may exist and threaten this province. Moreover, continuous 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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