

## Original Article

### Crimean-Congo Hemorrhagic Fever in the One-Humped Camel (*Camelus dromedarius*) in East and Northeast of Iran

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#### Abstract

**Background:** This comprehensive study was conducted on multi-purpose one-humped camel (*Camelus dromedarius*) sera and ticks to assess the epidemiological aspects of the Crimean-Congo hemorrhagic fever virus (CCHFV) in northeast Iran.

**Methods:** From May 2012 to January 2013, eleven cities were randomly selected in the Khorasan Provinces of Iran as “clusters,” and at least 14 one-humped camels were sampled from each area. Reverse transcriptase polymerase chain reaction was used for the detection of the CCHFV genome in ticks. Sera were analyzed using specific enzyme-linked immunosorbent assay tests.

**Results:** Four hundred and eighty ixodid ticks were collected, and the genome of the CCHFV was detected in 49 (10.2%) out of 480 ticks. The CCHFV genome was detected in two out of four tick species, and in tick samples from three cities in Khorassan-e-Jonoobi. All three provinces, and six out of eleven cities, were CCHFV-specific IgG-positive. In total, nine (5.3%) out of 170 one-humped camels were IgG-positive. The highest rate of IgG-positive samples was found in Nehbandan (16.67%).

**Conclusion:** Continued surveillance and strictly enforced importation and quarantine practices should be implemented to prevent human exposure and the on-going dispersal of infected ticks and livestock in these regions. It is recommended that acaricides be used to prevent CCHF transmission to humans, and to reduce the tick population. In addition, care should be taken by abattoirs workers and people who work with one-humped camels.

**Keywords:** Epidemiology, Survey, CCHFV, ELISA, RT-PCR, Iran

#### Introduction

“Crimean-Congo hemorrhagic fever virus belongs to the genus *Nairovirus* in the Bunyaviridae family, and is a human pathogen that can cause a severe, and often fatal, hemorrhagic fever.” (Ergonul 2006). CCHF virus is the most geographically widespread tick-borne virus of medical importance (Ergonul 2006). The disease is endemic in large areas of sub-Saharan Africa and the Middle and Far East, as well as in Eastern Europe. A significant increase of cases in countries such as Kosovo, Albania, Turkey, Iran, and Greece has recently been observed

(Alavi-Naini et al. 2006). The CCHFV genome has been isolated from at least 31 different tick species in the Ixodidae (hard ticks) and Argasidae (soft ticks).

*Hyalomma* spp ticks are considered the most important in the epidemiology of CCHFV; however, the virus has also been isolated from ticks of other genera (i.e., *Rhipicephalus*, *Boophilus*, *Dermacentor*, *Haemaphysalis*, and *Ixodes* spp, (Saijo et al. 2002, Tahmasebi et al. 2010). Infected animals are, however, asymptomatic (Papa et al. 2009). Humans in high-risk occupations

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(e.g., slaughterhouse workers, shepherds, health care workers, and veterinarians) are prone to CCHF infection (Garcia et al. 2006).

This study was performed to ascertain the prevalence of CCHFV in ticks present on the one-humped camel (*Camelus dromedarius*), and to estimate the prevalence of CCHFV IgG in the one-humped camel's sera in three provinces: Khorassan-e-Shomali, Khorassan-e-Razavi, and Khorassan-e-Jonoobi of Iran.

## Materials and Methods

### Study area

The study was conducted in three provinces: Khorassan-e-Shomali, Khorassan-e-Razavi, and Khorassan-e-Jonoobi located at 55°17', 61°15' E and 30°24', 38°17' N in northeastern and east of Iran (Fig. 1). Khorassan-e-Shomali is a mountainous region, with temperate, cold weather. Khorassan-e-Razavi is a semi-desert region that has mild weather. Khorassan-e-Jonoobi is a semi-desert region that experiences arid conditions. The average annual rainfall is approximately 300–400 mm in the northern areas (Khorassan-e-Shomali) and 150 mm in the central and southern areas (Khorassan-e-Razavi and Khorassan-e-Jonoobi). There are approximately 25 million camels in the world, and nearly 150,000 one-humped camels are in Iran, this is 0.6% of the world camel population, and 3.8% of the Asian camel population (FAO 2011). The majority of Iran's camels are dromedaries, and they are scattered across the country's provinces. In the Khorassan Provinces the camel population is 37,400 (Ministry of Agriculture Jihad, 2002), but in the authors' experience at present the actual number is several times greater, the majority of these are in Khorassan-e-Shomali and Khorassan-e-Jonoobi.

### Sampling

From May 2012 to January 2013, eleven cities and towns were randomly selected from

three provinces: Khorassan-e-Shomali, Khorassan-e-Razavi and Khorassan-e-Jonoobi as “clusters,” and at least 14 one-humped camels were sampled from each cluster. From each animal, two or three ticks were collected and placed in separate sterile tubes; the tubes were labeled with the date of collection, animal number, sex, age, and area. Collected ticks were sent to the laboratory and identified under a stereo microscope using general identification keys (Hoogstraal 1979, Apanaskevich and Filippova 2007). The samples were then pooled according to the area, sex and species of tick (each pool routinely contained 1–8 ticks, but occasionally the number was greater) and immediately sent to the Arboviruses and Viral Hemorrhagic Fevers Laboratory (National Reference Laboratory), Pasteur Institute of Iran, and stored at  $-70^{\circ}\text{C}$  until analysis. For the serum assay, 20 mL of blood collected from the jugular vein of each camel then the sample was labeled with the date of collection, animal number, sex, age, and area. The samples were immediately sent to a laboratory and centrifuged at 5000 rpm for 10 min. The sera were then separated and transferred into holding tubes and sent to the Arboviruses and Viral Hemorrhagic Fevers Laboratory and stored at  $-70^{\circ}\text{C}$  until analysis.

### Tick sample size calculation

According to previous studies in animals, tick infectious was 10% and with assumption 5% accuracy, 95% confidence interval and design effect equal 1.5 we need at least 207 ticks.

### Serum sample size calculation

With assumption 20% seropositivity in the studied animals, 8% accuracy, 95% degree of confidence, and design effect equal 1.5, the sample size will be 144, each cluster has at least 14 sera.

### Molecular detection

Reverse transcriptase polymerase chain reaction: Ticks were individually washed twice by PBS (PBS, pH 7.4) and crushed with a mortar and pestle in 200-300µl of PBS. Total RNA was extracted using an RNeasy mini kit (QIAGEN, Cat No. 2215716) according to the manufacturer's instructions. The extracted viral RNA was stored at -70 °C until analysis. For the RT-PCR, a master mix was prepared as follows: 28 µl of RNase free water, 10 µl of buffer (5 x conc), 2 µl of dNTP mixture, 2µl of enzyme mixture containing reverse transcriptase and Taq DNA polymerase enzymes, 1µl of primer F2 (5'-TGGACACCTTCACAACTC-3'), 1µl of Primer R3 (5'-GACAATTCCTACACC-3'), 1µl of RNase inhibitor and 5µl of extracted viral RNA as template. The F2 and R3 primers amplify a 536 bp fragment inside the S-segment of the CCHFV genome, 536 bp fragment is RT-PCR target. The thermal cycling program for the RT-PCR, included 30 min at 50 °C for reverse transcription reaction (cDNA synthesis), 15 min at 95 °C for activation of Hot Star Taq DNA polymerase and inactivation of reverse transcriptase, followed by 35 cycles of 95 °C for 30 sec, 50 °C for 30 sec, 72 °C for 45 sec, and a final extension at 72 °C for 5 min. For gel-based RT-PCR product analysis, 5µl of the PCR product was mixed with 1µl loading buffer (6 x conc). Then, the mixture was load in agarose gel 1.5%, and visualized with ethidium bromide (Chinikar et al. 2004, Chinikar et al. 2010).

### IgG-sandwich ELISA

For IgG antibody detection, the ELISA plates were coated overnight at 4 °C with mouse hyperimmune ascitic fluid diluted at 1:1000 in 0.05% Tween 20-PBS containing 5% skim milk as a saturating reagent. This solution was used to dilute antigen and sera. The native or the recombinant antigen (produced in our laboratory) diluted in PBSTM

(PBS containing 0.05% Tween and 3% skim milk) was added to the plates and the plates were incubated for 1h at 37 °C and extensively washed. Serum samples diluted in PBSTM were added, and the plates were incubated for 1h at 37 °C. After washing, the peroxidase-labeled anti-human or 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 3 times with PBS containing 0.5% Tween (PBST). Finally, hydrogen peroxide and tetramethylbenzidine (TMB) were added and the plates were incubated for 15 min at room temperature. The enzymatic reaction was stopped by the addition of sulphuric acid (4N) and the plates read by ELISA reader (Anathos 2020) at 450 and 620 nm. Taken together, an IgG-positive serum was considered as positive control and a negative serum taken as negative control in the IgG ELISA (Garcia et al. 2006, Duh et al. 2008, Chinikar et al. 2010).

### Statistical analysis

Data were analyzed using IBM/ SPSS version 20.0 statistics package. Descriptive statistics (i.e. prevalence and percentages) were used to summarize the quantitative variables. Location of noted research is shown on the map (Fig. 1).

### Results

A total of 200 one-humped multi-purpose camels (rearing for milk, meat, riding and offspring) were examined. Tick infestation was observed in 170 of them, and 480 ixodid ticks (133 females and 347 males) were collected from different regions in the Khorassan-e-Shomali, Khorassan-e-Razavi and Khorassan-e-Jonoobi (Table 1).

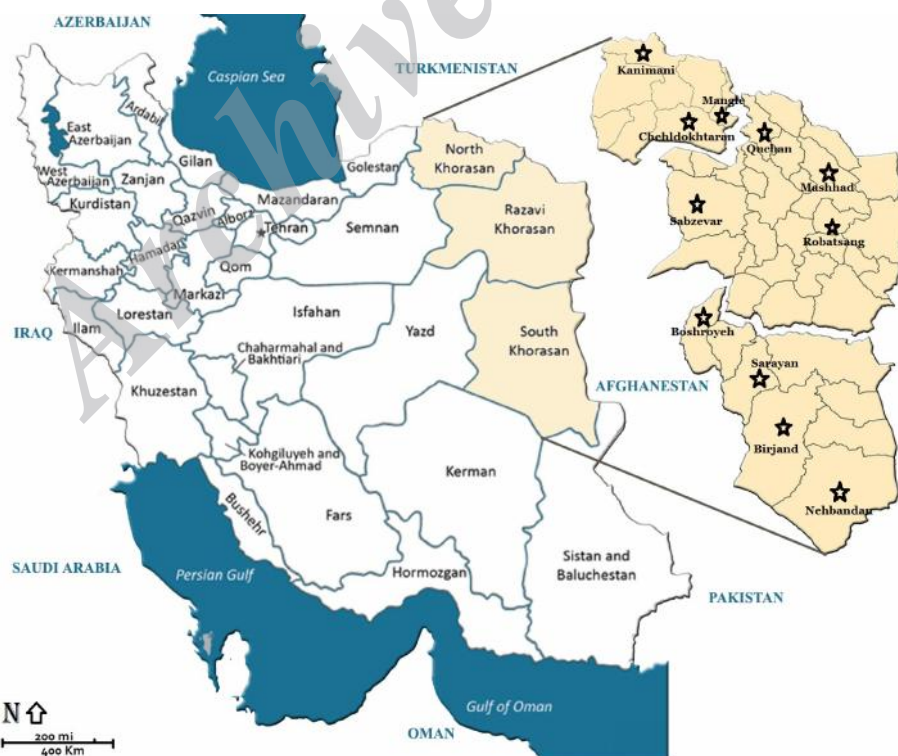
In the current study, only four species of *Hyalomma* genus were observed. Population frequency of *H. dromedarii* (90.7%) was

higher than others and *H. asiaticum* had the lowest frequency (0.4%). *Hyalomma marginatum* comprised about 2.9% and *H. anatolicum* accounted for 6% of total collected species. *H. dromedarii* is the most dominant tick species of camel in the Khorassan region and a one humped camel is a suitable host. The life cycle of this tick includes one, two, or three hosts. Immature ticks feed on small or large mammals, depending on their life cycle. *Hyalomma dromedarii*, *H. anatolicum* and *H. marginatum* were collected from all three provinces but in contrast, *H. asiaticum* only was collected from Khorassan-e-Razavi's one-humped camels (Table 2).

The CCHFV genome was found in 49 (10.2%) of 480 ticks, and three (6%) of 50 pools. Therefore, the tick prevalence was 10.2%. All of the CCHFV-positive ticks were male. The CCHFV genome was detected in two out of four tick species, and of these, 42 (85.7%) belonged to *H. dromedarii* and 7

(14.3%) belonged to *H. anatolicum* (Table 2). The viral genome was detected in tick samples from three cities in Khorassan-e-Jonoobi. The positivity rates were as follows: Boshroyeh, 25 out of 480 (51%), Birjand, 17 out of 480 (34.7%), and Nehbandan, 7 out of 480 (14.3%, Table 1).

Sera from 170 one-humped camels were collected. All three provinces, and six out of eleven cities and towns, were IgG-positive for CCHFV. Nine (5.3%) out of 170 camels were IgG-positive, this means that the IgG prevalence was 5.3%. The positivity rates for the provinces varied, and were as follows: Boshroyeh, 12.5%, Kanimani, 7.14 %, Birjand, 8%, Nehbandan, 16.67%, Chehl dokhtaran, 7.14%, and Sabzevar, 6.66%. The highest rate of IgG-positive samples was found in Nehbandan (16.67%, two out of 12 sera), Khorassan-e-Jonoobi. Eight out of the nine positive samples were collected from female camels (Table 3).



**Fig. 1.** Study area in Khorassan-e-Shomali, Khorassan-e-Razavi and Khorassan-e-Jonoobi are shown with asterisks

**Table 1.** Positivity rates, number, and sex of ticks collected from one-humped camels from Khorassan-e-Shomali, Khorassan-e-Razavi and Khorassan-e-Jonoobi in East and Northeast Iran

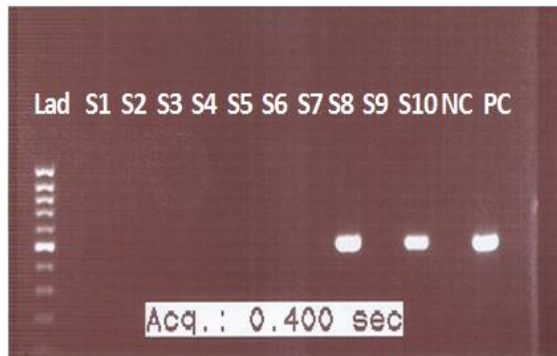
Area	No Males	No Females	Total	RT- PCR Positive
Nehbandan	27	9	36	7
Sarayan	28	23	51	0
Birjand	41	7	48	17
Kanimani	26	24	50	0
Boshroyeh	52	7	59	25
Robatsang	32	0	32	0
Quchan	28	13	41	0
Sabzevar	18	13	31	0
Mashhad	31	17	48	0
Chehl dokhtaran	28	6	34	0
Mangale	36	14	50	0
<b>Total</b>	<b>347</b>	<b>133</b>	<b>480</b>	<b>49</b>

**Table 2.** The sex, species and CCHF-positive rate of ticks infesting one-humped camels

Tick spp	Male	Female	Total	CCHF Positive
<i>H. dromedarii</i>	307	128	435	42
<i>H. marginatum</i>	10	4	14	0
<i>H. anatolicum</i>	29	0	29	7
<i>H. asiaticum</i>	1	1	2	0
<b>Total</b>	<b>347</b>	<b>133</b>	<b>480</b>	<b>49</b>

**Table 3.** IgG antibody-positive sera collected from one-humped camels from Khorassan-e-Shomali, Khorassan-e-Razavi and Khorassan-e-Jonoobi

Area	No. sera	No. IgG Positive	Local Positivity
Nehbandan	12	2	16.67
Sarayan	13	0	0
Birjand	25	2	8
Kanimani	14	1	7.14
Boshroyeh	16	2	12.5
Robatsang	16	0	0
Quchan	14	0	0
Sabzevar	15	1	6.66
Mashhad	16	0	0
Chehl dokhtaran	14	1	7.14
Mangale	15	0	0



**Fig. 2.** Amplification of the S segment of the CCHFV genome using RT-PCR, in tick samples from the Khorasan province. (PC: Positive Control, NC: Negative Control, S1-10: Samples, S1, S2, S3, S4, S5, S6, S7 and S9 are negative, S8 and S10 are positive)

## Discussion

In this study, *H. dromedarii* was the most dominant species of tick on one-humped camels, and this is in agreement with the results obtained by Salimabadi (2010) in Iran and Lawal et al. (2007) in Nigeria. Crimean-Congo hemorrhagic fever infection was detected in 49 (10.2%) of 480 tick samples and this is a higher percentage than that previously reported by Salim-Abadi (3.79%) in the Yazd Provinces of Iran (2011).

All the positive samples were obtained from *Hyalomma* spp. *Hyalomma* ticks are the primary vectors for the transmission of CCHFV throughout Europe, Asia, the Middle East, and Africa (Ergonul 2006). Although *Hyalomma* ticks are considered the most important vector and reservoir for the CCHFV, the virus has also been reported in other tick genera (Tahmasebi et al. 2010). The CCHFV genome was detected in two out of the four tick species that were collected (*H. dromedarii*, 85.7% and *H. anatolicum*, 14.3%). This result is similar to that found by Salim-Abadi et al. (2011), therefore, these results suggest that *H. dromedarii* and *H. anatolicum* act as vectors for CCHFV in one-humped camels.

*Hyalomma dromedarii* is distributed throughout the world wherever camels are present, and *H. anatolicum* is widely distributed throughout Iran. *Hyalomma anatolicum* transmits at least five *Arboviruses*, and is a significant vector of CCHFV to humans (Nabian and Rahbari 2008).

Telmadarraiy et al. (2010) detected the CCHFV genome in *Rhipicephalus bursa* (in one of the three ticks that were sampled), but we failed to discover *R. bursa* in our study. In the present study, we only found the CCHFV genome in one-humped camel ticks in Khorasan-e-Jonoobi (Birjand, Boshroyeh, and Nehbandan). This province has an unusual geographical location, as it borders Afghanistan to the east, the Sistan-va-Baluchistan Province of Iran in the south, and the Khorasan-e-Razavi Province of Iran in the north. Since 2000, the disease has infected 23 out of 31 provinces in Iran: Sistan-va-Baluchistan (283 confirmed human cases), Isfahan (44 confirmed cases), Fars (26 confirmed cases), Tehran (17 confirmed cases), and Khorasan (12 confirmed cases) (Chinikar et al. 2012). Notably, Sistan-va-Baluchistan Province (south of Khorasan-e-Jonoobi) has not just had the highest number of CCHFV cases, but CCHF infections have been observed in this area since 2000 (Chinikar et al. 2012), as it shares a border with two CCHF-endemic countries: Pakistan and Afghanistan (Chinikar et al. 2010). The unusual location of Khorasan-e-Jonoobi, which is connected in the north, south, and east to heavily infected or endemic areas of CCHF, may explain why it was the only CCHFV-positive area found in the study. We could not ascertain why all the reverse transcriptase polymerase chain reaction-positive ticks were male, but this may have been due to the presence of more males than females on the animals, and also in our samples (347 males vs 133 females).

During the last decade, an increasing number of human CCHFV infections have

been reported in various regions of Iran (Chinikar et al. 2008). IgG-positive serum samples collected from sheep, goats, cows, and humans have been frequently reported in different parts of the country (Saidi et al. 1975, Chinikar et al. 2005, Moradi et al. 2008). We found CCHFV IgG antibodies in one-humped camel sera in all three provinces studied, and these results, as well as those from a previous study by Chinikar et al. (2012), may indicate that CCHF is endemic to these regions, or that it has spread from neighboring countries. In total, nine (5.3%) out of 170 camels were IgG positive. This finding is not in accordance with the results obtained by Saidi et al. (1975), who found that CCHF samples were negative in all 157 camels that they had studied, from south and southeast Iran. However, Williams et al. (2000) found that 17 (16%) out of 109 camels sampled in Oman were tested positive for the CCHF IgG antibody.

Sistan-va-Baluchistan has been the most CCHFV-infected province in Iran since 2000, because, as mentioned previously, it shares a border with two CCHF-endemic countries, Pakistan and Afghanistan (Izadi et al. 2006, Chinikar et al. 2012). Khorasan, which is connected to heavily infected or endemic areas of CCHF, shares a large border area with neighboring countries, shares common pasture with herds from CCHF-endemic areas, and experiences the illegal importation of animals across the border, which may explain why it is a CCHF-positive area.

Another important issue is the presence of disease reservoirs and vectors, such as *Hyalomma* spp., in this area. The CCHFV genome has been isolated from at least 31 different tick species in Ixodidae (hard ticks) and Argasidae (soft ticks) (Saijo et al. 2002, Tahmasebi et al. 2010). As Champour (2013) noted, the main tick species that affect one-humped camels in this region are *H. dromedarii*, *H. anatolicum*, *H. marginatum*, and *H. asiaticum*. *Hyalomma* spp ticks are

considered the most important species in the epidemiology of CCHFV in camels in this area (Hoogstraal 1979). Six out of the eleven cities and towns studied yielded one-humped camel serum that was IgG-positive for CCHF, and it has been shown that CCHF is distributed across these three provinces.

We could not ascertain why almost all of the positive-IgG sera samples were collected from female camels (8/9), but this may have been due to the presence of more females than males in the samples (136 females vs 34 males), possibly because female camels remained in herds longer than did males at the time of sampling.

Our results reveal a lower prevalence of seropositivity in one-humped camels (5.3%) than in other domestic animals (30%) in Iran (Chinikar et al. 2009). However, due to the fact that camels remain in herds for longer than do other animals (Champour et al. 2013), and because camel pasture is very widely geographically distributed, this small percentage has a significant effect on the epidemiology of CCHF. The importance of camels in the epidemiology of CCHF in Russia and Astrakhan Oblast has been previously reported by Kurbanov, Berezin, and Chumakov (Steele 1994).

## Conclusion

Because CCHF is a serious threat to Iran, imported animals, particularly one-humped camels that carry a large number of ticks, should be inspected and treated carefully. Continued surveillance and strictly enforced importation and quarantine practices will be required to prevent human exposure and the on-going dispersal of infected ticks and livestock in these regions. The use of commercially available insect repellent, and the use of clothing impregnated with permethrin, can give some protection against tick bites (Telmadarraiy et al. 2010, Salim-Abadi et al.

2011). Further surveys of human and animal populations, particularly of those animals imported from neighboring countries to these regions, are recommended, in order to provide a better understanding of the distribution and epidemiology of the virus in these provinces.

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