ORIGINAL ARTICLE

Hard Ticks Infesting Domestic Ruminants, Species Composition and Infection with Crimean-Congo Hemorrhagic Fever Virus in a Highland Province, SW Iran

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Abstract

Background: Crimean-Congo hemorrhagic fever (CCHF) is a neglected tick-borne viral zoonotic disease. The aim was to detect CCHF virus (CCHFV) among wild ticks from Artiodactyla, *Bos taurus, Ovis aries*, and *Capra hircus*, in a previously declared CCHFV-free province of Kohgiluyeh Boyer-Ahmad, southwest Iran.

Methods: From April to November 2015, hard ticks were collected in a cross-sectional study and checked by microscope for species identity from ungulates in 51 study villages. About 55% of the ticks were then subjected to reverse-transcription polymerase chain reaction (RT-PCR) to detect CCHFV genome.

Results: Overall, 859 hard ticks were captured, from which 8 different species in two genera were identified. The genus *Rhipicephalus* was distributed in half (#26) of the study villages. It was the most frequent (≈60%) tick genus. *Hyalomma anatolicum, H. asiaticum, H. excavatum, H. marginatum, H. scupense, Rhipicephalus sanguineus, R. turanicus,* and *R. bursa* were identified on the ruminants. From 469 adult ticks subjected to RT-PCR, one (0.2%) tick, *R. bursa*, was positive with CCHFV genome. It was from a cold hardy highland village in Dena County. It had CCHFV RNA for the first time from this region.

Conclusion: The detection of CCHF viral RNA in one hard tick species, *R. bursa*, was confirmed in the southwest of Iran, thus partially indicating CCHFV presence of ticks in this region.

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Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a neglected tick-borne viral zoonotic disease endemic in many parts of the world, including Iran. With a case fatality rate (CFR) of about 14% in Iran, the detection of CCHF virus (CCHFV) genome in vectors is of fundamental importance to incriminate them as potential reservoirs of CCHFV infection. It is mainly transmitted to man by hard tick (family: Ixodidae) bites. CCHF is caused by a Nairovirus (family: Bunyaviridae) and transmitted, in particular, by the two-host tick, *Hyalomma* genus.[1]

Ticks can have a long duration of bloodsucking on briefly viremic vertebrate hosts.[2] This potentially

lethal arboviral infection presents unique challenges to public health infrastructure, particularly in tropical areas. Despite being an enzootic infection, its human sporadic epidemics have been recorded from many regions including the Balkan, the Middle East, Indo-Pakistan subcontinent, China, southwest Europe, Bulgarian-Turkish region, and southwest Russia.[3-6] CCHFV is asymptomatically transmitted to an extensive variety of vertebrates; it is, however, life-threatening only in humans.[3] The high burden of disease in Iran and its adjacent neighbors (>5000 cases in the first decade of this century) points to the magnitude of this concern.[7] Nosocomial transmission of CCHFV has also been reported with remarkable implications.[8]

This virus can be transmitted both vertically (from one generation to the next) and horizontally (simultaneous circulation of the virus from one blood sucking infected tick stage in close proximity to another uninfected one through synchrony in zoonotic niche conditions or co-feeding on a vertebrate host) among ticks.[2] Venereal (trans-sexual), trans-ovarial (through eggs), and trans-stadial (from one molting tick blood-feeding stage to the next developmental stage) passage of virus is also common.[2] The number of routes and directions of viral transmission attests to the extensive epidemiology of CCHF.[9]

In Iran, an early viral antibody survey using agar gel diffusion precipitation test on man and several animal species, including few rodents and Chiroptera species, showed differential positivity for CCHFV.[10] The commonest route of CCHFV transmission among Iranians is percutaneous contact with viremic tissues, blood, or other infected body fluids of contagious asymptomatic domestic and wild ungulates such as cattle, sheep, goats and camels.[11] Many of these livestock are illegally imported via eastern borders, bypassing official surveillance screening. Only a few sporadic cases of direct tick bites are normally reported.[12]

The zoonotic and ecological niche of CCHF points to those people who are involved in close proximity to the livestock (cattle, sheep, goats, etc.) and a huge proportion of shrub and grass land cover.[13] It is postulated that adequate threshold densities of both suitable vertebrate hosts with transient viremia and infected *Hyalomma* ticks are indispensable to establish a persistent natural CCHFV focus.[14] Furthermore, the assumption that non-*Hyalomma* ticks cannot maintain an active focus of natural CCHFV circulation remains contentious since no sine quae non-evidence exists for the persistence of the virus in the next generation of ticks.[2]

The main aim of this investigation was to detect CCHFV RNA relics using reverse transcription polymerase chain reaction (RT-PCR) in bloodsucking ticks collected from domestic herbivores (cattle, *Bos taurus*; sheep, *Ovis aries*; and goat, *Capra hircus*) in Kohgiluyeh Boyer-Ahmad province, Iran, in 2015. To the best of the authors' knowledge, this is the first report of detecting CCHF viral RNA in hard ticks of this province in Iran.

Materials and Methods

Study Area

Kohgiluyeh Boyer-Ahmad province is located in Southwestern Iran (30°25'-31°45'N; 49°57'-50°42'E) with an area of 26416 km^2 (Figure 1). This is an almost mountainous region (at a mean altitude of 2100 m) with many caves, rivers, and forest refuges, and a rich composition of wild and domestic animals including Reptilia, Aves, Artiodactyla, Rodentia, and Carnivora. Two types of climates (subtropical and temperate) are seen in this province. About 43% of its northeast flank enjoys a temperate climate. Average annual precipitation is <600 mm/year. The temperature ranges from -20°C to 36°C in winter and summer, respectively. The mean annual relative humidity is about 33%. This province is divided into 8 counties of Boyer-Ahmad, Dena, Gachsaran, Basht, Bahmai, Choram, Dehdasht and Lendeh (Figure 1). A total of 58 villages were randomly selected for tick collection. Most (#51) of these were infested with ticks. Recently, insecticide-treated husbandry units and those which did not consent to sampling were excluded from the study. The Zagros mountain range in Dena County has a peak of 4409 m above

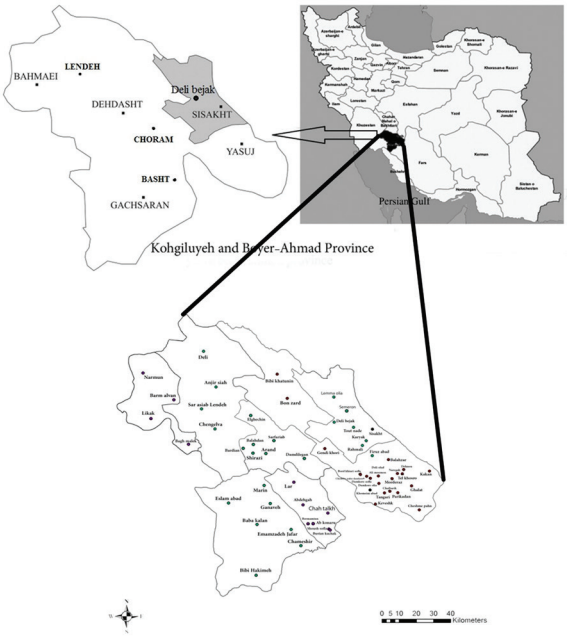

Figure 1: Map shows the location of Kohgiluyeh Boyer-Ahmad province in southwest region of Iran (top right map) and the approximate sites of eight counties (thick arrow; top left), including the county of Dena (grey area) where only one hard tick was positive with CCHFV RNA, and the distribution of 58 villages over these counties (solid black lines, bottom inset).

the sea level. This study area is dominantly covered with oak trees. Most residents of this province are involved in animal breeding husbandry and associated practices which consequently make them vulnerable to infectious diseases.

Collection and Identification of Ticks

Using finely curved-tip forceps, we promptly and safely removed the ticks from their hosts so as not to damage their mouth parts. They were gathered from domestic herbivores (cattle, *Bos taurus*; sheep, *Ovis aries*; and goat, *Capra hircus*) from April to November 2015. Each ungulate trunk was searched for the presence of tick infestations by palpation, principally on the ears and along the nape of the neck, perineum, scrotum or udder, and tail base. All ticks were handled with sterile gloves and fine forceps. They were put into labeled screw-capped vials. They were then deep frosted in a freezer at -20°C. All sampled specimens were identified by an expert acarologist to species level under a stereo-microscope and valid taxonomic keys,[15-17] and voucher specimens were registered at Entomology Museum (Shiraz School of Health, Shiraz, Iran).

RNA Extraction from Ticks

Each of the 469 adult ticks, being a random sample of the collected ticks, was separately washed twice with phosphate buffered saline (PBS; pH=7.4), and crushed with a pestle and mortar in 300µL of PBS. The tick suspension was then stored at -70°C. Viral RNA was subsequently extracted from tick suspensions, using RNAeasy Mini kit (QIAgen GmbH, Hilden, Germany) according to the instructions of the manufacturer.

RT-PCR

Reverse transcription of RNA was conducted using specific primers, which amplify a 536-bp fragment of the S-segment of the CCHF viral genome. Briefly, the extracted viral RNA was subsequently analyzed by gel-based and real time RT-PCR, using a one-step RT-PCR kit (QIAgen) with specific primers: F2 primer (5'-TGGACACCTTCACAAACTC-3'), and R3 primer (5'-GACAAATTCCCTGCACCA-3'), targeting the S-segment of viral RNA genome. The PCR reaction was done in 50µl total volume and 30 min at 50°C, 15 min at 95°C, and 40 cycles including 30s at 95°C, 30s at 50°C, 45s at 72°C, and finally 10 min at 72°C as a final extension. For real time RT-PCR, 1 µl of SYBR green dye at 1: 10,000 dilution was also used in the master mix. For gel-based analysis, 5µl of the PCR products was mixed with 1 µl loading buffer, and electrophoresis was carried out on 1.5% agarose gel in Tris-borate EDTA buffer (TBE). DNA bands were stained with ethidium bromide and visualized on a UV transilluminator.

Data Analysis

Data were analyzed using SPSS version 16 software. Descriptive statistics were used to analyze the results.

Results

A total of 859 hard ticks were collected from 51 villages in 8 counties. No hard ticks were found in 7 (12%) villages. The most (47%) and the least (4.5%) infested counties were Boyer-Ahmad and Lendeh, respectively (Table 1). Moreover, the most infested village was Lema olia in Dena County with 58 *Rhipicephalus* tick specimens. All study villages were infested with at least one and up to 5 different tick species. The latter condition occurred in Damkore sofla of Boyer-Ahmad County.

Eight different hard tick species (5 *Hyalomma* species and 3 *Rhipicephalus* species) in two distinct genera were identified. Both these genera were commonly found in Boyer-Ahmad and Basht counties. The genus *Rhipicephalus* was widely distributed in half (#26) of the mostly highland study villages of Boyer-Ahmad and Dena counties. It was also the most frequent (≈60%) tick genus. The preponderance of this genus in the cold highland villages of Boyer-Ahmad, Dena, and Basht counties was exemplary. No *Rhipicephalus* tick was found in the warm lowland counties of Gachsaran, Bahmai, Choram, Dehdasht and Lendeh. In contrast, the genus of *Hyalomma* was found in these as well as Basht County.

The second most abundant tick genus was *Hyalomma*. There were no *Hyalomma* species on ruminants in the commonly snow-covered peak villages of Dena County. Altitudinal variations appeared to influence the partial distribution of these two tick genera. Similarly, the brown-dog tick, *R. sanguineus* and the Anatolian tick, *H. anatolicum excavatum*, ranked the first and second most abundant (55%, 36%) species in this province, respectively (Figure 2). The rarest (0.2%) hard tick species, on the other hand, was *H. marginatum* found on sheep, *Ovis aries*, host.

A total of 303 ungulates were infested with each one of the eight hard tick species in this province. Of these, 20 were cattle (*Bos taurus*); 193 were sheep (*O. aries*); and 90 were goats (*Capra hircus*). Both tick genera were commonly collected from all these hoofed herbivores. The order of hard tick preponderance was directed from sheep to goats and then cattle. No species of naturally collected ticks was, however, absent from sheep. Furthermore, all species of *Rhipicephalus* were present on sheep and goats. This was not true for the cattle. Similarly, all species of *Hyalomma*, except for *H. marginatum*, were present on cattle. Unlike *Rhipicephalus*, only two species of *H. excavatum* and *H. anatolicum* were collected from goats.

The majority (52%) of ticks were found from the

Table 1: Abundance distribution of different hard tick species from livestock in different villages of Boyer Ahmad, Dena, Gachsaran, Basht, Bahmai, Choram, Dehdasht and Lendeh districts of Kohgiluye and Boyer Ahmad province, Iran

County village/Tick species	*H. excavatum*	*H. asiaticum*	*H. anatolicum*	*H. marginatum*	*H. scupense*	*R. sanguineus*	*R. bursa*	*R. turanicus*	Total
BOYER AHMAD									
Chat barik	0	0	0	0	0	31	0	0	31
Bord khiari sofla	3	0	1	0	0	45	0	1	50
Bon zard	0	0	0	0	0	10	0	0	10
Bibi khatunin	0	0	0	0	0	32	0	5	37
Firuz abad	0	0	0	0	1	0	0	0	1
Kakan	0	0	0	0	0	10	10	0	20
Keveshk	0	0	0	0	3	4	0	0	7
Nareh ghah	0	0	0	0	0	1	0	0	1
Tol khosro	0	0	0	0	0	4	0	0	4
Morderaz	13	0	1	0	0	0	0	0	14
Cheshme pahn dkr	0	0	0	0	0	18	0	0	18
Damkore sofla	1	0	0	1	1	34	5	0	42
Damkore olia	10	0	0	0	0	24	0	2	36
Deli olad	0	0	0	0	0	1	0	0	1
Parikadan	0	0	0	0	0	22	0	0	22
Dehno		0							11
Ghalat	0	0	0	0	0	2	0	0	2
Cheshme pahn	3	0	0	0	0	20	1	0	24
Tangari	7	0	0	0	0	6	0	0	13
Balahzar	0	0	0	0	0	10	0	0	10
Khomeini abad	0	0	0	0	0	44	0	4	48
subtotal	38	0	5	1	5	330	11	12	402
DENA									
Tut nade	0	0	0	0	0	8	0	0	8
Semeron	0	0	0	0	0	34	0	6	40
Karyak	0	0	0	0	0	20	0	3	23
Lema olia	0	0	0	0	0	54	0	4	58
Deli bejak	0	0	0	0	0	3	2	0	5
Rahmali	0	0	0	0	0	20	1	0	21
Sisakht	0	0	0	0	0	2	0	0	2
Subtotal	0	0	0	0	0	141	3	13	157
GACHSARAN									
Emamzade Djafar	22	0	0	0	0	0	0	0	22
Ganaveh	17	0	0	0	0	0	0	0	17
Marin	7	0	0	0	0	0	0	0	7
Eslam abad	6	0	1	0	0	0	0	0	7
Baba kalan	6	0	0	0	0	0	0	0	6
Subtotal	58	0	1	0	0	0	0	0	59
BASHT									
Chah talkh	2	7	7	0	0	0	1	0	17
Ab konaru	10	3	1	1	0	0	0	0	15
Bormamiun	13	0	1	0	0	0	0	0	14
Abdegah	20	0	0	0	0	0	0	0	20
Bustan kuchak	2	0	0	0	0	1	0	0	3
Shush sofla	38	0	0	0	0	0	0	0	38
Subtotal	87	10	8	1	0	0	1	0	107
BAHMAI									
Barm alvan	7	0	0	0	0	0	0	0	7
Narmun	5	0	0	0	0	0	0	0	5
Likak	29	0	0	0	0	0	0	0	29
Bagh malek	9	0	0	0	0	0	0	0	9
Subtotal	50	0	0	0	0	0	0	0	50
CHORAM									
Alghachin	7	0	0	0	0	0	0	0	7
Balahdan	5	0	0	0	0	0	0	0	5
Shirazi	6	0	0	0	0	0	0	0	6
Arand	21	0	0	0	0	0	0	0	21

Sarfariab	6	0	0	0	0	0	0	0	6
Subtotal	45	0	0	0	0	0	0	0	45
DEHDASH, LENDEH									
Anjir siah	11	0	1	0	0	0	0	0	12
Sar asiab	12	0	2	0	0	0	0	0	14
Bardian	10	0	3	0	0	0	0	0	13
Subtotal	33	0	6	0	0	0	0	0	39
Total	311	10	20	2	5	471	15	25	859

Figure 2: Habitus of *Hyalomma anatolicum excavatum* adult female

ears and perianal areas of these herbivores. Only 13 (2.5%) *Rhipicephalus* specimens were isolated from the cattle, while the rest were found on sheep and goats (Table 2). Similarly, only two different species of *H. excavatum* and *H. anatolicum* (15 specimens; 4.3%) were collected from goats, while the rest were attached to cattle and sheep (Table 2).

From 859 collected ticks, 469 tick specimens were randomly selected for molecular assays at the department of Arboviruses and Viral Hemorrhagic Fevers (National Reference Laboratory), Pasteur Institute of Iran, Tehran. The remnants of CCHFV RNA were recognized in one (0.2%) female tick on a cow (Table 2). This depleted hard tick was *R. bursa* isolated from *B. taurus* in Delibejak village of Dena County at an altitude of >3000 m above the sea level.

Discussion

Tick-borne diseases, caused by a wide spectrum of pathogenic agents, are of alarming public health significance in Iran.[11.18-21] Infestation of domestic herbivores with CCHF virus-infected ticks imposes a substantial and particularly persistent burden of disease on the public health infrastructure.[22] The detection of sporadic epidemics of CCHF points to the incremental clinical significance of this disease in recent years, in addition to a plethora of non-infectious[23] and other infectious diseases[24-27] in different parts of Iran. Although ticks of the genus *Hyalomma* are incriminated as the main reservoir and vector of CCHF virus, viral RNA has also been detected in other non-*Hyalomma* tick genera.[4]

Although eight different species of hard ticks were identified in this study, only two of them (*R. sanguineus* and *H. excavatum*) were predominantly associated with domestic ruminants in most villages. The remaining six hard tick species were sporadically distributed on different hosts in disparate places. In line with other studies conducted in Lorestan and Sistan-Baluchistan provinces,[28.29] the most abundant species of tick collected in this research was *R. sanguineus* followed by various species of *Hyalomma*. It is established that *Hyalomma* ticks are major vectors and reservoirs of CCHFV, and multiple non-*Hyalomma* hard tick species are reported to be involved in CCHFV circulation. As many as 28 tick species collected from vertebrate hosts have so far been reported in CCHFV circulation.[2]

The high abundance of hard ticks on sheep and goats, in particular, at high altitude counties of Boyer-Ahmad, Dena and Basht indicates that ticks, specifically the genus of *Rhipicephalus*, are well fitted to proliferate among the cold hardy moist microhabitats of vegetation in grasslands of mountain areas. It appears that altitudinal variations influence the partial distribution of this tick genus. The finding that the most infested village (with 58 *Rhipicephalus* specimen) and the most diversely tick-infested village (with 5 different tick species) were both in the highland counties of Dena and Boyer-Ahmad confirms this fitness. The range expansion and relatively higher abundance of *Rhipicephalus* over *Hyalomma* makes the natural circulation of CCHFV in this province highly contentious.

Although a wide and diverse range of vertebrate hosts could be naturally involved in CCHF virus passage, host specificity among hard ticks appears to be only partially elucidated. In the present study, the major role of sheep and goats being infested with hard ticks was confirmed in line with other previous studies.[4, 30]

One of the pitfalls in the present paper was the ambiguity on total number of ruminants. At any time of sampling, some herds were out onto the fields and no valid estimate could be taken. In Iran, it is utterly unknown if any convincing evidence exists on ruminants' spatiotemporal distribution with plausible value for academic research. It is, thus, essential to observe what comes when that will be indispensable in any investigations.

Table 2: RT-PCR results of infected tick species from livestock in different villages of Boyer-Ahmad, Dena, Gachsaran, Basht, Bahmai, Choram, Dehdasht and Lendeh counties of Kohgiluye and Boyer-Ahmad province, Iran

Tick species and host	Total no. of ticks	No. of selected ticks	No. (%) of positive/ species	No. (%) of positive/ genus	Collection station
H. excavatum					
Cattle	43	13	0	0	
Sheep	258	124	0	0	
Goats	10	2	0	0	
H. asiaticum					
Cattle	2	1	0	0	
Sheep	8	4	0	0	
Goats	0	0	0	0	
H. anatolicum					
Cattle	4	4	0	0	
Sheep	11	9	0	0	
Goats	5	3	0	0	
H. marginatum					
Cattle	0	0	0	0	
Sheep	2	1	0	0	
Goats	0	0	0	0	
H. scupense					
Cattle	3	3	0	0	
Sheep	2	2	0	0	
Goats	0	0	0	0	
R. sanguineus					
Cattle	11	7	0	0	
Sheep	284	192	0	0	
Goats	176	83	0	0	
R. bursa					
Cattle	2	1	1 (100.0)	1 (0.33)	Delibejak Dena
Sheep	11	7	0	0	
Goats	2	1	0	0	
R. turanicus					
Cattle	0	0	0	0	
Sheep	13	7	0	0	
Goats	12	5	0	0	
Total	859	469	1	1 (0.21)	

It is undeniable that a single RT-PCR-positive result in a hard tick species does not lead to its status as a competent vector. The present study has just revealed that a depleted female *R. bursa* collected from a cow was positive for CCHFV RNA. The tick melting curve displayed a mild undulating peak suppressed by the positive control spike which is likely attributed to a low CCHFV RNA titer in the tick body. This tick species, which normally uses similar host species for immature and adult stages, was also found to be positive in Turkey and Greece.[31-34]

In conclusion, the presence of CCHFV RNA in a depleted female *R. bursa* collected from a cow in the cold Delibejak village of Dena County in Kohgiluyeh Boyer-Ahmad province of Iran was confirmed, using RT-PCR method. To prevent and control the episodic outbreaks of the disease, persistent surveillance of livestock and hard ticks for CCHFV as well as serologic screening of high risk people is strongly recommended.

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Authorship Contributions

All authors contributed to different extents to the initial design, data collection, analyses and manuscript writing.

The first author, ZH, wrote the proposal, designed the method, collected the samples, identified them, implemented the PCR method, and collated all her findings in an MSc thesis. ZH wrote the initial MS draft. MS-V, SA, MRF, TJ, ZT, and MDM-F were all involved in the design, data screening, data analysis, and MS draft preparation. MS-V and MDM-F were also accountable in the technical assistance of molecular studies and final draft writing.

Compliance with Ethical Standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of Science and Ethics Committee of SUMS University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical permission was granted through the Science and Ethics Committee of SUMS University. Informed consent was obtained from each individual's family in this study.

Conflict of Interest: None declared.

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