

Crimean–Congo Hemorrhagic Fever Virus Clade IV (Asia 1) in Ticks of Western Iran

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ABSTRACT Crimean–Congo Hemorrhagic Fever virus (CCHFV) is transmitted through the bite of an infected tick, or by direct contact with CCHFV-infected patients' blood or the products of infected livestock. In 2012, ticks were collected in eight regions of Lorestan Province, Iran. In total, 434 ticks were collected. Reverse transcriptase polymerase chain reaction was used for the detection of CCHFV RNA. Of 434 ticks, 419 (96.6%) ticks were from the family Ixodidae (hard ticks) and 15 (3.5%) ticks were from the family Argasidae (soft ticks). The presence of CCHFV RNA was detected in 29 (6.7%) of 434 ticks. The infected tick species include *Hyalomma asiaticum* ($n = 7$, 7.4%), *Hyalomma anatolicum* ($n = 12$, 13.2%), *Hyalomma marginatum* ($n = 1$, 16.7%), and *Rhipicephalus sanguineus* ($n = 9$, 4.3%). These empirical data demonstrated that the majority of CCHFV-positive ticks belonged to the Ixodidae. None of the Argasidae and *Haemaphysalis sulcata* species was infected with CCHFV. The phylogenetic analyses of the tick-derived CCHFV strains revealed that all 29 viral strains fell in clade IV (Asia 1). The most abundant species of tick collected in this study was *R. sanguineus* followed by different species of *Hyalomma*. Given the infection rate among collected ticks, *H. marginatum* was the most abundant infected tick species (16.7%) followed by *H. anatolicum* (13.2%), *H. asiaticum* (7.4%), and *R. sanguineus* (4.3%).

KEY WORDS tick, Ixodidae, Argasidae, Crimean-Congo Hemorrhagic fever virus

Crimean–Congo Hemorrhagic Fever (CCHF) is caused by Crimean–Congo Hemorrhagic Fever virus (CCHFV). The virus is a member of the family Bunyaviridae and genus Nairovirus (Chinikar et al. 2014ab). CCHFV is a single stranded RNA virus with a segmented negative sense genome consisting of a small (S), a medium (M), and a large (L) segment. CCHFVs are relatively divergent in their genome sequence and grouped into seven distinct clades based

on the S-segment genome analysis (Han and Rayner 2011): West-Africa in clade I, Central Africa in clade II, South Africa and West Africa in clade III, Middle East and Asia in clade IV, Europe in clade V and Greece in clade VI (Hewson et al. 2004, Deyde et al. 2006). The clade IV may be divided into two distinct clades, Asia-1 and Asia-2 (Hewson et al. 2004).

CCHFV is usually transmitted in nature by tick bites. CCHFV has an enzootic cycle including vertebrates. CCHFV was first isolated from ticks of the genus *Hyalomma* in the 1960s (Begum et al. 1970). It has been detected in at least 31 species of ticks in the Ixodidae and Argasidae (Chinikar et al. 2013b, Champour et al. 2014).

The epidemiology of CCHF reflects the geographic distribution of the *Ixodid* ticks, particularly those of the genus *Hyalomma* (Ergönül 2006). The virus or disease is reported in >30 countries in Africa, southeast Europe, the Middle East, and Asia (Morikawa et al. 2007, Champour et al. 2014).

Epidemics usually coincide with the peak activity periods of *Hyalomma* ticks. Although *Hyalomma* spp. ticks are considered the most important in the epidemiology of CCHF, the virus has also been isolated from ticks of other genera (i.e. *Rhipicephalus*, *Boophilus*, *Dermacentor*, *Haemaphysalis*, and *Ixodes* spp.), which may contribute to its wide geographical distribution (Saijo et al. 2002, Tahmasebi et al. 2010).

In endemic areas, CCHFV is reported in domestic and wild animals. In animals, the infection is

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asymptomatic and lasts from a few days to a few weeks. In general, large cattle are the main host of mature *Hyalomma*, while smaller cattle are the main host of nymphal stages of ticks (Gonzalez et al. 1998, Zakhshvili et al. 2010).

Although surveys have shown CCHF antibodies in livestock and humans in Iran since 1970 (Saïdi et al. 1975, Sureau et al. 1980), the first clinical human case was reported in 1999 in the west of Iran. In 2008, a phylogenetic analysis in tick populations in Isfahan Province in central Iran demonstrated that a variant isolate was clustered within clade IV (Asia 1) with highest similarity to an Iraqi strain (Chinikar et al. 2012). Other phylogenetic studies on viruses from the north and northwest parts of Iran demonstrated similarity between Iranian isolates and Russian and Turkish isolates, which implicates the role of migratory birds and their ticks in CCHFV circulation (Chinikar et al. 2013a).

CCHF have been reported from 27 of 31 provinces in Iran (Chinikar et al. 2010). Understanding the ecology of ticks as the main vector of CCHFV is essential for implementation of the most effective strategies to control CCHFV in endemic areas. The hypothesis of this study was that Ixodidae and Argasidae ticks have a pivotal role in the maintenance and transmission of CCHFV in western Iran. Thus, the aims were to determine the infection rate, tick distribution and abundance, and the phylogeography of tick-derived CCHFV strains in eight regions of Lorestan Province, western Iran.

Materials and Methods

Study Area and Sample Collection. Lorestan Province is located in the west of Iran. Ticks were collected from 48 villages in eight different regions of Lorestan Province from April till June in 2012. During all of the field work, all participants used disposable personal protective equipment, i.e., latex gloves,

coverall suit, goggles, and a face mask. In each village, 20 livestock (cattle, sheep, and goat) and broiler chickens were checked randomly for tick collection. The total number of livestock (89%) and broiler chickens (11%) examined was 960. The entire body of each livestock and broiler chicken was checked. In total, 434 ticks were collected using forceps. All ticks were kept alive in separate labeled 2-ml Eppendorf tubes and then transferred to -80°C freezer in the laboratory for species identification. Morphological identifications and sex determination were conducted using the key of Estrada-Pena et al. 2004, using a chill table and a dissection microscope. Identified ticks were sent to the Arboviruses and Viral Haemorrhagic Fever Laboratory (National Reference Lab) at the Pasteur Institute of Iran on dry ice, for molecular detection of CCHFV (Fig. 1).

RNA Extraction. After transport into a BSL-3 facility, ticks were individually crushed with a mortar and pestle in 200–300 μl of PBS containing 10% fetal calf serum, 500 IU/ml penicillin, and 500 mg/ml amphotericin. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted total RNA was stored at -70°C until use (Chinikar et al. 2013a).

Amplification of Capsid Protein Gene (S-segment) Fragment. To further characterize the virus strains, a 536-bp length fragment of the nucleoprotein (S segment) gene was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using designed forward primer (PSF5'-GAATGTG-CATGGGTTAGCTC-3') and reverse primer (PSR 5'-GACATCACAATTCACCAGG-3'). For reverse transcription, a cycle of 50°C at 30 min was used. An initial enzyme activation step at 95°C for 5 min was succeeded by 40 reaction cycles carried out for 30 s at 94°C , 30 s at 50°C , and 45 s at 72°C followed by a final incubation at 72°C for 10 min. Products were

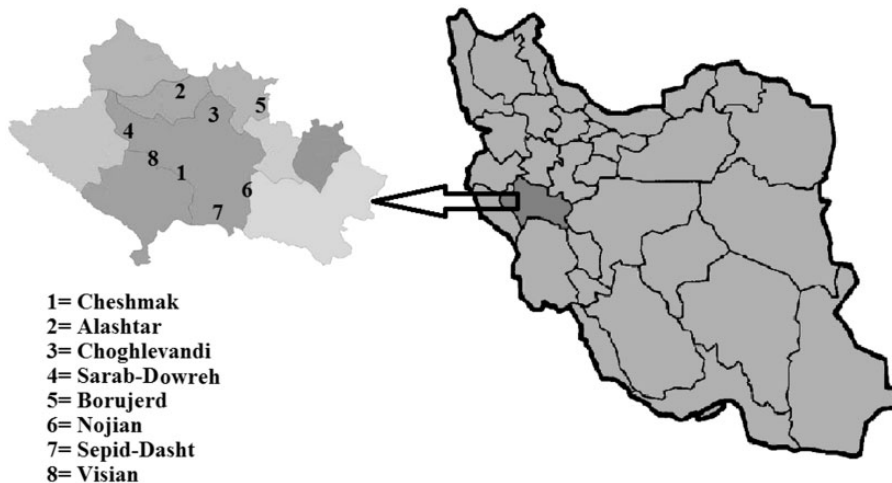


Fig. 1. Map of Iran (right): Province of Ardabil (A), Kurdistan Province (B), Hamadan Province (C), Province of Chaharmahal va Bakhtiari (D), Province of Yazd (E), and sampling regions of Lorestan Province are indicated by number (left).

subsequently analyzed by gel electrophoresis. In real-time analysis, both amplification and dissociation curves were evaluated with respect to negative and positive controls, as well as in the gel-based analysis (Escadafal et al. 2012). Then, positive samples were sent for sequencing using Big Dye Terminator v.3.1 Cycle sequencing Kit with Modified Sanger Sequencing Method by ABI Genetic Analyzer 3130 (Chinikar et al. 2013a).

Sequence Alignments and Phylogeny Analysis. In addition to the CCHFV sequences obtained in this study, 13 CCHFV strains including Baghdad12 (AJ538196), Kosovo1917 (JN173797), Kashmanov (DQ211644), SPU97-85 (DQ211646), ArD8194 (DQ211639), UG3010 (DQ211650), AP92 (DQ211638), 79121M18 (GU477494), Gujarat (JN108025), Beruwe (HQ849545), SPU431-85 (KJ682815), C-68031 (DQ211642), and SCTexAfghanistan (JX908640) retrieved from GenBank at www.ncbi.nlm.nih.gov were incorporated into the alignments for phylogenetic analyses. The sequence alignment was undertaken using ClustalW and phylogenetic trees were generated by the maximum likelihood (ML) method with Kimura two-parameter distance using the Mega 6 software. Bootstrap confidence limits were based on 1,000 replicates (Chinikar et al. 2013a).

Accession Numbers. The nucleotide sequences generated in this study have been deposited in GenBank under accession numbers KR136174–KR136202.

Statistical Analysis. Data were analyzed using the SPSS software version 15.0. Descriptive statistics (i.e., frequencies and percentages) were used to summarize the quantitative variables. Species, gender, and infection rate in ticks, and infection prevalence in each

sampling region were the parameters included in the analysis.

Results

Tick Species. In total, 434 ticks were collected from eight different regions of Lorestan Province. Of 434 ticks collected, 419 (96.6%) ticks were from Ixodidae (197 females and 222 males) and 15 (3.5%) ticks were from Argasidae (7 females and 8 males). Ixodidae ticks consist of *Hyalomma asiaticum* (95 ticks), *Hyalomma anatolicum* (91 ticks), *Hyalomma marginatum* (6 ticks), *Rhipicephalus sanguineus* (212 ticks), and *Haemaphysalis sulcata* (15 ticks) species. All ticks collected from broiler chickens were *Argas persicus* (15 ticks).

CCHFV Prevalence in the Tick Populations. The presence of CCHFV RNA was detected in 29 (6.7%) of 434 ticks. The infected tick species belonging to *H. asiaticum* ($n=7$, 7.4%), *H. anatolicum* ($n=12$, 13.2%), *H. marginatum* ($n=1$, 16.7%), and *R. sanguineus* ($n=9$, 4.3%). None of the species of *H. sulcata* and *A. persicus* were infected with CCHFV (Table1).

Among eight geographical regions of Lorestan Province, the highest rate of CCHFV infection was detected in tick populations of region-1/southwest (13.6%) followed by region-2/north (12.8%), region-3/northeast (9.7%), region-4/west (6.5%), region-5/east (5.6%), region-6/southeast (3.8%), region-7/south (2%), and region-8/southwest (1.7%; Table2).

Phylogenetic Analysis of Iranian CCHFV Strains from Ticks. The phylogenetic analysis of the tick-derived CCHFV strains was conducted using the ML algorithm. Phylogenetic analysis using the partial

Table 1. Details of infection of ticks by CCHF virus tested by RT-PCR in Lorestan Province, Iran, spring 2012

Family	Species	Infected ticks/ female ticks analyzed (%)	Infected ticks/ male ticks analyzed (%)	Total (%)
<i>Ixodidae</i>	<i>Hyalomma asiaticum</i>	5 of 39 (12.8)	2 of 56 (3.6)	7 of 95 (7.4)
	<i>Hyalomma anatolicum</i>	4 of 40 (10)	8 of 51 (15.7)	12 of 91 (13.2)
	<i>Hyalomma marginatum</i>	0 of 4 (0)	1 of 2 (50)	1 of 6 (16.7)
	<i>Rhipicephalus sanguineus</i>	4 of 106 (3.8)	5 of 106 (4.8)	9 of 212 (4.3)
	<i>Haemaphysalis sulcata</i>	0 of 8 (0)	0 of 7 (0)	0 of 15 (0)
Total		13 of 197 (6.6)	16 of 222 (7.2)	29 of 419
<i>Argasidae</i>	<i>Argas persicus</i>	0 of 7 (0)	0 of 8 (0)	0 of 15 (0)
Total		13 of 204 (6.4)	16 of 230 (6.95)	29 of 434 (6.7)

Table 2. Geographical prevalence of CCHFV genome among collected ticks in eight regions of Lorestan Province infected ticks by analysed ticks (% infected)

Regions	Species <i>H. asiaticum</i> (%)	Species <i>H. anatolicum</i> (%)	Species <i>H. marginatum</i> (%)	Species <i>R. sanguineus</i> (%)	Species <i>H. sulcata</i> (%)	Species <i>A. persicus</i> (%)	Total
1-Southwest	0 of 16 (0)	4 of 11 (36.4)	0 of 0 (0)	4 of 32 (12.5)	0 of 0 (0)	0 of 0 (0)	8 of 59 (13.6)
2-North	1 of 4 (25)	1 of 8 (12.5)	1 of 2 (50)	2 of 25 (8)	0 of 0 (0)	0 of 0 (0)	5 of 39 (12.8)
3-Northeast	2 of 6 (33.3)	0 of 11 (0)	0 of 0 (0)	2 of 24 (8.3)	0 of 0 (0)	0 of 0 (0)	4 of 41 (9.7)
4-West	0 of 2 (0)	4 of 44 (9.1)	0 of 0 (0)	0 of 16 (0)	0 of 0 (0)	0 of 0 (0)	4 of 62 (6.5)
5-East	3 of 10 (30)	1 of 3 (33.3)	0 of 4 (0)	0 of 42 (0)	0 of 13 (0)	0 of 0 (0)	4 of 72 (5.6)
6-Southeast	0 of 0 (0)	2 of 12 (16.7)	0 of 0 (0)	0 of 41 (0)	0 of 0 (0)	0 of 0 (0)	2 of 53 (3.8)
7-South	1 of 11 (9.1)	0 of 0 (0)	0 of 0 (0)	0 of 22 (0)	0 of 2 (0)	0 of 15 (0)	1 of 50 (2)
8-Southwest	0 of 46 (0)	0 of 2 (0)	0 of 0 (0)	1 of 10 (10)	0 of 0 (0)	0 of 0 (0)	1 of 58 (1.7)
Total	7 of 95 (7.4)	12 of 91 (13.2)	1 of 6 (16.7)	9 of 212 (4.3)	0 of 15 (0)	0 of 15 (0)	29 of 434 (6.7)

S-segment demonstrated that all 29 sequenced CCHFV obtained in this study were located within clade IV (Asia 1), where 22 sequences formed a cluster with each other and the rest clustered with two previously known CCHFV strains from Iraq (Baghdad12) and Afghanistan (SCTexAfghanistan; Fig. 2).

Discussion

Virus survival in infected ticks and the ability of *Hyalomma* species to survive at least 800 d without a blood-meal indicates the important role of ticks in CCHFV transmission (Nasiri et al. 2010).

The most abundant species collected was *R. sanguineus* followed by different species of *Hyalomma*, while *H. sulcata* and *A. persicus* had the lowest number among the ticks collected. Regarding *H. sulcata* and *A. persicus*, sampling regions would be an explanation for the low number of sampling and the host preference for these ticks, for instance, all soft ticks were found only on chickens in investigated regions.

Based on the results of the present study, the prevalence of infected ticks with CCHFV in Lorestan Province was 6.7%. These data showed that the majority of ticks that tested positive by RT-PCR for the presence of CCHFV RNA belonged to the Ixodidae. Four tick

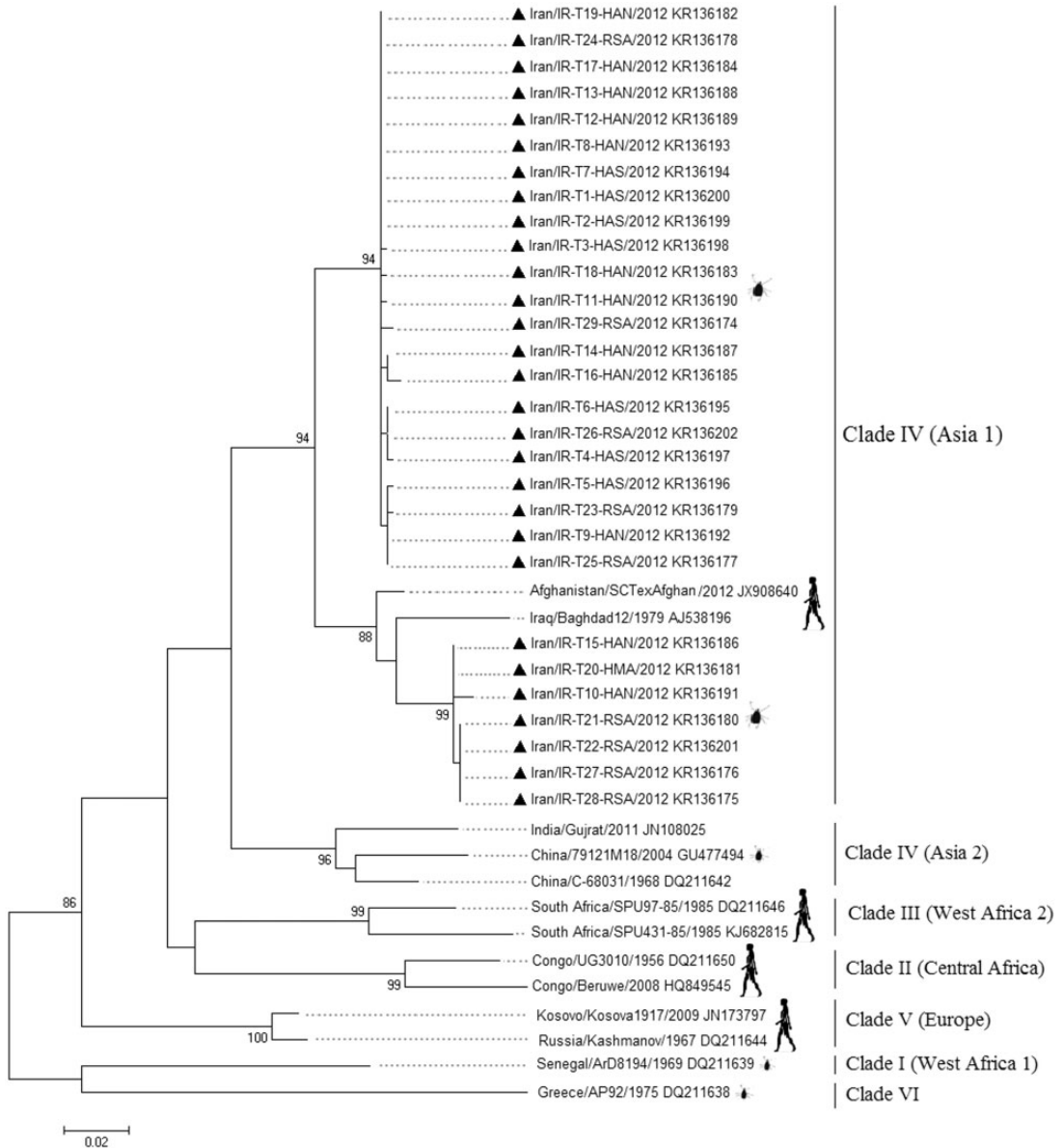


Fig. 2. Phylogenetic tree based on the partial sequence of the S-segment of CCHFVs. The tree was constructed by using the ML method with Mega 6. The sequences obtained from this study are marked with ▲. The numbers above the branches indicate the bootstrap values in percentages of 1,000 replicates.

species were reported with CCHFV infection in Lorestan Province. *H. marginatum* had 16.7%, *H. anatolicum* had 13.2%, *H. asiaticum* had 7.4%, and *R. sanguineus* had 4.3% infection rate. None of the specimens of *H. sulcata* and *A. persicus* were infected with CCHFV.

The prevalence of infection among ticks varied in different regions of Lorestan Province. The majority infected ticks were collected in the Northern region of Lorestan Province, which borders with Hamadan Province. These data are in accordance with previously reported CCHFV prevalence data in Hamadan Province (Chinikar 2009).

In a previous study undertaken in the Province of Ardabil, north-western Iran, 28% of ticks, including *Hyalomma* sp., *Rhipicephalus* sp. and *Ornithodoros* sp., were infected with CCHFV, which is the highest rate of infected ticks among all tick surveys in Iran (Telmadarray et al. 2010). In a study in Hamadan Province, Northern Province of Lorestan, results showed 19.2% of all collected hard and soft ticks (*Hyalomma dromedarii*, *Hyalomma anatolicum*, *R. sanguineus*, *Argas reflexus*) were RNA positive for CCHFV. These data suggest that the infection rate among collected ticks in Hamadan Province is about three times that in Lorestan Province (Tahmasebi et al. 2010).

In the north-western neighbor of Lorestan, Kurdistan Province, the infection rate among ticks was 5.6%, which is similar to data reported in this current study (6.7%). From a tick survey in Kurdistan Province, *Hyalomma* ticks were considered to be the most prominent vectors (70%), and both *Haemaphysalis* and *Rhipicephalus* were negative for CCHFV RNA (Fakoorziba et al. 2012).

In a previous survey undertaken using molecular (RT-PCR) methods in the Province of Chaharmahal va Bakhtiari, southern neighbor of Lorestan Province, 22.8% of soft ticks were reportedly infected with CCHFV, which is considerably higher than our results regarding infected soft ticks (Shirani et al. 2004). An investigation undertaken in the Province of Yazd showed that 5.7% of hard ticks were infected, including the species *H. marginatum*, *H. dromedarii*, *Hyalomma asiaticum*, *Hyalomma detritum*, *H. anatolicum* (Yaser et al. 2011).

The results of a tick survey in Bulgaria showed that of 911 collected ticks from livestock between 2006 and 2010, 2.1% were infected by CCHFV. The most commonly collected tick species was *H. marginatum marginatum*. Also, the highest rate of CCHFV infection was detected in tick species of *H. m. marginatum* (4.9%) followed by *R. sanguineus* (2.3%) and *Ixodes* spp. 1.0%. According to these findings in Bulgaria, *H. marginatum* was considered as the main reservoir for CCHFV, which is in accordance with Iranian and Turkish surveys (Gunes et al. 2011, Gergova et al. 2012). In another study in the southern region of Turkey, a higher infection rate among tick populations (12.3%) was shown compared to the Bulgarian tick survey (Yesilbag et al. 2013).

Our phylogenetic results are in accordance with previous studies that reported the genetic heterogeneity of CCHFV worldwide and found that the existing isolates

can be grouped into seven main clades. The study provides insights into the genetic variability of CCHFV in tick populations from Lorestan Province. Phylogenetic analysis of CCHFVs in tick populations reveals clustering in clade IV (Asia 1), which is in consistent with previous reports given circulating genomic variants of CCHFV in Iran (Chinikar et al. 2014a).

Tick species identified in this study was most similar to other tick species collected from Iran and Eastern Europe specifically Turkey (Mehravaran et al. 2013). Both *Hyalomma* and *Rhipicephalus* showed the highest prevalence of CCHFV RNA. Given the low number of collected *A. persicus* in this study, we suggest the collection and testing of more soft ticks in the future studies.

As CCHF is a serious health problem in Iran. Increased effort should be taken into account by health centers in this province to develop strategies to decrease tick populations to provide public awareness of the exposure from tick bites and to alert medical professionals about the public health risks of CCHF.

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