ORIGINAL ARTICLE

First phylogenetic analysis of a Crimean-Congo hemorrhagic fever virus genome in naturally infected *Rhipicephalus appendiculatus* ticks (Acari: Ixodidae)

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Abstract Crimean-Congo haemorrhagic fever (CCHF) is a potentially fatal systemic viral disease in many parts of the world, including Iran. The nationwide incidence of human CCHF in endemic areas was 870 confirmed cases with 126 deaths (case fatality rate, CFR = 17.6 %) in the decade leading to 2012. The detection of the CCHF virus (CCHFV) genome in tick vectors is of fundamental importance for identifying these ticks as potential reservoirs of CCHFV infection. From May to October 2013, following detection of four new clinical cases resulting in two deaths in the city of Mashhad (northeast Iran), hard ticks were recovered from infested livestock in 40 villages in Khorasan-Razavi province and examined by the microscopic method for species identification. About a quarter of the ticks were then subjected to reverse-transcription polymerase chain reaction (RT-PCR) to detect the CCHFV genome. The PCR products were then sequenced, and their phylogenetic lineages were determined. A total of 407 hard ticks were captured, representing seven different species in two distinct genera. Members of the genus *Hyalomma* were widely distributed in all but two of the villages studied, and this was also the most frequent (83.3 %) tick genus. Of 105 adult ticks subjected to RT-PCR, four (3.8 %) ticks were found positive for the CCHFV genome. One brown ear tick, *Rhipicephalus appendiculatus*, was found to be naturally infected for the first time anywhere in the world. Ticks of *Hyalomma asiaticum*, *Hyalomma marginatum*, and *Rhipicephalus turanicus* were also found to be naturally infected with CCHFV. CCHFV found in these four different tick species were clustered in the same lineage with the Matin and SR3 strains from Pakistan and some other strains from Iran, indicating that these tick species were naturally infected with genetically closely related CCHFV in the region. The presence of CCHFV infection in four different hard tick species was confirmed using RT-PCR in northeast Iran. Part of this infection was attributed to *Rh. appendiculatus*, which is thus a potential new natural vector of CCHFV in Iran. It is also confirmed by phylogenetic analysis that CCHFV in this region is genetically closely related, even in the different tick species.

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Introduction

Crimean-Congo hemorrhagic fever (CCHF) is caused by members of the genus *Nairovirus* in the family *Bunyaviridae* [1]. Despite being an enzootic infection,

outbreaks and sporadic small clusters of disease cases occur focally in humans after direct bites of infected ticks, which are competent natural reservoirs, or via skin contact with viremic tissues, blood, or other contaminated body fluids of patients or infectious asymptomatic domestic and wild livestock such as cattle, camels, sheep and goats [2, 3]. Although CCHF virus (CCHFV) is transmitted to a wide variety of animals, it is severely pathogenic only for humans [4]. The large burden of human CCHF cases in Iran and neighbouring countries ($\approx$5000 patients in the first decade of this century) points to the serious importance of this challenge [5].

Specific antibodies against CCHFV antigens in the sera of humans, cattle and sheep were first demonstrated back in the early 1970s [6]. Current molecular epidemiology and phylogenetic data indicate that the widest genetic diversity of CCHFV in any country occurs in Iran, where there are at least four (genotype 1 [Africa 1]; genotype 2 [Asia 2]; genotype 4 [Europe 1]; genotype 7 [Africa 1]) or five different circulating genomic variants out of a total of seven distinct genotypes [7–9].

Viruses were isolated from hard (ixodid) ticks [10]. The first human cases were seen in 1999 and have since been recorded in all but four (87 %) Iranian provinces [8, 11]. Nosocomial infection with CCHFV is also being reported, with immense and alarming implications [11]. As a result of a recent hospital outbreak of CCHF in the city of Mashhad, Khorasan-Razavi province in northeast Iran, four patients were hospitalized, two of whom (one of them a nurse) died later due to clinical complications [11].

Ticks are one of the most medically important groups of bloodsucking arthropods, which may carry a wide variety of pathogens responsible for many tropical zoonotic diseases, including those caused by the arboviruses in the genus *Nairovirus*. The genus of hard tick, *Rhipicephalus*, includes numerous species that are important vectors of infectious agents. *Rhipicephalus appendiculatus* (known as the "brown ear tick") is a three-host bloodsucking inornate species of hard tick.

This study describes the first identification of the CCHFV genome in the *Rh. appendiculatus* species of hard tick in northeastern Iran. While CCHFV has been experimentally shown to infect members of this species, [12] and other species of *Rhipicephalus* have been shown to be infected in nature, this is the first demonstration of naturally occurring infection in this particular species of *Rhipicephalus* tick. While this may then appear to be an incremental increase in understanding, the identification of additional tick species that are able to serve as vectors for CCHFV in the endemic areas is an important and neglected area of research with significant public health implications for the transmission dynamics of this disease.

This outbreak of CCHF prompted us to conduct research on faunal identification of hard ticks feeding on livestock and their likely infection with CCHFV using a reverse transcription polymerase chain reaction (RT-PCR) method. To the best of our knowledge, this is the first global report on natural detection of CCHFV in a brown ear hard tick, *Rh. appendiculatus*, in northeastern Iran. The natural presence of these viruses in wild-caught *Rh. appendiculatus* has never been reported before.

Materials and methods

Study area

Khorasan-Razavi province is located on the northeastern frontier of Iran (Fig. 1). This study focused on the three counties of Torbat-Heydariyeh (35°15′N, 59°132′E at 1333 m above sea level), Roshtkhar (34°58′N, 59°38′E at 1215 m a.s.l.), and Khaf (34°38′N, 60°10′E at 970 m a.s.l.). For the present study, 15 villages each from Torbat-Heydariyeh and Roshtkhar, and 10 villages from Khaf that each had at least 10 households, were randomly selected.

Collection of ticks

Hard ticks were collected from infested livestock for six months from May to October 2013, when peak densities of ticks were expected [13]. Adult ticks were collected from different parts of the bodies of sheep, goats and cows by clustered random sampling in the 40 sampled villages. The exclusion criteria were recently insecticide-sprayed animal husbandry units and those that did not consent to sampling. The whole body of each ruminant host was examined for the presence of tick infestation by palpation, principally on the ears and along the nape of the neck, perineum, scrotum or udder, and tail base. With the aid of experienced animal breeders, 3 hours were spent searching for ticks on infested animals at each station. All ticks were handled with sterile gloves and fine forceps. They were identified at the species level by trained acarologists. Each tick was cautiously dislodged and transferred alive into a separate screw-capped sterile vial with a small piece of cotton soaked in 1 % mycostatin solution to inhibit desiccation and mold growth. Each vial was labeled accordingly, frozen at -40 °C, and subsequently, only depleted (non-engorged) adult ticks were checked for the CCHF virus genome at Pasteur Institute of Tehran, Iran.

RNA extraction from ticks

Each of 105 adult ticks, chosen as a random sample of the collected ticks, was separately washed twice with
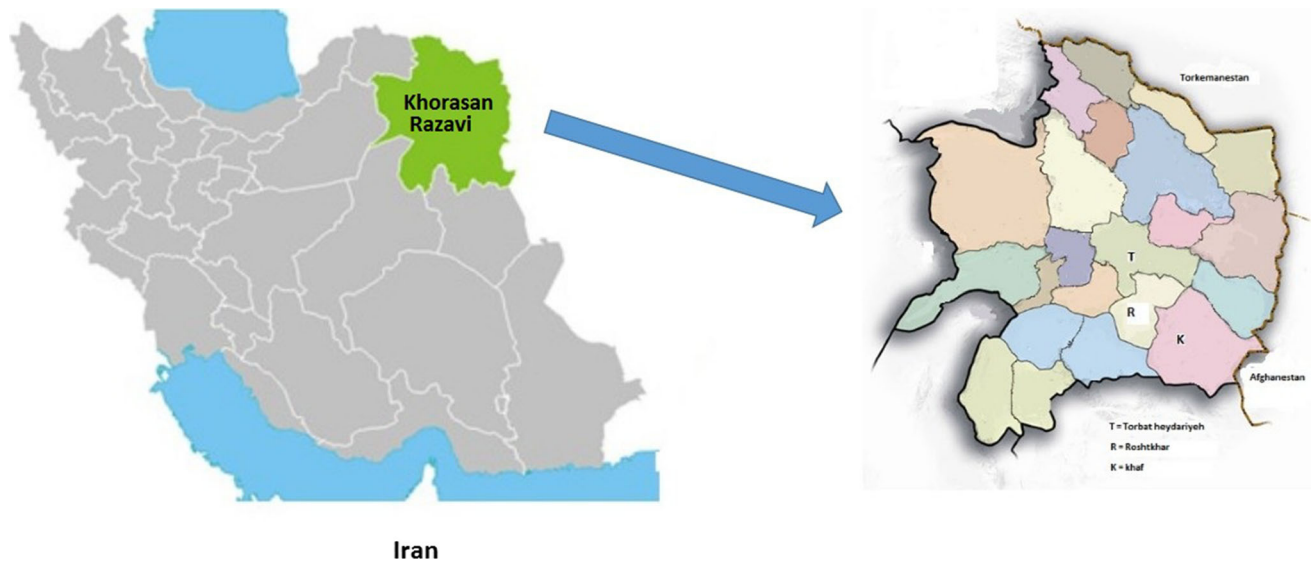
Fig. 1 Map of the study area showing the position of Khorasan-Razavi province in northeastern Iran and the locations of the 40 villages that were selected for tick sampling

phosphate-buffered saline (PBS, pH = 7.4) and crushed with a pestle and mortar in 300 μL of PBS. Viral RNA was extracted from 140 μL of phenol-extracted tick suspensions using a QIAamp RNA Mini kit (QIAGEN GmbH, Hilden, Germany) according to the instructions of the manufacturer.

RT-PCR

Reverse transcription of RNA was conducted using previously described primers [14] that amplify a 536-bp fragment of the S segment of the CCHF viral genome. Briefly, the extracted viral RNA was analyzed by gel-based and real-time RT-PCR using a one-step RT-PCR kit (QIAGEN) with specific primers: F2 primer (5′-TGGACACCTTCA-CAAACTC-3′) and R3 primer (5′-GACAAATTCCCTG-CACCA-3′) [15]. The PCR reaction was done in a total volume of 50 μL. The reaction conditions were 30 min at 50 °C, 15 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 45 s at 72 °C, and finally, 10 min at 72 °C as a final extension step. For qualitative analysis, 1 μL of SYBR green dye at 1:10,000 dilution was also used in the master mix. For gel-based analysis, 5 μL of the PCR products were mixed with 1 μL of loading buffer, and electrophoresis was carried out in a 2 % agarose gel in Tris-borate EDTA buffer (TBE). DNA bands were stained with ethidium bromide and were visualized on a UV transilluminator.

Sequencing and gene sequence submission

Amplified PCR products were sequenced directly by a modified Sanger sequencing method using an ABI 3130

Genetic Analyzer and a BigDye terminator cycle sequencing reaction kit V3.1. Four new sequences (Nad-8-494c, *Hy. asiaticum*; Nad-20-510c, *Hy. marginatum*; Nad-71-496c, *Rh. appendiculatus*; and Nad-83-519c, *Rh. turanicus*) were submitted to GenBank and assigned the accession numbers KP223318, KP223319, KP223320 and KP223321, respectively.

Phylogenetic and sequence analysis

The sequences of the CCHFV amplicons from this study and 44 sequences retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/) (Table 3) were aligned using the Clustal W Multiple Sequence Alignment (MSA) tool with the following parameters: gap opening penalty, 15; gap extension penalty, 6.66; DNA weight matrix IUB and transition weight, 0.5.

A phylogenetic tree was constructed by maximum-likelihood statistical method using the Kimura 2-parameter model. The reliability of the phylogenetic tree was evaluated using the bootstrap test based on 1000 replicates at a cutoff value of 70 %. Pairwise distances were computed using MEGA 5 software. Phylogenetic and sequence analyses were conducted using MEGA version 5 [16].

Results

A total of 407 adult ticks were collected from the three counties of Torbat-Heydaryieh (160 ticks, 39.3 %), Roshtkhar (144 ticks, 35.4 %) and Khaf (103 ticks, 25.3 %) during the study period. All the villages in this study were infested with no fewer than three and no more

Table 1 Distribution of different tick species in different villages of Torbat-Heydariyeh, Roshtkhar and Khaf districts of Khorasan-Razavi province, Iran

	Hy. asiaticum	*Hy. anatolicum*	*Hy. marginatum*	*Rh. bursa*	*Rh. turanicus*	*Rh. sanguineus*	*Rh. appendiculatus*	**Total**
Khaf								
Chehardeh	0	0	4	0	0	0	0	4
Kalshoor	0	0	0	4	10	1	0	15
Khaf	15	0	0	0	0	0	0	15
Khalilabad	7	1	0	0	0	0	0	8
Khargerd	2	0	1	0	0	0	0	3
Nashtifan	10	0	1	0	0	0	0	11
Qasemabad	1	0	0	12	0	0	0	13
Sangan	13	0	4	0	0	0	0	17
Sarcheshmeh	0	0	3	4	0	0	0	7
Tizab	10	0	0	0	0	0	0	10
Subtotal	58	1	13	20	10	1	0	103
Roshtkhar								
Ahmadabad	11	0	0	0	1	0	0	12
Alinaqi	0	0	8	0	0	0	0	8
Bandozbak	10	0	0	7	0	0	0	17
Basfar	7	0	1	0	0	0	0	8
Cat	6	0	0	0	0	0	0	6
Chahshurak	8	0	2	0	0	0	0	10
Dochahi	5	0	1	2	0	0	0	8
Fazelmand	5	0	4	0	0	0	0	9
Hasanabad	7	0	1	0	0	0	0	8
Janatabad	2	0	0	0	5	0	3	10
Jangal	1	0	7	2	0	0	0	10
Kudeh	6	0	3	0	0	0	0	9
Mohammadabad	7	0	0	1	0	0	0	8
Salami	8	0	3	0	0	0	0	11
Shaebeh	10	0	0	0	0	0	0	10
Subtotal	93	0	30	12	6	0	3	144
Torbat-Heydaryieh								
Benhang	12	0	0	0	0	0	0	12
Booriabad	12	0	0	0	0	0	0	12
Ciuki	0	0	15	0	0	0	0	15
Dehnotalkhi	9	0	3	0	0	0	0	12
Fahandar	0	0	0	0	10	0	0	10
Federd	14	0	0	0	0	0	0	14
Hajiyan	0	0	3	2	0	0	0	5
Hesar	12	0	0	0	0	0	0	12
Kajderakht	12	0	0	0	0	0	0	12
Kalateali	4	0	0	0	0	0	0	4
Mooreshk	6	0	3	0	0	0	0	9
Qalenoo	10	0	3	0	0	0	0	13
Safiabad	4	0	0	0	0	0	2	6
Shadmehr	5	0	5	0	0	0	0	10
Sorkhabad	12	0	0	0	0	2	0	14
Subtotal	114	0	32	2	10	2	0	160
Total	263	1	75	34	26	3	5	407

Table 2 RT-PCR results of infected tick species from livestock in different villages of Torbat-Heydariyeh (T), Roshtkhar (R) and Khaf (K) districts of Khorasan-Razavi province, Iran

Tick species and host	Total no. of ticks	No. of selected ticks	No. (%) of positives/species	No. (%) of positives/genus	Collection station
Hy. asiaticum					
Cattle	120	23	1 (4.35)	2 (2.27)	Benhang T
Sheep	106	27	0	0	
Goats	37	8	0	0	
Hy. marginatum					
Cattle	11	8	1 (12.5)	0	Basfar R
Sheep	57	17	0	0	
Goats	7	4	0	0	
Hy. anatolicum					
Cattle	0	0	0	0	
Sheep	1	1	0	0	
Goats	0	0	0	0	
Rh. bursa					
Cattle	0	0	0	0	
Sheep	13	2	0	0	
Goats	21	5	0	0	
Rh. turanicus					
Cattle	0	0	0	0	
Sheep	21	5	1 (20.0)	0	Fahandar T
Goats	5	0	0	0	
Rh. appendiculatus					
Cattle	0	0	0	0	
Sheep	2	2	1 (50.0)	2 (11.76)	Safiabad T
Goats	3	2	0	0	
Rh. sanguineus					
Cattle	0	0	0	0	
Sheep	1	1	0	0	
Goats	2	0	0	0	
Total	407	105	4	4 (3.8)	

than 17 different tick specimens (Table 1). Only six villages among the 40 study stations, including the village of Safiabad in the county of Torbat-Heydariyeh, had no more than six ticks in their sampling collections.

At Torbat-Heydaryieh, different numbers of tick species were removed from cattle (71 ticks, 44.3 %), sheep (57 ticks, 35.6 %), and goats (32 ticks, 20 %). At Roshtkhar, multiple tick species were isolated from cattle (18 ticks, 12.5 %), sheep (99 ticks, 68.7 %), and goats (27 ticks, 18.8 %). At Khaf, these values were 43 (41.8 %), 44 (42.7 %) and 16 ticks (15.5 %), respectively.

Two distinct tick genera were represented, from which seven different tick species (three *Hyalomma* and four *Rhipicephalus*) were identified on infested livestock in the three counties of Khorasan-Razavi province that were studied (Table 1). Ticks of the genus *Hyalomma* was found

to be the most widely distributed in all but two of the villages. It was also the most frequent (83.3 %) tick genus. The least abundant tick species (1 tick, 0.24 %) was *Hy. anatolicum*, which was found in only one village, while the least common (3 ticks, 0.74 %) tick species in the genus *Rhipicephalus* was the brown dog tick *Rh. sanguineus*. The most abundant species were *Hy. asiaticum* and *Hy. marginatum*, with 66.6 % and 18.5 %, respectively. The latter species was present in only half of the villages surveyed, whereas the largest number of *Hy. marginatum* was recorded in a village known as Ciuki in Torbat-Heydariyeh county. Although both tick species (*asiaticum* and *marginatum*) were found to be widely distributed in the surveyed region, neither of them was present in all sampled locations in the study area.

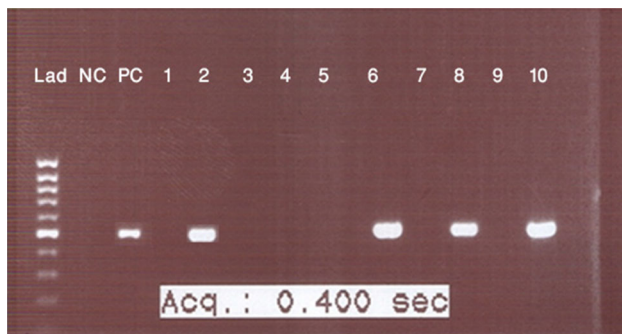The other tick genus was *Rhipicephalus*, which was represented by four different species comprising only

Fig. 2 Gel-based RT-PCR of CCHFV samples. PC, positive control; NC, negative control; S1-10, samples 1-10. S1, S3, S4, S5, S7 and S9 were negative; S2 (*Hyalomma marginatum*), S6 (*Rhipicephalus appendiculatus*), S8 (*Hyalomma asiaticum*) and S10 (*Rhipicephalus turanicus*) were positive

16.7 % of the total sample size. The first and second most abundant species in the genus of *Rhipicephalus* were *Rh. bursa* (34 ticks, 8.3 %) and *Rh. turanicus* (26 ticks, 6.4 %), respectively. The other two rarely found species were the brown dog tick, *Rh. sanguineus*, referred to above, and the brown ear tick, *Rh. appendiculatus*. Only five (1.2 %) specimens of the latter species were found in the villages of Safiabad and Janatabad.

Using reverse transcription PCR on a selection of collected ticks (~ 26 %), it was revealed that a small number (4, 3.8 %) of ticks from both genera, *Hyalomma* and *Rhipicephalus*, removed from infested hosts were naturally infected with CCHFV (Table 2). A total of 105 depleted adult male and female ticks from two distinct genera were included in the molecular assay in this study. Most of the selected ticks were of the species *Hy. asiaticum* (58 ticks, 55.2 %) and *Hy. marginatum* (29 ticks, 27.6 %). The number of *Hy. anatolicum*, *Rh. bursa*, *Rh. turanicus*, *Rh. sanguineus*, and *Rh. appendiculatus* ticks identified was 1 (0.95 %), 7 (6.7 %), 5 (4.8 %), 1 (0.95 %), and 4 (3.8 %), respectively.

All of the selected ticks were subjected to nested RT-PCR. The natural presence of the CCHFV genome was only detected in one sample each of *Hy. asiaticum* (1.7 %), *Hy. marginatum* (3.4 %), *Rh. turanicus* (20 %), and *Rh. appendiculatus* (25 %) (Fig. 2). The positivity rate in *Rhipicephalus* ticks was 12 %, and that of *Hyalomma* ticks was about 2 %. No *Hy. anatolicum*, *Rh. bursa* or *Rh. sanguineus* ticks gave positive nested RT-PCR results for CCHF virus.

Current molecular epidemiology and phylogenetic data have indicated the existence of seven distinct clusters of CCHFV (Tables 3 and 4). All four of the newly found strains of CCHFV identified in *Hyalomma asiaticum*, *Hy. marginatum*, *Rhipicephalus turanicus*, and *Rh. appendiculatus* in Khorasan-Razavi province of Iran were clustered in the same lineage of Clade IV (Asia 1) with Matin;

and SR3 strains in Pakistan; Afg09-2990 in Afghanistan; and Iran-52, Iran-53, and 786/02 strains in Iran (Fig. 3). These four strains were genetically distinguishable from the Baghdad strain in Iraq, the Oman strain in Oman, and SCT ex Afghanistan in Afghanistan (Fig. 3). These results indicate that CCHFV in Khorasan-Razavi province of Iran is genetically closely related, even in the different tick species.

Discussion

Tick-borne fevers, which are caused by a wide range of pathogens, are of increasing public-health significance in Iran [17–19]. CCHFV is a polythetic and zoonotic microparasite of engorging hard and soft ticks with a wide spectrum host specificity. This virus can be transmitted by the venereal, trans-stadial and trans-ovarial routes in ticks [20]. Infections due to viruses being transmitted via haematophagous arthropods (arthropod-borne viruses or arboviruses such as CCHFV) impose a particularly persistent and substantial burden of disease, in addition to those from non-infectious [21] or other infectious diseases [22–24] on the lives of healthy Iranians. Co-infections with these infectious agents could simultaneously be found in endemic regions [25].

The present study was conducted in a northeast Iranian Khorasan-Razavi province where a recent sero-epidemiological survey of CCHFV in slaughterhouse workers indicated an infection rate of 17.5 % [26]. This province was also shown to be one of the hot spots of CCHFV infection in livestock [27]. The national incidence of this clinical disease in endemic areas was 870 confirmed cases with 126 deaths (case fatality rate [CFR], 17.6 %) in the decade leading to the year of 2012 [9]. On the other hand, a CFR of only 5 % was reported in the northwestern neighbouring country of Turkey. The gap in CFR values of Iran and Turkey was suggested to be due to different surveillance (sporadic case reports in Iran *vs.* a large case series including mild disease cases in Turkey) or better laboratory diagnosis of the patients in Turkey, which also included patients with mild symptoms [1, 28]. It is also possible that the gap is partly due to the difference in supportive care operating in those adjacent countries.

The detection of CCHFV in tick vectors and confirmation of their vector competence through transovarial and venereal transmission of CCHFV is of paramount importance for determining whether they are potential reservoirs of infection [1]. However, few attempts have been made to identify tick vectors of CCHFV, despite their clinical indispensability. In a study in the west of Iran, Hamadan province, researchers found three of four, two of three, and one of three different species in the genera of *Hyalomma*,

Table 3 Accession numbers, locations and clade numbers of new isolates (names beginning with NAD) from this study and other CCHF virus strains used for sequence analysis

Strain name	Location	Accession no.	Clade no.
AnD15786	Senegal	DQ211640.1	Clade I
ArD8194	Senegal	DQ211639	Clade I
3010	Uganda	DQ144418.1	Clade II
Congo_3010	DRC	DQ144418.1	Clade II
AB1-2009	Sudan	HQ378179.1	Clade III
Al-Fulah	Sudan	GQ862371.1	Clade III
ArD39554	Mauritania	DQ211641	Clade III
Daral_2012	Mali	KF793333.1	Clade III
IbAr 10200	Nigeria	U88410	Clade III
SPU_55687	South Africa	KJ682817.1	Clade III
SPU4/81	South Africa	DQ076416.1	Clade III
Afghanistan (Afg09-2990)	Afghanistan	HM452305.1	Clade IV, Asia 1
Baghdad	Iraq	AJ538196.1	Clade IV, Asia 1
Ex_Afghanistan	Afghanistan	JX908640.1	Clade IV, Asia 1
Iran_52	Iran	DQ446212.1	Clade IV, Asia 1
Iran_53	Iran	DQ446213.1	Clade IV, Asia 1
Iran_786	Iran	AY366378.1	Clade IV, Asia 1
Matin	Pakistan	AF527810.1	Clade IV, Asia 1
Nad-8-494c: Hy. asiaticum	Iran	KP223318	Clade IV, Asia 1
Nad-20-510c: Hy. marginatum	Iran	KP223319	Clade IV, Asia 1
Nad-71-496c: Rh. appendiculatus	Iran	KP223320	Clade IV, Asia 1
Nad-83-519c: Rh. turanicus	Iran	KP223321	Clade IV, Asia 1
Oman	Oman	DQ211645.1	Clade IV, Asia 1
SR3	Pakistan	AJ538198.1	Clade IV, Asia 1
7803	China	AF354296.1	Clade IV, Asia 2
8402	China	AJ010649.1	Clade IV, Asia 2
66019	China	AJ010648.1	Clade IV, Asia 2
75024	China	AF362080.1	Clade IV, Asia 2
Dubai_616	UAE	JN108025.1	Clade IV, Asia 2
Gujrat	India	JX051650.1	Clade IV, Asia 2
HY_13	China	U88413.1	Clade IV, Asia 2
NIV_112143	India	JN572089.1	Clade IV, Asia 2
NIV_10405051	India	JF922681.1	Clade IV, Asia 2
TAJ/HU8975	Tajikistan	AY297692.2	Clade IV, Asia 2
TAJUK_7	Tajikistan	JN086996.1	Clade IV, Asia 2
UzbekTI10145	Uzbekistan	AF481799.1	Clade IV, Asia 2
6608-Rodopi	Greece	EU871766.2	Clade V
Cankiri_4255-2009	Turkey	HQ166288.1	Clade V
CTF-Hu2706	Turkey	DQ983744.1	Clade V
Drozdov	Russia	DQ211643	Clade V
Erzurum	Turkey	HQ664913.1	Clade V
Kastamonu	Turkey	HQ173895.1	Clade V
Kelkit06	Turkey	GQ337053.1	Clade V
Kosovo	Kosovo	AF404507	Clade V
STV-HU29223	Russia	AF481802	Clade V
V5909	Bulgaria	JF807428.1	Clade V
AP92	Greece	DQ211638.1	Clade VI
Dugbevirus (strain KT281/75)	-	AF434165	Out-group
Hazaravirus (strain JC280M86624)	Pakistan	M86624	Out-group

Table 4 Distance table of CCHF virus, partial S segments (503 nucleotides)

Group	Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
New Isolates	Nad-8-494c_(*Hy. asiaticum*)	0.000															
	Nad-20-510c_(*Hy. marginatum*)	0.004															
	Nad-71-496c_(*Rh. appendiculatus*)	0.004	0.004	0.000													
	Nad-83-519c_(*Rh. turanicus*)	0.004	0.004	0.000	0.000												
Clade I	Senegal/ArD8194(U15021)	0.175	0.175	0.169	0.169												
Clade II	DRC(Congo_3010)_gblDQ144418.1l	0.150	0.150	0.150	0.150	0.183											
Clade III	South_Africa(SPU_55687_)_gblKJ682817.1l	0.120	0.120	0.114	0.114	0.204	0.167										
	Mali(Daral_2012_)_gblKF793333.1l	0.145	0.145	0.139	0.139	0.192	0.191	0.074									
	Nigeria(IbAr)10200(U88410)	0.145	0.145	0.139	0.139	0.205	0.165	0.089	0.097								
Clade IV Asia1	Afghanistan(Afg09-2990)gblHM452305.1l	0.004	0.004	0.000	0.000	0.169	0.150	0.114	0.139	0.139							
	Pakistan_(Matin)_gblAF527810.1l	0.011	0.011	0.007	0.007	0.172	0.147	0.122	0.148	0.142	0.007						
	Pakistan(SR3)_emblAJ538198.1l	0.004	0.004	0.000	0.000	0.169	0.150	0.114	0.139	0.139	0.000	0.007					
	Iran_52_gblDQ446212.1l	0.007	0.007	0.002	0.002	0.166	0.153	0.111	0.142	0.142	0.002	0.009	0.002				
	Iran_53_gblDQ446213.1l	0.007	0.007	0.002	0.002	0.172	0.153	0.117	0.142	0.142	0.002	0.009	0.002	0.002			
	Iran_786_gblAY366378.1l	0.004	0.004	0.000	0.000	0.169	0.150	0.114	0.139	0.139	0.000	0.007	0.000	0.002	0.002		
Clade IV Asia 1	Ex_Afghanistan_gblJX908640.1l	0.046	0.046	0.046	0.046	0.187	0.136	0.128	0.142	0.134	0.046	0.049	0.046	0.049	0.049	0.046	
	Iraq(Baghdad)_emblAJ538196.1l	0.087	0.087	0.081	0.081	0.196	0.165	0.153	0.180	0.171	0.081	0.079	0.081	0.084	0.084	0.081	0.046
	Oman_gblDQ211645.1l	0.046	0.046	0.046	0.046	0.172	0.156	0.128	0.163	0.160	0.046	0.049	0.046	0.044	0.049	0.046	0.037
Clade IV Asia 2	India(Gujrat)gblJX051650.1l	0.108	0.108	0.108	0.108	0.212	0.142	0.149	0.173	0.164	0.108	0.105	0.108	0.105	0.110	0.108	0.102
	China(7803)gblAF354296.1l	0.094	0.094	0.089	0.089	0.199	0.135	0.136	0.147	0.157	0.089	0.086	0.089	0.086	0.091	0.089	0.104
	China(8402)emblAJ010649.1l	0.097	0.097	0.097	0.097	0.208	0.138	0.148	0.159	0.169	0.097	0.094	0.097	0.094	0.099	0.097	0.107
	Tajikistan(TAJ/HU8975)_gblAY297692.2l	0.102	0.102	0.097	0.097	0.184	0.135	0.157	0.145	0.142	0.097	0.094	0.097	0.099	0.099	0.097	0.094
	UAE(Dubai_616)_gblJN108025.1l	0.107	0.107	0.107	0.107	0.187	0.144	0.150	0.168	0.171	0.107	0.110	0.107	0.104	0.110	0.107	0.118
	Uzbekistan(UzbekTI10145)_gblAF481799.1l	0.105	0.105	0.105	0.105	0.196	0.127	0.163	0.157	0.154	0.105	0.102	0.105	0.102	0.108	0.105	0.116
Clade V	Russia/STV-HU29223(AF481802)	0.167	0.167	0.161	0.161	0.213	0.183	0.173	0.198	0.189	0.161	0.164	0.161	0.164	0.164	0.161	0.152
	Kosovo(AF404507)	0.160	0.160	0.154	0.154	0.197	0.179	0.166	0.175	0.169	0.154	0.157	0.154	0.157	0.157	0.154	0.129
	Greece(6608-Rodopi)_gblEU871766.2l	0.152	0.152	0.152	0.152	0.194	0.173	0.172	0.181	0.175	0.152	0.155	0.152	0.155	0.155	0.152	0.129
	Bulgaria(V5909)gblJF807428.1l	0.155	0.155	0.150	0.150	0.188	0.180	0.166	0.182	0.170	0.150	0.152	0.150	0.152	0.152	0.150	0.124
	Turkey_Kelkit06_gblGQ337053.1l	0.167	0.167	0.167	0.167	0.206	0.179	0.184	0.193	0.178	0.167	0.170	0.167	0.170	0.170	0.167	0.140
Clade VI	Greece(AP92)_gblDQ211638.1l	0.234	0.234	0.234	0.234	0.219	0.228	0.239	0.249	0.220	0.234	0.238	0.234	0.231	0.238	0.234	0.238
Out group	Dugbevirus(strain KT281/75AF434165)	0.564	0.564	0.554	0.554	0.547	0.543	0.521	0.555	0.566	0.554	0.549	0.554	0.559	0.559	0.554	0.570
	Hazaravirus(strain JC280M86624)	0.637	0.637	0.632	0.632	0.594	0.634	0.631	0.631	0.624	0.632	0.626	0.632	0.626	0.637	0.632	0.633

Column index continuation (columns 17–31):

	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31

Group	Isolate
New Isolates	Nad-8-494c_(*Hy. asiaticum*)
	Nad-20-510c_(*Hy. marginatum*)
	Nad-71-496c_(*Rh. appendiculatus*)
	Nad-83-519c_(*Rh. turanicus*)
Clade I	Senegal/ArD8194(U15021)

Table 4 continued

		17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Clade II	DRC(Congo_3010)_gb|DQ144418.1|															
Clade III	South_Africa(SPU_55687_)_gb|KJ682817.1|															
	Mali(Daral_2012_)_gb|KF793333.1|															
	Nigeria(IbAr)10200(U88410)															
Clade IV Asia1	Afghanistan(Afg09-2990)gb|HM452305.1|															
	Pakistan_(Matin)_gb|AF527810.1|															
	Pakistan(SR3)_emb|AJ538198.1|															
	Iran_52_gb|DQ446212.1|															
	Iran_53_gb|DQ446213.1|															
	Iran_786_gb|AY366378.1|															
Clade IV Asia 1	Ex_Afghanistan_gb|JX908640.1|															
	Iraq(Baghdad)_emb|AJ538196.1|															
	Oman_gb|DQ211645.1|	0.066														
Clade IV Asia 2	India(Gujrat)gb|IX051650.1|	0.121	0.097													
	China(7803)gb|AF354296.1|	0.121	0.099	0.063												
	China(8402)emb|AJ0I0649.1|	0.129	0.102	0.066	0.011											
	Tajikistan(TAJ/HU8975)_gb|AY297692.2|	0.113	0.113	0.074	0.037	0.039										
	UAE(Dubai_616)_gb|JN108025.1|	0.132	0.102	0.063	0.066	0.063	0.066									
	Uzbekistan(UzbekTI10145)_gb|AF481799.1|	0.135	0.119	0.082	0.039	0.042	0.034	0.074								
Clade V	Russia/STV-HU29223(AF481802)	0.163	0.138	0.155	0.135	0.141	0.138	0.155	0.144							
	Kosovo(AF404507)	0.145	0.126	0.146	0.129	0.135	0.126	0.149	0.144	0.030						
	Greece(6608-Rodopi)_gb|EU871766.2|	0.152	0.126	0.143	0.126	0.126	0.124	0.140	0.141	0.030	0.011					
	Bulgaria(V5909)gb|JF807428.1|	0.140	0.124	0.150	0.132	0.138	0.130	0.153	0.148	0.037	0.016	0.018				
	Turkey_Kelkit06_gb|GQ337053.1|	0.157	0.137	0.152	0.140	0.140	0.132	0.152	0.152	0.044	0.025	0.023	0.032			
Clade VI	Greece(AP92)_gb|DQ211638.1|	0.247	0.252	0.258	0.246	0.244	0.244	0.246	0.244	0.251	0.231	0.227	0.231	0.233		
Out group	Dugbevirus(strain KT281/75AF434165)	0.559	0.570	0.552	0.581	0.576	0.562	0.586	0.582	0.559	0.570	0.579	0.574	0.580	0.548	
	Hazaravirus(strain JC280M86624)	0.651	0.627	0.652	0.625	0.636	0.647	0.619	0.625	0.682	0.669	0.670	0.676	0.692	0.625	0.652

The first four rows relate to the new isolates found in this study. The next 28 items are available sequences in GenBank, which are classified in seven groups, clade I to clade VI, and an out-group including Dugbe virus (strain KT281/75AF434165) and Hazara virus (strain JC280M86624). The boxed cells of clade IV, Asia 1 pairwise-distance values show the most closely related strains to the ones identified in the present study
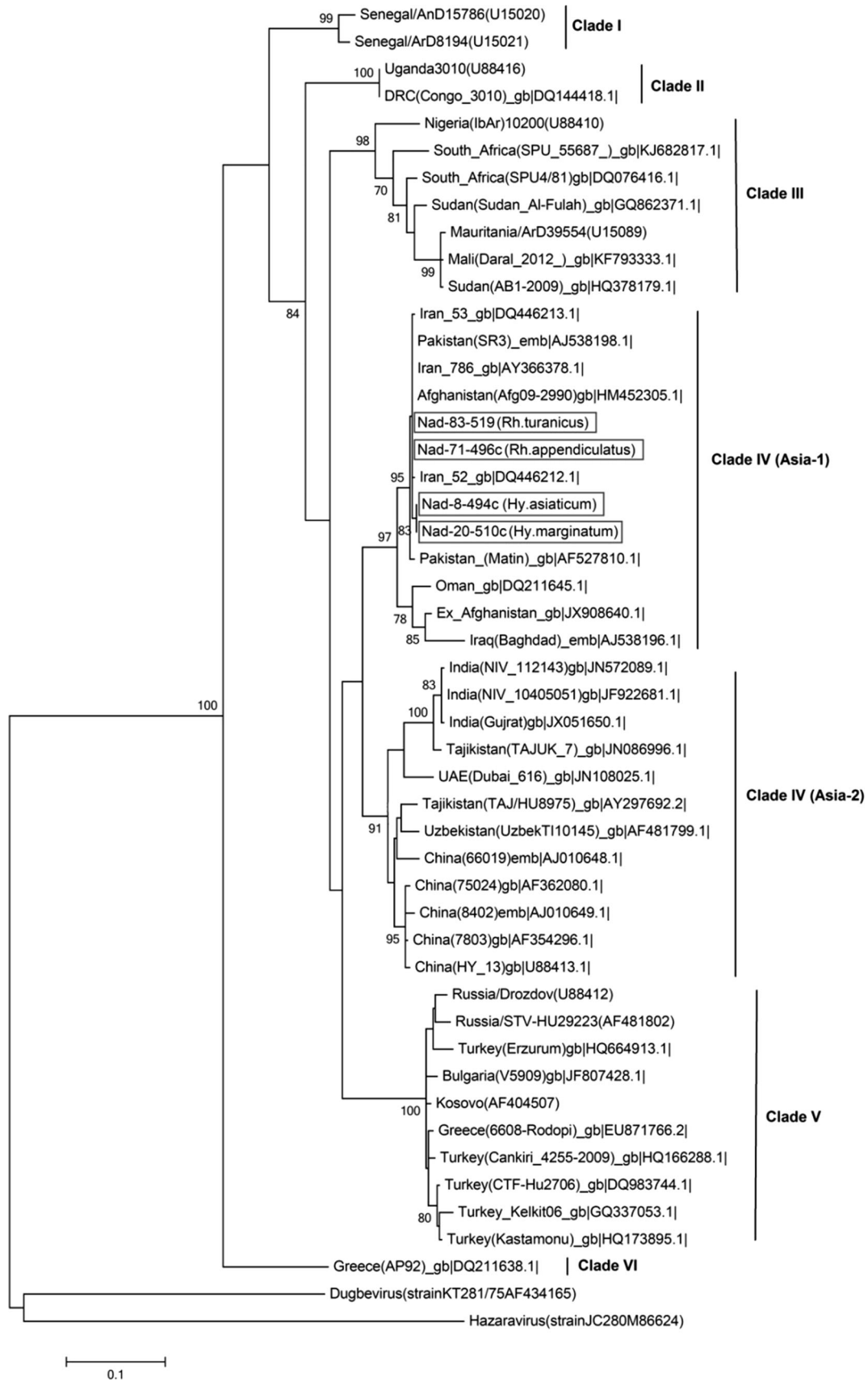
◄**Fig. 3** Unrooted phylogenetic tree based on a portion of the S segment (503 nucleotides) of CCHF virus, constructed by the maximum-likelihood using Mega5 software. Dugbe virus (strain KT281/75AF434165) and Hazara virus (strain JC280M86624) were used as outgroups. The new sequences from this study are shown in a box. The numbers at nodes indicate the bootstrap values for 1000 replicates. Clade I, W. Africa 1; Clade II, C. Africa; Clade III, S. Africa and/or W. Africa 2; Clade IV, Asia 1; Clade IV, Asia 2; and Clade VI, Greece AP92

Rhipicephalus, and *Haemaphysalis*, respectively, to be positive for the CCHFV genome by RT-PCR, [29]. In a later report from the same province, both hard and soft ticks were positive for the CCHFV genome [30]. These viruses were all genetically clustered in the main strain group circulating in Iran and closely related to the Pakistani Matin strain, except one isolate from *Rh. sanguineus*, which showed more divergence from the other isolates, possibly correlated to its geographical range [30]. The current study also found members of two species each in the genera *Hyalomma* and *Rhipicephalus* to be positive for the CCHFV genome. Most (75 %) of the CCHFV-positive samples originated from the northernmost study area, Torbat-Heydariyeh county. In an earlier report from Kurdistan province, northwest Iran, only *Hyalomma* ticks were found to be infected with CCHFV. Three remaining genera of ticks were all negative in the RT-PCR assay [18].

The eco-climatic conditions in Iran and neighbouring countries are favorable for many ticks to transmit CCHFV in nature [4, 5]. Similarly, disease outbreaks have recently progressed against a background of optimal climatic parameters and ambient changes suitable for the presence of ticks and their infested hosts [31, 32]. In addition, transborder transmission has also been suggested to be involved in CCHFV circulation [7, 33]. In these endemic regions, most tick-borne multi-host zoonotic infections of domesticated animals are treated with the use of acaricides. These infested ungulates are mostly in contact with wildlife, so transmission of the infection could hardly be prevented [34]. It has been proposed that more spatio-temporal studies are needed to elucidate the natural history of the CCHFV-tick-host system, and transmission modeling is required to better understand the maintenance of CCHFV foci in all Iranian provinces [35].

Four tick species (*Hy. asiaticum*, *Hy. marginatum*, *Rh. turanicus*,, *Rh. appendiculatus*) that were positive for the CCHFV genome were naturally caught from infested ungulates in the study region. The brown ear tick, *Rh. appendiculatus*, was positive for the CCHFV genome by RT-PCR. No evidence of its natural infection with the arbovirus causing CCHF has so far been provided. This is the first report of CCHFV infection of brown ear ticks anywhere in the world. Ticks of the genus *Hyalomma* are

still considered to be the main vectors of CCHFV, but increasing reports of infection of *Rhipicephalus* ticks suggest a potentially supportive role in viral transmission of CCHF. In accordance with the results of previous studies conducted elsewhere in different parts of the region, [15, 36] it is speculated that *Rhipicephalus* spp. could play an auxiliary role in the epidemiology of CCHFV in Iran.

It is well known that *Hyalomma* ticks are major vectors of CCHFV, and multiple hard tick species are presumed to be involved in transmission of CCHF viruses. Brown dog ticks (*Rh. sanguineus*) were found to be infected with CCHFV in Bulgaria, Iran, and Turkey [30, 37, 38]. *Rh. turanicus* was also reported to be positive for the CCHFV genome in Turkey [36, 38, 39]. *Rh. bursa* was positive in Turkey and Greece [38–40]. *Rh. guilhoni* and *Rh. evertsi evertsi* in Senegal and *Rh. evertsi mimeticus* in Africa were also reported to have the CCHFV genome [41, 42]. The new evidence of a CCHFV genome in *Rh. appendiculatus* is thus an important observation that needs to be considered in any novel reappraisal of the molecular epidemiology and transmission dynamics of CCHFV in southwest Asia.

All four strains of CCHFV identified in *Hyalomma asiaticum*, *Hy. marginatum*, *Rh. turanicus*, and *Rh. appendiculatus* in Khorasan-Razavi province were clustered in the same lineage with the Matin and SR3 strains from Pakistan; Afg09-2990 from Afghanistan; and the Iran-52, Iran-53, and 786/02 strains from Iran (Fig. 3). These four strains were genetically distinguishable from the Baghdad strain from Iraq, the Oman strain from Oman, and the SCT ex Afghanistan strain from Afghanistan (Fig. 3). These results showed that CCHFV in this province of Iran is genetically closely related, even in the different tick species. This finding is in line with other reports [8, 30, 43]. However, the Oman and Iraq lineages showed higher divergence from the virus sequences in the present study.

There is no doubt that a single RT-PCR-positive finding in a tick species does not necessarily equate to its status as a competent vector. Since the tick samples selected for RT-PCR were depleted, this rules out the possibility of ingestion of a CCHFV-positive blood meal from an infected ungulate host without actual infection of the tick itself. Furthermore, even if a tick species is legitimately CCHFV positive, this does not mean that it is competent as a vector. It should be noted that several other criteria have to be met in order for a given arthropod species to have status of vector. Some of these criteria are (1) natural bloodsucking of the suspected tick on humans or other hosts, (2) sustained survival of the virus at different life cycle stages (eggs, larvae, nymphs) of the vector, (3) exact identity of the virus in the vector and vertebrate host, (4) the ability of the vector to transmit the virus by bite, and (5) vector abundance.

In conclusion, the presence of CCHFV in four different species of hard ticks in northeast Iran was confirmed using RT-PCR. For the first time, the viral genome was found in the brown ear tick *Rh. appendiculatus*, suggesting that it is a potential new natural vector of CCHFV in Iran. It was also shown by phylogenetic analysis that CCHFV isolates in this region are genetically closely related, even in ticks of different species. They were clustered in the same lineage with some strains from Afghanistan, Iran and Pakistan.

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