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ORIGINAL PAPER

Molecular detection of Crimean-Congo hemorrhagic fever virus in ticks, Greece, 2012–2014

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Abstract Crimean-Congo hemorrhagic fever virus (CCHFV) is transmitted to humans mainly through the bite of infected ticks. In Greece, only one clinical case has been observed, in 2008, but the seroprevalence in humans is relatively high (4.2%). To have a first insight into the circulation of CCHFV in Greece, 2000 ticks collected from livestock during 2012–2014 were tested. CCHFV was detected in 36 of the 1290 (2.8%) tick pools (1–5 ticks per pool). Two genetic lineages were identified: Europe 1 and Europe 2. Most Europe 1 sequences were obtained from *Rhipicephalus sanguineus* sensu lato ticks, while most Europe 2 sequences were recovered from *Rhipicephalus bursa* ticks. The number of collected *Hyalomma marginatum* ticks (the principal vector of CCHFV) was low (0.5% of ticks) and all were CCHFV negative. Since it is not known how efficient ticks of the *Rhipicephalus* genus are as vectors of the virus, laboratory studies will be required to explore the role of *Rhipicephalus* spp. ticks in CCHFV maintenance and transmission.

Keywords Crimean-Congo hemorrhagic fever virus · Ticks · Greece · Lineage

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Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) is an orthonairovirus (family *Nairoviridae*) which causes a potentially severe febrile disease (CCHF) in humans, with fatality rates up to 40% (World Health Organization, 2013). The virus circulates in nature in an enzootic tick-vertebrate-tick cycle, and it is transmitted to humans by bite of infected ixodid ticks (mainly of the genus *Hyalomma*), or by direct contact with blood or tissues from viremic patients or animals (Bente et al. 2013). Ticks are vectors and reservoirs of the virus, which is maintained in ticks by transovarial, transstadial, and venereal transmission, while co-feeding (infection of an uninfected tick that is feeding simultaneously with an infected tick on the same host in the absence of systemic infection) is an additional epidemiologically significant way for virus transmission (Gargili et al. 2017). Immature *Hyalomma* ticks feed on small animals, such as hares, hedgehogs, and ground-feeding birds, while adult ticks feed on larger animals, such as livestock. Infected animals present a short viremia (up to 2 weeks) while showing no signs of illness (OIE 2014).

Although CCHF endemic foci are present in the Balkan Peninsula, only one human case has been reported in Greece. The case occurred in 2008 and was caused by the Rodopi strain which clusters in Europe 1 lineage, together with pathogenic strains from other Balkan countries and Turkey (Papa et al. 2008). However, the first detection of CCHFV in Greece was much earlier, in 1975, when the AP92 strain was isolated from *Rhipicephalus bursa* ticks collected from goats in the northern part of the country (Papadopoulos and Koptopoulos 1978). AP92 is the prototype strain of lineage Europe 2, one of the seven genetic lineages in which CCHFV strains are grouped on the basis of S RNA segment sequences (Papa et al. 2015). A relatively high CCHFV seroprevalence in humans has been reported in

Greece (4.2%), varying from 0 to 14.5% among prefectures (Sidira et al. 2012). Increased age, agro-pastoral activities, slaughtering livestock, and living or working in close proximity with livestock have been associated with seropositivity (Papa et al. 2013). A spatial cluster meta-analysis study showed that CCHFV seroprevalence in the western part of Greece is significantly higher than that in the eastern part, and that altitude, land cover type, transitional woodland/shrubland per person, and number of livestock (goats, sheep, and cattle) per person are related significantly with seropositivity (Papa et al. 2016). In the absence of human cases, it was suggested that high seroprevalence is related to the circulation of low-pathogenicity strain(s) (Sidira et al. 2012). AP92-like strains have recently been detected in ticks in Kosovo, Bulgaria, and Turkey, while very few mild CCHF cases associated with the AP92-like strain have been reported in Turkey (Elevli et al. 2009; Midilli et al. 2009; Ozkaya et al. 2010; Panayotova et al. 2016; Papa et al. 2014; Sherifi et al. 2014). In an effort to identify the circulating CCHFV strains in Greece, we collected ticks from livestock in various regions of the country and tested them for CCHFV infection.

Materials and methods

Tick collection and identification

Greece is a Balkan country in southeastern Europe with a surface area of 131,960 km^2. According to the 2001 census, the population of Greece was 10,964,020. The country is divided into nine districts: Thrace, Macedonia, Epirus, Thessaly, Central Greece, Peloponnese, Aegean islands, Ionian islands, and Crete. Each district is further divided into prefectures.

Tick collection was performed in various locations in eight of the nine districts (all except the Ionian islands). Two thousand adult ticks were collected from sheep and goats in farms located in 222 villages in 26 prefectures in 8 districts. The collection was conducted from April to July, 2012 (1091 ticks), from March to November, 2013 (865 ticks), and in June, 2014 (44 ticks). Ticks were transported and refrigerated to the laboratory and were stored at − 80 °C until testing. The identification was performed morphologically under a stereomicroscope using taxonomic keys (Estrada-Peña et al. 2004). Ticks were grouped into pools (1–5 per pool) according to the collection date, location, animal host, tick species, and sex.

RNA extraction and molecular screening

Before testing, ticks were washed with distilled water and were homogenized in PBS in a FastPrep FP120 cell disrupter (Bio-101, Thermo Savant; Q-Biogene, Carlsbad, CA, USA) using glass beads (150–212 μm in diameter). RNA extraction was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An RT-nested PCR was applied which amplifies a partial fragment of the CCHFV S RNA segment (Rodriguez et al. 1997). All PCR products were sequenced. A real-time RT-PCR (Wolfel et al. 2007) was applied to those positive samples from which a sequence could not be obtained. Nucleotide sequences were tested using the Basic Local Alignment Search Tool (BLAST). The CCHFV sequences were aligned with respective sequences from the GenBank database using Clustal W. A phylogenetic tree was constructed using the maximum likelihood method. The evolutionary distances were computed using the Kimura 2-parameter method using MEGA 7 (Kumar et al. 2016).

Results

A total of 2000 adult ticks were collected from sheep and goats in Greece. Most (55.9%) were female. The ticks belonged to four genera: *Rhipicephalus* (1894, 94.7%), *Hyalomma* (58, 2.9%), *Dermacentor* (47, 2.4%), and *Ixodes* (1, 0.1%). Most of the ticks were *R. sanguineus* sensu lato (s.l.) (1370, 68.5%) and *R. bursa* (524, 26.2%) (Table 1). Grouping by collection date, location, animal host, tick species, and sex resulted in 1290 pools.

CCHFV RNA was detected in 36 (2.8%) of the tick pools (Table 2). CCHFV-positive ticks were detected in 28 of 222 (12.6%) villages located in 6 of the 8 districts tested. The percentages of CCHFV-positive tick pools per district and prefecture are seen in Table 2 and Fig. 1. For comparison purposes, the respective seroprevalence rates in humans, as estimated in a previous study (Sidira et al. 2012), are also shown in Fig. 1.

All CCHFV-positive ticks belonged to the genus *Rhipicephalus*. Among the 36 positive tick pools, 27 consisted

Table 1 Ticks collected from sheep and goats in Greece during 2012–2014 and tested for CCHFV

Tick genus	*N* (%)	Tick species	*N* (%)
Rhipicephalus	1894 (94.7)	*R. sanguineus* s.l.	1370 (68.5)
		R. bursa	524 (26.2)
Hyalomma	58 (2.9)	*H. dromedarii*	37 (1.9)
		H. marginatum	10 (0. 5)
		H. rufipes	8 (0.4)
		H. excavatum	2 (0.1)
		H. impeltatum	1 (0.1)
Dermacentor	47 (2.4)	*D. marginatus*	47 (2.4)
Ixodes	1 (0.1)	*I. ricinus*	1 (0.1)
Total	2000 (100)		2000 (100)

Table 2 Number (*N*) of CCHFV positive tick pools per district and prefecture of Greece

District	*N* of ticks (pools)	Positive pools (%)	Prefecture	*N* of ticks (pools)	*R. sanguineus* s.l. Positive pools/ *N* of pools (%)	*R. bursa* Positive pools/ *N* of pools (%)	Other tick species Positive pools/ *N* of pools (%)	Total positive pools/ *N* of pools (%)
Thrace	636 (418)	6 (1.4)	Evros	98 (66)	3/42 (7.1)	0/17	0/7	3/66 (4.5)
			Rodopi	439 (288)	3/208 (1.4)	0/67	0/13	3/288 (1.0)
			Xanthi	99 (64)	0/47 (0)	0/9	0/8	0/64 (0)
Macedonia	412 (251)	22 (8.8)	Kavala	23 (14)	0/9 (0)	0/5	0	0/14 (0)
			Serres	47 (27)	0/20 (0)	0/7	0	0/27 (0)
			Thessaloniki	56 (26)	1/19 (5.3)	0/7	0	1/26 (3.8)
			Chalkidiki	63 (41)	0/31 (0)	0/9	0/1	0/41 (0)
			Pella	160 (103)	1/57 (1.8)	0/31	0/15	1/103 (1.0)
			Imathia	86 (54)	2/34 (5.9)	0/18	0/2	2/54 (3.7)
			Kozani	84 (49)	1/30 (3.3)	0/19	0	1/49 (2.0)
			Kastoria	157 (150)	5/90 (5.6)	9/56 (16.1)	0/4	14/150 (9.3)
			Florina	71 (41)	3/26 (11.5)	0/15	0	3/41 (7.3)
Epirus	137 (81)	1 (1.2)	Thesprotia	48 (28)	1/20 (5.0)	0/6	0/2	1/28 (3.6)
			Ioannina	89 (53)	0/38 (0)	0/15	0	0/53 (0)
Thessaly	162 (98)	3 (3.1)	Larissa	68 (29)	2/20 (10.0)	0/9	0	2/29 (6.9)
			Trikala	38 (28)	0/15 (0)	0/13	0	0/28 (0)
			Karditsa	56 (41)	1/24 (4.2)	0/17	0	1/41 (2.4)
Central Greece	123 (87)	0	Fokida	50 (38)	0/20 (0)	0/18	0	0/38 (0)
			Evia	35 (15)	0/12 (0)	0/3	0	0/15 (0)
			Evritania	3 (3)	0/2 (0)	0/1	0	0/3 (0)
			Fthiotida	37 (31)	0/17 (0)	0/14	0	0/31 (0)
Peloponnese	80 (41)	2 (5.7)	Corinthia	71 (35)	2/26 (7.7)	0/9	0	2/35 (5.7)
		0	Ilia	9 (6)	0/6 (0)	0/0	0	0/6 (0)
Aegean Islands	59 (28)	0	Lesvos	41 (18)	0/0 (0)	0/0	0/18	0/18 (0)
		0	Cyclades	18 (10)	0/7 (0)	0/3	0	0/10 (0)
Crete	56 (32)	2 (6.2)	Rethymno	56 (32)	2/23 (8.7)	0/9	0	2/32 (6.3)
Total	2000 (1290)	36 (2.8)		2000 (1290)	27/843 (3.2)	9/377 (2.4)	0/70	36/1290 (2.8)

of *R. sanguineus* s.l. ticks and 9 consisted of *R. bursa* ticks. Overall, CCHFV was detected in 27 of the 843 (3.2%) *R. sanguineus* s.l. tick pools and 9 of the 377 (2.4%) *R. bursa* tick pools (Table 2).

Sequences were obtained from 27 tick pools (Fig. 2), but for 9 positive pools, sequencing was not successful. When these pools were tested by real-time PCR, the Ct value was ≥ 37, suggesting that the viral load was low. BLAST and phylogenetic analyses showed that sequences clustered into two genetic lineages, Europe 1 and Europe 2. Eight sequences belonging to Europe 2 were 100% identical and were obtained from ticks collected in Kastoria prefecture; 6 were taken from *R. bursa* ticks, and two were taken from *R. sanguineus* s.l. ticks. All the rest of the 19 sequences clustered into lineage Europe 1, and most (16/19) came from *R. sanguineus* s.l. pools.

A phylogenetic tree based on a 220-bp fragment of the S RNA segment was constructed (Fig. 2). The mean genetic difference between the two lineages (Europes 1 and 2) is 23% at the nucleotide level. The mean genetic diversity among Europe 1 sequences of the present study is 1.4%, while the Europe 2 sequences were identical, differing by 10.4% from the prototype AP92 strain (Ac. No. DQ211638).

Sequences from the study were submitted to the GenBank dataBase under accession numbers KF146306, MF004261-MF004267, and MF780718-MF780723.

Discussion

In order to obtain insight into CCHFV strains circulating in Greece, ticks were collected from livestock in several

Fig. 1 Map of Greece showing the percentage of CCHFV-positive tick pools per prefecture. The percentages in parentheses indicate the CCHFV IgG seroprevalence in humans, based on previous studies (Sidira et al. 2012). The map was obtained from commons.wikimedia.org

geographical regions and tested for CCHFV infection. Viral RNA was detected in 3.2 and 2.4% of the pools of *R. sanguineus* s.l. and *R. bursa* ticks, respectively. The percentage of positive pools in districts of Greece ranged from 0 to 9.3% (mean 2.8%) which correlates quite well with results from previous seroprevalence studies in humans (Fig. 1) (Papa et al. 2016). The respective percentage in endemic countries such as Turkey, Kosovo, and Bulgaria is 2 to 11%, depending on the endemicity level of the area (Panayotova et al. 2016; Sherifi et al. 2014; Tekin et al. 2011; Tonbak et al. 2006). However, to avoid biases in comparison among studies, several parameters should be taken into account, such as methods used for tick collection (from the ground or from livestock), seasonality, tick and host species, number of ticks collected per animal, farm and location, husbandry conditions, usage of repellents, and the number of ticks in the tested pools.

Two genetic lineages, Europe 1 and Europe 2, were detected. The genetic variability among sequences was relatively high, reflecting a long history of virus co-evolution with its tick reservoirs (Honig et al. 2004). Europe 2 sequences were detected mainly in *R. bursa* ticks (6 of the 8 Europe 2-positive pools consisted of *R. bursa* ticks). This finding, together with previous reports from the Balkans and Turkey, suggests that this lineage is strongly associated with *R. bursa* ticks, at least in this geographic area. The percentage of Europe 2-positive *R. bursa* ticks in Kastoria prefecture was high (7/56 tick pools, 16.1%), suggesting that the environmental conditions and the presence of suitable hosts are favorable for the maintenance of

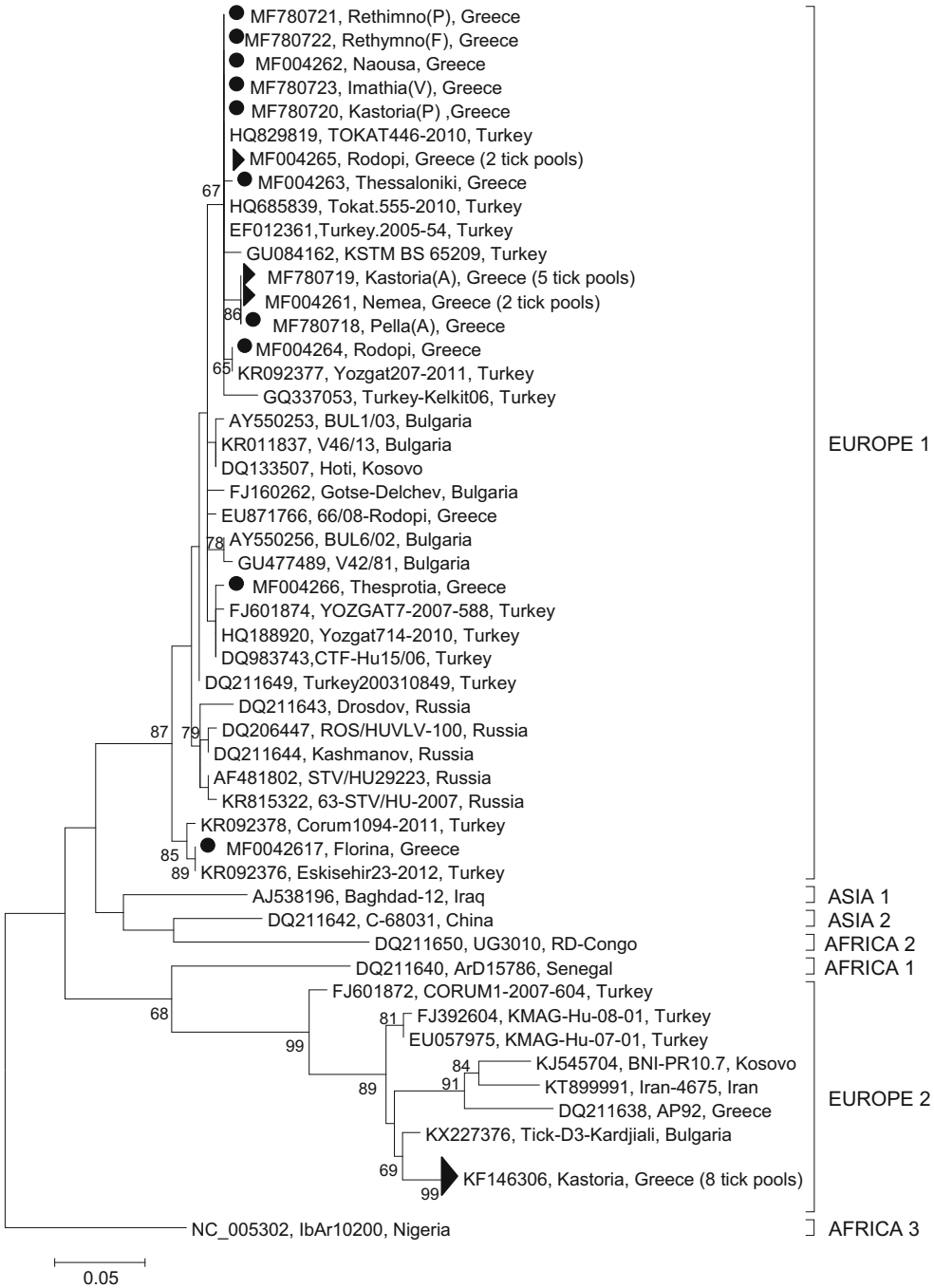

Fig. 2 Maximum likelihood phylogenetic tree, based on a 220-nt fragment of the S RNA segment of CCHFV. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Virus strains are labeled by GenBank accession number, name, and country of origin. Bootstrap values were obtained based on 1000 replicates; only values > 60% are shown. Sequences of the current study are marked. Identical sequences from the same region are shown with a triangle

this strain in the region. Analogous focused circulation of AP92-like strains (Europe 2) was observed in Kosovo and Bulgaria (Panayotova et al. 2016; Sherifi et al. 2014). Especially in a study in Kosovo, AP92-like sequences were obtained exclusively from *R. bursa* ticks in a non-endemic region, while Europe 1 sequences were detected in *H. marginatum* ticks or in humans, predominantly in the hyper-endemic regions (Sherifi et al. 2014). These observations strengthen previous suggestions that AP92-like strains are of low pathogenicity (Antoniadis and Casals 1982; Papa et al. 2014; Sidira et al. 2012). A recent report on a fatal case in Iran associated with an AP92-like strain (Salehi-Vaziri et al. 2016) suggests that full genome sequences are needed for in-depth analysis of strains of this lineage, to identify possible markers of increased pathogenicity.

Europe 1 sequences were detected mainly in *R. sanguineus* s.l. ticks. The number of collected *H. marginatum* ticks, the principal vectors of CCHFV, was low (only 2.9% of the collected ticks), and they were CCHFV negative. The low number may be due to the fact that the ticks were collected from

sheep and goats, and not from cattle, the main hosts of adult *H. marginatum* ticks. The collection was not done from cattle because in Greece, they are usually enfarmed and have less chances to become infested by ticks. Furthermore, a recent study showed that small ruminants (sheep, goats) are more suitable indicator animals than cattle for the detection of CCHFV circulation in an area (Schuster et al. 2016). This was also shown in a spatial cluster analysis of CCHFV seroprevalence in humans in Greece: CCHFV seropositivity was significantly associated with living or working in close proximity to sheep, goats, and cattle ($p < 0.001$, OR 4.62). However, the risk was higher when working with sheep ($p < 0.001$, OR 4.72) or goats ($p < 0.001$, OR 3.73), than that with cattle ($p = 0.006$, OR 2.14) (Papa et al. 2016).

Until recently, the high CCHFV seroprevalence in the Greek population in the absence of clinical cases was attributed to the probable circulation of low-pathogenic strain(s) (Sidira et al. 2012). The current study shows that, in addition to Europe 2, strains of Europe 1 lineage circulate in Greece and cluster together with pathogenic strains. However, they were detected in *Rhipicephalus* spp. ticks, which might be less efficient virus vectors than *H. marginatum*. It is known that for an arthropod to be incriminated as an actual vector, several criteria must be met, including vector competence in laboratory studies; evidence that the arthropod species feeds in nature on a host that develops an appropriate viremia; and evidence that it is active at the time of year when viral transmission is occurring (Reeves 1957; Turell 2007). It is also well accepted that the detection of virus in wild-caught ticks, especially in ticks collected from animals, may only indicate that the ticks have recently fed on a viremic mammal, and not that they are competent vectors (Shepherd et al. 1991), since some tick species may harbor the virus and transmit it among mammalian animals, without serving as a source of human infection. Laboratory studies on vector competence will be required to determine the role of *Rhipicephalus* spp. ticks in CCHFV maintenance and transmission.

Regardless of whether ticks transmit virus transovarially, adult ticks which become infected by feeding may be an important source of human infection if removed by hand or squashed (Shepherd et al. 1991). Given that ticks are vectors of several pathogens, including CCHFV, avoidance of tick habitat is recommended, while preventive measures should be taken especially by persons working in the livestock industry. These include wearing protective clothing and gloves, use of repellents, regular examination of clothing and skin for ticks, and the use of acaricides on livestock and other domestic animals.

The present study provides a baseline for the circulation of CCHFV in ticks in Greece and shows that many issues remain to be elucidated to explain the enigmatic epidemiology of the disease in Greece. Studies using next-generation sequencing and proteomic analysis will identify genetic differences among virus strains and among tick species and show how they affect virus entry into the host cell, the host immune response, and general virus-tick-host interactions.

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