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Comparison of the prevalence of Crimean-Congo hemorrhagic fever virus in endemic and non-endemic Bulgarian locations

Ivanka Gergova & Bozhin Kamarinchev

Department of Military Epidemiology and Hygiene, Military Medical Academy, Sofia, Bulgaria

ABSTRACT

Background & objectives: The Balkans is an endemic region for Crimean-Congo *hemorrhagic* fever (CCHF), caused by the CCHF virus (CCHFV). Several Bulgarian regions comprised of smaller locations are categorized either as endemic or non-endemic for CCHF. However, little is known about the dynamics that underlie the development of endemicity within the locations throughout the years.

Methods: Seven locations categorized as endemic in one central Bulgarian region (Stara Zagora) were compared to seven non-endemic areas. During the period 2006–12, a total of 1775 blood samples from cattle, were tested for anti-CCHFV antibodies using an indirect immunofluorescence antibody assay. Also, the infestation of 617 mature ticks for CCHFV was studied using a combination of an immunofluorescence haemocytes assay and molecular-virological methods.

Results: Anti-CCHFV antibodies were established in 7.89% (140/1775) of the sera. The average CCHFV-infestation in the ticks was 1.46% (9/617). CCHFV was detected in three tick species: *H.m. marginatum* (3.73%, 6/161), being the main vector of the infection; *R. sanguineus* (1.63%, 2/123); and *I. ricinus* (1.96%, 1/51).

Interpretation & conclusion: The data for the endemic and non-endemic locations did not reveal significant differences for the prevalence of CCHFV. Mosaic dispersion of the virus was determined in the studied region and the results did not vary significantly throughout the investigated years.

Key words Cattle; CCHFV; IFA; RT-PCR; tick

INTRODUCTION

The Crimean-Congo hemorrhagic fever virus (CCHFV) belongs to the genus *Nairovirus* (Family *Bunyaviridae*) and causes a tick-borne disease characterized in humans by the severe hemorrhagic syndrome[1]. The virion of CCHFV is with a spherical shape and a diameter of 90 nm. The three negative-sense single-stranded RNA segments (S, M and L segments) comprising the viral genome, encode the nucleocapsid (N) protein, the two surface glycoproteins—G1 and G2, and the viral RNA polymerase[2]. CCHFV is transmitted to humans through bites from infected ticks or after direct contact with blood or tissues from viremic humans or animals[1]. The virus causes the disease called Crimean-Congo hemorrhagic fever (CCHF), a zoonotic disease with clinical features recognized in humans by the severe hemorrhagic fever with toxic syndrome, a rapid progression and potential for transmission from human to human[3]. High mortality rate (reaching 50%) makes CCHF a significant public health concern[2, 4–5].

CCHFV circulates in the nature through asymptomatic infections between the vector (ticks) and reservoirs of infection (small vertebrate mammalians and domestic animals)[1]. Different ruminants possess similar susceptibility to CCHFV and surveys of animal sera are useful to determine the prevalence of infection[6]. Ticks are noteworthy, not only as viral vectors, but also as reservoirs, making them an important factor for the epidemiology of CCHF. Infected ticks can be used as an indicator to determine the intensity of the viral circulation in nature. Factors that influence the transmission of CCHFV include the density of competent vector ticks, particularly of the genus *Hyalomma*, and the abundance of vertebrates that serve both as hosts to these ticks and possible reservoirs of the virus[1].

Studies concerning the prevalence of CCHFV (reservoirs and vector) were performed in Bulgaria during the 1970s[7] and 1980s[8], for arbitrary areas by complement fixation and immunofluorescence hemocytes (IFH) assay. These studies only covered separate regions from the country and since then similar investigations were not performed. Thus, the relationship between the diversity of tick species and the magnitude of CCHFV transmission within particular geographic regions has not been systematically studied in Bulgaria and little is known about the epidemiological processes that drive the evolution of the virus.

The study compares the prevalence of anti-CCHFV antibodies in livestock and CCHFV-infestation among ticks that were collected in central Bulgaria from areas previously described as endemic and non-endemic (control) ones. The distribution of ticks positive for CCHFV among the different tick species was also outlined. Both classical methods—Indirect immunofluorescence antibody (IFA) assay, IFH assay and a modern molecular technique (RT-PCR) were used.

MATERIAL & METHODS

Study area

There have been more than 760 endemic locations registered and mapped in details in the northeastern and central parts of the country. Locations were considered as endemic according to the registered cases with CCHF and their distribution in Bulgaria[6]. The survey was conducted in the governmental administrative region Stara Zagora (central Bulgaria, Fig. 1) in 14 locations. Half of them were already established as endemic and the remaining seven were considered as control locations (CCHF cases were not previously documented). The region of Stara Zagora is situated in a Front-Mediterranean climate zone with an average precipitation of 550 to 600 mm/yr, plain and low mountainous relief and an altitude between 100 and 200 m above the sea level.

Animal serology

A total of 1775 blood samples from domestic animals were obtained from 2006–12. Cattle were preferably selected, because they can be 10-fold more heavily infected by ticks than sheep and thus, represent a more sensitive indicator of low level CCHFV circulation[9]. Moreover, on an average cattle are longer-lived. Blood specimens were obtained from each animal in sterile test tubes after slaughter. Tubes were labeled with collection points noted and age of the animals.

Collection of ticks

A total of 617 tick specimens were detached from domestic ruminants (cattle). Sampling of ticks was done during the period 2006–10. After removal from the animals each tick was kept alive in a separate vial which was labeled with collection points noted, and sent to the laboratory for species identification and IFH assay. Only ticks in mature stages were selected and morphologically identified in the laboratory of "Medical Arachnoentomology and Zoology" of the National Center of Infectious and Parasitic Diseases (NCIPD) Sofia, Bulgaria. After IFHA testing, ticks were frozen and stored at –70°C, and used for investigation by RT-PCR.

Immunofluorescence assays

The indirect IFA assay was performed with an antigen prepared as previously described by Kamarinchev *et al*[8]. Brain suspensions from suckling mice intracerebrally infected with the viral strain CCHFV-V42/81 were used. After inactivation with 0.4% formalin, Teflon-templated microscope slides were prepared with 12 spots per slide, air-dried, fixed in acetone for 10 min, and stored at –70°C prior to examination.

The indirect IFA assay[8], included a two-stage "sandwich" procedure. During the first stage, 25 µl from each diluted serum was placed to a slide, which was incubated in a humid closed chamber (30 min, 37°C). Following incubation, the slides were triple washed with PBS to remove unbound serum antibodies. In the second stage, each antigen well was overlaid with anti-cattle hyper immune serum conjugated with fluorescein isothiocyanate (FITC)-

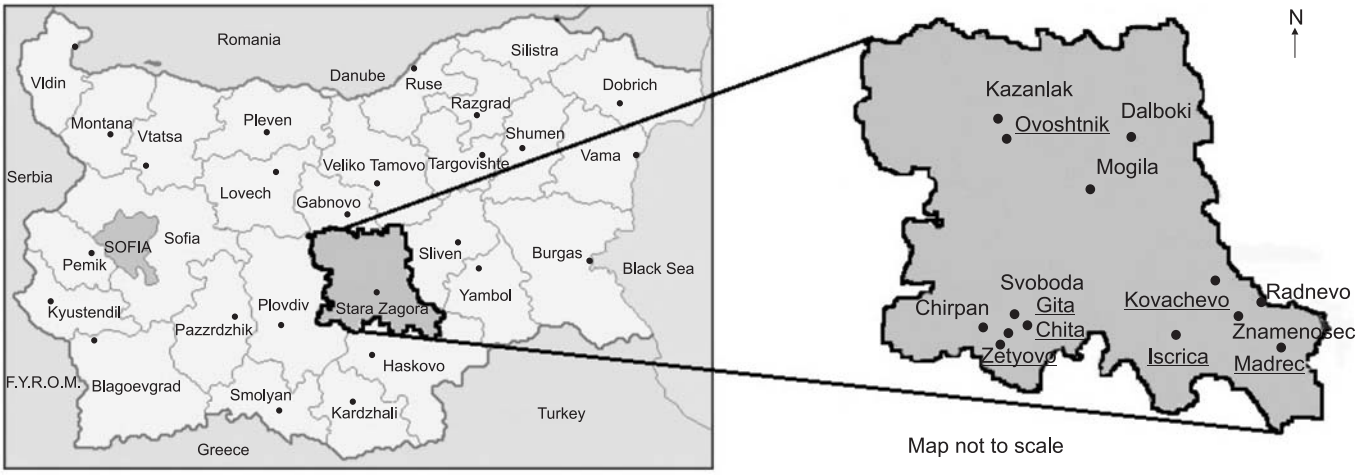

Fig. 1: Map of the studied region (Stara Zagora) showing endemic and non-endemic (underlined names) locations.

labeled antibody with Evans blue dye (concentration 1:10000) incorporated as a counter stain. The stained slides were incubated and washed under the same conditions as in the first stage. Each run of the assay included positive, negative and buffer controls.

Detection of specific CCHFV antigen was performed with IFH assay according to Rehacek *et al*[10] after modification by Kamarinchev *et al*[8], in order that the technique is applied to arboviruses. In brief, live ticks were surface disinfected (3% hydrogen peroxide and 70% ethanol for 10 min). Next, hemolymph was obtained by sectioning a tick's leg and a drop was used to directly smear 12-spot slides, which were air-dried for 30 to 60 min and fixed in cold acetone for 20 min. FITC-labeled goat antibodies to CCHFV (NCIPD, Sofia, Bulgaria) were added and the stained hemolymph preparations were incubated for 1 h at 37°C in a humid closed chamber, triple washed with PBS, mounted on microscope slides and examined by fluorescence microscopy. An appropriate barrier and excitation filters for FITC visualization were applied.

PCR assays

Homogenates were obtained from smashed ticks after centrifugation at 1500 rpm for 15 min at 4°C in sterile conditions. Trizol-LS (Invitrogen) reagent and chloroform, followed by isopropanol precipitation were used according to the manufacturer's recommendations for total-RNA extraction from 250 µl of the clarified supernatant.

ThermoScript RT-PCR System (Invitrogen) was used for reverse transcription (RT) of the extracted RNA following the manufacturer's instructions. Next, a two-round nested PCR was conducted as described by Tang *et al*[11] with slight modifications of the cycling conditions. The primers were targeted for partial amplification of the viral S segment. A total of 2 µl of the cDNA template were used in the first-round-PCR. The reaction was conducted in a 50 µl with 22 mM Tris-HCl (pH 8.4), 55 mM potassium chloride, 1.65 mM magnesium chloride, 20 pmol F2C primer (5'-TGG ATA CTT TCA CAA ACT C-3'), 20 pmol R3 primer (5'-GAC AAA TTC CCT GCA CCA-3'), 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP and 1.1 U recombinant Taq DNA Polymerase/ml (Invitrogen). The following cycling conditions were used: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 60 sec, and a final extension at 72°C for 10 min. The primers in the second-round-PCR were: F3C primer (5'-GAG TGT GCC TGG GTT AGC TC-3') and R2C primer (5'-GAC ATT ACA ATT TCG CCA GG-3'), under the same conditions as for round one. RNA extracted from the CCHFV strain V42/81 was used as a positive control in each RT-PCR run.

The PCR-products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis (100 mV for 90 min at room temperature) under UV illumination.

RESULTS

Prevalence of anti-CCHFV antibodies in animal sera

A total of 1775 cattle sera were tested by indirect IFA assay for the presence of anti-CCHFV antibodies (Table 1). In all surveyed locations, an average prevalence of 7.89% (140/1775) was established. The detection rate for anti-CCHFV antibodies varied from 1.04% (1/96) to 16.54% (22/133). The presence of anti-CCHFV antibodies in the endemic and non-endemic locations was 7.68% (76/989) and 8.14% (64/786), respectively. The prevalence of anti-CCHFV antibodies in endemic and non-endemic locations was similar and CCHFV-circulation was relatively constant during the years (data not shown in detail).

The spatial pattern of CCHFV transmission, as indi-

Table 1. Prevalence of CCHFV-positive ticks and anti-CCHFV antibodies in central Bulgaria

Location	No. of CCHFV positive ticks	No. of CCHFV-positive *H.m.marginatum* ticks	Anti-CCHFV antibodies positive sera
Chirpan	0/40 (0)	0/16 (0)	6/154 (3.9)
Dalboki	2/20 (10)	1/12 (8)	14/215 (6.51)
Kazanlak	1/97 (1.03)	0/14 (0)	8/146 (5.48)
Mogila	1/162 (0.62)	1/47 (2.1)	18/122 (14.75)
Radnevo	0/40 (0)	0/14 (0)	0/46 (0)
Svoboda	0/2 (0)	–	1/62 (1.61)
Znamenosec	1/48 (2.08)	1/17 (6)	29/244 (11.89)
Total for endemic locations	5/409 (1.22)	3/120 (2.5)	76/989 (7.68)
Chita	0/12 (0)	–	18/179 (10.06)
Gita	0/13 (0)	0/6 (0)	1/96 (1.04)
Iscrica	0/32 (0)	–	2/66 (3.03)
Kovachevo	0/13 (0)	–	0/59 (0)
Madrec	0/61 (0)	0/16 (0)	21/183 (11.48)
Ovoshtnik	1/24 (4.17)	1/11 (9.1)	0/70 (0)
Zetyovo	3/60 (5)	2/8 (25)	22/133 (16.54)
Total for non-endemic locations	4/208 (1.92)	3/41 (7.3)	64/786 (8.14)
Total for region of Stara Zagora	9/617 (1.46)	6/161 (3.73)	140/1775 (7.89)

Figures in parentheses indicate percentages.

cated by antibody prevalence, varied among the natural areas. A higher than the average prevalence of anti-CCHFV antibodies was documented in some locations, whereas in other locations anti-CCHFV antibodies were not found.

Prevalence of CCHFV in the tick population

A total of 617 mature ticks were collected from cattle (region of Stara Zagora) during 2006–10. Nine (9 out of 617, 1.46%) from all of the investigated ticks were determined as positive for CCHFV using IFH assay and confirmed through RT-nested PCR. All the nine examined samples produced bands of the expected size (262 bp) with the F2C/R3 and F3C/R2C primer sets. In addition, all IFH assay-negative ticks, pooled into groups of 2 to 10 according to species, hosts, and geographic origin were also tested by RT-nested-PCR. All pools were negative for CCHFV, using RT-PCR (Table 1). The prevalence of CCHFV in the separate locations studied in this region varied from 0 to 10%, it was relatively constant during the years, i.e. 1.12% for 2006, 1.08% for 2007 and 2.63% for 2010.

Prevalence of CCHFV-distribution of tick species

The ticks that were collected from the investigated areas belonged to eight different tick species, but CCHFV was only found in three of them — *H.m. marginatum*, *R. sanguineus* and *I. ricinus* (Fig. 2). These three tick species were found in all locations in the surveyed region.

The most abundant species was *H.m. marginatum* which constituted 26% (161/617) of the studied ticks and 3.73% (6/161) of them were infected by CCHFV. *R. sanguineus* was the second most abundant tick species in this investigation with 20% of the total number of ticks (123/617). CCHFV was found in 1.63% (2/123) of the tested *R. sanguineus* ticks. From the entire group of ticks 51 out of 617 (8%) were identified as *I. ricinus*. Among these CCHFV was found in 1 out of 51 ticks (1.96%).

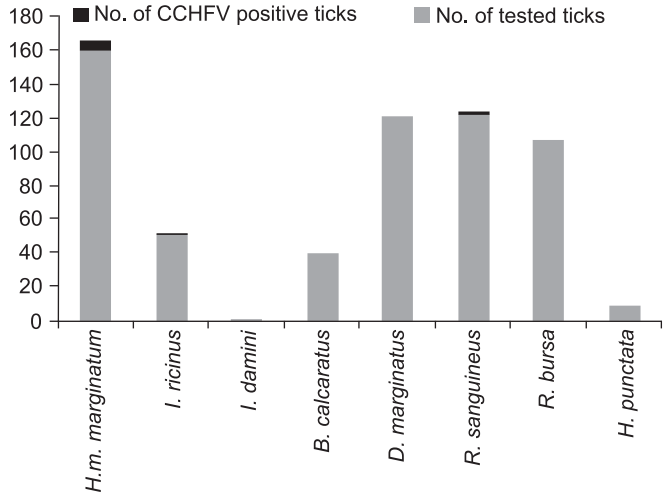

Fig. 2: CCHFV-infestation among ticks from the region of Stara Zagora—Tick species distribution and CCHFV-positive ticks.

DISCUSSION

Several epidemiological investigations reported the widespread geographic distribution of CCHF in over 30 countries situated in southeastern Europe, the Middle East, Africa and Asia[1,4,12] since the disease was first described in the Crimean region of Russia in 1944[13]. During the last decade there have been an increasing number of publications on CCHF including large outbreaks in community and nosocomial settings. The Balkan Peninsula is an endemic region for the disease, where Turkey and Bulgaria are countries with the majority of the cases[14]. In its natural hosts (cattle, sheeps, goats and small mammals including hares) CCHFV causes inapparent infection which lasts for a maximum of one week. Viral infection has been documented through viremia or antibody production in numerous wild and domestic vertebrates worldwide[4].

CCHF was first recognized in Bulgaria in 1952 (region of Stara Zagora) and it became a reportable disease in 1953[15]. Over 1600 cases with CCHF were registered for the period from 1953 to 2012 (as reported by Bulgarian Ministry of Health). The data not only depict the country as an endemic region for CCHF but also shows that CCHFV is a significant public health concern in Bulgaria. However, systematic research concerning seroepidemiology among animals and infestation of ticks with CCHFV in Bulgaria was not carried out since late 1970s. Thus, in the country it is accepted that an endemic location is defined as one where cases were registered.

Prevalence of anti-CCHFV antibodies among cattle from endemic and non-endemic locations

This study reports the prevalence of anti-CCHFV antibodies among livestock from central Bulgaria for the first time. Locations previously described as endemic according to the accepted criterion were compared to non-endemic (control) areas, where CCHF cases were not documented. However, the detected prevalence of anti-CCHFV antibodies and tick infestations were similar (Fig. 3). Moreover, the antibody prevalence among cattle in this study was similar to the average rates previously reported from other sites in the country (from 1.9 to 13.6%, determined with complement fixation assay)[7].

There are various factors that influence the dynamics of the CCHFV life cycle. Thus, the presented data may

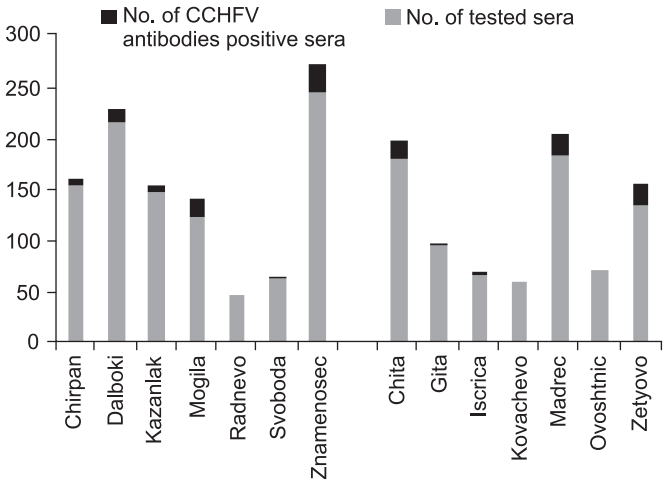

Fig. 3: Prevalence of anti-CCHFV antibodies among cattle from endemic and non-endemic locations in the region of Stara Zagora. Columns in the left and right half of the diagram represent data from endemic and non-endemic (control) locations, respectively.

reflect an increase in the area where the virus circulates due to climate changes, urbanization, shift in the typical human activities, etc. However, another explanation may be related to the diagnostic limitations during the last decades of 20th century. Recently, the widespread of contemporary molecular techniques allowed research to be carried out that revealed a clearer picture. Thus, it might be just a matter of time before cases originating from the control locations are registered among humans. Additional in depth investigations with sequencing and phylogenetic analysis are needed.

Similar levels of anti-CCHFV antibodies were documented in Senegal (average 10.4%, varying among the different locations from 0 to >20%)[9]. The prevalence of anti-CCHF antibodies in domestic and wild animals in Sultanate of Oman was determined as 22%[16]. A low level of anti-CCHF antibodies was detected in different animal species in Egypt (3.31%), while among cattle sera the sero-prevalence rate was 3.83%[17].

Prevalence of CCHFV among the tick population and distribution of tick species

The geographic distribution of CCHF coincides with the global distribution of *Hyalomma* ticks. Being a principal vector and reservoir of CCHFV they are considered appropriate for monitoring the viral activity in the endemic areas[4]. The results for CCHFV infestation in ticks from the investigated region for five successive years remained relatively stable in the separate studied locations and can be considerably different in relation to the average for the region. The screening of all 617 ticks revealed a low prevalence with nine ticks positive for CCHFV (1.46%) probably due to testing of individual ticks and the use of IFH assay. Previous studies which were performed in the same manner detected CCHFV in 32 out of 3038 tested ticks in other Bulgarian regions (1.05%)[8]. Also, an infestation rate of 2% among *Ixodes* ticks (15/745) was established in Turkey with a real-time PCR assay[18]. Although, other studies in various countries showed higher prevalence in tick populations, these investigations examined variable number of pooled ticks[12, 19] which may be assumed to be less accurate.

The study found CCHFV in 6 out of 161 (3.73%) of the tested *H.m. marginatum* ticks. A similar prevalence of CCHFV among ticks (8/271, 2.95%) was described in a previous investigation for other Bulgarian regions[8]. The established prevalence for CCHFV among the *H.m. marginatum* infected ticks in Bulgaria was similar to that in neighboring areas: Turkey (3.22%) and Kosovo (15.8%)[12,14,19]; as well as to some more distant countries: Kenya (2%)[18] and 6.5 to 12.3% for regions of European Russia-Stavropol, Volgograd, Astrakhan and Rostov[20].

Infected ticks may be transferred through importation of livestock from endemic to non-endemic areas[21]. The established levels for CCHFV-circulation in endemic and non-endemic locations that are geotropically alike were similar, which may confirm an expansion in the area of the infection. Thus, there is a potential threat for emergence of the disease and outbreaks in novel areas.

CONCLUSION

Like all other vector-borne diseases, the presence and persistence of zoonotic foci of infection depend on biological and ecological relationships between three different kinds of organisms: virus, ticks, and vertebrates. These three must interact not only physically and biologically to permit each complete act of transmissions, but ecologically to permit continuing cycles of transmissions in nature in tick-vertebrate-tick. The results of this study demonstrated a positive correlation between the distribution of the *H.m. marginatum* ticks and of the prevalence of viral antibodies in domestic animals.

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Correspondence to: Prof. Bozhin Kamarinchev, Department of Military Epidemiology and Hygiene, Military Medical Academy, Georgi Sofijsky blvd. 3, 1606 Sofia, Bulgaria.
E-mail: kamarinchev.b@abv.bg

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