

SURVEY OF CRIMEAN-CONGO HAEMORRHAGIC FEVER VIRUS IN *RHIPICEPHALUS* AND *DERMACENTOR* SPECIES TICKS FROM SOUTH-EASTERN ROMANIA

INVESTIGAȚII PRIVIND VIRUSUL FEBREI HEMORAGICE CRIMEEA-CONGO ÎN CĂPUȘELE *RHIPICEPHALUS* ȘI *DERMACENTOR* SPECIES DIN SUD-ESTUL ROMÂNIEI

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ABSTRACT | REZUMAT

Few previous studies suggested the circulation of Crimean-Congo haemorrhagic fever virus (CCHFV) in Romania, but were based on serological techniques.

The present work investigated the presence of CC HFV in *Rhipicephalus* sp. and *Dermacentor* sp. ticks, in south-eastern Romania. In July 2019, 127 ticks were collected from the environment and from domestic ruminants (sheep and goats) in five different locations from Tulcea county. All tick samples were tested using Real Time RT-PCR based on SYBR Green with the purpose to detect the CCHFV. For this purpose, specific primer sets for each of the six known CCHFV genotypes and one degenerate primer pair for the detection of all genotypes were used. None of the samples was identified as positive (prevalence <2.5%, p=0.05), highlighting the nil or the low prevalence of CCHFV in *Dermacentor* sp. and *Rhipicephalus* sp. ticks from Tulcea county, in 2019. Further studies based on the serological surveillance of animals and more comprehensive ticks testing by molecular biology methods will indicate if CCHFV circulates in Romania.

Key words: CCHFV, Real-Time RT-PCR, ticks, Tulcea county

În România, există puține studii privind circulația virusului febrei hemoragice Crimeea-Congo (FHCCV) dar care s-au bazat pe tehnici serologice.

Prezentul studiu analizează prezența FHCCV în căpușele *Rhipicephalus* sp. și *Dermacentor* sp. în sud-estul României. În iulie 2019, au fost colectate 127 căpușe din mediul înconjurător, dar și de pe rumegătoare domestice (oi și capre), din cinci locații diferite din județul Tulcea. Toate probele au fost testate folosind SYBR Green Real Time RT-PCR pentru a detecta FHC CV. În acest scop, au fost utilizate perechi de primeri specifici pentru fiecare dintre cele șase genotipuri FHCCV, dar și o pereche degenerată de primeri pentru detectarea tuturor genotipurilor. Niciuna dintre probele testate nu a fost pozitivă (prevalența <2.5%, p=0.05%), evidențiind o prevalență scăzută a FHCCV în căpușele *Dermacentor* sp. și *Rhipicephalus* sp. din județul Tulcea, în 2019. Studii ulterioare bazate pe supravegherea serologică a animalelor, dar și pe testarea căpușelor prin metode de biologie moleculară, vor indica dacă virusul FHCCV circulă în România.

Cuvinte cheie: FHCCV, RT-PCR în timp real, căpușe, județul Tulcea

Crimean-Congo haemorrhagic fever virus (CCHFV), a orthonairovirus of the *Nairoviridae* family is the causative agent of a severe human haemorrhagic fever disease characterized by fever, weakness, myalgia, and haemorrhagic signs (2). The disease, named Crimean haemorrhagic fever was first observed in the Crimean Peninsula in 1944, and the causative agent which was isolated in 1967, was found to be identical to Congo virus isolated in 1956 from a febrile child in

the Belgian Congo hence the names Crimean and Congo are used in combination (3). The mean of CCHF fatality rates in Africa (22.0%) is lower than Asia (33.5%) and Europe (33.8%) (20). The natural vector and reservoir have been identified as *Hyalomma* sp. ticks, and the distribution of human cases closely mirrors vector distribution CCHFV has been detected in more than 30 species of ticks, including *Dermacentor* sp. and *Rhipicephalus* sp. ticks (18). Studies conducted since then have consistently found a relationship between various tick species (*Hyalomma*, *Rhipicephalus*, *Boophilus*, *Dermacentor* and *Ixodes*) and the presence of CCHFV in ticks are believed to be the principal means of viral transmission and persistence in nature (7, 30). Nevertheless, the ability to transmit infection

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has been demonstrated for ixodid ticks of several genera, and transovarial transmission of the virus from adult females to the succeeding generation of larval ticks has been shown to occur in a few members of the *Hyalomma*, *Dermacentor*, and *Rhipicephalus* genera (31).

In south-eastern Romania, the presence of *Hyalomma* was confirmed in 2014 (8, 27). The coincidence in distribution of CCHF virus and *Hyalomma* ticks implies that members of this genus are important vectors of the virus (25, 28). Arboviruses usually show low levels of genome diversity, perhaps since they have to be adapted both to an arthropod vector and a vertebrate host species, but CCHFV does not follow this rule and shows a high nucleic acid diversity (29). This virus is a negative-sense, single-stranded RNA virus with a genome of approximative 19.2 kb in length. The genome contains three segments: small (S), medium (M) and large (L). The L segment encodes the RNA-dependent RNA polymerase, the M segment encodes the precursor of the two envelope glycoproteins Gn and Gc, and the S segment encodes the nucleocapsid protein (1, 11, 14). CCHFV is an enveloped virus characterized by a degree of sequence diversity of 20%, 22%, and 31% among the S-, L-, and M-segments of the virus genome (2). M and L RNA segments of nairoviruses are larger than other bunyaviruses (hantaviruses and phleboviruses) (10, 15). As other members of the family *Bunyaviridae*, CCHFV glycoproteins target the Golgi apparatus, where most viral assembly takes place. Recent studies have revealed a new strain which shows unique S-segment phylogenies and which constitutes a unique clade (provisionally identified as clade VII) (5). Crimean-Congo haemorrhagic virus circulates in nature in unnoticed enzootic tick-vertebrate-tick cycles. Asymptomatic CCHFV infection has been reported in numerous vertebrate species and appears to be pervasive in both wild and domestic animals. Asymptomatic viremia last up to 7–15 days in several vertebrate animal species, and CCHFV has been isolated from livestock and small mammals (horses, donkeys, goats, cattle, sheep, and pigs) (9).

In Romania there is still not sufficient data concerning CCHFV and the virus has never been detected in ticks. Virus detection by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) is essential to prove an actual circulation of CCHFV in a country and can be used to identify animals and humans CCHFV infections, together with viral detection in ticks. The predominant problem in CCHFV RT-qPCR development has always been the high genetic diversity of the virus (6).

The aim of this study was to assess the prevalence of CCHFV in *Dermacentor sp.* and *Rhipicephalus sp.*

ticks collected from Romania, more precisely in Tulcea county, where in previous studies IgG antibodies anti-CCHFV were found in sheep and goats (4, 23).

MATERIAL AND METHODS

Ticks were collected during July 2019 from different locations in south-eastern Romania. The sampling consisted in environmental and body-surface of domestic ruminants, from five sites across Tulcea county. Tulcea county (latitude: 44.64°S–45.42°N; longitude: 28.00°W–29.65°E) is situated in southeast Romania and forms the northern part of Dobrogea province. Vegetation is represented by sub-Mediterranean broadleaf forests and xerophytic pastures with shrubs. The county is bordered by the river Danube (W and N), and the Black Sea (E), and is neighbouring Ukraine (N). Ground-feeding birds and small and larger mammals, including livestock, are abundant in this area. Questing tick's collection was performed by dragging. The surface covered at each collection sites was ~1 hectare, and the flag was examined after every 10 m of dragging, at the end of each segment. Between every 10 m of dragging, every transect was separated by 20 m. Tick sampling in Tulcea county was performed only once at each site (Table 1).

Engorged ticks were collected on domestic ruminants. The whole body of each sampled animal was inspected for the presence of tick infestations by palpation, mainly on the ears and along the nape of the neck, perineum, scrotum or udder, and tail base. Ticks collected from each animal were kept alive in separate vials and labelled. Live ticks were transported in vials (within a closed secondary container) to the laboratory. Prior handling, ticks were cooled by placing the vials in ice. Developmental stages were identified under a stereomicroscope using standard morphological identification keys. After the identification, each tick was cut lengthwise, using sterile scalpels on an ice-cold surface Petri dish, in a class II laminar flow hood. Half was used for additional processing and the remainder was stored at –80°C. Ticks were transferred to Institut Pasteur (Paris, France) and analysed by real time RT-qPCR. For maintaining the integrity of RNA, ticks were introduced in RNA later solution (Invitrogen, France), according to best security practices of storing and transport.

RNA extraction

Extraction steps were conducted in a BSL-3 laboratory. The ticks were pooled based on the geographical region before extraction, resulting a final number of 13 pools (Table 2).

The tick pools were homogenized in 2 mL reinforced tubes MK28-R from Precellys containing 1 ml of TRIzol Reagent for cells.

Table 1

The distribution of tick collection sites in Tulcea County

Locality	Number of ticks	Tick species
Cataloi	18	<i>Rhipicephalus sp.</i>
Slava Rusă	27	<i>Rhipicephalus sp.</i>
Slava Cercheză	17	<i>Rhipicephalus sp.</i>
Somova	18	<i>Rhipicephalus sp.</i>
Sat Pescăresc Zărești	47	<i>Dermacentor sp.</i>
TOTAL	127	

Table 2

Pooling protocol (grouping by sample place, species and site)

Sample type	Site	Ticks number	Ticks genus	Sampling origin
1. Tick pool	Sat Pescăresc Zărești, Tulcea	7	<i>Dermacentor sp.</i>	Environment
2. Tick pool	Sat Pescăresc Zărești, Tulcea	10	<i>Dermacentor sp.</i>	Environment
3. Tick pool	Sat Pescăresc Zărești, Tulcea	10	<i>Dermacentor sp.</i>	Environment
4. Tick pool	Sat Pescăresc Zărești, Tulcea	5	<i>Dermacentor sp.</i>	Environment
5. Tick pool	Sat Pescăresc Zărești, Tulcea	5	<i>Dermacentor sp.</i>	Environment
6. Tick pool	Sat Pescăresc Zărești, Tulcea	10	<i>Dermacentor sp.</i>	Environment
7. Tick pool	Cataloi, Tulcea	5	<i>Rhipicephalus sp.</i>	Adult sheep
8. Tick pool	Cataloi, Tulcea	15	<i>Rhipicephalus sp.</i>	Adult goats
9. Tick pool	Slava Rusă, Tulcea	11	<i>Rhipicephalus sp.</i>	Adult goats
10. Tick pool	Slava Rusă, Tulcea	16	<i>Rhipicephalus sp.</i>	Adult sheep
11. Tick pool	Slava Cercheză, Tulcea	15	<i>Rhipicephalus sp.</i>	Adult goats
12. Tick pool	Somova, Tulcea	5	<i>Rhipicephalus sp.</i>	Adult sheep
13. Tick pool	Somova, Tulcea	13	<i>Rhipicephalus sp.</i>	Adult goats

Ticks were shaken two to three times for 30s using the Minilys (Bertin, France). Between each shaking procedure the samples were placed on ice for 1 minute. The homogenates were used for total RNA extraction. The lysates were centrifuged at 12,000 g for 2 min at 4°C. RNA was purified with a RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including on-column DNase treatment and further analysed using an Agilent Bioanalyzer. The RNA recovered in nuclease-free water was stored at -80°C.

Detection of CCHFV in *Dermacentor sp.* and *Rhipicephalus sp.* ticks using Real Time RT-PCR based on SYBR Green

In the present study specific primer sets for each of the six known CCHFV genotypes and one degenerate primer pair for the detection of all genotypes, described by others were used (24). These genotypes were assigned to different geographic areas: I – West Africa, II – Central Africa, III – South and West Africa, IV – Asia and Middle East, V- South and East Europe,

VI – Europe. The region targeted by these primers was from nucleotide 1068 to 1248 for the S-sequence. Viral RNA was transcribed to cDNA using SuperScript IV Reverse Transcriptase (ref. 18090050, Invitrogen, France) reagent kit and a 6-mer random primer (ref. N8080127, Invitrogen, France).

One µl of DNase, 0.2 µl of each CCHF-deg primer, 0.2 µl of each genotype-specific CCHF-primer, 10 µl SybrGreen reagent and 8.6 µl H₂O were used, in a total reaction volume of 20 µl. The real time RT-qPCR was performed with Roche LightCycler 96 System (Roche Diagnostics, Germany). The cycling conditions used as follows: 95°C for 300 s preincubation, followed by 45 cycles at 95°C for 10s (denaturation), 60°C for 10 s (annealing) 72°C for 30 s and 37°C for 30 s (cooling), the annealing temperature being adjusted at 60°C depending on the primers temperature. For regulatory reasons regarding bioterrorism, it was not possible to use a positive control. A negative control nuclease - free water was used to rule out cross contamination of reagents and surfaces. Analysis of the fluorescence data was conducted with the Light

Table 3

Results obtained using RT-qPCR for the presence of CCHFV in ticks from Tulcea county

Locality	Tick genus	No. of CCHFV positive ticks
Cataloi	<i>Rhipicephalus sp.</i>	0/18
Slava Rusă	<i>Rhipicephalus sp.</i>	0/27
Slava Cercheză	<i>Rhipicephalus sp.</i>	0/17
Somova	<i>Rhipicephalus sp.</i>	0/18
Sat Pescăresc Zărești	<i>Dermacentor sp.</i>	0/47

Cycler 96 software. Fifteen μ l of PCR product was mixed with 3 μ l of loading buffer and then electrophoresed on 2% agarose gel in Tris-acetate-EDTA buffer.

Statistical analysis

The upper range of prevalence was calculated using VS Outbreak SurveillanceToolbox. The maximal prevalence of viral infection in ticks was estimated assuming a sensitivity of 100% and a confidence level of 95%.

RESULTS AND DISCUSSION

During this study a total of 127 ticks were collected. Of these, 47 were identified as *Dermacentor sp.* questing ticks from the environment and 80 were confirmed as *Rhipicephalus sp.* engorged ticks, detached from adult goats and sheep. All ticks were tested for detection of the six CCHFV genotypes by real time qRT-PCR. For this purpose, real time RT-qPCR based on SybrGreen with specific primer sets for the detection of all the six known CCHFV genotypes (24) in questing and engorged ticks were used. The detection of CCHFV consisted in the amplification of a 180 bp region from S segment that is coding for NP. CCHFV is classified as a risk group 4 pathogen, being considered to have bioterrorism potential due its aerosol infectivity. For regulatory reasons a positive control was not included in reaction. By using the negative control, we have not encountered any false-positive RT-PCR results due to contamination. In all collection sites from Tulcea county, the tested samples using real time qRT-PCR were identified as negative for the six genotypes of CCHFV. The results are listed in Table 3.

As no positive result was recorded, this defines a maximal prevalence $< 2.5\%$ ($p=0.05$). We consider this result as robust because of the good sensitivity of the RT-qPCR used in this study. Two copies/ μ l were detected for genotypes II (DR Congo), IV (Afghanistan), V (Kosovo) and VI (Greece), while the limits of detection for genotypes I and III were 200 copies/ μ l respectively (24). During the same study the specificity was favourably evaluated using ten viruses from the order

Bunyavirales as none of the viruses was detected.

The present study highlight the nil or at most low prevalence of CCHFV RNA in *Dermacentor sp.* and *Rhipicephalus sp.* questing ticks and engorged ticks from south-eastern Romania, in 2019 from the same region (Tulcea county) where the presence of CCHFV antibodies in animals has been detected previously (4, 23).

Crimean Congo haemorrhagic fever has a wide-spread geographic distribution, the disease being reported in many regions of Africa, Middle East, Europe, and Asia, and outbreaks caused by CCHFV have been recorded in several countries (5). A detailed review about the epidemiology of CCHF in Asia, Europe and Africa was published in 1979 by Hoogstraal (15).

The Balkan Peninsula is an endemic region for the disease, where Turkey and Bulgaria are countries with the majority of the cases (10, 14, 21, 22, 31). In Bulgaria, country that is on the border with Romania, CCHFV has an endemic evolution (13) which could suggest the circulation of the virus in Romania, possibly by ticks carried on migratory birds or through the international livestock trade. Migratory birds' role in spreading Crimean-Congo haemorrhagic fever virus (CCHFV) through attached ticks was demonstrated in Turkey (17). The geographic area of our present investigations comprises one of the most active bird migration pathways in south-eastern Europe, for both spring and fall migrations.

In livestock the CCHFV does not cause clinical disease. However, domestic animals are very important for the epidemiology of the virus. Sheep have been recognized as CCHFV reservoirs in certain endemic regions, and have been epidemiologically linked to human cases on several occasions (16, 19). Not only are they hosts for adult vectors, but they also may amplify the virus and infect other ticks during their short-lived viremia, and thus may introduce the virus into new areas via movement and importation of tick-infested and virus-infected livestock ().

So far, the presence of the CCHFV in ticks from Ro-

mania was not confirmed and, consistently with our results, CCHFV human disease has not been reported to date. There are few serological studies regarding the circulation of CCHFV on Romanian territory. In 2012, Ceianu tested 471 sheep serum samples from different localities in Tulcea county and obtained a prevalence of 27.8% for IgG antibodies against CCHFV (4). However, a more recent study conducted by Raileanu in 2015, reported an overall prevalence of 74% IgG antibodies by testing 90 domestic ruminants (23). In the study conducted by Raileanu 74 *Rhipicephalus* sp. ticks, collected from the positive animals, were tested using rRT-PCR method obtaining negative results, which suggest the seroconversion process occurred before the ticks were attached and the transmission of the virus to vectors was not achieved (23).

Many studies suggest that the viral RNA in attached ticks does not directly indicate transmission to host species, and vice versa: infected ticks have been found on seronegative animals and uninfected ticks on seropositive animals (31).

The results of this study together with previous published data may suggest a silent tick-vertebrate-tick cycle of CCHFV in Romania. Clinical cases occur when a range of factors related to tick, vertebrate host, and human behaviour align, increasing the level of viral circulation, the interactions between humans and sources of infection. Thus, detection of CCHFV antibodies in domestic animals has been important in providing initial evidence of circulating virus and localizing CCHFV and increased risk for human infection (26).

There are many factors that can influence the circulation of CCHFV. For example, abiotic variation by season and region is reported in CCHFV evolution (26). Longitudinal studies demonstrated considerable variation when repeated sampling was performed in the same location. These studies reported September as the optimum period for detecting antibodies with a notable decrease in seroprevalence in the winter-spring period (12). This could also partially explain our negative results, because we collected the ticks only once, in July 2019. Variation in seroprevalence is often associated with competent vector distribution, host preference of competent tick vectors, and tick load on a particular animal species. *Hyalomma* sp. ticks were not tested, which is a limit of our study. This study can therefore be extended by exploring different tick species, areas and seasons of sampling.

CONCLUSIONS

Our results highlight that in 2019, the presence of the CCHFV RNA was not detected in *Rhipicephalus* sp. and *Dermacentor* sp. ticks collected from five different sites in Tulcea county, even if the CCHFV IgG antibo-

dies were identified in animals from this area, during researches undertaken in previous years.

Further studies should focus on increasing the number of collected ticks and also in expanding the geographical area of research, correlated with serological investigations in animals in order to accomplish a longitudinal research for better understanding CCHFV epidemiology throughout Romania.

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