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Original article

Serologic survey of the Crimean-Congo haemorrhagic fever virus infection among wild rodents in Hungary

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ABSTRACT

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne pathogen, which causes an increasing number of severe infections in many parts of Africa, Asia and in Europe. The virus is primarily transmitted by ticks, however, the spectrum of natural hosts regarding CCHFV includes a wide variety of domestic and wild animals. Although the presence of CCHFV was hypothesized in Hungary, data in support of CCHFV prevalence has thus far, proven insufficient. In the present study, rodents belonging to four species, the yellow-necked mouse (*Apodemus flavicollis*), the striped field mouse (*A. agrarius*), the wood mouse (*A. sylvaticus*) and the bank vole (*Myodes glareolus*), were all systematically trapped in the Mecsek Mountain region (Southwest Hungary), from 2011 through 2013. Rodent sera were collected and screened for CCHFV antibodies with dot-blot pre-screening and immunofluorescence assay. Among the 2085 tested rodents, 20 (0.96%) were positive for IgG antibody against CCHFV. Seroprevalence was the highest (1.25%) in *A. flavicollis* serum samples. Distinctly, we now provide the first data regarding CCHFV occurrence and seroprevalence among wild rodents in Hungary. This observation represents a need for large-scale surveillance to effectively assess the enzootic background and the potential public health risk of CCHFV in Hungary.

1. Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is categorically associated with the *Orthonairovirus* genus, the *Nairoviridae* family in the *Bunyavirales* order. CCHFV is a human pathogen and may cause severe haemorrhagic fever with a high fatality rate.

Among tick-borne viral diseases, CCHF is the most extensively disseminated. Notably, CCHFV has spread worldwide to more than 30 countries, in strict accordance with the distribution area of *Hyalomma* ticks. CCHFV has appeared in several areas of Africa (Zaire, Uganda, Kenya, Tanzania), Asia (China, Kazakhstan, Pakistan), the Middle East (United Arab Emirates, Iraq, Iran, Saudi Arabia), Europe (Bulgaria, Turkey, Portugal, France, Hungary and Greece). Next to the Dengue virus, CCHFV is the most widespread of all medically relevant arboviruses (Ergönül, 2006).

The natural distribution and spreading of CCHFV is characterized by a tick-vertebrate-tick cycle. Thus far, more than 30 species of ticks have been identified as potential carriers, which likely is attributed in the spreading of the virus (*Hyalomma, Rhipicephalus, Ornithodoros, Dermacentor, Ixodes*). Although the virus has been associated with a wide variety of tick species, it is believed that members of the *Hyalomma* genus are mainly involved in transmitting the causative agent (Ergönül, 2006; Estrada-Peña et al., 2012; Panayotova et al., 2016). Although *Hyalomma marginatum* was reported in Hungary originating from migratory birds (Hornok and Horváth, 2012), the ticks have established populations only in neighbouring countries (Hoogstraal, 1979). Due to global warming, the distribution of Mediterranean tick species has shifted to the north (Maltezou and Papa, 2010). Additionally, the spread of Mediterranean tick species is also assisted by the movement of migratory birds through disseminating the ticks under favourable climatic conditions (De Liberato et al., 2018).

The presence of CCHFV was hypothesized in Hungary during the last decades (Horváth, 1976; Molnár E., 1982). To the best of our knowledge, the occurrence of CCHFV in Hungary is limited and based

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on historical observations (Horváth, 1976; Molnár E., 1982). Serological studies conducted in the late 1960s identified sheep, cattle, and human sera positive for CCHFV in 31%, 0.9%, and 2.9%, respectively (Horváth, 1976). As a part of a national survey for endemic foci of arboviruses, two strains of the virus were isolated from *I. ricinus* ticks in suckling mice during the early 1970s in Hungary (Molnár, 1982). In 2011, 12 positive brown hare serum samples were found out of 198 samples, and (6%) tested parallel with ELISA and IFAT (Németh et al., 2013). Since then, no systematic examination has been conducted regarding CCHFV, raising the question of whether these viruses still circulate throughout Hungary. Furthermore, the highly lethal nature associated with CCHFV has restricted research to biosafety level-4 (BSL-4) laboratories and has consequently limited research investigation. The perception indicates a need for large-scale active surveillance to effectively estimate the potential public health risk regarding CCHFV in Hungary. Moreover, genetic detection of the virus should provide information referencing the strains circulating throughout the country.

In the present study, we aimed to determine serological evidence of CCHFV infection in wild rodents throughout Hungary using two aspects of serologic assays: dot-blot analysis (DBA) and immunofluorescent assay (IFA). Detection of CCHFV antibody is a good indicator of virus presence and circulation and can baseline targeted studies in order to retrieve genetic information in the future. Moreover, Hungary lies along the northern boundary line of the spread of CCHFV based on WHO's data. Although there have been no molecularly proven cases of CCHFV infection among humans so far, the possibility of the debilitating illness may occur in this non-endemic region based on the previous serological investigations (Horváth, 1976; Molnár, 1982).

We targeted wild rodents to detect CCHFV antibodies since these mammals can be infected by larvae, nymphs and adult ticks (i.e. *I. ricinus*), thus, they are highly exposed to tick bites (Perkins et al., 2003; Szekeres et al., 2015). Additionally, the investigation focused on rodents due to their high population rates (Hayes, 2000).

Moreover, to the best of our knowledge, this is the first serological survey among wild rodents in Europe regarding the CCHFV antibody (Spengler et al., 2016). Although CCHFV has been isolated from wild animal species, most field data is primarily restricted to serosurvey, in areas in which the disease is endemic and the seroprevalence is high (Gunes et al., 2009). In the present study, we aimed to determine serological evidence of CCHFV infection throughout Southwest Hungary. This European country is known to be a non-endemic, however, high-risk area for the introduction of CCHFV. Large-scale active surveillance is required to assess the enzootic patterns of the virus prior to the occurrence of spill over events and its potential eruption threatening the local human population.

2. Materials and methods

2.1. Study area and sampling method

The forest reserve of Kőszegi-forrás (46°09′28.88″ N, 18°17′09.90″ E) is located in the Mecsek Middle Mountain Range in Southwest Hungary and includes a total area of 149 ha (Fig. 1). The forest reserve is located upon a plateau surrounded by a deep valley, with an elevation of 320–400 m above sea level.

No fewer than 13 sampling plots were selected in 2011, 2012 and 2013 in an area roughly measuring 11 ha. The sampling was part of a previous ecological study, therefore sampling points were set up based on the former investigation. Capture–mark-recapture method (CMR) was used, implementing the use of live trapping plastic box traps. Various forms of cereals and bacon mixed with aniseed extract and vegetable oil were used in the form of bait. Each sampling plot included a grid comprised of 6 × 6 trap stations, 5 m apart. The monitoring of small mammals was performed in seven monthly trapping periods during an eight-month period, spanning from March through October, in each of the corresponding years. In each month, standard five-night capture events were carried out. The traps were examined twice a day at 7 AM, then again at 7 PM. Blood samples were taken from trapped animals each month using retro-orbital sampling. In each month during the five-night capture period, a blood sample from a given individual animal was taken once.

2.2. Mice immunization with recombinant CCHFV protein

The 1448-bp-long nucleocapsid protein fragment of the IbAr10200 Nigerian strain of CCHFV was produced with an *Escherichia coli* protein expression system, as described previously (Németh et al., 2011).

Thereafter, C57BL/6 wild-type mice were immunized to produce polyclonal antibodies against the recombinant capsid protein of the CCHFV. The purified recombinant CCHFV nucleocapsid protein was diluted in 1xphosphate-buffered saline (PBS) (Lonza) and was mixed with complete Freund adjuvant (Sigma) in a 1:1 ratio. Four sample mice were inoculated intraperitoneally using 100 µl mixture for each specimen. A negative control mouse was inoculated with the same mixture without the recombinant protein. Following four weeks, the recombinant CCHFV nucleocapsid protein was diluted in a solution consisting of 1xPBS and was mixed with incomplete Freund adjuvant (Sigma) in a 1:1 ratio. Four mice were inoculated again, intraperitoneally, with 100 µl mixture per specimen. A negative control mouse was inoculated with the same mixture without the CCHFV protein. Following two weeks, the tail vein blood collection was performed in support of testing the CCHFV-specific antibody response for Western-blot. Following one week, mice with a positive immune response were given a booster inoculation. 1xPBS and recombinant peptide 2:1 ratio was inoculated intraperitoneally consisting of 100 µl mixture per specimen.

Seven days later, the total blood volume was collected from mice through the use of cardiac puncture. Blood was centrifuged (10,000 × g for 10 min) to separate the serum from the cells. Immunized mice serum samples were used as positive controls in future experiments.

The immunization protocol was executed in accordance to personal communication with Dr. Péter Engelmann (Department of Immunology and Biotechnology, University of Pécs, Pécs, Hungary).

2.3. Dot-blot assay for pre-screening

Rodent samples were pre-screened using the standard 96 well Bio-Dot® (Bio-Rad) apparatus, based on the manufacturer's instructions regarding the presence of anti−CCHFV immunoglobulin G (IgG) antibodies. In reference to the recombinant CCHFV protein binding, 100 ng/well recombinant protein was used. Rodent samples, positive and negative control mouse samples were used in a 1:100 dilution. Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Dako) was used as a secondary antibody in a 1:1000 dilution. The colour development was achieved using a 3.3′-diamino-benzidine colour development solution (Bio-Rad) based on the manufacturer's instructions.

In regards to the positive control, an immunized mouse serum sample was used as the primary antibody. The negative control was performed using the serum from an uninfected mouse.

2.4. Immunofluorescence

To effectively confirm the dot-blot positive samples, CCHFV-specific IgG antibodies were detected from rodent serum samples using immunofluorescence assay (IFA).

All laboratory manipulations associated with CCHFV was performed in a BSL-4 suit laboratory, aligned to the University of Pécs. 96 well plates were seeded using A549 cells (25.000 cell/well) and infected with a known CCHFV Kosova Hoti strain (Duh et al., 2008). Three days following infection, cells were fixed for 20 min with ice-cold absolute methanol. Following air-drying and 20 min UV radiation, the plates

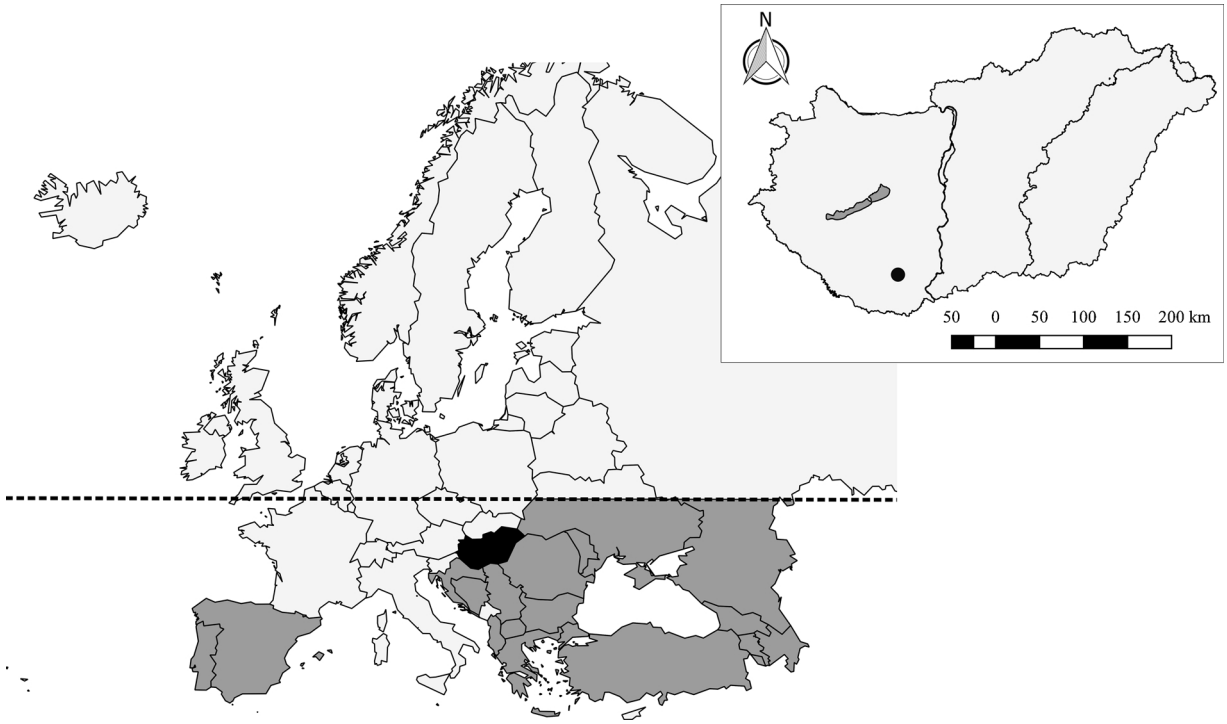

Fig. 1. Location of the study area in Hungary is represented by the black background. The Kőszegi-forrás forest reserve is annotated in the use of a solid black dot. Climatic and geographical distribution limits of *Hyalomma* ticks are depicted in the use of a dashed line. Grey countries have virological or serological evidence and vector presence based on the WHO's data.

were removed the BSL-4 laboratory and placed in storage at 4 °C until further use.

The plates were equilibrated to room temperature and 1xPBS was added to wash cells. 50 µl 1% BSA diluted in 1xPBS was next added to block false-positive reactions. The plates were incubated for 30 min at room temperature. Following the blocking step 50 µl, 1:50 diluted rodent serum samples were added, and the plates were again incubated at 37 °C for 30 min. Following primary antibody binding, 100 µl 1xPBS was used for 5 min at three instances, to effectively rinse the cells. 50 µl goat anti-mouse IgG Alexa Fluor® 488 (Abcam) was used as secondary antibody in 1:1000 dilution, followed by three rinse cycles in 100 µl 1xPBS for 5 min. Plates were evaluated using a Nikon Eclipse Ti-U microscope system, at an excitation wavelength of 480 nm.

In regards to positive control, an immunized mouse serum sample was used as a primary antibody. Serum from an uninfected mouse was used as negative control. For negative cell control, uninfected cells were used with immunized mouse serum as the primary antibody.

2.5. Statistical analysis

Pearson's chi-squared test was performed using the RStudio software (RStudio: Integrated Development for R. RStudio, Inc., B., 2015).

2.6. Ethical statement

The South-Transdanubian Inspectorate of Environment Protection, Nature and Waters Conservation (Hungary) provided an ethical statement allowing the trapping and marking of the rodents throughout the nature reserve area of Kőszegi-forrás.

Immunized mice were housed in accordance to the University of Pécs, Szentágothai Research Centre animal house ethical rules, and all experimental protocols were approved by the regional Ethical Committee regarding animal research (study project permission number: BA02/2000-10/2018).

3. Results

Out of the 3207 collected serum samples, 2085 were selected for CCHFV antibody detection study. In the case of animals with multiple serum samples, only the samples taken the latest month were used, due to the persistence of antibody titer following infection.

We tested all 2085 individual rodents, dating from 2011 through 2013. Captured animals were categorized to four species: 1439 yellow-necked mice (*Apodemus flavicollis*), 448 bank voles *(Myodes glareolus),* 156 wood mice *(A. sylvaticus)* and 42 striped field mice (*A. agrarius)* (Table 1). Out of the four tested species, *A. flavicollis* was captured more significantly frequently in the span of the 3-year-long screening ($\chi^2 = 301.61$; df = 1; $p < 2.2\times10^{-16}$).

In 2012, the number of specimens nearly doubled compared with that of 2011, with a significant difference ($\chi^2 = 125.13$; df = 1; $p < 2.2\times10^{-16}$), while in 2013 the number of specimens was significantly lower compared to that of the previous two years ($\chi^2 = 616.02$; df = 1; $p < 2.2\times10^{-16}$).

Serological evidence of CCHFV was determined using two aspects of serologic assays: DBA pre-screening and IFA confirmation. 20 out of the 2085 (0.96%) tested rodents were positive for CCHFV IgG antibody using both methods.

During the IFA, the positive cell control, which stained primer antibodies as immunized mouse serum showed strong green fluorescence, while negative cell control failed to exhibit any specific staining. Thus, the reaction is not the result of non-specific binding between the cell and the antibody. Additionally, during the screening of wild rodent samples, a clearly distinct positive signal was obtained.

Out of the 20 CCHFV antibody-positive rodent sera samples, 18 were *A. flavicollis*. One positive sample originated from *A. agrarius* and one from *M. glareolus*. None of the *A. sylvaticus* samples demonstrated seropositivity. Total seroprevalence per species was as follows: 1.25% (18/1439) in *A. flavicollis*, 0.22% (1/448) in *M. glareolus*, and 0.24% (1/42) in *A. agrarius*.

In 2011, 5 out of 684 rodent samples (0.73%) were positive. All five

Table 1
Seroprevalence of tested rodents in south-western Hungary from 2011 to 2013.

		Apodemus flavicollis			*Apodemus agrarius*			*Myodes glareolus*			*Apodemus sylvaticus*			Total tested	Total positive	Total seroprevalence
		Tested (n)	Positive (n)	%	Tested (n)	Positive (n)	%	Tested (n)	Positive (n)	%	Tested (n)	Positive (n)	%			
2011	March	19	1	5.3	0	0	0.0	9	0	0.0	3	0	0.0	**684**	**5**	**0.7%**
	April	91	1	1.3	1	0	0.0	9	0	0.0	9	0	0.0			
	May	95	0	0.0	0	0	0.0	5	0	0.0	9	0	0.0			
	June	112	0	0.0	1	0	0.0	1	0	0.0	18	0	0.0			
	July	150	3	2.0	5	0	0.0	18	0	0.0	19	0	0.0			
	August	58	0	0.0	8	0	0.0	25	0	0.0	3	0	0.0			
	September	12	0	0.0	0	0	0.0	4	0	0.0	0	0	0.0			
	Subtotal	**537**	**5**	**0.9**	**15**	**0**	**0.0**	**71**	**0**	**0.0**	**61**	**0**	**0.0**			
2012	March	51	0	0.0	3	0	0.0	7	0	0.0	6	0	0.0	**1165**	**10**	**0.1%**
	April	62	1	1.6	6	0	0.0	22	0	0.0	10	0	0.0			
	May	95	0	0.0	9	0	0.0	17	0	0.0	7	0	0.0			
	June	99	1	1.0	2	0	0.0	47	0	0.0	6	0	0.0			
	July	65	1	1.5	3	1	33.3	31	0	0.0	7	0	0.0			
	August	167	5	2.9	0	0	0.0	57	1	1.8	24	0	0.0			
	September	85	0	0.0	1	0	0.0	92	0	0.0	10	0	0.0			
	October	113	0	0.0	1	0	0.0	55	0	0.0	5	0	0.0			
	Subtotal	**737**	**8**	**1.1**	**25**	**1**	**4.0**	**328**	**1**	**0.3**	**75**	**0**	**0.0**			
2013	March	16	1	6.3	0	0	0.0	13	0	0.0	5	0	0.0	**236**	**5**	**2.1%**
	April	8	1	12.5	0	0	0.0	15	0	0.0	2	0	0.0			
	May	13	0	0.0	0	0	0.0	7	0	0.0	1	0	0.0			
	June	28	0	0.0	1	0	0.0	5	0	0.0	3	0	0.0			
	July	30	1	0.3	1	0	0.0	1	0	0.0	1	0	0.0			
	August	24	1	4.2	0	0	0.0	5	0	0.0	2	0	0.0			
	September	18	0	0.0	0	0	0.0	2	0	0.0	0	0	0.0			
	October	28	0	0.0	0	0	0.0	1	0	0.0	6	0	0.0			
	Subtotal	**165**	**5**	**3.0**	**2**	**0**	**0.0**	**49**	**0**	**0.0**	**20**	**0**	**0.0**			
Total		**1439**	**18**	**1.3**	**42**	**1**	**0.2**	**448**	**1**	**0.2**	**156**	**0**	**0.0**	**2085**	**20**	**0.9%**

rodent sera originated from *A. flavicollis*. In 2012, 10 out of 1165 rodent samples (0.09%) were positive against CCHFV antibodies. Out of 10 positive rodent samples, 8 sera originated from *A. flavicollis*, one from *A. agrarius* and one from *M. glareolus*. In 2013, 5 out of 236 rodent samples (2.12%) were positive. Both positive rodent sera originated from *A. flavicollis* (Table 1).

Sexual distribution of all tested and seropositive rodents was determined. During the three-year period, the ratio of males to females for all tested rodents was approximately equal (994:1087) and the seroprevalence also depicted this phenomenon (9:11).

Two outstanding seasons were observed during the three-year research era. The first peak appeared in the spring (March and April) followed by the second peak in the summer (June, July and August).

4. Discussion

The current study reports data regarding CCHFV IgG antibody prevalence in four rodent species in existence, in Hungary, from 2011 through 2013. To the best of our knowledge, this is the first report of indirect serological evidence regarding CCHFV presence among wild rodents throughout Europe (Spengler et al., 2016).

The dominant species in the study area include the yellow-necked mouse (*A. flavicollis*), the striped field mouse (*A. agrarius*), the wood mouse (*A. sylvaticus*) and the bank vole (*M. glareolus*). Among these rodents, *A. flavicollis* is the most predominant species. Our data acquired over the three-year period corroborate this fact since the majority of our samples originated from this species. There were also a large number of *M. glareolus*, especially in 2012, while *A. agrarius* and *A. sylvaticus* occurred in smaller numbers each year. There are a plethora of studies in support of the fact in which rodents are likely the primary distributors or hosts of many viruses throughout Hungary (Jakab et al., 2008; Németh et al., 2011; Oldal et al., 2015). Due to the inherent risk among rodents undergoing exposure to ticks, they may also be one of the important carriers of the CCHFV or even other nairoviruses.

In this study, the rodent sample numbers nearly doubled between 2011 and 2012. Density was the highest in 2012. Rodent population fluctuations can be driven by predators, climate, weather, and source of food (Cavanagh et al., 2004). From 2012–2013, the number of samples decreased by nearly 80%. It is possible, in which the population experienced a breakdown and entered a phase of collapse.

In the current study, 20 (0.96%) rodent samples were positive for CCHFV IgG antibody during the three-year period using two different methods (DBA and IFA). Antibodies against CCHFV were detected in three rodent species: *A. flavicollis*, *M. glareolus* and *A. agrarius*. The mainly affected species was *A. flavicollis* (18/20) due to its being the most abundant species in the study area. Significantly, lower seropositivity was detected in the *M. glareolus* (1/20) and *A. agrarius* (1/20). Serological studies showed rodent species possess a lower prevalence regarding CCHFV antibodies (Spengler et al., 2016). Factually, the lower prevalence (0.96%) associated throughout Hungary in consideration of wild rodents is not surprising and snugly fits into other areas of research (De Liberato et al., 2018; Németh et al., 2013). Although, Hungary is a non-endemic area regarding CCHFV, IgG antibodies against the virus were previously observed among domestic animals (cattle and sheep) (Horváth, 1976; Molnár, 1982), similar to other endemic regions (Chinikar et al., 2012; Mustafa et al., 2011; Yadav et al., 2014). Moreover, in adjacent regions of Romania, which also is not an endemic area, similar seroprevalence (27.8%) has been detected compared with that of other regions (Turkey and Iran) in which CCHFV is endemic, as shown by the examination of sheep serum samples (Ceianu et al., 2012; Gunes et al., 2009; Humolli et al., 2010).

In consideration of many pathogens, especially vector-borne microorganisms, transmission is generally seasonal. Rechav found the immature stages of two species of *Hyalomma* ticks ticks fed on hares and other small mammals. During these stages, they were infected with CCHFV. However, mostly adult ticks transfer the virus to humans. (Rechav, 1986). In our study, two peaks were observed in each year (in spring and summer) regarding seroprevalence.

Although *I. ricinus* ticks are, as of yet, an unsubstantiated species regarding CCHFV transmission, two strains were isolated from this tick species in suckling mice during the early 1970s in Hungary (Molnár, 1982). Based on this historical observation along with the previous serological studies, we hypothesized in which a new Central European CCHFV strain of the virus transmitted by *I. ricinus* may, in fact, exist today. *I. ricinus* larvae and nymphs in Hungary show two peaks in seasonal activity, namely in April and July (spring and summer). On the other hand, preliminary experiments have shown small mammals are naturally parasitized by *I. ricinus* ticks in Hungary (Földvári et al., 2011; Szekeres et al., 2015).

Notably, DBA and IFA can lead to the detection of cross-reactive antibodies against CCHFV-like viruses, especially since we used polyclonal antibodies. Nevertheless, antibodies regarding related nairoviruses (Hazara virus and Dugbe virus) or the other viruses originating from the family *Bunyaviridae,* as seen in the Puumala virus, have been shown not to cross-react with the recombinant nucleoprotein of CCHFV (Garcia et al., 2006; Marriott et al., 1994). However, the L and S segments of CCHFV which encode the RNA polymerase and nucleocapsid proteins, respectively, have a strong similarity to Lassa virus (family *Arenaviridae*) (Guterres et al., 2017). However, we have no molecular evidence in support of viruses originating from the *Arenaviridae* family, such as the lymphocytic choriomeningitis (LCM) virus, originating from wild rodents throughout Hungary, thus far. The indirect IFA proved to be a more specific method. Based on the results of the current serologic investigation, we assume the presence regarding CCHFV throughout region, yet, admittedly, further molecular-based studies are needed.

Virus presence and re-emergence continue to be main topics of national and international health security. The potential entry regarding CCHFV into new geographic areas are to be considered and requires appropriate and effective measures of surveillance to best acquire knowledge regarding virus ecology, transmission dynamics and possible reservoir hosts and vectors in so far non-endemic regions.

5. Conclusion

Emerging infectious diseases are a growing threat to public health, domesticated and wild animals. CCHF is one of the several viral diseases, which was identified by the WHO as a likely cause of a future epidemic and recognized as a possible agent regarding bioterrorism. In non-endemic areas, serological experiments are suitable for testing the appearance of CCHFV. Detection of antibodies may be a good indicator of the presence of CCHFV in the study area. Rodents were tested for CCHFV in the present study since they are highly exposed to tick bites. Moreover, Hungary lies along the northern boundary line of the spread of CCHFV based on the WHO's data. Therefore, possible illness may occur in this non-endemic region. Widespread active monitoring is required to assess the spread of the virus prior to the risk of infection threatening the local population.

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