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Survey for Hantaviruses, Tick-Borne Encephalitis Virus, and *Rickettsia* spp. in Small Rodents in Croatia

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Abstract

In Croatia, several rodent- and vector-borne agents are endemic and of medical importance. In this study, we investigated hantaviruses and, for the first time, tick-borne encephalitis virus (TBEV) and *Rickettsia* spp. in small wild rodents from two different sites (mountainous and lowland region) in Croatia. In total, 194 transudate and tissue samples from 170 rodents (*A. flavicollis,* $n=115$; *A. agrarius,* $n=2$; *Myodes glareolus,* $n=53$) were tested for antibodies by indirect immunoflourescence assays (IIFT) and for nucleic acids by conventional (hantaviruses) and real-time RT-/PCRs (TBEV and *Rickettsia* spp.). A total of 25.5% (24/94) of the rodents from the mountainous area revealed specific antibodies against hantaviruses. In all, 21.3% (20/94) of the samples from the mountainous area and 29.0% (9/31) from the lowland area yielded positive results for either Puumala virus (PUUV) or Dobrava–Belgrade virus (DOBV) using a conventional RT-PCR. All processed samples ($n=194$) were negative for TBEV by IIFT or real-time RT-PCR. Serological evidence of rickettsial infection was detected in 4.3% (4/94) rodents from the mountainous region. Another 3.2% (3/94) rodents were positive for *Rickettsia* spp. by real-time PCR. None of the rodents ($n=76$) from the lowland area were positive for *Rickettsia* spp. by real-time PCR. Dual infection of PUUV and *Rickettsia* spp. was found in one *M. glareolus* from the mountainous area by RT-PCR and real-time PCR, respectively. To our knowledge, this is the first detection of *Rickettsia* spp. in small rodents from Croatia. Phylogenetic analyses of S- and M-segment sequences obtained from the two study sites revealed well-supported subgroups in Croatian PUUV and DOBV. Although somewhat limited, our data showed occurrence and prevalence of PUUV, DOBV, and rickettsiae in Croatia. Further studies are warranted to confirm these data and to determine the *Rickettsia* species present in rodents in these areas.

Key Words: Hantavirus—*Rickettsia*—Rodents—Tick-borne encephalitis virus—Zoonosis.

Introduction

CROATIA IS ENDEMIC FOR VARIOUS zoonotic agents. Due to biological and ecological diversity, differences in occurrence and prevalence of various pathogens in small rodent populations can be expected throughout the country (Markotić et al. 2009).

Hantaviruses (family *Bunyaviridae*) may be the most important rodent-borne pathogens in the country. Human infections caused by hantaviruses are endemic, with hemorrhagic fever with renal syndrome (HFRS) cases varying between sporadic annual reports of 30 cases (Epidemiology Unit of the Croatian National Institute of Public Health) up to epidemic occurrence with 400 human cases (Cvetko et al. 2005). In Croatia, as well as in Europe, several hantaviruses are circulating with Puumala virus (PUUV) and Dobrava–Belgrade virus (DOBV) being of medical importance (Markotić et al. 2002, Cvetko et al. 2005). Tula (TULV) and Saaremaa (SAAV) viruses have also been reported, but the medical importance is unknown (Scharninghausen et al. 2002, Plyusnina et al. 2011).

Tick-borne encephalitis virus (TBEV, family *Flaviviridae*), transmitted by *Ixodes* ticks, is endemic in parts of Europe, including northern Croatia. It causes human infections of the central nervous system (Mansfield et al. 2009, Dobler et al. 2012), with up to 50 reported cases annually in

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Croatia (Borcić et al. 1999). For Croatia, only few seroepidemiological data regarding TBEV in humans are available (Borcić et al. 1999, Miletić-Medved et al. 2011). Limited data have suggested small rodents as a reservoir of this virus (Achazi et al. 2011, Knap et al. 2012). Rodents may also be key hosts for amplification of TBEV in the natural transmission cycle (Süss 2003, Dobler et al. 2012). However, this aspect has not been investigated in Croatia so far.

Rickettsiae (genus *Rickettsia*) are obligate intracellular bacteria transmitted by arthropods, causing human diseases of various severities (Roux and Raoult 2000). Rodents have been suggested to act as reservoir hosts for certain rickettsiae (Schex et al. 2011). In Croatia, data regarding rickettsioses in humans are very limited. Due to unspecific clinical symptoms, human cases may be misdiagnosed and undetected. On average, seven cases are recorded annually (Epidemiology Unit of the Croatian National Institute of Public Health). The human seroprevalence against spotted fever group (SFG) rickettsiae varies between $\leq 44\%$ in southern Croatia (Punda-Polić et al. 2003) and $\leq 7.1\%$ in continental Croatia (Pandak et al. 2011). So far, no epidemiological data exists for the mountainous area of the country.

Identification of reservoir hosts of zoonotic agents is a prerequisite for an effective prevention of human infections. Therefore, this study was conducted to investigate the occurrence and prevalence of *Rickettsia* spp. and TBEV and to determine the prevalence of hantaviruses in small wild rodents in two geographically and ecologically distinct localities in Croatia.

Materials and Methods

Study sites

Gerovo is located in a mountainous area of Gorski kotar adjacent to the border of Slovenia, approximately 150 km southwest of the Croatian capital of Zagreb (Fig. 1). The area is covered in deciduous (beech) and mixed coniferous forests (beech and fir). Žutica lies within a lowland area in central Croatia, approximately 50 km southeast of Zagreb (Fig. 1). This area is characterized as a floodplain deciduous common oak forest. Both localities are being exploited for timber and are known recreational areas for tourists and sportsmen.

FIG. 1. Geographic location of the trapping sites in Croatia— Gerovo (45°30′53″N, 14°38′32″E) in mountainous area and Žutica (45°37′48″N, 16°26′18″E) in lowland area.

Animal samples

During November, 2007, 76 rodents were trapped at Žutica, and from April to May, 2008, 94 rodents were collected at Gerovo using snap traps. Guidelines by Gannon et al. (2007) were followed. Trapping was performed along linear transects at 100 meters above sea level (a.s.l.) in Žutica and from 360 to 1220 meters a.s.l. in Gerovo. Tissue samples ($n = 194$) available for investigation in this study were lung ($n = 75$) and kidney ($n = 25$) from rodents trapped at Žutica and heart tissue ($n = 94$) from animals captured at Gerovo. Hearts were stored in 0.5 mL of phosphate-buffered saline solution. All samples were stored at $-80°C$ until further investigation.

*Detection of anti-hantavirus, anti-TBEV, and anti-*Rickettsia *spp. antibodies*

Transudate was collected from heart tissue ($n = 94$), and 10 μL were tested undiluted for the presence of immunoglobulin G (IgG) antibodies by indirect immunofluorescence tests (IIFTs) as recommended by the manufacturer. Hantavirus Mosaic 1 (Euroimmun AG, Lübeck, Germany), TBE virus (Euroimmun AG), *Rickettsia conorii* IgG IFA Kit (Fuller Laboratories, Fullerton, CA), and *Rickettsia typhi* IgG IFA Kit (Fuller Laboratories) were used. As secondary antibody fluorescein isothiocyanate (FITC)-conjugated polyclonal rabbit anti-mouse IgG was used (dilution 1:20; Dako, Glostrup, Denmark) together with Evans Blue counterstaining (BioMerieux, Marcy l'Etoile, France). Slides were read on a fluorescent microscope Eclipse 50i (Nikon Instruments Inc., Japan) by two independent examiners.

Nucleic acid isolation

Each sample (total $n = 194$) was homogenized in cell culture medium (minimum essential medium [MEM] + GlutaMAX; Invitrogen Life Technologies, Carlsbad, CA) using the FastPrep 24 (MP Biomedicals, Santa Ana, CA). Nucleic acids were extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions.

Detection and phylogenetic analysis of hantaviral RNA

A total of 94 heart and 31 lung samples were screened for hantaviruses by a reverse transcription polymerase chain reaction (RT-PCR) targeting the partial S-segment of PUUV, DOBV, and TULV (Table 1) as described previously (Essbauer et al. 2006). The remaining 44 lung samples (*Apodemus flavicollis*, $n = 28$; *Myodes glareolus*, $n = 16$) from Žutica had been screened and published before (Tadin et al. 2012). For amplification of almost complete S-segment sequences of DOBV, new primer pairs were designed (Table 1). For PUUV, previously published primers (Mertens et al. 2011) were used (Table 1). Partial M-segments of PUUV and DOBV were gained from screening-positive heart samples using

TABLE 1. OLIGONUCLEOTIDES USED IN THIS STUDY

Genomic target	*Oligonucleotide name*	*Forward (5′-3′)*	*Oligonucleotide name*	*Reverse (5′-3′)*	*Reference*
Hantaviruses S-segment	DOBV-M6	AGYCCWGTNATGRGWGTRATTGG	DOBV-M8	GAKGCCATRATNGTRTTYCKCATRTCCTG	Essbauer et al. 2006
	PUU Fpuni	TAGTAGACTCCTTGAARAGCTRCTACGA	cPUUV1122	GCCATDATDGTTTYCTCAT	Mertens et al. 2011
	PUU M6	AGYCCWGTNATGGGDGTNATTGG	cPUUV 1910	TAGTAGTAKVCTCCTTGAAA	Essbauer et al. 2006
	DOBV Fpuni	TAGTAGTAKRCTCCCTAAARAG	cDOB_S_ FP	CACGGGAGGTCAAGCCA	This paper
	DOBV_S_957F	TCTGGGTCTTTGCAGGAGCA	cDOBV_S_1620R	AGGTAGTGTTGTTGTTTGAGGTA	This paper
Hantaviruses M-segment	PUU B3	CARTTACARAAYCCIGCMAATGA	PUU C2	CCAACTCCTGAACCCCATGC	Asikainen et al. 2000
	DOBV_M_kroa1	Available upon request	DOBV_M_kroa2	Available upon request	Plyusnina et al. 2011
Rickettsia spp.	PanRick_2_for	ATAGGACAACCGTTTATTT	PanRick_2_rev	CAAACATCATATGCAGAAA	Wölfel et al. 2008, Schex et al. 2011
	Probe: PanRick_3_taq	FAM-CCTGATAATTCGTTAGATTTTACCG-TMR			
	ompB -120-2788	AAACAATAATCAAGGTACTGT	ompB-120-3599	TACTTCCGGTTACAGCAAAGT	Roux and Raoult 2000
TBEV	F-TBE1	GGGCGGTTCTTGTTCTCC	R-TBE1	ACACATCACCTCCTTGTCAGACT	Schwaiger and Cassinotti 2003
	Probe: TBE-WT	FAM-TGAGCCACCATCACCCAGACACA-TMR			
mt-Cytb	L14648	TGAATYTGAGGRGGATTCTCAGTA	H16	CWGGTTGRCCTCCRATTCAWGT	Essbauer et al. 2006
	CB_F	ATGACARTCATCCGAAARAAACAC	CB_R	TTARTCTAGGTCYAKRATGTYGTTTTC	Dekonenko et al. 2003

TBEV, tick-borne encephalitis virus; *mt-Cytb*, mitochondrial cytochrome *b* gene.

published primer pairs (Asikainen et al. 2000, Plyusnina et al. 2011) (Table 1). All S- and M-segment sequences were submitted to GenBank (accession no. KC676589-635).

The phylogeny was inferred using the maximum likelihood method based on the Tamura–Nei model implemented in MEGA5 software (Tamura et al. 2011) with additional sequences from GenBank.

Detection of Rickettsia *spp. DNA*

Real-time screening PCR targeting citrate synthase gene (*gltA*) was performed for all samples ($n = 194$) using LightCycler FastStart DNA Master HybProbe (Roche Applied Science, Indianapolis, IN) on a LightCycler 1.5 instrument (Roche) as published previously (Wölfel et al. 2008, Schex et al. 2011). A conventional PCR targeting the partial outer membrane protein B gene (*ompB*) was performed to determine the *Rickettsia* species in positive samples as described (Roux and Raoult 2000).

Detection of TBEV RNA

Real-time RT-PCR targeting a fragment of the 3′ noncoding region of the TBEV was performed for all samples ($n = 194$) using QuantiTect Virus RT-PCR-Kit System (Qiagen) on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA) following a published protocol (Schwaiger and Cassinotti 2003). Langat virus RNA served as a positive control.

Rodent species determination

Rodent species were determined morphologically and confirmed genetically on rodents from Gerovo ($n = 94$) and *Apodemus* mice from Žutica ($n = 17$) using PCR targeting the mitochondrial cytochrome *b* gene (*mt-Cytb*) (Dekonenko et al. 2003, Essbauer et al. 2006). Part of *mt-Cytb* sequences were submitted to GenBank (accession no. KC676636-655).

Results

A total of 194 heart, lung, and kidney samples of 170 rodents from two localities in Croatia were analyzed (Table 2). At Žutica ($n = 76$), 15 *A. flavicollis* and two *A. agrarius* were genetically identified, genetic determination of 28 *A. flavicollis* was done before by Tadin et al. (2012), and 31 *M. glareolus* were morphologically identified. From Gerovo ($n = 94$), 72 *A. flavicollis* and 22 *M. glareolus* were genetically confirmed.

Hantaviruses

Specific antibodies to hantaviruses were found in 24/94 (25.5%) investigated heart transudates from Gerovo (Table 2). Seventeen of these originated from *A. flavicollis* and seven from *M. glareolus*. Fewer animals from Gerovo, 20/94 (21.3%), were positive by RT-PCR, all of which were positive by IIFT; 7/22 (31.8%) *M. glareolus* revealed PUUV and 13/72 (18.1%) *A. flavicollis* were DOBV positive. From Žutica, nine of 31 (29.0%) rodents were positive by RT-PCR, including one of 15 (6.7%) *M. glareolus* (PUUV) and eight of 14 (57.1%) *A. flavicollis* (Table 2). In five of seven PUUV positives from Gerovo, almost complete S-segment sequences (1766 base pairs, nucleotides 52–1830) were recovered showing 98.4–100% similarity to each other, whereas deduced amino acid sequences were 98.1–100% similar. The PUUV sequence obtained from Žutica was 345 base pairs (nucleotides

Table 2. Number of Positive and Number of Tested Rodents from Gerovo and Žutica on Hantaviruses and *Rickettsia* spp.

		Hantaviruses		Rickettsia *spp.*	
Locality	*Rodent species*	*IIFT*[d]	*RT-PCR*	*IIFT*	*PCR*
Gerovo (mountainous Croatia)[a]	*Apodemus flavicollis*	17/72	13/72[e] (DOBV)	2/72	2/72
	Myodes glareolus	7/22	7/22[e] (PUUV)	2/22	1/22
	Total Gerovo	**24/94 (25.5%)**	**20/94 (21.3%)**	**4/94 (4.3%)**	**3/94 (3.2%)**
Žutica (lowland Croatia)[b]	*Apodemus flavicollis*	nd	8/14 (DOBV)	nd	0/43[g]
	Apodemus agrarius	nd	0/2	nd	0/2
	Myodes glareolus[c]	nd	1/15 (PUUV)	nd	0/31
	Total Žutica	**nd**	**9/31[f] (29.0%)**	**nd**	**0/76 (0%)**
Total		**24/94 (25.5%)**	**29/125 (23.2%)**	**4/94 (4.3%)**	**3/170 (1.8%)**

[a]Heart samples were available for testing.
[b]Lung and kidney samples were available for testing.
[c]Rodents were morphologically determined but not confirmed by *mt-Cytb* PCR.
[d]In IIFT a cross-reactivity pattern was seen: Bank vole samples reacted to all six tested hantaviruses (Hantaan, Sin Nombre, Puumala, Dobrava-Belgrade, Seoul, Saaremaa), whereas yellow-necked mice samples were only reactive on DOBV, Hantaan virus, and Seoul virus. Serial dilutions of the positive samples were not performed.
[e]The same animals were positive by IIFT.
[f]A total of 44 animals were already tested for hantaviruses (16 PUUV-positive *M. glareolus* and 28 positive *A. flavicollis)* by Tadin et al. (2012) and are therefore not included in this table.
[g]Includes one more *Apodemus flavicollis* that was not tested for hantaviruses in this paper or by Tadin et al. (2012), as samples were not available.
DOBV, Dobrava–Belgrade virus; IIFT, indirect immunofluorescence test; nd, not determined; PCR, polymerase chain reaction; PUUV, Puumala virus; RT-PCR, reverse transcription polymerase chain reaction.

714–1058) due to unsuccessful amplification of further S-segment sequences by additional RT-PCRs and shared 94.2–95.5% similarity to the sequences from Gerovo, while the deduced amino acid sequences were 97.3–98.2% similar. Therefore, two phylogenetic trees were constructed, one for the almost complete S-segment sequences from Gerovo and other European PUUV strains (nucleotides 52–1674) and the second comparing partial sequences from Gerovo, Žutica and other strains (nucleotides 714–1058). The phylogenetic analysis for the almost complete S-segment (Fig. 2) revealed eight PUUV lineages. The Alpe-Adrian PUUV lineage formed a sister group to the Central European lineage. Within the Alpe-Adrian lineage, the subgroup A, with good bootstrap support, included only sequences from Gerovo that subdivided into distinct inferior branches. The subgroup B included sequences of previously described PUUV strains from Austria, Hungary, and the Balkans. The phylogenetic analysis for a partial S-segment of 345 nucleotides (with the sequences in this analysis adjusted to the same length; Fig. 2, inset) revealed that PUUV sequences from Gerovo and Žutica lay within two separate subgroups within the Alpe-Adrian lineage. Subgroup A only included the sequences from Gerovo, whereas subgroup B included the sequence from Žutica and sequences of previously described PUUV strains from Austria, Hungary, and the Balkans.

Almost complete S-segment sequences (1567 base pairs, nucleotides 23–1677) were gained in seven of 13 DOBV positive samples from Gerovo and revealed 98.7–100% similarity among themselves, whereas deduced amino acid sequences showed 96.6–100% similarity. In four of eight DOBV positive samples from Žutica, almost complete S-segment sequences (1568 base pairs) were obtained showing a 100% similarity among each other, and the similarity to sequences from Gerovo was 96.6–96.8%. Interestingly, the sequences from Gerovo have one less nucleotide (nucleotide 1333) in the noncoding region compared to sequences from Žutica and sequences from Greece or Slovakia. The phylogenetic analysis for the almost complete DOBV S-segment revealed four lineages (nucleotides 23–1590; Fig. 3). The phylogenetic analysis of DOBV showed that the sequences from Gerovo and Žutica were situated in three well-supported subgroups A–C within genotype Dobrava. Subgroup A comprised sequences from Gerovo that subdivide into inferior branches. Subgroup B included one sequence from Gerovo together with sequences from Slovenian genotype Dobrava strains. This sequence from Gerovo had more nucleotide differences (1.3%) compared to other sequences from Gerovo. Subgroup C represented sequences from Žutica together with sequences of previously described genotype Dobrava strains from Greece and Slovakia.

Partial M-segment sequences of PUUV and DOBV were obtained from rodents trapped at Gerovo. The seven PUUV M-segment sequences of 850 base pairs (nucleotides 2161–3010) had 99.4–100% similarity to each other, whereas deduced amino acid sequences were 95.5–100% identical. The gained 11 DOBV M-segment sequences of 520 base pairs (nucleotides 1489–2008) bore 98–100% resemblance to one another, whereas deduced amino acid sequences showed 93.9–100% identity. The phylogenetic analysis of PUUV and DOBV M-segment sequences for the partial corresponding region is in line with the phylogenetic analysis of the S-segment sequences but is not shown due to insufficient bootstrap support in the branches.

TBEV

All processed samples were negative for TBEV by IIFT ($n = 94$) and real-time RT-PCR ($n = 194$). All respective

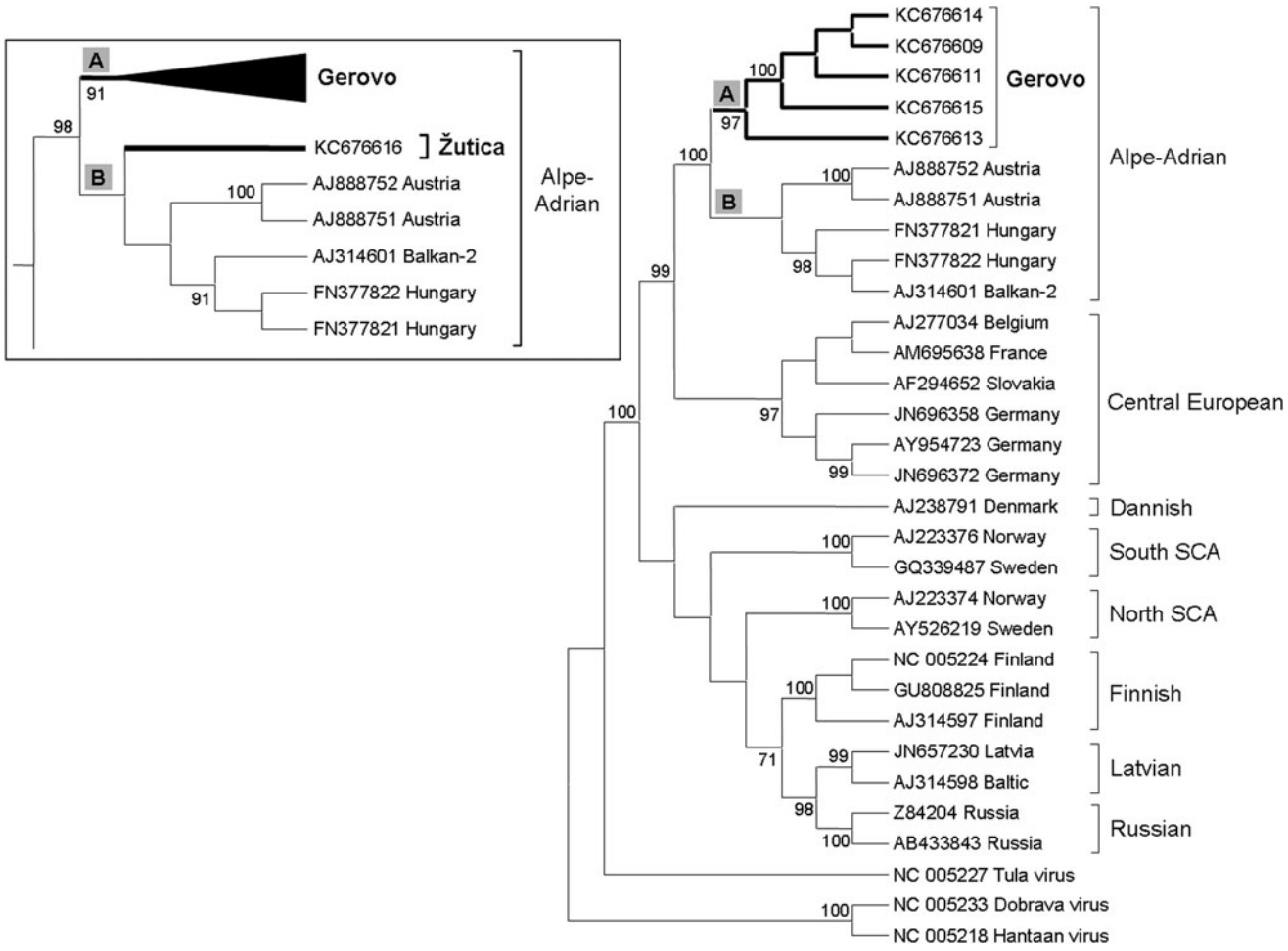

FIG. 2. Phylogenetic analysis of the almost complete Puumala virus (PUUV) S-segment (corresponding to nucleotides 52–1674 of the reference strain, accession no. NC_005224) using the maximum likelihood method based on the Tamura–Nei model. The inset part of the tree is calculated for the PUUV partial S-segment region of 345 base pairs (corresponding to nucleotides 714–1058 of the reference strain, accession no. NC_005224). Only bootstrap values >70%, calculated from 1000 replicates, are shown at the tree branches.

controls on all IIFT slides and in real-time RT-PCR reactions were accurate.

Rickettsia *spp.*

Only animals from Gerovo were positive for *Rickettsia* spp. Four out of 94 (4.3%, from two *M. glareolus* and two *A. flavicollis*) heart transudates showed serological evidence of reactivity with SFG rickettsiae (Table 2). Additionally, another three individuals (three of 94, 3.2%) from Gerovo (one *M. glareolus*, two *A. flavicollis*) were positive for *Rickettsia* spp. by *gltA* real-time PCR. Species determination could not be confirmed by *ompB* PCR due to lesser sensitivity of the conventional PCR.

Multiple infections

All four samples reactive for antibodies against *Rickettsia* spp. were also positive for hantavirus antibodies and hantavirus RNA (two PUUV and two DOBV positive) (data not shown). Additionally, one *M. glareolus* from Gerovo revealed simultaneous detection of rickettsial DNA and PUUV RNA (data not shown).

Discussion

The high percentage (25.5% by IIFT, 23.2% by RT-PCR) of hantavirus-positive rodents found in this study is in accordance with previous studies on hantaviruses in Croatia (Cvetko et al. 2005, Tadin et al. 2012) and other countries (Avsic-Zupanc et al. 2000, Essbauer et al. 2006, Heyman et al. 2012), although the sampling was performed in different years/seasons and in nonepidemic periods in Croatia. Overall, 21.3% of the rodents from Gerovo and 29.0% of the animals from Žutica were positive. In 2008, 13 cases of HFRS were recorded from the mountainous area of Gorski kotar (Institute of Public Health of the Primorsko-goranska county) where Gerovo is situated. Moreover, Gerovo is adjacent to Slovenia, where a higher number of human infections had been noticed in 2008 (Heyman et al. 2011). As indicated by Borcić et al. (1991), the area of Gorski kotar can be considered as HFRS endemic with a seroprevalence of 4.4% in the human population. Compared to the seroprevalence (25.5%) measured in rodent transudates from mountainous Gerovo, 43% of the rodents from lowland Žutica reacted seropositive in another study (Tadin et al. 2012).

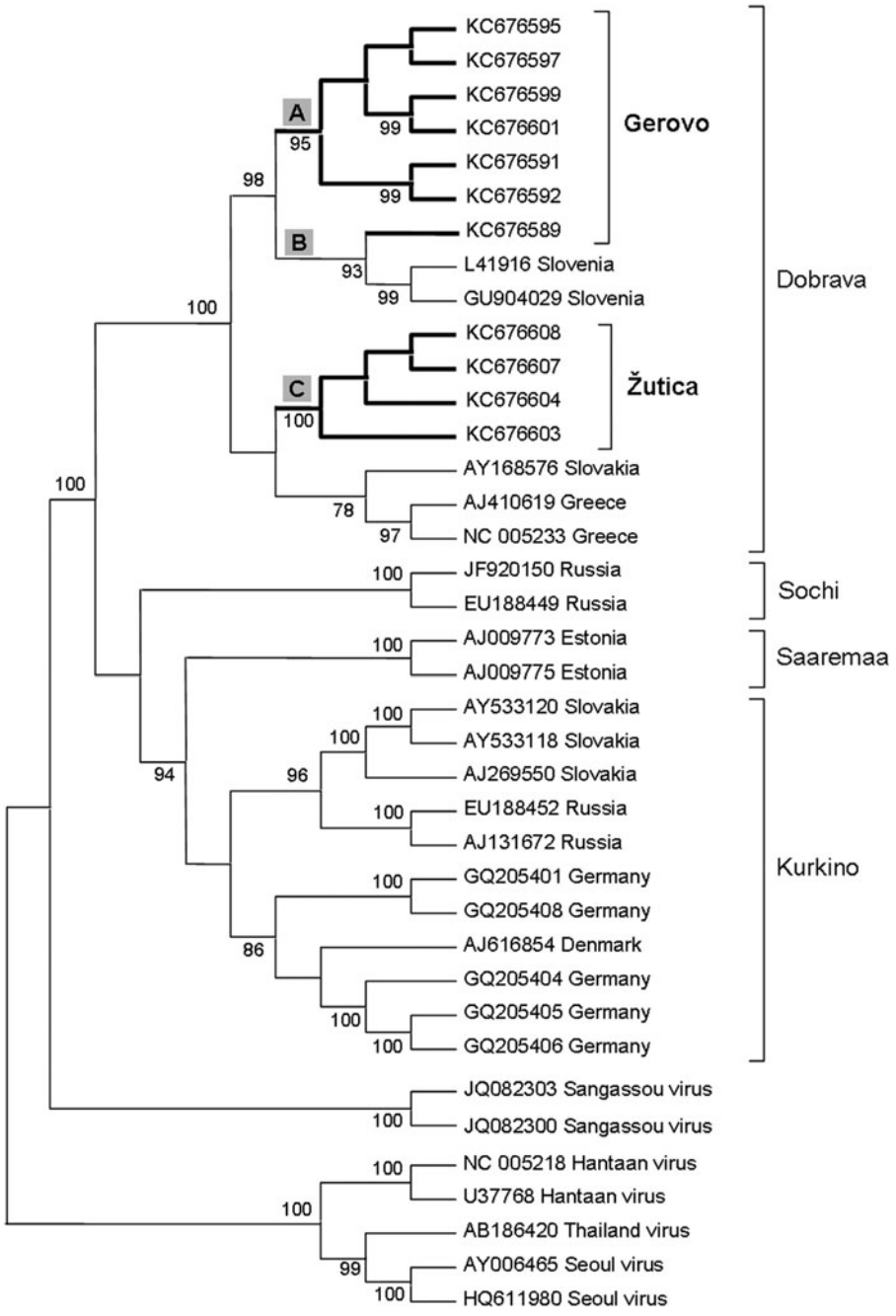

FIG. 3. Phylogenetic analysis of the almost complete Dobrava–Belgrade virus (DOBV) S-segment (corresponding to nucleotides 23–1590 of the reference strain, accession no. NC_005233) using the maximum likelihood method based on the Tamura–Nei model. Only bootstrap values >70%, calculated from 1000 replicates, are shown at the tree branches. DOBV genotypes within the tree were termed according to the subdivision of DOBV suggested by Klempa et al. (2013).

For Žutica, human seroprevalence data regarding hantavirus infections were not investigated so far.

The samples available from Gerovo unfortunately only comprised heart tissue. Due to lower detection rates of hantavirus RNA in heart specimens in other studies or depending on the molecular method used (Essbauer et al. 2006, Korva et al. 2009), these results may not allow definite conclusions on hantavirus prevalence in Gerovo. They rather suggest that the percentage of hantavirus positives would be even higher if other tissue (*e.g.*, lung) had been used (Essbauer et al. 2006).

Phylogenetic analyses of partial S- and M-segments revealed that the sequences from Gerovo and Žutica formed two well-supported distinct genetic subclusters in Croatian PUUV and DOBV. Separation into genetic geographic clusters where the genetic distances of DOBV and PUUV strains increase with increasing geographical separation has been described (Avsic-Zupanc et al. 2000, Ettinger et al. 2012). The genetic distance between virus sequences obtained from mountainous Gerovo and lowland Žutica was demonstrated by the two subclusters within PUUV and

DOBV and might be explained by geographical barriers (mountainous area between the two localities) that prevent the spreading of rodents and virus. Gerovo lies within the Dinarides (Dinaric Alps) that form a mountain chain in a northeast–southwest direction. Žutica is located in the Panonic-peripanonic area in a Sava river valley. The two sites are approximatly 200 km apart. The two strains of PUUV and DOBV could also suggest that there were two routes of rodent postglacial colonization dispersing from Balkan Peninsula refugia for both *Myodes* and *Apodemus* rodents, respectively (Bilton et al. 1998, Michaux et al. 2004). A similar situation has been postulated in Fennoscandia (Asikainen et al. 2000) for bank vole populations and PUUV. Further studies are needed to discriminate whether rodent host genetic lines at our study sites are different. Moreover, data from other geographic areas in Croatia are needed to prove this hypothesis.

To date, there are no available data about the presence of rickettsiae in wild rodents from Croatia and only limited data are available about *Rickettsia* spp. in small rodents worldwide (Schex et al. 2011, Dantas-Torres et al. 2012). In our study, 4.2% of the rodents (two *A. flavicollis*, two *M. glareolus*) from Gerovo revealed anti-*Rickettsia* antibodies. From Germany, 29.3% of wild rodents were positive for anti-*Rickettsia* antibodies (Schex et al. 2011). Three out of 170 (1.8%) rodents in our study were positive for *Rickettsia* DNA by real-time PCR, with only hearts being positive. In contrast, only ear tissue (5.8%) and spleen samples (5.2%) were positive in other studies (Kim et al. 2006, Schex et al. 2011). The low percentage of *Rickettsia* spp.–positive samples in the present study might be explained by the suboptimal tissue choice. However, it might also suggest that both sampling sites are within areas of low *Rickettsia* spp. circulation, which coincides with low seroprevalence in humans in continental (Pandak et al. 2011) compared to coastal Croatia (Punda-Polić et al. 2003).

None of the samples examined tested positive for TBEV RNA or antibodies against TBEV. The real-time RT-PCR used is specific for all Western subtypes of TBEV strains published so far (Schwaiger and Cassinotti 2003, Essbauer, unpublished data). Although there are reports about TBEV detected in rodents in Europe (Achazi et al. 2011, Knap et al. 2012), either none of the captured rodents from this study were infected with TBEV or viral infection could not be demonstrated. TBEV RNA in heart tissue is detectable only at late days postinfection (Achazi et al. 2011). Furthermore, TBE natural foci seem to be very focal (Kupča et al. 2010) and therefore TBE may be nonexistent in the areas tested, which might have hampered TBEV detection in this study with animals of unknown infection status.

Although one might expect simultaneous infections with the pathogens investigated in this study, only one *M. glareolus* from Gerovo was found harboring both *Rickettsia* DNA and PUUV RNA. Comparable studies showed, by proving etiological agents, dual infections of PUUV and *Leptospira* (Cvetko et al. 2006) and even triple infections with hantaviruses, *Leptospira*, and *Babesia* in Croatian rodents (Tadin et al. 2012).

Conclusions

Herein we have presented data on the occurrence and prevalence of three zoonotic pathogens in wild rodents from mountainous and lowland Croatia. Overall, nearly 30% of rodents showed evidence of hantavirus infection. The study areas constitute HFRS risk areas due to the high percentage of hantavirus-positive rodents. Almost complete hantavirus S-segment sequences were gained for PUUV from a mountainous region and DOBV from both study sites. Within the phylogenetic trees, the sequences from the two sites formed well-supported subgroups revealing two variants in Croatian PUUV and DOBV for S- and M-segment with the sequences from the mountainous locality separating into distinct subgroups. To our knowledge, this is the first description of the detection of *Rickettsia* spp. in Croatian rodents. Our data also demonstrate that *Myodes* carry and may transmit different zoonotic agents (*Rickettsia* spp. and PUUV). Regarding TBEV, further investigations are needed to clarify whether the virus is present at the study sites. Because Gerovo and Žutica are situated near protected recreational areas with high numbers of annual visitors, the current and future data will contribute to risk assessment and prevention strategies.

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Author Disclosure Statement

No competing financial interests exist.

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