Am. J. Trop. Med. Hyg., 94(2), 2016, pp. 466–473
doi:10.4269/ajtmh.15-0517

Molecular Survey of Zoonotic Agents in Rodents and Other Small Mammals in Croatia

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Abstract. Croatia is a focus for many rodent-borne zoonosis. Here, we report a survey of 242 rodents and small mammals, including 43 *Myodes glareolus*, 131 *Apodemus flavicollis*, 53 *Apodemus agrarius*, three *Apodemus sylvaticus*, six *Sorex araneus*, four *Microtus arvalis*, one *Microtus agrestis*, and one *Muscardinus avellanarius*, collected at eight sites in Croatia over an 8-year period. Multiplex MassTag polymerase chain reaction (PCR) was used for detection of *Borrelia*, *Rickettsia*, *Bartonella*, *Babesia*, *Ehrlichia*, *Anaplasma*, *Francisella tularensis*, and *Coxiella burnetii*. Individual PCR assays were used for detection of *Leptospira*, lymphocytic choriomeningitis virus, orthopoxviruses, flaviviruses, hantaviruses, and *Toxoplasma gondii*. Of the rodents, 52 (21.5%) were infected with *Leptospira*, 9 (3.7%) with *Borrelia miyamotoi*, 5 (2%) with *Borrelia afzelii*, 29 (12.0%) with *Bartonella*, 8 (3.3%) with *Babesia microti*, 2 (0.8%) with *Ehrlichia*, 4 (1.7%) with *Anaplasma*, 2 (0.8%) with *F. tularensis*, 43 (17.8%) with hantaviruses, and 1 (0.4%) with an orthopoxvirus. Other agents were not detected. Multiple infections were found in 32 rodents (13.2%): dual infections in 26 rodents (10.7%), triple infections in four rodents (2.9%), and quadruple infections in two rodents (0.8%). Our findings indicate that rodents in Croatia harbor a wide range of bacteria and viruses that are pathogenic to humans.

INTRODUCTION

Rodents are hosts to a wide range of zoonotic pathogens. Rodent-borne agents can be transmitted to humans directly through contact with rodents and their excretions or through rodent bites and indirectly by arthropod vectors including ticks, fleas, and mites. Croatia is a natural focus for many rodent-borne zoonoses because of its diverse forest ecology and wide range of rodent species. Different rodent species in Croatia have been implicated as reservoirs to several clinically important bacteria, protozoa, and viruses. The main rodent-associated bacteria include species of *Anaplasma*, *Borrelia*, *Bartonella*, *Coxiella*, *Ehrlichia*, *Francisella*, *Leptospira*, and *Rickettsia*. Infections with *Leptospira* and *Borrelia* are the most frequently reported from among this group. With a mean yearly infection incidence of 1.7 per 100,000 inhabitants, Croatia has one of the highest recorded incidences of human leptospirosis in the world.[1] *Borrelia* infections result in two distinct tick-transmitted diseases, Lyme borreliosis and relapsing fever (RF). Both are endemic in Croatia with majority of cases of borreliosis reported in the northwest of the country.[2] Q fever, caused by *Coxiella burnetii* is endemic in parts of southern Croatia.[3,4] Limited data exist on tick-transmitted *Rickettsia*, *Anaplasma*, and *Ehrlichia*.[5] Human cases of anaplasmosis and rickettsiosis have been reported in Croatia, but little data are available on ehrlichiosis.[6,7] Infections with *Francisella tularensis*, the agent of tularemia, are occasionally reported in Croatia, particularly in Sava Valley in the central part of the country.[8] *Bartonella* spp. are hemotropic bacteria detected in many mammals, predominantly rodents.[9] Many species and subspecies of *Bartonella* circulate in European rodents, although the pathogenicity of many of these species is as yet undetermined. In addition, the diversity of *Bartonella* spp. present in rodents in Croatia has not been examined.

Hantaviruses are the viruses most frequently associated with rodents in Croatia. These include Dobrava (DOBV) and Puumala (PUUV) viruses, implicated in hemorrhagic fever with renal syndrome, and the nonpathogenic Tula (TULV) and Saaremaa viruses.[10–14] Rodents can also be infected with flaviviruses, orthopoxviruses, and lymphocytic choriomeningitis virus (LCMV); however, to our knowledge, there are no published reports of investigation for the presence of these viruses in Croatia.[15–17] Rodents in Croatia are also reservoirs of protozoan *Apicomplexa* parasites, including *Toxoplasma* and *Babesia* that cause toxoplasmosis and babesiosis, respectively, mainly in immunocompromised individuals.

Here, we report results of analysis of rodents and small mammals trapped in eight localities in Croatia for the presence of a wide range of known zoonotic human pathogens.

MATERIALS AND METHODS

Sample collection. This survey was an extension of a project designed to assess the distribution of small rodents in diverse forest ecosystems in Croatia. Between 2003 and 2011, rodents were trapped in eight different localities in Croatia: Ivanić-Grad, Mikanovci, Ilok, Cerna, Papuk, Žutica, Draganić, and Sušica (Figure 1). In each of three selected geographic transects at each location, 100 traps were placed. The distance between the traps was approximately 7 m and the distance between the transects was approximately 150 m. Each transect was at least 50 m away from the edge of the forest stands. Each of these localities includes forest ecosystems without proximity to urban areas. All rodents were trapped using Sherman-type live traps and were morphologically characterized to species.[18] Species identification of *Apodemus flavicollis* and *Apodemus sylvaticus* was confirmed by PCR (polymerase chain reaction) targeting the mitochondrial cytochrome *b* gene and sequencing of PCR products.[19,20] A total of 242 animals were collected, including 131 yellow-necked mouse (*A. flavicollis*), 53 striped field mouse (*Apodemus agrarius*), three wood mouse (*A. sylvaticus*),

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FIGURE 1. Map in Croatia indicating the regions where the rodents were trapped.

43 bank voles (*Myodes glareolus*), six common shrews (*Sorex araneus*), four common voles (*Microtus arvalis*), one field vole (*Microtus agrestis*), and one common dormouse (*Muscardinus avellanarius*). Animal experimentation guidelines of the American Society of Mammologists was followed during all animal work.[21] After euthanasia, kidney and lung samples were collected from individual specimens and stored at −80°C.

Nucleic acid extraction and complementary DNA synthesis. A total of 323 organ specimens were available for PCR analysis. These consisted of lung samples acquired from 237 animals and kidney samples from 86 animals. At least one organ (lung or kidney) was available for PCR analysis from all 242 animals. In cases where both lung and kidney samples were available from an individual animal, both were tested by PCR. Tissues were homogenized using TissueLyser and 5-mm beads for homogenization (Qiagen, Hilden, Germany). DNA and RNA were extracted from each sample using the AllPrep DNA/RNA Mini Kit (Qiagen). DNA from each sample was eluted in 100 μL EB Buffer and RNA from each sample was eluted in 40 μL RNase-free water. Nucleic acid concentration was assessed with the NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA). Complementary DNA (cDNA) was prepared in a 20 μL reaction using 5 μL extracted RNA and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA).

Polymerase chain reaction. MassTag PCR was performed using primers for detection of Lyme borreliosis–associated *Borrelia* spp., *Bartonella* spp., *Ehrlichia* spp., *Anaplasma* spp., *F. tularensis*, *C. burnetii*, *Rickettsia* spp., RF *Borrelia* spp., and *Babesia* spp. (Table 1).[22,23,29] Of template DNA, 2 μL was used in all reactions. Reaction conditions were 94°C for 15 minutes; one cycle at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, followed by 11 cycles with annealing temperature decreased by 1°C in each cycle. The final PCR was run for 38 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds.

All samples that were positive on the MassTag PCR assay were retested in individual PCR assays for the microbe(s) of interest using the same primer pairs. The presence of relevant sequence in the resulting PCR product was required as confirmation before a sample was declared agent positive. In cases where species differentiation was required, we designed primers within a discriminatory region of the genome to amplify partial sequences that were then compared with existing genomic data from GenBank. To discriminate among *Bartonella* spp., we designed primer pairs that amplified 513 nucleotides (nts) of the *gltA* gene and 800 nts of the *16SrRNA* gene. For *Babesia* spp., we used a primer pair that amplified 607 nts of the *18SrRNA* gene, and for *Borrelia* spp., an assay targeting the *flaB* gene amplified a sequence of 966 nts (Table 1). Sequences for *Baronella* spp. and *Borrelia miyamotoi* were deposited in GenBank under accession nos. KT452901–KT452935. All alignments were generated in Geneious v 6.1.5 (Biomatters Ltd., Auckland, New Zealand). Maximum-likelihood phylogenetic tree for *Bartonella* was constructed using the Jukes–Cantor model with 1,000 bootstrap replicates in Mega 6.0.[30]

TABLE 1
Primer sequences used for pathogen detection

Pathogen	Gene target	Primer sequence	Reference
Lyme borreliosis–associated *Borrelia* spp.	*flaB*	Fwd: GCAATGACAAAACATATTGRGGAASTTGA Rev: YACAATGACMGATGAGGTTGTRGC	22
Relapsing fever *Borrelia* spp.	*flaB*	Fwd: GCTGAAGAGCTTGGAATGCAAC Rev: GCAATTGCYTCATCCTGATTTG	23
Bartonella spp.	*hbpA*	Fwd: CTTCTGCRGCACAAGCTGCTGAT Rev: CCACCAATATARAAACCTGTCCAAGA	22
Ehrlichia spp.	*16SrRNA*	Fwd: CGTAAAGGGCACGTAGGTGGACTA Rev: CACCTCAGTGTCAGTATCGARCCA	22
Anaplasma spp.	*16SrRNA*	Fwd: GGGCATGTAGGCGGTTCGGT Rev: TCAGCGTCAGTACCGGACCA	22
Francisella tularensis	*fopA*	Fwd: ATGTTTCGGCATGTGAATAGTTAA Rev: ACCACTGCTTTGTGTAGTAGCTGAA	22
Coxiella burnetii	*IS1111*	Fwd: GCTCCTCCACACGCTTCCAT Rev: GGTTCAACTGTGTGGAATTGATGAGT	22
Rickettsia spp.	*ompB*	Fwd: YAAAAGTCAGCGTTACTTCTTYGA Rev: YGCTTRTTTGCAACTGTTGTACC	22
Babesia spp.	*18SrRNA*	Fwd: CGACTAGDGATTGGDGGTCGTC Rev: CTHTGTCTGGACCTGGTGAGKTT	This study
Lymphocytic choriomeningitis virus	*RdRp*	Fwd: CCACTYTTGTCTGCACTGTCTAT Rev: CTTTTTGATGCGCAATGGAT	This study
Orthopoxvirus	*VETFS*	Fwd: ACCAACTATATTACCTCATCAGTTA Rev: TTAAACAAGTTCATAGCTACACCCA	This study
Flavivirus	*NS5*	Fwd: ATGGCHATGACNGACACNAC Rev: TTCTTYTCTCTYTTNCCCATCAT	This study
Toxoplasma gondii	*B1*	Fwd: GAAGAGATCCAGCAGATCTCGT Rev: TGAGAGGAGGCAGCACAAG	This study
Puumala/Tula hantavirus	*N*	Fwd1: TATGGIAATGTCCTTGATGT Rev1: GCACAIGCAAAIACCCA Fwd2: CCIAGTGGICAIACAGC Rev2: AAICCIATIACICCCAT	24 24
Dobrava hantavirus	*G*	Fwd1: GGACCAGGTGCAGCTTGTGAAGC Rev1: ACCTCACAAACCATTGAACC Fwd2: ATGCCAGCGAGTCGACCAA Rev2: GAGCTATTATGTAAGATTGC	25 26
Leptospira spp.	*LA0322*	FwdA: CATTCATGTTTCGAATCATTTCAAA RevA: GCCCAAGTTCCTTCTAAAAG	27
	secY	FwdB: GAATTTCTCTTTTGATCTTCG RevB: GAGTTAGAGCTCAAATCTAAG	28
Bartonella spp.*	*gltA*	Fwd: GGTCTATCAYGACTCKATTGATATTA Rev: GCACGTGGRTCATAATTTTTATARAC	This study
	16SrRNA	Fwd: ATAGCAAGTCGAGCGCRCT Rev: GCCCGACGGCTAACATTC	This study
Borrelia spp.*	*flaB*	Fwd: GGGGATGATYATMAATCATAATAC Rev: TGCAATCATWGCCATTGC	This study
Babesia spp.*	*18SrRNA*	Fwd: TTCGACGGTAKGGTATTGGC Rev: GGGAATTTCACCTCTGACAGTYAA	This study

*Consensus genus primers used for species discrimination.

All samples were additionally tested for LCMV, orthopoxvirus, flaviviruses, hantaviruses, and *Toxoplasma gondii* in single-agent PCR assays. For detection of RNA viruses, 2 μL cDNA were used as template. For the detection of orthopoxviruses and *T. gondii*, 2 μL DNA was used. Nested PCR assays were used to detect the presence of hantaviruses (PUUV, DOBV, and TULV).[24–26] All primer sequences are listed in Table 1. PCR products were size-fractionated in 1% agarose gels and visualized using ethidium bromide before purification for dideoxy sequencing.

Detection of *Leptospira* by renal tissue culture and PCR. Kidney tissue from each animal ($N = 242$) was available for *Leptospira* detection. Tissue was inoculated into Korthof's medium for isolation and stabilization of *Leptospira* isolates. Isolates were grown to a density of 2–4 × 10^8/mL and harvested by centrifugation at 14,000 rpm. Genomic DNA was extracted from each kidney tissue using the QIAamp DNA Mini Kit (Qiagen). Two PCR assays were used for species identification. In the first assay, real-time PCR was used as described by Merien and others[27] with a primer set that amplified 331 nts of the locus LA0322 obtained from the complete genome sequence of *Leptospira interrogans* serovar Lai. All specimens were tested 10 times and mean melting temperatures were calculated to distinguish *Leptospira* genomic species. In a second assay, PCR was done with primers that amplified a 658-nt segment of *secY* that includes a 245-nt fragment suitable for rapid phylogenetic determination.[28] Amplified products were visualized in a 1.5% agarose gel, and their sizes were estimated by comparison with a 100-bp ladder (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom). PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and sequenced. DNA sequence clustal alignments were done using the LaserGene software package (DNASTAR, LaserGene, Madison, WI). In addition, a primer set that amplified a 563-nt DNA fragment of *Leptospira kirschneri* was used to confirm the speciation of *L. kirschneri* isolates.[31]

RESULTS

In our survey, 114 of 242 rodents (47%) were positive for at least one agent. Polymicrobial infections were detected in 32 rodents (13%). Dual infections were detected in 26 rodents (11%), triple infections in four rodents (3%), and quadruple infections in two rodents (0.8%) (Table 2).

Leptospira. *Leptospira* species were the most frequently detected agents with 52 *Leptospira*-positive rodents (21%). *Apodemus* spp. have previously been implicated as reservoirs of *Leptospira* in Croatia.[32,33] Consistent with this earlier work, we found that 49 of 52 *Leptospira*-positive rodents represented *Apodemus* spp. Bacterial isolation from kidney tissue was successful in 40 PCR-positive rodents, and speciation assays indicated that 22 were *L. interrogans*, 16 *L. kirschneri*, and two *Leptospira borgpetersenii*.

Borrelia. Five rodents were positive for Lyme borreliosis–associated *Borrelia* spp. All were identified as *Borrelia afzelii* (> 99% identity with *B. afzelii* PKo strain, accession no. CP002933). The presence of this species in Croatian rodents has been reported previously.[34] Although *Borrelia garinii* is known to circulate in Croatia, we did not detect any rodents infected with this species. RF *Borrelia* was detected in nine rodents (4%) from four different localities. All RF *Borrelia* were identified as *B. miyamotoi* (> 99% identity with LB-2001 strain, accession no. CP006647) with the majority (7/9) detected in *A. agrarius.* Two *B. miyamotoi*-positive rodents originated from Ivanić-Grad in the central part of Croatia, and the remaining seven were trapped in the eastern end of the country.

Bartonella. *Bartonella* spp. were detected in 29 animals (12%) in six different localities with the highest prevalence in Ilok and Draganić (26.7% and 25.0%, respectively). Although *A. flavicollis* was the most frequent reservoir of *Bartonella* in this study (9 of 29 *Bartonella*-positive rodents) and in earlier studies,[35] *A. agrarius* as well as *M. glareolus* and *S. araneus* were also positive for *Bartonella*. To determine the phylogeny of *Bartonella* in these rodents, we obtained partial sequences of *16SrRNA* and *gltA* genes by PCR. Previous reports have indicated that sequence analysis of *16SrRNA* gene alone is inadequate for species/genotype demarcation in *Bartonella*, and an additional loci need to be analyzed, with *gltA* the preferred gene used to establish *Bartonella* phylogeny.[36] Our analysis based on a 388-nt *gltA* sequence indicated that representatives of six putative *Bartonella* species were present in the these rodents (Figure 2). Five rodents were infected with strains representative of *Bartonella grahamii* and nine rodents were infected with strains of *Bartonella taylorii*. Both species are distributed throughout Eurasia and are frequently detected in *Apodemus* and *Myodes* rodents.[37,38] A single *A. flavicollis* was infected with a strain that clustered with *Bartonella elizabethae* species

TABLE 2
Summary of all agents and coinfections detected in different rodent species

	N	*Apodemus flavicollis*	*Apodemus agrarius*	*Apodemus sylvaticus*	*Myodes glareolus*	*Sorex araneus*	*Microtus arvalis*	*Microtus agrestis*	*Muscardinus avellanarius*
Total	242	131	53	3	43	6	4	1	1
Borrelia afzelii	5	2	1	–	3	–	–	–	–
Bartonella spp.	29	16	7	–	5	1	–	–	–
Babesia microti	8	4	3	–	–	–	–	1	–
Ehrlichia spp.	2	1	1	–	–	–	–	–	–
Borrelia miyamotoi	9	1	7	–	–	1	–	–	–
Anaplasma spp.	4	3	–	–	1	–	–	–	–
Francisella tularensis	2	–	2	–	–	–	–	–	–
Leptospira spp.	52	37	12	–	1	–	1	–	1
Hantavirus	43	36	2	–	4	–	1	–	–
DOBV	38	36	2	–	–	–	–	–	–
PUUV	4	–	–	–	4	–	–	–	–
TULV	1	–	–	–	–	–	1	–	–
Orthopoxvirus	1	–	–	–	1	–	–	–	–
Infections with two agents									
Leptospira, *B. afzelii*	1	1	–	–	–	–	–	–	–
Leptospira, *Ehrlichia*	1	–	1	–	–	–	–	–	–
Leptospira, *B. microti*	1	1	–	–	–	–	–	–	–
Leptospira, *Bartonella*	3	3	–	–	–	–	–	–	–
Leptospira, *Anaplasma*	1	1	–	–	–	–	–	–	–
Leptospira, *B. miyamotoi*	3	1	2	–	–	–	–	–	–
Leptospira, TULV	1	–	–	–	–	–	1	–	–
Leptospira, DOBV	10	10	–	–	–	–		–	–
Bartonella, DOBV	3	3	–	–	–	–	–	–	–
B. microti, *B. miyamotoi*	1	–	1	–	–	–	–	–	–
Anaplasma, DOBV	1	1	–	–	–	–	–	–	–
Infections with three agents									
Leptospira, *B. afzelii*, *B. microti*	1		1	–	–	–	–	–	–
Bartonella, *Anaplasma*, DOBV	1	1	–	–	–	–	–	–	–
B. microti, *Leptospira*, DOBV	2	2	–	–	–	–	–	–	–
Infections with four agents									
Bartonella, *B. afzelii*, *Leptospira*, DOBV	1	1	–	–	–	–	–	–	–
Bartonella, *F. tularensis*, *Leptospira*, DOBV	1	–	1	–	–	–	–	–	–

DOBV = Dobrava virus; PUUV = Puumala virus; TULV = Tula virus.

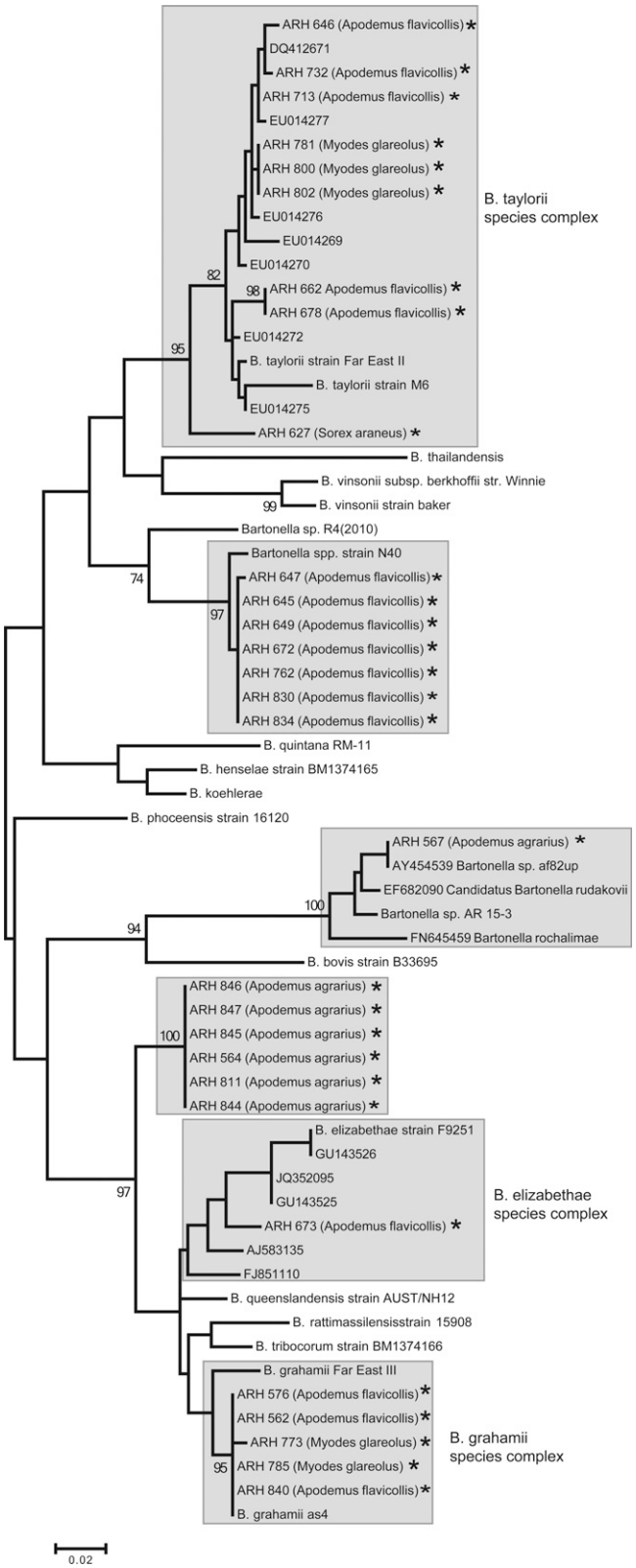

FIGURE 2. Maximum likelihood phylogenetic tree based on a 388-nucleotide fragment of *Bartonella gltA* gene. All sequences obtained in this study are indicated by * and the six species they cluster into are shown in gray. Only select relevant sequences from GenBank are shown in the tree.

group. Seven *A. flavicollis* were infected with a genotype similar to strain N40 isolated from *A. sylvaticus* from the United Kingdom.[39] On the basis of high sequence divergence relative to other strains and a distinct *gltA* phylogenic clade formed by this group, we speculate that this genotype constitutes a new *Bartonella* species. Sequences obtained from six *A. agrarius* formed a monophyletic clade and likely constitute another potential novel *Bartonella* species. We also detected a single *Bartonella* strain (ARH567) in *A. agrarius* that clustered with AR15-3 strain from the United States (FN645480) and strains from Sweden (AY454539), Siberia (EF682090). Analysis of the *16SrRNA* sequence indicated that ARH567 was > 99% identical to *B. rochalimae* (strain BAA-1498, accession no. FN645459) and thus may constitute a *B. rochalimae* subspecies.

Babesia. Eight rodents from five localities were infected with *Babesia,* all identified as *Babesia microti* (100% identity to strain Jena, accession no. EF413181). Previous work has implicated *A. flavicollis* and *M. glareolus* as the primary reservoirs for *B. microti* in Europe.[40] Of the eight positive rodents in our study, three were *A. agrarius* and one *M. agrestis*, implicating these species as additional reservoirs of *Babesia* spp.

Hantaviruses. Hantaviruses were the second most frequently detected pathogens. Of 43 hantavirus-positive rodents, 38 were infected with DOBV, four with PUUV, and a single rodent was infected with TULV. Hantavirus-positive rodents originated from seven different locations with the highest prevalence in Žutica (34.5%) and Ilok (20%) (Table 3). Among *Apodemus* species, 28% of *A. flavicollis* and 2% of *A. agrarius* were DOBV positive. The highest prevalence of DOBV-positive rodents (30%) was in Žutica (Table 3). PUUV was detected in *M. glareolus* and TULV was detected in *M. arvalis*, similar to previous reports.[12]

Other agents. *Francisella tularensis* was detected in two *A. agrarius* from the Draganić site. One rodent was positive for orthopoxvirus. This is the first report of *F. tularensis* and orthopoxvirus infection in rodents in Croatia. Four rodents were positive for *Anaplasma* and two for *Ehrlichia*. *Rickettsia*, *C. burnetii*, LCMV, flaviviruses, and *T. gondii* were not detected. Among the agents detected, RF *Borrelia*, *Bartonella*, *Babesia*, *Ehrlichia*, and hantaviruses were detected in nucleic acids isolated from both lung and kidney tissues; *Anaplasma*, *Francisella*, and *B. afzelii* were detected in nucleic acids isolated only from lung tissue.

DISCUSSION

In this study, we demonstrate the presence of multiple zoonotic pathogens in rodent species present in Croatia. Although this study was not designed to demonstrate a linkage between the presence of these agents and human disease, the data presented here may provide insight into assessing the risk of rodent-borne zoonoses in Croatia.

Leptospira spp. and hantaviruses were the human pathogens most frequently detected in our study. The high prevalence of *Leptospira*-positive rodents parallels recent surveys in Croatia that revealed *Leptospira* infection in > 20% of rodents. The majority of *Leptospira*-positive rodents reported here were *Apodemus* species. In our previous work, *Leptospira* spp. were isolated from 13 of 28 *A. flavicollis* captured in Žutica forest.[41] *Leptospira interrogans*, *L. kirschneri*, and *L. borgpetersenii* were identified as the *Leptospira* species in our study, similar to

TABLE 3
Number of agent-positive rodents at each trapping site

	Ivanić-Grad	Mikanovci	Ilok	Cerna	Papuk	Žutica	Draganić	Sušica	Total
	N = 39	*N* = 28	*N* = 30	*N* = 9	*N* = 18	*N* = 87	*N* = 16	*N* = 15	242
Borrelia afzelii	–	1	–	–	–	3	0	1	5
Bartonella	4	1	8	–	2	10	4	–	29
Babesia microti	1	2	–	1	–	3	1	–	8
Ehrlichia	1	1	–	–	–	–	–	–	2
Borrelia miyamotoi	2	4	1	2	–	–	–	–	9
Anaplasma	–	–	–	–	3	1	–	–	4
Francisella tularensis	–	–	–	–	0	0	2	–	2
Leptospira	4	11	9	1	1	24	–	1	52
Hantaviruses	1	1	6	–	3	30	1	1	43
DOBV	1	–	6	–	3	26	1	1	38
PUUV	–	–	–	–	–	4	–	–	4
TULV	–	1	–	–	–	–	–	–	1
Orthopoxvirus	–	–	–	–	–	1	–	–	1

DOBV = Dobrava virus; PUUV = Puumala virus; TULV = Tula virus.

previous reports from Croatian rodents.[32,33] Hantavirus and *Leptospira* also represented the most frequent coinfections. We previously showed coinfections with both agents in humans[10] and rodents[41,42] highlighting the potential risk for simultaneous transmission of these pathogens. The clinical underestimation of hantavirus and *Leptospira* coinfections was recently emphasized.[43]

The genus *Bartonella* contains over 30 named species. Approximately 20 are adapted to wild rodents and six of these have caused documented human infections.[37] We detected the presence of six putative species present in Croatian rodents. Our data provide insight into the diversity of *Bartonella* in Croatia and the high overall genetic diversity of this genus worldwide. The extent of human infections associated with rodent-borne *Bartonella* is unknown as data regarding the incidence of zoonotic human *Bartonella* infections in Europe are not available. However, despite a relatively high prevalence of infected rodents, based on existing reports of infections due to rodent-borne *Bartonella*, we anticipate that the risk of human infection is low.

In this study, we report the first evidence of *Borrelia miyamotoi* in Croatia and have implicated *Apodemus* mice as reservoirs, similar to recent report from Switzerland.[44] *Borrelia miyamotoi* is an emerging tick-borne pathogen phylogenetically classified within the RF *Borrelia* group. Originally detected in Asia, it was subsequently detected in North America and Europe.[23,45,46] Recent work has also implicated it as a potential cause of meningoencephalitis.[47,48]

The prevalence of *Anaplasma* spp. infection in our study was 1.7%. *Anaplasma* spp. were detected in only two locations in central Croatia (Papuk and Žutica) among *A. flavicollis* and *M. glareolus*. *Anaplasma* spp. were previously reported in small rodents elsewhere in Europe,[49] but not in Croatia. *Ehrlichia* spp. were detected in only two rodents (0.8%) of genus *Apodemus* in two locations (Ivanić-Grad and Mikanovci).

A limitation of this study was the unavailability of organs other than lungs and kidneys for analysis. This is because we used samples banked during the course of a hantavirus and *Leptospira* surveillance project wherein only lungs and kidneys were collected. Thus, we may have missed some bacteria and viruses that concentrate in other organs. Furthermore, we did not screen for all rodent-associated agents, nor did we pursue unbiased sequencing. Nonetheless, our data indicate that small rodents in Croatia are reservoirs for a wide range of viral, bacterial, and protozoan pathogens. We also demonstrated a high rate of coinfections that may have important implications for simultaneous transmission to humans.

Received July 10, 2015. Accepted for publication November 2, 2015.

Published online December 28, 2015.

Financial support: Work reported here was supported by awards from the NIH (AI057158, Northeast Biodefense Center-Lipkin), USAID-PREDICT, DTRA, and by the grants 143-1430115-0103, 068-1430115-2119, 053-1430115-2116 from the Ministry of Science, Education and Sports of Republic Croatia and the project "The role of biotic agents on vitality of narrow-leafed ash in Croatian floodplain forests" from Croatian Science Foundation.

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