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First records of *Borrelia burgdorferi* sensu lato in voles and selected *Apodemus* species in Serbia

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Abstract

Background: Voles and Apodemus mice are recognized as important hosts for numerous parasites and pathogens. Understanding the occurrence of zoonoses, key host characteristics, and the factors influencing their population dynamics and distribution is essential for predicting disease occurrence and assessing the risks of transmission and spread. Determining the role of voles and field mice, in maintaining B. burgdorferi s.l. in the environment clarifies its transmission during tick activity periods and highlights the need for timely monitoring and control measures.

Results: We examined the prevalence of *Borrelia burgdorferi* sensu lato in solid heart tissue of small rodent species (*Clethrionomys glareolus, Apodemus* spp*., Microtus* spp.). Detection and molecular typing of bacteria was completed using real-Time PCR and Sanger sequencing. The presence of the pathogen *Borrelia afzelii* in voles (*Microtus arvalis*, *C. glareolus*) and mice (*Apodemus agrarius*, *Apodemus sylvaticus*) was recorded in Serbia for the first time during this

study. The prevalence of *B. burgdorferi* s.l. infection in the collected *M. arvalis* and *C. glareolus* was 12.9%. and 8.2%, respectively. The prevalence of infected *A. agrarius* and *A. sylvaticus* was 66.7% and 20%, respectively.

Conclusions: The presence of *B. burgdorferi* s.l. in the sampled animals suggests that rodent species may act as hosts for this pathogen and contribute to its maintenance in the environment.

Keywords: rodent, *Borrelia burgdorferi* sensu lato, *Borrelia afzelii*, vole, *Apodemus* spp., zoonoses

Introduction

Lyme borreliosis is a cosmopolitan disease caused by the spirochete *Borrelia burgdorferi* sensu lato, which includes a complex of more than 20 species. A wide range of domestic and wild animals, including rodents, participate in the maintenance and dissemination of spirochetes [1-5]. The most important tick hosts, which represent key factors in the transmission of Lyme disease in Europe, are rodents and deer [2]. Pathogenic species known to infect humans are primarily associated with transmission involving small rodents and birds [6]. Rodents serve as hosts of *Borrelia afzelii*, one of the causative agents of Lyme borreliosis alongside *Borrelia burgdorferi* sensu stricto and *Borrelia garinii*, which are mainly maintained by larger mammals and birds [2-5,7]. An important feature of rodents, in terms of maintenance of the pathogen in nature, is that the infection has no effect on the survival or general their body conditions, which secures perpetuation of the causal agent as the source of infection development and transmission [8,9].

The role of rodents in the transmission chain of *Borrelia* is very significant. It is especially important that rodents are suitable *Borrelia* reservoirs because bacteria develop in their bodies

and are then spread by ticks. In contrast to rodents, certain large mammals, such as deer, owing to their body size, function as highly competent hosts to vectors. However, they do not serve as competent reservoirs for the maintenance and amplification of *Borrelia*. Accordingly, their role in the transmission dynamics of *Borrelia* is restricted to supporting infected tick populations and facilitating their subsequent dispersal [10,11]. Uninfected tick larvae get infected through feeding on *Borrelia* infected hosts, mostly rodents, who are both suitable hosts and reservoirs. Once infected, rodents remain infective for life [12-16]. Research data for striped field mice (*A. agrarius*) showed that *B. afzelii* had the highest infection rate in the rodent heart, followed by spleen, kidneys and lungs, while *B. burgdorferi* s.s. and *B. garinii* were detected only in spleen [12-14,17,18].

Small mammals are suitable hosts for immature tick stages (larvae and nymphs), both as feeding sources and as facilitators of their spatial distribution due to their low body size [14,15, 19,20]. Small rodents are also important for spreading *Borrelia* because they are able to host larvae and nymphs simultaneously, in contrast to larger mammals [21]. Both *Apodemus* mice and voles are important hosts for immature stages of ticks, although infestation levels vary depending on habitat, host abundance and season [22,23]. Some researchers suggest that *Apodemus* mice are recognized as more competent reservoirs for *B. afzelii* than voles. In accordance with studies, vole has demonstrated a strong immune response to enzyme secretion by ticks [24,25]. The result is slower blood sucking by ticks and delayed development of nymphs [14,26].

The overall risk of spreading zoonotic pathogens by small rodents, such as *Borrelia*, is generally considered to be higher in natural habitats than in urban environments. The difference between natural and urban areas is probably related to differences in the abundance and diversity of arthropod vectors and the composition of vertebrate community [27]. Additionally,

some researchers have indicated that there is no significant difference between *Borrelia* spp. prevalence in natural and urban environments [28].

The most prevalent species of the *B. burgdorferi* s.l. complex in Europe are *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* [29,30]. In Serbia, the presence of *B. burgdorferi* s.s., *B. garinii*, and *Borrelia lusitaniae* was confirmed by PCR in analyzed red foxes [5]. Analysis of jackal tissues revealed the presence of *Borrelia valaisiana*, *B. garinii*, and *B. lusitaniae* [4]. The analysis of ticks confirmed the presence of *Borrelia bavariensis*, *B. lusitaniae*, *B. garinii* and *B. valaisiana* strains [31], and according to Potkonjak et al. [32], presence of *Borrelia miyamotoi* in ticks collected in vegetation is also present. In 2018, Lyme borreliosis was put on the European Union epidemiological surveillance list [33]. Current Serbian legislation regarding the protection of the population from infectious diseases does not mandate the reporting of Lyme borreliosis in humans or animals, as it is not currently subject to formal epidemiological surveillance [34].

The aim of this study was to determine the presence of *Borrelia* in tissues of different small rodent species as primary hosts, collected form locations in Serbia that are geographically distant or separated by natural barriers. Generally, establishing the role of small rodent species in the pathogen maintenance cycle further helps to identify the risk of transmission during tick activity periods and the rationale for establishing timely monitoring and control measures.

Materials and Methods

Samples Collection

Animals were collected over the spring and autumn of 2021 on three locations in Serbia: Bogatić (44°49'N, 19°30'E), Putinci (44°59'N, 19°57'E) and Bavanište (44°49'N, 20°53'E). The locations were selected based on an assumption that there would be no mixing of populations.

Rivers were natural barriers which separated the three locations (Fig. 1). Animals were trapped in alfalfa crops at field edge during the period of greatest activity of rodent populations.

Every night at each location 49 snap traps (7x7) were set. To increase trap success and species diversity, the traps were placed near the peripheral edges of alfalfa field. In total there were 147 trap nights per location. The number of trap nights in calculations of relative density index was corrected for the number of traps found to be inactive. A total of 5, 10 and 11 traps were inactive on the locations Putinci, Bavanište and Bogatić, respectively. All trapped animals were transferred to the Laboratory for Applied Zoology, Institute of Pesticides and Environmental Protection, Belgrade. The species were determined based on their external characteristics, body measurements and cranial morphometrics [35,36]. Autopsy and further analysis of animal hearts were conducted in the Scientific Veterinary Institute 'Novi Sad'. All samples were transported and kept under conditions of -20 °C before preparation for analysis.

Sample Preparation and DNA Extraction

For each of the 150 samples analyzed for the presence of *B. burgdorferi* s.l., a sample of solid heart tissue was suspended in 1 mL of phosphate-buffered saline along with quartz sand and a stainless-steel bead. The tissues were then homogenized for three minutes at 50 Hz using a TissueLyser LT (Qiagen, USA). Following homogenization, the samples were centrifuged at 12,000 rpm, and 200 µL of the supernatant was used in subsequent DNA extraction procedures, following the manufacturer's protocol (PureLink™ Genomic DNA Mini Kit, Invitrogen, USA, REF K182002). After extraction, the sample DNA was either used immediately for real-time PCR analysis or stored at -20° C until further analysis.

Molecular analysis

Real-time PCR

All samples were analyzed for the presence of *B. burgdorferi* s.l. Detection of the presence of this pathogen was done by real-time PCR (Mx3005P® QPCR System (Agilent, CA 95051, United States), using commercially available kit- BactoReal® Kit *B. burgdorferi* s.l. (Ingenetix GmbH, Vienna, Austria), according to the manufacturer's instructions. The test is based on multiplex real-time PCR by 5'-nuclease-assay technology for qualitative DNA detection of the ospA gene of *B. burgdorferi* s.l. The assay targets the ospA gene and includes an internal positive control (IPC) detected in the HEX channel, which verifies successful amplification and absence of PCR inhibition. Amplification was performed on an Mx3005P® QPCR System (Agilent, USA). Samples showing a detectable Ct (Cp) value in the FAM channel together with a valid IPC signal and no amplification in the negative control were considered positive. The fluorescence threshold was set manually above the background of the negative control. The kit we use BactoReal® Kit *B. burgdorferi* s.l. (Ingenetix GmbH, Vienna, Austria) gives qualitative results, not quantitative.

Molecular Typing of B. burgdorferi s.l.

The polymorphic outer surface protein C (OspC) gene was used for molecular typing. After DNA extraction from tissue samples, a 617-bp region of the OspC gene was amplified using the primers OC6(+) and OC623(-) [37] and Q5® High-Fidelity PCR Kit (New England BioLabs) according to the manufacturer's instructions with primer annealing temperature of 54°C. The amplified regions were sequenced using Sanger sequencing (Macrogen Europe). Raw sequences were processed and consensus sequences were generated using the Staden package [38]. To verify species identity, each consensus sequence was queried using BLASTn against a custom local database containing all publicly available *B. burgdorferi* s.l. OspC reference sequences downloaded from GenBank. Species identification was confirmed when the top hit matched a known *B. burgdorferi* s.l. OspC allele with ≥98% identity across the full

alignment length. The sequences were submitted in GenBank under the accession numbers PQ676528 and PQ676529.

Data Analysis

The number of collected small rodent species was used to calculate the population density index (I) [39] and the percent of species presence on all locations. The mean prevalence and corresponding 95% confidence intervals (CI) for *B. burgdorferi* s.l. in rodents were calculated using the Clopper–Pearson method.

Results

Rodent Species

A total of 150 animals were caught: 62 common voles (*Microtus arvalis*), 49 bank voles (*Clethrionomys glareolus*), 22 European pine voles (*Microtus subterraneus*), five wood mice (*Apodemus sylvaticus*), nine yellow-necked mice (*Apodemus flavicollis*) and three striped field mice. The population density index indicated that the highest abundance of small rodent species was recorded at Bogatić. Voles were proportionally more abundant than *Apodemus* mice at all locations (Table 1). At the Bogatić, 97.6% of the captured individuals were voles. Only voles were captured at the Putinci, while at the Bavanište the proportion of captured voles and *Apodemus* mice was 71.7% and 28.3%, respectively.

Results of Molecular Typing of *B. burgdorferi s.l.*

All 150 samples were analyzed for the presence of pathogenic *B. burgdorferi* s.l. Considering the heart tissue, *B. burgdorferi* s.l. was detected in a total of 15 out of 150 animals (10.0, 95% CI: 5.71–15.96) upon amplification by real-time PCR**.** Our samples showed the greatest similarity with sequences belonging to *B. afzelii* (99.3%). *B. burgdorferi* s.l. was confirmed in

animals from Bogatić with 3.7% prevalence, and locality Bavanište with 22.6% prevalence (Table 1). *B. burgdorferi* s.l. was not confirmed in samples from the location Putinci.

The prevalence of *B. burgdorferi* s.l infection in voles was 12.9% and 8.2% for common vole and bank vole, respectively. The prevalence for striped field mouse and wood mouse was 66.7% and 20%, respectively. *B. burgdorferi* s.l. was not detected in European pine voles and yellow-necked mice.

Discussion

Small mammals are important hosts and reservoirs of *B. burgdorferi* s.l.. Infected ticks are the main vectors of the pathogen within rodent populations, as well as to humans and domestic and wild animals. The risk of *B. burgdorferi* s.l. being transmitted to the human population correlates with the presence of pathogen reservoirs and infected ticks as vectors [2,14,31,40-45].

The lowest population density of small rodent species was recorded at the Putinci site, where only the common vole and bank vole were present. No pathogen presence was detected at this site, whereas it was confirmed at Bavanište and Bogatić at small rodent densities ranging from 38.7 to 60.2. Despite a twofold higher population density index of bank voles at Bogatić compared to Bavanište, the number of pathogen-positive individuals was identical between the two sites. Rodent community diversity was highest at Bavanište (six species), compared to Bogatić (four species). Mixed populations of small rodent species are usually present in Serbia [46,47]. Future studies should confirm the role of small rodent community structure, interspecific interactions, and tick abundance in pathogen maintenance.

Our results present the first evidence of *B. burgdorferi* s.l. in voles (common vole and bank vole) and mouse species (striped field mouse and wood mouse) in Serbia. Previous studies in Serbia suggested a potential role of rodents in the *Borrelia* transmission cycle, based solely on

the confirmation of its presence in yellow-necked mice. Stajković et al. [48] confirmed the presence of *B. burgdorferi* s.l. in yellow-necked mice, reporting two animals collected in an urban area of Belgrade, one of which tested positive. Similarly, Veinović et al. [49] detected *B. afzelii* in the same species, with one out of 30 yellow-necked mice collected in Belgrade's Avala suburb testing positive (3.3%). As demonstrated in our study, both *Apodemus* mice and voles serve as hosts for the pathogen.

A study in Croatia has confirmed the presence of *B. afzelii* in *Apodemus* mice and bank voles. The prevalence of *B. afzelli* in striped field mice was 3.8% and bank vole prevalence (7%) corresponds to our results [50]. The presence of *B. afzelii* in Croatian rodents had also been reported previously in fat dormouse (*Glis glis*) with 8.9% *B. afzelli* positive results [51]. Similar research in Romania reported a lower overall prevalence (4.9%) than we found in our study [52]. In general, there is a lack of data on the presence of *B. burgdorferi* s.l. in rodents in the Balkans [53].

The overall prevalence for *B. burgdorferi* s.l. in small mammals in our study of 10% is in line with results reported from France that showed 11% prevalence [54]. Overall prevalence of *Borrelia* in Germany (6.2%) is consistent with previous results reported from Poland (6.8%) and slightly lower than the results in our research [55,56]. On the other hand, the percentage of rodent infestation was significantly lower at the Bogatić location, while *B. burgdorferi* s.l. was not confirmed at all at Putinci.

The incidence of *Borrelia* is higher in areas with plenty of vertebrate animals are serving as host for infected ticks [41,57,58]. In accordance with Lassen et al. [59] the type of vegetation has an influence on *B. burgdorferi* s.l. prevalence and genodiversity. The area in which specimens were collected at Putinci is surrounded by arable fields. Many areas lacked suitable habitats, such as bushes or forest belts, for other vertebrate-host animals, and some plots were bordered by roads. In contrast, taller vegetation at Bavanište and Bogatić provided adequate

shelter for larger vertebrate-host species. Some next studies could investigate how vegetation structure, the presence of other vertebrate hosts, and rodent abundance affect the emergence and spread of zoonotic pathogens, including *Borrelia*.

Research on Lyme borreliosis in Serbia has primarily focused on analyzing samples collected from ticks or from the blood of patients and voluntary blood donors [31,40,44,45,60,]. According to more recent studies, *B. lusitaniae* has been reported as the most prevalent species, followed by *B. afzelii* [31,32,40, 61].

Conclusion

The results of this research confirm rodents as hosts of the pathogen of interest and represent the first detection of *B. burgdorferi* s.l. in voles (common vole and bank vole) and *Apodemus* mice (striped field mouse and wood mouse) in Serbia, suggesting their potential role in the perpetuation and transmission of *B. burgdorferi* s.l. across the country.

Furthermore, it is necessary to investigate the causes of the uneven spatial distribution of pathogens across different locations as well as effects of different habitats on pathogen prevalence, and relationship between number of infected animals, animal species and infected tick. When feasible, such insights should be utilized to prevent pathogen spread with infected ticks, and conduct the measures to minimize infection risks among human populations.

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Author contributions

G.J. led the research design. G.J., T.B. and L.B. contributed to data and sample collection. M.Ž. and V.G. performed sample preparation and molecular analysis. T.P., S.S., M.Ž. and V.G. contributed reagents/materials/analysis. T.B. wrote the first draft of the manuscript. T.P., L.B. and S.S. provided critical revisions in the writing process. All authors have reviewed and approved final version of the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate with the approval number: No. 323-07-04943/2020-05, dated 29.5.2020.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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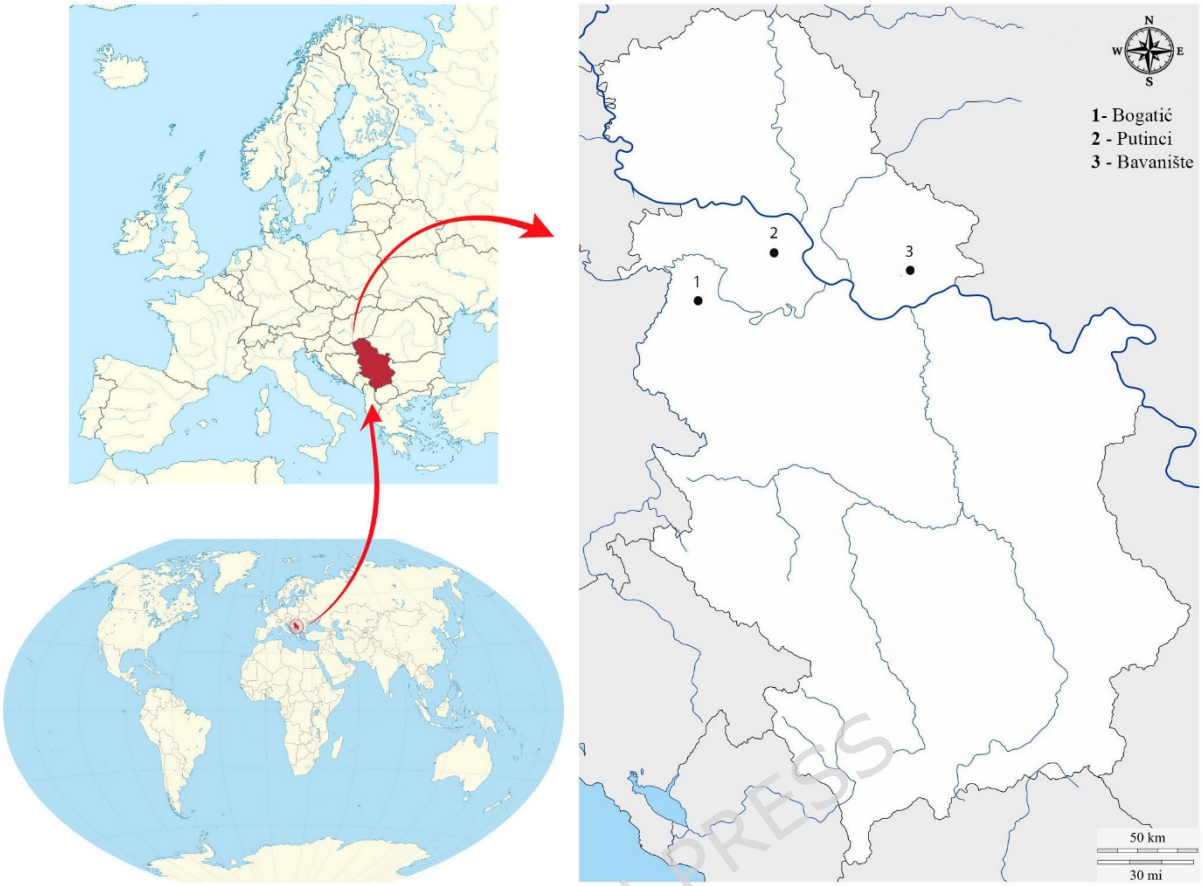

Figure 1. Location of the study area and small rodent trapping sites in Serbia

Table 1. Relative abundance (%), population density index (I) and Clopper-Pearson confidence interval (CI) of small rodents and *Borrelia burgdorferi* sensu lato prevalence (%) across sampling locations

Locality/species	Relative abundance	I	n positive/ n tested	CI	Prevalence
Bogatić					
Microtus subterraneus	24.4	14.7	0/20	-	-
Microtus arvalis	36.6	22.0	1/30	0.08-17.22	3.33
Clethrionomys glareolus	36.6	22.0	2/30	0.82-22.07	6.67
Apodemus flavicollis	2.4	1.5	0/2	-	-
Total	100	60.2	3/82	0.76-10.32	3.66
Putinci					
Microtus arvalis	66.7	7.0	0/10	-	-
Clethrionomys glareolus	33.3	3.5	0/5	-	-
Total	100	10.6	0/15	-	-
Bavanište					
Microtus subterraneus	3.8	1.5	0/2	-	-
Microtus arvalis	41.5	16.0	7/22	13.86-54.87	31.82
Clethrionomys glareolus	26.4	10.2	2/14	1.78-42.81	14.28
Apodemus agrarius	5.7	2.2	2/3	9.43-99.16	66.67
Apodemus sylvaticus	9.4	3.7	1/5	0.51-71.64	20
Apodemus flavicollis	13.2	5.1	0/7	-	-
Total	100	38.7	12/53	12.28-36.21	22.64

First records of *Borrelia burgdorferi* sensu lato in voles and selected *Apodemus* species in Serbia

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Abstract

Background: Voles and Apodemus mice are recognized as important hosts for numerous parasites and pathogens. Understanding the occurrence of zoonoses, key host characteristics, and the factors influencing their population dynamics and distribution is essential for predicting disease occurrence and assessing the risks of transmission and spread. Determining the role of voles and field mice, in maintaining B. burgdorferi s.l. in the environment clarifies its transmission during tick activity periods and highlights the need for timely monitoring and control measures.

Results: We examined the prevalence of *Borrelia burgdorferi* sensu lato in solid heart tissue of small rodent species (*Clethrionomys glareolus, Apodemus* spp*., Microtus* spp.). Detection and molecular typing of bacteria was completed using real-Time PCR and Sanger sequencing. The presence of the pathogen *Borrelia afzelii* in voles (*Microtus arvalis*, *C. glareolus*) and mice (*Apodemus agrarius*, *Apodemus sylvaticus*) was recorded in Serbia for the first time during this

study. The prevalence of *B. burgdorferi* s.l. infection in the collected *M. arvalis* and *C. glareolus* was 12.9%. and 8.2%, respectively. The prevalence of infected *A. agrarius* and *A. sylvaticus* was 66.7% and 20%, respectively.

Conclusions: The presence of *B. burgdorferi* s.l. in the sampled animals suggests that rodent species may act as hosts for this pathogen and contribute to its maintenance in the environment.

Keywords: rodent, *Borrelia burgdorferi* sensu lato, *Borrelia afzelii*, vole, *Apodemus* spp., zoonoses

Introduction

Lyme borreliosis is a cosmopolitan disease caused by the spirochete *Borrelia burgdorferi* sensu lato, which includes a complex of more than 20 species. A wide range of domestic and wild animals, including rodents, participate in the maintenance and dissemination of spirochetes [1-5]. The most important tick hosts, which represent key factors in the transmission of Lyme disease in Europe, are rodents and deer [2]. Pathogenic species known to infect humans are primarily associated with transmission involving small rodents and birds [6]. Rodents serve as hosts of *Borrelia afzelii*, one of the causative agents of Lyme borreliosis alongside *Borrelia burgdorferi* sensu stricto and *Borrelia garinii*, which are mainly maintained by larger mammals and birds [2-5,7]. An important feature of rodents, in terms of maintenance of the pathogen in nature, is that the infection has no effect on the survival or general their body conditions, which secures perpetuation of the causal agent as the source of infection development and transmission [8,9].

The role of rodents in the transmission chain of *Borrelia* is very significant. It is especially important that rodents are suitable *Borrelia* reservoirs because bacteria develop in their bodies

and are then spread by ticks. In contrast to rodents, certain large mammals, such as deer, owing to their body size, function as highly competent hosts to vectors. However, they do not serve as competent reservoirs for the maintenance and amplification of *Borrelia*. Accordingly, their role in the transmission dynamics of *Borrelia* is restricted to supporting infected tick populations and facilitating their subsequent dispersal [10,11]. Uninfected tick larvae get infected through feeding on *Borrelia* infected hosts, mostly rodents, who are both suitable hosts and reservoirs. Once infected, rodents remain infective for life [12-16]. Research data for striped field mice (*A. agrarius*) showed that *B. afzelii* had the highest infection rate in the rodent heart, followed by spleen, kidneys and lungs, while *B. burgdorferi* s.s. and *B. garinii* were detected only in spleen [12-14,17,18].

Small mammals are suitable hosts for immature tick stages (larvae and nymphs), both as feeding sources and as facilitators of their spatial distribution due to their low body size [14,15, 19,20]. Small rodents are also important for spreading *Borrelia* because they are able to host larvae and nymphs simultaneously, in contrast to larger mammals [21]. Both *Apodemus* mice and voles are important hosts for immature stages of ticks, although infestation levels vary depending on habitat, host abundance and season [22,23]. Some researchers suggest that *Apodemus* mice are recognized as more competent reservoirs for *B. afzelii* than voles. In accordance with studies, vole has demonstrated a strong immune response to enzyme secretion by ticks [24,25]. The result is slower blood sucking by ticks and delayed development of nymphs [14,26].

The overall risk of spreading zoonotic pathogens by small rodents, such as *Borrelia*, is generally considered to be higher in natural habitats than in urban environments. The difference between natural and urban areas is probably related to differences in the abundance and diversity of arthropod vectors and the composition of vertebrate community [27]. Additionally,

some researchers have indicated that there is no significant difference between *Borrelia* spp. prevalence in natural and urban environments [28].

The most prevalent species of the *B. burgdorferi* s.l. complex in Europe are *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* [29,30]. In Serbia, the presence of *B. burgdorferi* s.s., *B. garinii*, and *Borrelia lusitaniae* was confirmed by PCR in analyzed red foxes [5]. Analysis of jackal tissues revealed the presence of *Borrelia valaisiana*, *B. garinii*, and *B. lusitaniae* [4]. The analysis of ticks confirmed the presence of *Borrelia bavariensis*, *B. lusitaniae*, *B. garinii* and *B. valaisiana* strains [31], and according to Potkonjak et al. [32], presence of *Borrelia miyamotoi* in ticks collected in vegetation is also present. In 2018, Lyme borreliosis was put on the European Union epidemiological surveillance list [33]. Current Serbian legislation regarding the protection of the population from infectious diseases does not mandate the reporting of Lyme borreliosis in humans or animals, as it is not currently subject to formal epidemiological surveillance [34].

The aim of this study was to determine the presence of *Borrelia* in tissues of different small rodent species as primary hosts, collected form locations in Serbia that are geographically distant or separated by natural barriers. Generally, establishing the role of small rodent species in the pathogen maintenance cycle further helps to identify the risk of transmission during tick activity periods and the rationale for establishing timely monitoring and control measures.

Materials and Methods

Samples Collection

Animals were collected over the spring and autumn of 2021 on three locations in Serbia: Bogatić (44°49'N, 19°30'E), Putinci (44°59'N, 19°57'E) and Bavanište (44°49'N, 20°53'E). The locations were selected based on an assumption that there would be no mixing of populations.

Rivers were natural barriers which separated the three locations (Fig. 1). Animals were trapped in alfalfa crops at field edge during the period of greatest activity of rodent populations.

Every night at each location 49 snap traps (7x7) were set. To increase trap success and species diversity, the traps were placed near the peripheral edges of alfalfa field. In total there were 147 trap nights per location. The number of trap nights in calculations of relative density index was corrected for the number of traps found to be inactive. A total of 5, 10 and 11 traps were inactive on the locations Putinci, Bavanište and Bogatić, respectively. All trapped animals were transferred to the Laboratory for Applied Zoology, Institute of Pesticides and Environmental Protection, Belgrade. The species were determined based on their external characteristics, body measurements and cranial morphometrics [35,36]. Autopsy and further analysis of animal hearts were conducted in the Scientific Veterinary Institute 'Novi Sad'. All samples were transported and kept under conditions of -20 °C before preparation for analysis.

Sample Preparation and DNA Extraction

For each of the 150 samples analyzed for the presence of *B. burgdorferi* s.l., a sample of solid heart tissue was suspended in 1 mL of phosphate-buffered saline along with quartz sand and a stainless-steel bead. The tissues were then homogenized for three minutes at 50 Hz using a TissueLyser LT (Qiagen, USA). Following homogenization, the samples were centrifuged at 12,000 rpm, and 200 µL of the supernatant was used in subsequent DNA extraction procedures, following the manufacturer's protocol (PureLink™ Genomic DNA Mini Kit, Invitrogen, USA, REF K182002). After extraction, the sample DNA was either used immediately for real-time PCR analysis or stored at -20° C until further analysis.

Molecular analysis

Real-time PCR

All samples were analyzed for the presence of *B. burgdorferi* s.l. Detection of the presence of this pathogen was done by real-time PCR (Mx3005P® QPCR System (Agilent, CA 95051, United States), using commercially available kit- BactoReal® Kit *B. burgdorferi* s.l. (Ingenetix GmbH, Vienna, Austria), according to the manufacturer's instructions. The test is based on multiplex real-time PCR by 5'-nuclease-assay technology for qualitative DNA detection of the ospA gene of *B. burgdorferi* s.l. The assay targets the ospA gene and includes an internal positive control (IPC) detected in the HEX channel, which verifies successful amplification and absence of PCR inhibition. Amplification was performed on an Mx3005P® QPCR System (Agilent, USA). Samples showing a detectable Ct (Cp) value in the FAM channel together with a valid IPC signal and no amplification in the negative control were considered positive. The fluorescence threshold was set manually above the background of the negative control. The kit we use BactoReal® Kit *B. burgdorferi* s.l. (Ingenetix GmbH, Vienna, Austria) gives qualitative results, not quantitative.

Molecular Typing of B. burgdorferi s.l.

The polymorphic outer surface protein C (OspC) gene was used for molecular typing. After DNA extraction from tissue samples, a 617-bp region of the OspC gene was amplified using the primers OC6(+) and OC623(-) [37] and Q5® High-Fidelity PCR Kit (New England BioLabs) according to the manufacturer's instructions with primer annealing temperature of 54°C. The amplified regions were sequenced using Sanger sequencing (Macrogen Europe). Raw sequences were processed and consensus sequences were generated using the Staden package [38]. To verify species identity, each consensus sequence was queried using BLASTn against a custom local database containing all publicly available *B. burgdorferi* s.l. OspC reference sequences downloaded from GenBank. Species identification was confirmed when the top hit matched a known *B. burgdorferi* s.l. OspC allele with ≥98% identity across the full

alignment length. The sequences were submitted in GenBank under the accession numbers PQ676528 and PQ676529.

Data Analysis

The number of collected small rodent species was used to calculate the population density index (I) [39] and the percent of species presence on all locations. The mean prevalence and corresponding 95% confidence intervals (CI) for *B. burgdorferi* s.l. in rodents were calculated using the Clopper–Pearson method.

Results

Rodent Species

A total of 150 animals were caught: 62 common voles (*Microtus arvalis*), 49 bank voles (*Clethrionomys glareolus*), 22 European pine voles (*Microtus subterraneus*), five wood mice (*Apodemus sylvaticus*), nine yellow-necked mice (*Apodemus flavicollis*) and three striped field mice. The population density index indicated that the highest abundance of small rodent species was recorded at Bogatić. Voles were proportionally more abundant than *Apodemus* mice at all locations (Table 1). At the Bogatić, 97.6% of the captured individuals were voles. Only voles were captured at the Putinci, while at the Bavanište the proportion of captured voles and *Apodemus* mice was 71.7% and 28.3%, respectively.

Results of Molecular Typing of *B. burgdorferi s.l.*

All 150 samples were analyzed for the presence of pathogenic *B. burgdorferi* s.l. Considering the heart tissue, *B. burgdorferi* s.l. was detected in a total of 15 out of 150 animals (10.0, 95% CI: 5.71–15.96) upon amplification by real-time PCR**.** Our samples showed the greatest similarity with sequences belonging to *B. afzelii* (99.3%). *B. burgdorferi* s.l. was confirmed in

animals from Bogatić with 3.7% prevalence, and locality Bavanište with 22.6% prevalence (Table 1). *B. burgdorferi* s.l. was not confirmed in samples from the location Putinci.

The prevalence of *B. burgdorferi* s.l infection in voles was 12.9% and 8.2% for common vole and bank vole, respectively. The prevalence for striped field mouse and wood mouse was 66.7% and 20%, respectively. *B. burgdorferi* s.l. was not detected in European pine voles and yellow-necked mice.

Discussion

Small mammals are important hosts and reservoirs of *B. burgdorferi* s.l.. Infected ticks are the main vectors of the pathogen within rodent populations, as well as to humans and domestic and wild animals. The risk of *B. burgdorferi* s.l. being transmitted to the human population correlates with the presence of pathogen reservoirs and infected ticks as vectors [2,14,31,40-45].

The lowest population density of small rodent species was recorded at the Putinci site, where only the common vole and bank vole were present. No pathogen presence was detected at this site, whereas it was confirmed at Bavanište and Bogatić at small rodent densities ranging from 38.7 to 60.2. Despite a twofold higher population density index of bank voles at Bogatić compared to Bavanište, the number of pathogen-positive individuals was identical between the two sites. Rodent community diversity was highest at Bavanište (six species), compared to Bogatić (four species). Mixed populations of small rodent species are usually present in Serbia [46,47]. Future studies should confirm the role of small rodent community structure, interspecific interactions, and tick abundance in pathogen maintenance.

Our results present the first evidence of *B. burgdorferi* s.l. in voles (common vole and bank vole) and mouse species (striped field mouse and wood mouse) in Serbia. Previous studies in Serbia suggested a potential role of rodents in the *Borrelia* transmission cycle, based solely on

the confirmation of its presence in yellow-necked mice. Stajković et al. [48] confirmed the presence of *B. burgdorferi* s.l. in yellow-necked mice, reporting two animals collected in an urban area of Belgrade, one of which tested positive. Similarly, Veinović et al. [49] detected *B. afzelii* in the same species, with one out of 30 yellow-necked mice collected in Belgrade's Avala suburb testing positive (3.3%). As demonstrated in our study, both *Apodemus* mice and voles serve as hosts for the pathogen.

A study in Croatia has confirmed the presence of *B. afzelii* in *Apodemus* mice and bank voles. The prevalence of *B. afzelli* in striped field mice was 3.8% and bank vole prevalence (7%) corresponds to our results [50]. The presence of *B. afzelii* in Croatian rodents had also been reported previously in fat dormouse (*Glis glis*) with 8.9% *B. afzelli* positive results [51]. Similar research in Romania reported a lower overall prevalence (4.9%) than we found in our study [52]. In general, there is a lack of data on the presence of *B. burgdorferi* s.l. in rodents in the Balkans [53].

The overall prevalence for *B. burgdorferi* s.l. in small mammals in our study of 10% is in line with results reported from France that showed 11% prevalence [54]. Overall prevalence of *Borrelia* in Germany (6.2%) is consistent with previous results reported from Poland (6.8%) and slightly lower than the results in our research [55,56]. On the other hand, the percentage of rodent infestation was significantly lower at the Bogatić location, while *B. burgdorferi* s.l. was not confirmed at all at Putinci.

The incidence of *Borrelia* is higher in areas with plenty of vertebrate animals are serving as host for infected ticks [41,57,58]. In accordance with Lassen et al. [59] the type of vegetation has an influence on *B. burgdorferi* s.l. prevalence and genodiversity. The area in which specimens were collected at Putinci is surrounded by arable fields. Many areas lacked suitable habitats, such as bushes or forest belts, for other vertebrate-host animals, and some plots were bordered by roads. In contrast, taller vegetation at Bavanište and Bogatić provided adequate

shelter for larger vertebrate-host species. Some next studies could investigate how vegetation structure, the presence of other vertebrate hosts, and rodent abundance affect the emergence and spread of zoonotic pathogens, including *Borrelia*.

Research on Lyme borreliosis in Serbia has primarily focused on analyzing samples collected from ticks or from the blood of patients and voluntary blood donors [31,40,44,45,60,]. According to more recent studies, *B. lusitaniae* has been reported as the most prevalent species, followed by *B. afzelii* [31,32,40, 61].

Conclusion

The results of this research confirm rodents as hosts of the pathogen of interest and represent the first detection of *B. burgdorferi* s.l. in voles (common vole and bank vole) and *Apodemus* mice (striped field mouse and wood mouse) in Serbia, suggesting their potential role in the perpetuation and transmission of *B. burgdorferi* s.l. across the country.

Furthermore, it is necessary to investigate the causes of the uneven spatial distribution of pathogens across different locations as well as effects of different habitats on pathogen prevalence, and relationship between number of infected animals, animal species and infected tick. When feasible, such insights should be utilized to prevent pathogen spread with infected ticks, and conduct the measures to minimize infection risks among human populations.

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Author contributions

G.J. led the research design. G.J., T.B. and L.B. contributed to data and sample collection. M.Ž. and V.G. performed sample preparation and molecular analysis. T.P., S.S., M.Ž. and V.G. contributed reagents/materials/analysis. T.B. wrote the first draft of the manuscript. T.P., L.B. and S.S. provided critical revisions in the writing process. All authors have reviewed and approved final version of the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate with the approval number: No. 323-07-04943/2020-05, dated 29.5.2020.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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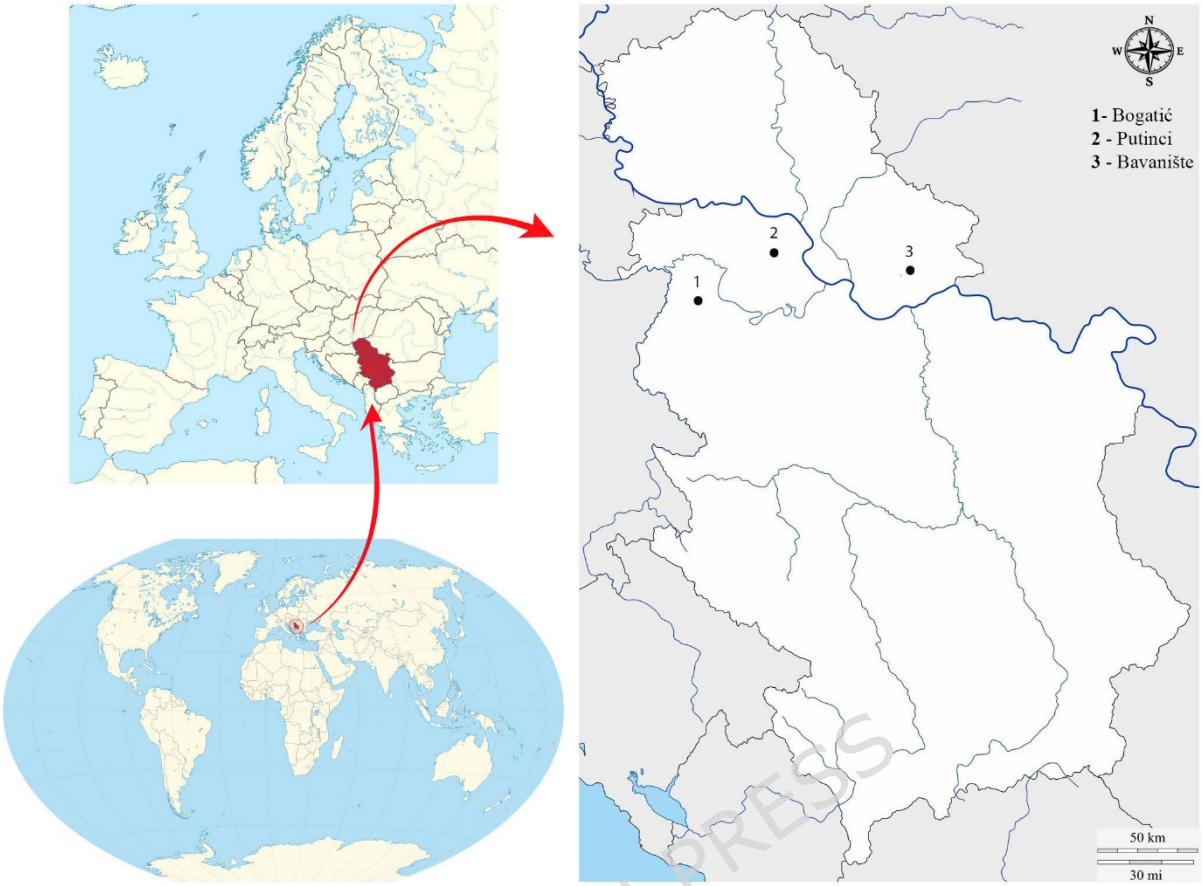
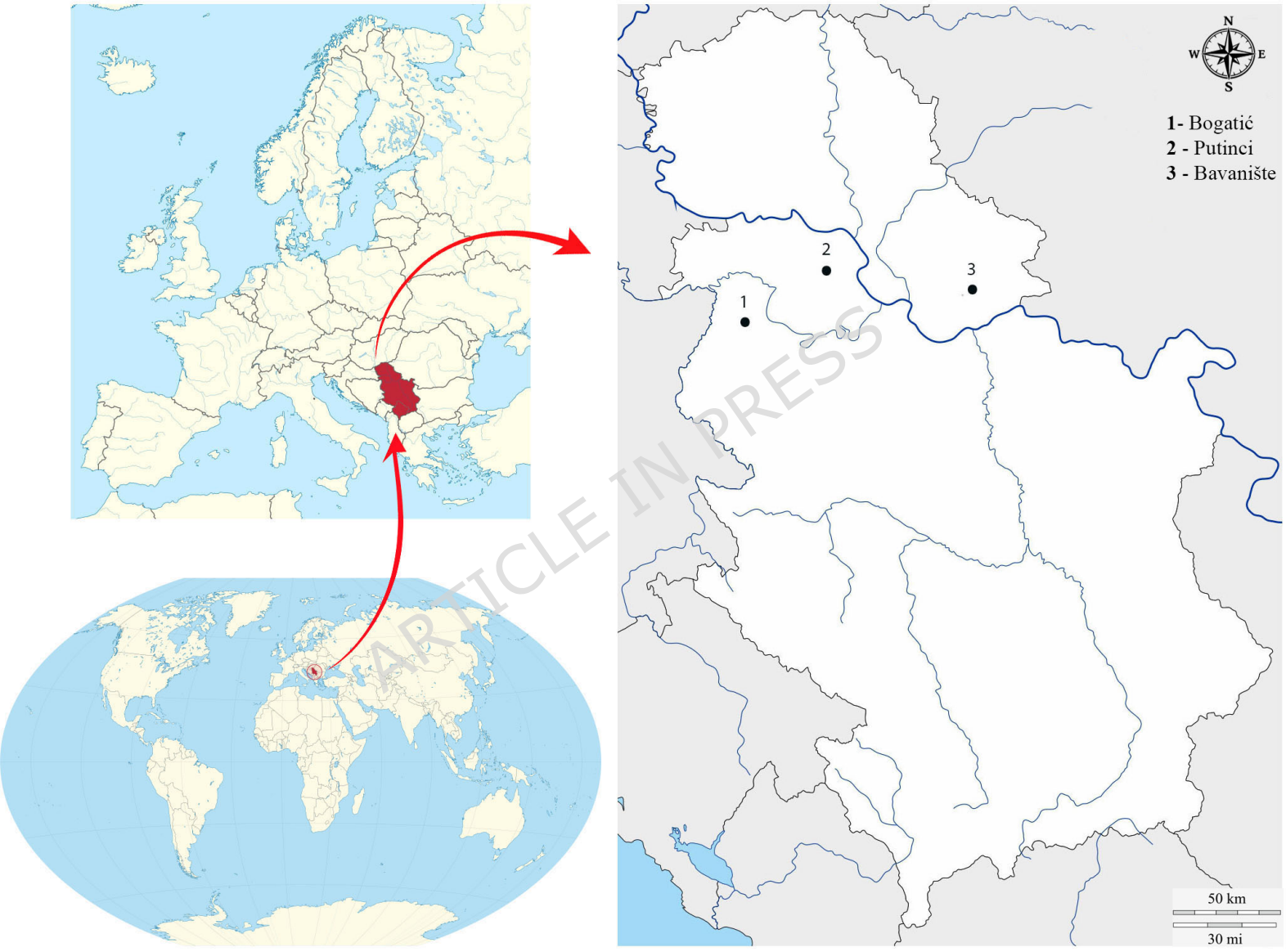


Figure 1. Location of the study area and small rodent trapping sites in Serbia

Table 1. Relative abundance (%), population density index (I) and Clopper-Pearson confidence interval (CI) of small rodents and *Borrelia burgdorferi* sensu lato prevalence (%) across sampling locations

Locality/species	Relative abundance	I	n positive/ n tested	CI	Prevalence
Bogatić					
Microtus subterraneus	24.4	14.7	0/20	-	-
Microtus arvalis	36.6	22.0	1/30	0.08-17.22	3.33
Clethrionomys glareolus	36.6	22.0	2/30	0.82-22.07	6.67
Apodemus flavicollis	2.4	1.5	0/2	-	-
Total	100	60.2	3/82	0.76-10.32	3.66
Putinci					
Microtus arvalis	66.7	7.0	0/10	-	-
Clethrionomys glareolus	33.3	3.5	0/5	-	-
Total	100	10.6	0/15	-	-
Bavanište					
Microtus subterraneus	3.8	1.5	0/2	-	-
Microtus arvalis	41.5	16.0	7/22	13.86-54.87	31.82
Clethrionomys glareolus	26.4	10.2	2/14	1.78-42.81	14.28
Apodemus agrarius	5.7	2.2	2/3	9.43-99.16	66.67
Apodemus sylvaticus	9.4	3.7	1/5	0.51-71.64	20
Apodemus flavicollis	13.2	5.1	0/7	-	-
Total	100	38.7	12/53	12.28-36.21	22.64



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Bavanište					
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