Institute of Parasitology, Biology Centre CAS
Folia Parasitologica 2023, 70: 007
doi: 10.14411/fp.2023.007

Folia Parasitologica
http://folia.paru.cas.cz

Research Article OPEN ACCESS

Detection, identification and genotyping of *Borrelia* spp. in ticks of Coastal-Karst and Littoral-Inner Carniola regions in Slovenia

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Abstract: The density and spread of tick vector species have increased throughout Europe in the last 30 years, leading to an increase of Lyme borreliosis cases, including in Slovenia. The aim of this study was to isolate *Borrelia* strains and determine the prevalence of *B. burgdorferi* sensu lato and *B. miyamotoi* in adults of *Ixodes ricinus* (Linnaeus) collected in 2019 in the two regions of the country (Coastal-Karst and Littoral-Inner Carniola) by cultivation and PCR. We isolated *B. burgdorferi* s.l. by culture method in 28/559 (5%) ticks from both regions. Culture-negative samples (531/559, i.e., 95%) were additionally tested by real-time PCR. In 155/531 (29.2%) PCR-positive samples, a fragment of *flaB* or *glpQ* was amplified and further sequenced to identify species of the *Borrelia*. Using both methods, cultivation and PCR, *Borrelia* spp. prevalence was 32.7% in the Coastal-Karst region and 33.0% in the Littoral-Inner Carniola region. Genotyping of the *Borrelia* spp. isolates revealed that 17/28 (60%) were *B. garinii* subtype Mlg2. Of all tick samples tested for *B. miyamotoi* 8/398 (2%) were PCR positive. Based on previous studies in these regions, we had expected more ticks to be infected with *B. afzelii*, but genotyping revealed that *B. garinii* was the most abundant.

Keywords: *Borrelia burgdorferi* s.l., *Borrelia miyamotoi*, tick vector, *Ixodes ricinus*, isolation, Central Europe

Ticks (Ixodidae) transmit a wide variety of pathogens, surpassing all other arthropods in the diversity of pathogens they transmit (Jongejan and Uilenberg 2004). Among them, *Ixodes ricinus* (Linnaeus) is undoubtedly the best-known tick species in Europe and epidemiologically the most important vector of pathogenic microorganisms. This species is also a major vector of the causative agents of Lyme borreliosis (LB), the most common vector-borne disease in the Northern Hemisphere, and tick-borne encephalitis (TBE) (Steere et al. 2016, Černý et al. 2020). In endemic regions of Europe and Asia, *B. burgdorferi* genospecies circulate between hard ticks of the *I. ricinus* complex and vertebrate hosts. The main hosts for larvae and nymphs are small mammals and ground-feeding birds.

Members of the *Borrelia burgdorferi* sensu lato complex (*B. burgdorferi* s.l.) are spiral-shaped bacteria from the phylum Spirochaetes (see Barbour and Hayes 1986). The complex currently includes 22 named and proposed genospecies in the Americas and Eurasia (Majerová et al. 2020, Wolcott et al. 2021). To maintain a complex enzootic cycle, *Borrelia* sp. must adapt to distinctly different environments (ticks and vertebrates). When a tick feeds on its host, it can become infected if a pathogenic bacterium is present in the infected tissue of the vertebrate (Estrada-Peña 2015). Reservoirs include small rodents, hares and birds. The key role in the successful transmission of *Borrelia* from one host to another is played by outer membrane proteins of the membrane of *B. burgorferi* s.l. (Schwan and Piesman 2000). It is also crucial that the borrelia remain in the tick after molting until the next developmental stage, which increases the chances that the bacteria will be transferred to the vertebrate host during the feeding period (Hartemink et al. 2008). Population composition of *Borrelia* spp. is influenced by host and vector population dynamics and demographic processes, as well as external abiotic factors (combination of temperature and humidity, climate type, and landscape), which also influence the geographic distribution of vectors and hosts (Margos et al. 2011).

Lyme borreliosis, the most common tick-borne zoonosis in Europe, is also a significant epidemiological problem in North America and Asia (Steere 2001, Masuzawa 2004, Steere et al. 2004). The highest prevalence rates in Europe are found in Central European countries, including Slovenia (Lindgren and Jaenson 2006, Rizzoli et al. 2011). Hot

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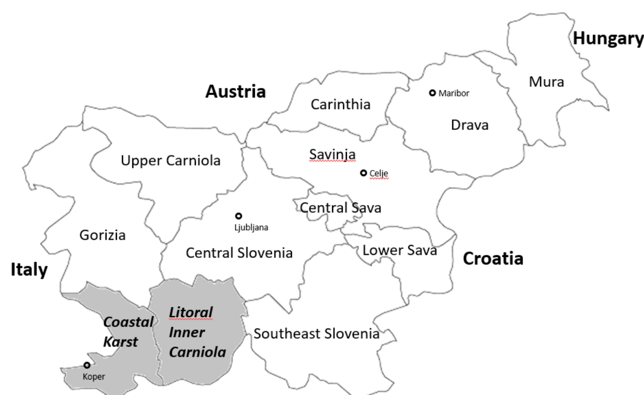

Fig. 1. Study regions in Slovenia, Coastal-Karst and Littoral-Inner Carniola

spots with more than 100 cases per 100,000 inhabitants per year have been reported in Austria, Slovenia, Germany, the Baltic Sea coast, and southern Sweden (Rizzoli et al. 2011).

In Europe, of the 21 reported *Borrelia* species (Cutler et al. 2017, Margos et al. 2019), only some can cause LB in humans, such as *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s.s.), *B. bavariensis* and *B. spielmanii* (Comstedt et al. 2011, Stanek et al. 2012, ECDC 2016). Infections with *B. afzelii* and *B. garinii* account for most LB cases in Europe, whereas *B. garinii* is predominant in Asia. *Borrelia afzelii* is mostly associated with skin manifestations, *B. garinii* appears to be the most neurotropic pathogen, and *B. burgdorferi* s.s. appears to be the most arthritogenic (Stanek et al. 2012). In Slovenia, all human pathogenic species have been isolated from patients, some have been isolated from ticks and patients, and some have been isolated only from ticks (Ružić-Sabljić et al. 2008, Cerar et al. 2015).

Borrelia miyamotoi, a relapsing fever species of *Borrelia*, was first isolated in Japan in 1994 from the tick *Ixodes persulcatus* Schulze and from the small Japanese field mouse *Apodemus argenteus* Temminck (Fukunaga et al. 1995). Later, this species was isolated from many other tick species and is widely distributed in Europe, Asia and North America (Scoles et al. 2001, Barbour et al. 2009, Gugliotta et al. 2013). In 2011, the pathogenic potential of *B. miyamotoi* was reported from Russia (Platonov et al. 2011). Today, it is considered an emerging pathogen that causes relapsing fever (RF) in humans, a febrile viral-like illness that relapses in up to 10% of patients (Platonov et al. 2011, Stanek et al. 2012, Ravagnan et al. 2018, Cutler et al. 2019). This genospecies was also confirmed in Slovenia in 2015, detected by PCR in two heart samples from *Apodemus flavicollis* Melchior, resulting in a low overall prevalence (Cerar et al. 2015).

In the clinical diagnosis of LB, isolation is a direct confirmation of *B. burgdorferi* s.l., especially in confirming unclear cases (Stanek et al. 2012). Culture is considered the gold standard for confirmation of active infection (Aguero-Rosenfeld et al. 2005). Qualitative and quantitative results can be obtained by PCR, with real-time PCR considered more sensitive than classical or nested PCR. Both culture and PCR are commonly used to detect *Borrelia* spirochaetes not only in humans but also in ticks and reservoirs (Cerar et al. 2015).

The aim of the present study was to isolate *Borrelia* strains and determine the prevalence of *B. burgdorferi* s.l. and *B. miyamotoi* in adult *I. ricinus* in the two regions of Slovenia (Coastal-Karst and Littoral-Inner Carniola) for which we currently have no data on tick infection.

MATERIALS AND METHODS

Tick sampling

Ticks were collected in May 2019 during their peak seasonal activity in the Coastal-Karst and Littoral-Inner Carniola regions of Slovenia (Fig. 1). Sites were selected based on suitable habitat type (grassland, forest or forest edge) along hiking and walking trails where most people come into contact with ticks. Ten sites were selected in the Coastal-Karst region and 12 in the Littoral-Inner Carniola region. At each location, ticks were collected by dragging a flag over vegetation for 30 minutes. The flag was checked periodically after approximately 10 m. Ticks were collected in sterile tubes and taken to laboratory. The species, stage and sex of the ticks were determined by a professional entomologist using the existing taxonomic key ''Ticks of Europe and North Africa'' (Estrada-Peña et al. 2017).

Cultivation and genotyping of *Borrelia burgdorferi* s.l.

Ticks were decontaminated in 70% ethanol and rinsed in sterile double distilled water. After decontamination, the whole tick was transferred to liquid modified Kelly-Pettenkofer medium (MKP) according to a published protocol (Ružić-Sabljić et al. 2014). In the medium, the tick was punctured with a sterile needle to release the bacteria from the tick's digestive system into the culture medium. All media were incubated at 33 °C for 9 weeks. The media were examined weekly for the presence of spirochetes using a dark field microscopy (Aguero-Rosenfeld et al. 2005, Ružić-Sabljić et al. 2014). After 9 weeks of incubation, all contaminated and negative media were subjected to additional molecular analysis. Spirochetes from positive media were further genotyped with restriction fragment length polymorphism with *MluI* (*MluI*-LRFP) according to the previously published protocol (Ružić-Sabljić et al. 2008). In addition, identification by sequencing of *flaB* was performed.

DNA extraction, PCR detection and sequencing

Each tick with its corresponding contaminated or negative MKP medium was subjected to DNA extraction using the MagNA Pure 24 Total Nucleic Acid Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. A whole tick, together with 600 µl of MKP medium and 200 µl of Bacteria Lysis Buffer (Roche, Germany) was mechanically beated in the MagNA Lyser instrument (Roche, Germany). After beating, 50 µl of Proteinase K (Roche, Germany) was added and incubated overnight at 65°C. After incubation, 400 µl of the lysate suspension was used in DNA extraction protocol using the MagNA Pure 24 System (Roche, Germany). For culture positive samples, 400 µl of MKP medium was used for extraction. EAV control was

Table 1. Determination of *Borrelia* spp. in ticks collected in May 2019 in two Slovenian regions using cultivation and real-time PCR.

	Littoral-Inner Carniola				Coastal-Karst				Total			
	No. ticks	Culture pos	PCR positive	Neg/ Inh	No. ticks	Culture pos	PCR positive	Neg/Inh	No. ticks	Culture pos	PCR positive	Neg/ Inh
Ixodes ricinus	452	22	127	291/11	103	6	28	65/3	**555**	**28**	**155**	**356/14**
Haemaphysalis punctata	0	0	0	0	4	0	0	4	**4**	**0**	**0**	**4**
Total	**452**	22	127	291/11	**107**	6	28	69/3	**559**	**28**	**155**	**360/14**

Determination of *Borrelia* spp. in ticks collected in May 2019 in two Slovenian regions using cultivation and real-time PCR (total number of ticks in bold).

used for extraction control (LightMix Modular EAV RNA Extraction control diagnostic kit, Roche, Germany).

Duplex real-time PCR was performed for the detection of *Borrelia* DNA and internal EAV control. For the detection of *B. burgdorferi* s.l., the LightMix Modular diagnostic kit for the detection of *Borrelia* sp. (TIB MOLBIOL, Berlin, Germany) was used together with a diagnostic kit for internal EAV control extraction (LightMix Modular EAV RNA Extraction control diagnostic kit, Roche, Germany). The kit for detection of *Borrelia* spp. contains lyophilized primers and a probe for amplification and detection of part of the 23S rRNA gene of *Borrelia* spp. Each step of molecular detection was performed in a separate room to avoid contamination. Real-time PCR was performed on a LightCycler 480 Instrument II (Roche, Germany) according to the manufacturer's instructions (Tib MolBiol, Roche, Germany). After amplification, detection of *Borrelia* DNA and possible inhibition were monitored at corresponding optical channels: *Borrelia* spp. at 530 and internal EAV control at 660. If inhibition occurred, the DNA sample was diluted 1 : 10 and the assay was repeated. If inhibition occurred despite dilution, the sample was labeled as inhibited. All PCR-positive samples were subjected to amplification and sequencing of a part of the *flab* gene (Clark et al. 2005) to determine species of *Borrelia*.

All samples were also tested for the presence of *B. miyamotoi*. Specific primers and probes targeting a part of the 16S rRNA gene were used for real-time PCR, as described by Platonov et al. 2011. All positive samples were also confirmed by conventional PCR targeting *glpQ* gene (Hovius et al. 2013) and sequenced. The amplicons of *flaB* and *glpQ* genes were sequenced on both strands using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied BioSystems, Waltham, USA). Sequences were assembled using the CLC Main Workbench 7 software package (Qiagen Aarhus, Aarhus, Denmark) and homology searches were performed using the NCBI BLAST service (National Institutes of Health, Bethesda, Maryland, USA).

RESULTS

In May 2019, 559 ticks were collected in the Coastal-Karst and Littoral-Inner Carniola region and two tick species were identified, *Ixodes ricinus* (555) and *Haemaphysalis punctata* Canestrini et Fanzago, 1878 (4) (Table 1).

Isolation of *B. burgdorferi* s.l. by culture was performed in all 559 sampled ticks. *B. burgdorferi* s.l. was successfully isolated from 28/559 (5%) ticks (Table 1). Genotypic characterization based on *MluI*-LRFP revealed that 17/28 (61%) isolates belonged to *B. garinii*. Most of them belonged to the Mlg2 subtype. New *B. garinii* subtypes, never before isolated in Slovenia, were Mlg8 and Mlg9 (Table 2). Six isolates were determined to be *B. afzelii* (all of subtype Mla1), four were *B. valaisiana* (three subtype Mlv1, one Mlv2) and one was *B. burgdorferi* s.s. (subtype Mlb2). Five samples were contaminated and therefore excluded from genotyping. The results of both genotyping approaches, *MluI*-LRFP and *flaB* sequencing, were concordant (Table 2).

Table 2. Genotyping of *Borerelia burgdorferi* s.l. isolates from ticks sampled in 2019 by *Mlu*I-LRFP and *flaB* sequencing (ND – not done).

Region	Location	*Mlu*I-LRFP (No. of isolates)	*Fla seq* (No. of isolates)
Littoral-Inner Carniola	Bloška Polica	*B. garinii* Mlg2 (2)	*B. garinii* (2)
	Kozarišče	*B. garinii* Mlg2 (2)	*B. garinii* (2)
	Dane	*B. garinii* Mlg2 (1) *B. afzelii* Mla1 (1) *B. burgdorferi* s.s. Mlb2 (1)	*B. garinii* (1) *B. afzelii* (1) *B. burgdorferi* s.s. (1)
	Lož	*B. garinii* Mlg2 (1) *B. valaisiana* Mlv2 (1) ND (1)	*B. garinii* (1) *B. valaisiana* (1) ND (1)
	Sveta Ana	*B. garinii* Mlg6 (1) *B. afzelii* Mla1 (1)	*B. garinii* (1) *B. afzelii* (1)
	Grahovo	*B. garinii* Mlg2 (2) *B. garinii* Mlg6 (1) *B. afzelii* Mla1 (1) ND (1)	*B. garinii* (3) *B. afzelii* (1) negative (1)
	Begunje	*B. afzelii* Mla1 (1)	*B. afzelii* (1)
	Volčje	*B. garinii* Mlg2 (1) *B. afzelii* Mla1 (1)	*B. garinii* (1) *B. afzelii* (1)
	Ravnik	*B. garinii* Mlg2 (1) *B. afzelii* Mla1 (1)	*B. garinii* (1) *B. afzelii* (1)
Coastal-Karst	Sveti Vid	*B. valaisiana* Mlv1 (1)	*B. valaisiana* (1)
	Sv. Anton	*B. garinii* Mlg 7 (1)	*B. garinii* (1)
	Osp	*B. garinii* Mlg4 (1) *B. garinii* Mlg8 (1) *B. valaisiana* Mlv1 (1)	*B. garinii* (2) *B. valaisiana* (1)
	Koper	*B. garinii* Mlg2 (1) *B. garinii* Mlg9 (1) *B. valaisiana* Mlv2 (1)	*B. garinii* (2) *B. valaisiana* (1)

Of 531 ticks from negative or contaminated cultures, 155 were PCR positive, 360 were negative, while 14 samples were inhibited. Sequencing of the *flaB* gene allowed determination of *Borrelia* species in 64 ticks: *B. garinii* was found in 32 ticks, *B. afzelii* in 22 ticks, *B. valaisiana* in nine ticks and *B. lusitaniae* in one tick. The rest of the ticks were *flaB* gene PCR-negative, due to the lower sensitivity of a conventional PCR compared to real-time PCR; the Ct values of most *flaB* gene PCR-negative samples were above 30. Using both methods (culture and real-time PCR), the prevalence of B. burgdorferi s.l. in May 2019 was 32.85% (183/559) in both regions combined.

Due to the insufficient amount of DNA isolates, real-time PCR was performed to detect *B. miyamotoi* in 398/559 samples. This species was detected in 8/398 (2%) samples, 6/317 (1.9%) from the Littoral-Inner Carniola region and

2/81 (2.5%) from the Coastal-Karst region (Table 3). In 4 samples, the *glpQ* gene was successfully sequenced and *B. miyamotoi* was confirmed. One of these ticks was co-infected with *B. garinii* and *B. miyamotoi* simultaneously.

DISCUSSION

The castor bean tick is the most common and epidemiologically most important tick species in Central Europe, including Slovenia (ECDC 2016, Estrada-Peña et al. 2017). Moreover, all previous studies of the prevalence of *Borrelia burgdoferi* s.l. in *Ixodes ricinus* confirm that the area of Central Europe is of particular epidemiological importance for the occurrence of Lyme disease in humans (ECDC 2016). LB and tick-borne meningoencephalitis (TBE) are the most common tick-borne diseases in Europe (ECDC 2022). In Slovenia, between 3,000 and more than 7,000 patients with LB and 170 TBE cases per year were registered from 2010 to 2019 (NIJZ 2020).

Recent epidemiological data from Slovenia show that the Littoral-Inner Carniola region was among the areas with the highest number of LB infections, but the Coastal-Karst region has not been extensively studied (NIJZ 2020). In the Coastal-Karst region, the onset of tick activity was reported in the early spring months, so we expected to collect sufficient numbers of adult ticks in May. The Littoral-Inner Carniola region is strongly influenced by the alpine climate, which means that seasonal activity begins one month later and the greatest abundance occurs from April to June (Knap et al. 2009). As in the northeast and northwest of Italy (Otranto et al. 2014), *I. ricinus* ticks in the two Slovenian regions studied show a high affiliation to woodland areas, where this species finds optimal conditions in terms of temperature and relative humidity for its development.

The distribution pattern as well as the host preferences of the different *Borrelia* genospecies in Slovenia is also interesting. For example, *B. afzelii* is the predominant genospecies in human samples (skin, blood and cerebrospinal fluid) in Slovenia (Ružić-Sabljić et al. 2008). This genospecies has been isolated from humans, but also from ticks and rodents (Ružić-Sabljić et al. 2002, Lagal et al. 2003, Stanek and Strle 2003, Logar et al. 2004, Cerar et al. 2015).

This finding is supported by several studies that have shown that erythema migrans (EM) in humans is most frequently caused by *B. afzelii* (up to 96%) and less frequently by *B. garinii* (up to 33%). The last such study in Slovenia showed that of 488 skin isolates from Slovenian patients with EM, 433 (89%) were *B. afzelii*, 53 (11%), *B. garinii* and only 2 *B. burgdorferi* s.s. (Ružić-Sabljić et al. 2002). However, in 82 patients from Finland who had EM, 21.5% of skin cultures were positive, and all isolates were identified *B. garinii* (Oksi et al. 2001). Strle and Stanek (2009) speculate that *B. afzelii* is prevalent in western and central Europe but may not be in eastern Scandinavia, eastern Europe and Asia.

It is interesting to note that the proportions of the main *Borrelia* species isolated from EM skin lesions do not completely match the proportions of *Borrelia* genospecies found in ticks. Studies in Slovenia and Germany found that *B. garinii* and *B. burgdorferi* s.s. were isolated from ticks relatively more frequently than *B. afzelii* (Strle and Stanek 2009). Genotyping results in our study and sequencing of the *flaB* gene also showed that *B. garinii* was the most common genospecies detected in host-seeking *I. ricinus* specimens.

Another study (Schwarz et al. 2012) on the prevalence of different *Borrelia* genospecies in *I. ricinus* at three sites in the Siebengebirge, Germany, found that the most common *Borrelia* genospecies were also *B. garinii* and *B. afzelii* genotyped by reverse line blotting. The infection rate in that study was almost twice as low as our results (19.5% in 2007 and 16.5% in 2008), mainly due to the proportion of life stages of the ticks. Namely, we examined only adults, while in the study of Schwarz et al. (2012) the proportion of adults was 4.8%.

A similar study conducted in Serbia found that 52 of 115 adult host-seeking *I. ricinus* were PCR positive and two *Borrelia* genospecies were isolated, *B. lusitaniae* and *B. afzelii* (Radulović et al. 2010). Interestingly, the prevalence of *B. burgdorferi* s.l. in adult ticks collected in Serbia (45%) was even higher than in samples collected in two Slovenian regions studied (33%). Another study from Slovakia (Gern et al. 1999) addressed the distribution of different *B. burgdorferi* species in adult *I. ricinus* ticks collected in an endemic area in Slovakia. Immunofuorescence was used to determine that 56/114 (49%) ticks were infected with *B. burgdorferi* s.l.. RFLP identification revealed 25 *B. afzelii* (68%), 5 *B. garinii* (14%), 5 *B. valaisiana* (14%), and 2 *B. lusitaniae* (5%) out of 37 isolates.

Different prevalence of *Borrelia* genospecies could be influenced by many factors, such as the sampling period, changes in microclimatic conditions, the natural dynamics of *Borrelia* and the abundance of reservoir hosts. Several studies indicate that genospecies of *B. burgdorferi* s.l. are maintained in nature by different vertebrate hosts. For example, in Europe, *B. afzelii* is commonly found in association with mice and voles, while seabird and songbird populations often carry *B. garinii* (Kurtenbach et al. 1998, 2002).

Moreover, *B. garinii* was the most common genospecies in ticks removed from birds, especially from ground-foraging species (Comstedt et al. 2006). Birds do not choose their migration routes randomly, but follow established routes. In general, European migratory birds move along four migration routes (Vrezec et al. 2006). In this respect,

Table 3. Presence of *Borrelia miyamotoi* in ticks (ND/INH number are lost or inhibited samples).

Region	Total ticks (No.)	Positive (No.)	Negative (No.)	ND/INH (No.)	Prevalence
Littoral-Inner Carniola	317	6	311	135	1.9%
Coastal-Karst	81	2	79	26	2.5%
Total	398	8	390	161	2.0%

Slovenia has a special geographical position, as it is located at the crossroads of three migratory routes, which is reflected both in the number and diversity of bird species (Vrezec et al. 2006). Since many of them are ground-foraging species, higher infection of nymphs and adult forms of *I. ricinus* with *B. garinii* was expected. Recent studies are attempting to determine the relationship between birds and different genotypes. For example, in Slovakia, identical *ospA* alleles of *B. garinii* were found in ticks that had fed on birds and in questing ticks. (Hanincová et al. 2003).

Previous studies suggest that *B. garinii* is less present in nymphs (Mysterud et al. 2019). Nevertheless, the results of another study from Scotland showed that 20% of *I. ricinus* nymphs collected from birds were positive for *B. burgdorferi* s.l. and all belonged to *B. garinii*. The authors of this study also showed that ground-foraging species were more important than arboreal species in hosting *I. ricinus* nymphs and *B. burgdorferi* s.l. particularly common blackbirds (*Turdus merula* Linnaeus) (James et al. 2011), a species that is also very abundant in both study areas in Slovenia. On the other hand, our results showed a significant infection rate with this genospecies in adult forms of *I. ricinus*.

The results of all the above studies, including this one, indicate that it is relatively difficult to determine the association of individual genospecies of *B. burgdorferi* s.l. with hosts. In any case, they suggest that the infection ratio of different stages of *I. ricinus* with *B. garinii* depends primarily on the availability of suitable hosts and their abundance, but less on climatic factors. Based on the previous studies, as well as information received from the Slovenian Society for Bird Observation (DOPPS-Birdlife Slovenia), the main cause of the high prevalence of the *B. garinii* genospecies is most likely the high abundance of ground-foraging bird species during the 2019 spring migration.

Borrelia miyamotoi DNA was detected in eight *I. ricinus* specimens from all sampled ticks, representing 2% infected ticks. Using the same method as described by Cerar et al. (2015), *B. miyamotoi* was first detected in infected rodents in Slovenia; in the prospective and retrospective studies, it was detected in 1/251 (0.4%) and 1/46 (2.2%) heart samples, respectively. All attempts to isolate this species from PCR-confirmed samples have been unsuccessful. A similar percentage, 2.1% of infected castor bean ticks, was reported in a study from the Netherlands (Wagemakers et al. 2017). *Borrelia miyamotoi* also occurs in the United Kingdom, but in a lower percentage. It was first detected in this country in 2014 and later confirmed in another study from the same year. The infection rate was 0.3% and 0.7%, respectively (Hansford et al. 2015, Layzell et al. 2018). All these results suggest a wide distribution of *B. miyamotoi* in Europe, but a higher infection rate in Central European regions.

There may be several reasons for differences in *Borrelia* spp. prevalence between studies: different sampling procedures, sample size, methods used to detect *Borrelia* spp., different geographic locations and time of collection (Mannelli et al. 2012). A reliable and standardised approach for such studies has not yet been defined. However, studies are helpful to assess the risk of *Borrelia* spp. transmission and to decide on antibiotic treatment.

In conclusion, the incidence of *B. burgdorferi* s.l. in questing *I. ricinus* ticks collected in Slovenia is similar in both regions studied. The results of the occurrence of *Borrelia* genotypes confirm previous similar studies conducted in Slovenia, i.e., that molecular methods are more sensitive than isolation by culture. The prevalence of *Borrelia* genotypes in questing ticks was 32.7% in both regions combined for 2019, and *B. garinii* was the dominant isolated genospecies in both sampling areas. Two new subtypes of *B. garinii* were found for the first time in Slovenia. *Borrelia miyamotoi* was confirmed in ticks at low frequency (2%).

Acknowledgements. The field research and laboratory analyses were funded by Slovenian Research Agency (ARRS) project J3-8197 (Vector surveillance of emerging microorganisms in Slovenia).

Author contribution. VI and TCK designed research, JŠ and KSS performed research, VI, TCK, KSS, KK and JŠ analysed data, and VI, ERS and TCK wrote the paper.

REFERENCES

Aguero-Rosenfeld M.E., Wang G., Schwartz I., Wormser G.P. 2005: Diagnosis of Lyme borreliosis. Clin. Microbiol. Rev. 484–509.

Barbour A.G., Bunikis J., Travinsky B., Gatewood Hoen A., Diuk-Wasser M.A., Fish D., Tsao J.I. 2009: Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am. J. Trop. Med. Hyg. 81: 1120–1131.

Barbour A.G., Hayes S.F. 1986: Biology of *Borrelia* species. Microbiol. Rev. 50: 381–400.

Černý J., Lynn G., Hrnková J., Golovchenko M., Rudenko N., Grubhoffer L. 2020: Management options for *Ixodes ricinus*-associated pathogens: a review of prevention strategies. Int. J. Environ. Res. Publ. Health 17: 1830.

Cerar T., Korva M., Avšič-Županc T., Ružić-Sabljić E. 2015: Detection, identification and genotyping of *Borrellia* spp. in rodents in Slovenia by PCR and culture. BMC Vet. Res. 11: 188.

Clark K., Hendricks A., Burge D. 2005: Molecular identification and analysis of *Borrelia burgdorferi* sensu lato in lizards in the southeastern United States. Appl. Environ. Microbiol. 71: 2616–2625.

Comstedt P., Bergström S., Olsen B., Garpmo U., Marjavaara L., Mejlon H., Barbour A.G., Bunikis J. 2006: Migratory passerine birds as reservoirs of Lyme borreliosis in Europe. Emerg. Infect. Dis. 12: 1087–1095.

Comstedt P., Jakobsson T., Bergström S. 2011: Global ecology and epidemiology of *Borrelia garinii* spirochetes. Infect. Ecol. Epidemiol. 1: 9545.

Cutler J.S., Ruzic-Sabljic E., Potkonjak A. 2017: Emerging borreliae – expanding beyond Lyme borreliosis. Mol. Cell. Probes. 31: 22–27.

Cutler S., Vayssier-Taussat M., Estrada-Peña A., Potkonjak A., Mihalca A.D., Zeller H. 2019: A new *Borrelia* on

the block: *Borrelia miyamotoi* – a human health risk? Euro Surveill. 24: 1800170.

ECDC: European Centre for Disease Prevention and Control 2016: Factsheet about Borreliosis, https://www.ecdc.europa.eu/en/borreliosis/facts/factsheet.

ECDC: European Centre for Disease Prevention and Control 2022: Tick-borne diseases, https://www.ecdc.europa.eu/en/tick-borne-diseases.

Estrada-Peña A. 2015: Ticks as vectors: taxonomy, biology and ecology. Rev. Sci. Tech. 34: 53–65.

Estrada-Peña A., Mihalca A.D., Petney T.N. 2017: Ticks of Europe and North Africa. A Guide to Species Identification. Springer, Cham, 404 pp.

Fukunaga M., Takahashi Y., Tsuruta Y., Matsushita O., Ralph D., McClelland M., Nakao M. 1995: Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int. J. Syst. Evol. Microbiol. 45: 804–810.

Gern L., Hu C. M., Kocianova E., Vyrostekova V., Rehacek J. 1999: Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia. Eur. J. Epidemiol. 15: 665–669.

Gugliotta J.L., Goethert H.K., Berardi V.P., Telford S.R. 2013: Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. N. Engl. J. Med. 368: 240–245.

Hanincová K., Taragelová V., Koci J., Schäfer S.M., Hails R., Ullmann A.J., Piesman J., Labuda M., Kurtenbach K. 2003: Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl. Environ. Microbiol. 69: 2825–2830.

Hansford K.M., Fonville M., Jahfari S., Sprong H., Medlock J.M. 2015: *Borrelia miyamotoi* in host-seeking *Ixodes ricinus* ticks in England. Epidemiol. Infect. 143: 1079–1087.

Hartemink N.A., Randolph S.E.., Davis S.A., Heesterbeek J.A.P. 2008: The basic reproduction number for complex disease systems: defining R0 for tick-borne infections. Amer. Natur. 171: 743–754.

Hovius J.W.R., de Wewre B., Maaike S., Matthijs B., Jeroen C., Alex W., Anneke O., Henrike K. Sukanya N., Caspar H., Seta J., Steven P., Hugo H., Erol F., Hein S., Marinus O. 2013: A case of meningoencephalitis by the relapsing fever spirochaete *Borrelia miyamotoi* in Europe. Lancet 382: 658.

James M.C., Furness R.W., Bowman A.S., Forbes K.J., Gilbert L. 2011: The importance of passerine birds as tick hosts and in the transmission of *Borrelia burgdorferi*, the agent of Lyme disease: a case study from Scotland. Ibis 153.2: 293–302.

Jongejan F., Uilenberg G. 2004: The global importance of ticks. Parasitology 129: S3–S14.

Knap N., Durmiši E., Sakida A., Korva M., Petrovec M., Avšič-Županc T. 2009: Influence of climatic factors on dynamics of questing *Ixodes ricinus* ticks in Slovenia. Vet. Parasitol. 164: 275–281.

Kurtenbach K., De Michelis S., Etti S., Schfer S.M., Sewell H.S., Brade V., KraiczyP. 2002: Host association of *Borrelia burgdorferi* sensu lato – the key role of host complement. Trends Microbiol. 10:74–79.

Kurtenbach K., Peacey M., Rijpkema S.G.T., Hoodless A.N., Nuttall P.A., Randolph S.E. 1998: Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl. Environ. Microbiol. 64: 1169–1174.

Lagal V., Postic D., Ruzić-Sabljić E., Baranton G. 2003: Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. J. Clin. Microbiol. 41: 5059–5065.

Layzell S.J., Bailey D., Peacey M., Nuttall P.A. 2018: Prevalence of *Borrelia burgdorferi* and *Borrelia miyamotoi* in questing *Ixodes ricinus* ticks from four sites in the UK. Ticks Tick Borne Dis. 9: 217–224.

Lindgren E., Jaenson T.G.T. 2006: Lyme borreliosis in Europe: influences of climate change, epidemiology, ecology and adaptation measures, CCASHH Project Report, EUR/04/5046250. WHO Regional Office for Europe, Copenhagen.

Logar M., Ružić-Sabljić E., Lotric-Furlan S., Cimperman J., Jurca T., Strle F. 2004: Comparison of erythema migrans caused by *Borrelia afzelii* and *Borrelia garinii*. Infection. 3: 15e19.

Majerová K., Hönig V., Houda M., Papežík P., Fonville M., Sprong H., Rudenko N., Golovchenko M, Černá Bolfíková B., Hulva P., Růžek D., Hofmannová L., Votýpka J., Modrý D. 2020: Hedgehogs, squirrels, and blackbirds as sentinel hosts for active surveillance of *Borrelia miyamotoi* and *Borrelia burgdorferi* complex in urban and rural environments. Microorganisms 8: 1908.

Mannelli A., Bertolotti L., Gern L., Gray J. 2012: Ecology of *Borrelia burgdorferi* sensu lato in Europe: transmission dynamics in multi-host systems, influence of molecular processes and effects of climate change. FEMS Microbiol. Rev. 8: 633–44.

Margos G., Fingerle V., Reynolds S. 2019: *Borrelia bavariensis*: vector switch, niche invasion, and geographical spread of a tick-borne bacterial parasite. Front. Ecol. Evol. 7: 401.

Margos G., Vollmer S.A., Ogden N.H., Fish D. 2011: Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi* sensu lato. Infect. Gen. Evol. 7: 1545–1563.

Masuzawa T. 2004: Terrestrial distribution of the Lyme borreliosis agent *Borrelia burgdorferi* sensu lato in East Asia. Jpn. J. Infect. Dis. 57: 229–235.

Mysterud A., Heylen D. J. A., Matthysen E., Garcia A. L., Jore S., Viljugrein H. 2019: Lyme neuroborreliosis and bird populations in northern Europe. Proc. R. Soc. B. 286: 20190759.

NIJZ (National Institute of Public Health, Republic of Slovenia) 2020: http://www.nijz.si/sl.

Oksi J., Marttila H., Soini H., Aho H., Uksila J., Viljanen M.K. 2001: Early dissemination of *Borrelia burgdorferi* without generalized symptoms in patients with erythema migrans. APMIS 109: 581–588.

Otranto D., Dantas-Torres F., Giannelli A., Latrofa M.S., Cascio A., Cazzin S., Ravagnan S., Montarsi F., Zanzani S.A., Manfredi M.T., Capelli G. 2014: Ticks infesting humans in Italy and associated pathogens. Parasit. Vector. 7: 328.

Platonov A.E., Karan L.S., Kolyasnikova N.M., Makhneva N.A., Toporkova M.G., Maleev V.V., Fish D., Krause P.J. 2011: Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg. Infect. Dis. 17: 1816–1823.

Radulović Ž., Milutinović M., Tomanović S., Mulenga A. 2010: Detection of *Borrelia*-specific 16S rRNA sequence in total RNA extracted from *Ixodes ricinus* ticks. Arq. Bras. Med. Vet. Zootec. 62: 862–867.

Ravagnan S., Tomassone L., Montarsi F., Krawczyk A.I., Mastrorilli E., Sprong H., Milani A., Rossi L., Capelli G. 2018: First detection of *Borrelia miyamotoi* in *Ixodes ricinus* ticks from northern Italy. Parasit. Vector. 11: 130.

Rizzoli A., Hauffe H.C., Carpi G., Courc'h G.I., Neteler M., Rosà R. 2011: Lyme borreliosis in Europe. Eurosurveillance 16: 19906.

Ružić-Sabljić E., Maraspin V., Cimperman J., Strle F., Lotrič-Furlan S., Stupica D., Cerar T. 2014: Comparison of isolation rate of *Borrelia burgdorferi sensu lato* in two different culture media, MKP and BSK-H. Clin. Microbiol. Infect. 20: 636–641.

Ružić-Sabljić E., Maraspin V., Lotric-Furlan S., Jurca T., Logar M., Pikelj-Pecnik A., Strle F. 2002: Characterization of *Borrelia burgdorferi sensu lato* strains isolated from human material in Slovenia. Wien. Klin. Wochenschr. 114: 544–550.

Ružić-Sabljić E., Zore A., Strle F. 2008: Characterization of *Borrelia burgdorferi* sensu lato isolates by pulses-field gel electrophoresis after *MluI* restriction of genomic DNA. Res. Microbiol. 159: 441–448.

Schwan T.G., Piesman J. 2000: Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J. Clin. Microbiol. 38: 382–388.
Schwarz A., Hönig V., Vavrušková Z., Grubhoffer L., Balczun C., Albring A., Schaub A.G. 2012: Abundance of *Ixodes ricinus* and prevalence of *Borrelia burgdorferi* s.l. in the nature reserve Siebengebirge, Germany, in comparison to three former studies from 1978 onwards. Parasit. Vector. 5: 268.
Scoles G.A., Papero M., Beati L., Fish D. 2001: A relapsing fever group spirochete transmitted by *Ixodes scapularis* ticks. Vector-Borne. Zoonotic Dis. 1: 21–34.
Stanek G., Strle F. 2003: Lyme borreliosis. Lancet, 362, 1639–1647.
Stanek G., Wormser G.P., Gray J., Strle F. 2012: Lyme borreliosis. Lancet 379: 461–473.
Steere A.C. 2001: Lyme disease. N. Engl. J. Med. 345: 115–125.
Steere A.C., Coburn J., Glickstein L. 2004: The emergence of Lyme disease. J. Clin. Investig. 113: 1093–1101.
Steere A.C., Strle F., Wormser G.P., Hu L.T., Branda J.A., Hovius J.W.R., Li X., Mead P.S. 2016: Lyme borreliosis. Nat. Rev. Dis. Primers 2: 16090.
Strle F., Stanek G. 2009: Clinical manifestations and diagnosis of Lyme borreliosis. Curr. Probl. Dermatol. 37: 51–110.
Vrezec A., Tome D., Denac D. 2006: [Migration and exceptional migratory phenomena with birds]. Ujma 20: 125–136. (In Slovenian, with English abstract.)
Wagemakers A., Jahfari S., De Wever B., Spanjaard L., Starink V.M., Henry J.C. De Vries HJC, Sprong H, Hovius WJ. 2017: *Borrelia miyamotoi* in vectors and hosts in The Netherlands. Ticks Tick-borne Dis. 8: 370–374.
Wolcott A.K., Margos G., Fingerle V., Becker S.N. 2021: Host association of *Borrelia burgdorferi* sensu lato: a review. Ticks Tick Borne Dis. 12: 101766.

Received 13 June 2021 Accepted 13 January 2023 Published online 31 March 2023

Cite this article as: Šušnjar J., Kišek T.C., Smrdel K.S., Ružić-Sabljić E., Adam K., Ivović V. 2023: Detection, identification and genotyping of *Borrelia* spp. in ticks of Coastal-Karst and Littoral-Inner Carniola regions in Slovenia. Folia Parasitol. 70: 007.