University of South Bohemia in České Budějovice

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Detection and isolation of relapsing fever spirochete, *Borrelia miyamotoi*, from *Ixodes ricinus* collected in the Czech Republic: separation of relapsing fever and Lyme disease spirochetes by cultivation on solid medium

Bachelor Thesis

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Annotation:

This study is focused on a recently described tick-borne pathogen *Borrelia miyamotoi*, which belongs to the relapsing fever group of spirochetes and is transferred by the same *Ixodes* species that transmit Lyme disease spirochetes. The knowledge about the cultivation of *B. miyamotoi in vitro* is limited. Therefore, our interest was aroused to apply and optimize techniques successfully used for cultivating Lyme disease spirochetes, for *in vitro* cultivation of *B. miyamotoi* in modified liquid and on solid media. The aim of this project was to detect the presence of *Borrelia miyamotoi* in hard ticks collected from different areas of the Czech Republic and to obtain a monoclonal population of *B. miyamotoi*.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it, I have used the sources and literature displayed in the list of used sources only.

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Angela Kienberger

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List of abbreviation

Abbreviation	Definition
ALT	Alanine aminotransferase
ampR	Ampicillin resistant
AST	Aspartate aminotransferase
ATB	Antibiotics
BLAST	Basic Local Alignment Search Tool
BMD	*Borrelia miyamotoi* disease
BSA	Bovine serum albumin
BSK medium	Barbour-Stoenner-Kelly medium
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic acid
EDAC	1-Ethyl-3-(3-dimethyl aminopropyl)carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Erythema migrans
FLA	Gene for flagellin
gDNA	genomic DNA
glpQ	Glycerol phosphodiester phosphodiesterase
gyrB	Gene for gyrase
IgM	Anti-*Borrelia* immunoglobulin
IGS	Intergenic spacers
IL-6	Interleukin 6
JHR	Jarisch-Herxheimer reaction
LB medium	Luria-Bertani medium

LBRF	Louse-borne relapsing fever
LD	Lyme disease
MKP	Modified Kelly-Pettenkofer medium
MKP-F	Modified Kelly-Pettenkofer medium – with fetal calf serum
MLSA	Multi-Locus-Spacer-Analysis
MLST	Multi Locus Sequence Typing
ospC	Outer surface protein C
PCR	Polymerase Chain Reaction
RF	Relapsing fever
RLB	Reverse line blotting
rRNA	ribosomal Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
TBRF	Tick-borne relapsing fever
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-a	Tumor necrosis factor a
TOT	Transovarian transmission
Vlps	Variable large proteins
VlsE	Variable major protein-like sequence expressed
Vmps	Variable major proteins
Vsps	Variable small proteins

Abstract

Pathogenic members of the genus *Borrelia* consist of two main groups. The first group includes spirochetes responsible for causing Lyme disease and the second group includes spirochetes causing relapsing fever. Recently discovered tick-born pathogen *Borrelia miyamotoi* stands somewhere in between those two groups and is the only known agent causing relapsing fever transmitted by the same *Ixodes* species that also transmit Lyme disease spirochetes. The first case of *Borrelia miyamotoi* disease was described in 2011 in Russia and included some common for the disease symptoms. One of the key features of *B. miyamotoi* is the existence in co-infections with *B. burgdorferi* s.l. in tick vectors and certain reservoirs hosts. In this study, we aimed to detect the presence of human pathogenic *Borrelia miyamotoi* in hard ticks collected from different areas of the Czech Republic and to obtain a monoclonal population of *B. miyamotoi*, separating the co-infected cultures on the plates with solid medium. A total of 10 *Borrelia* isolates, cultivated from the ticks, were analyzed by PCR and sequencing, and gave a positive result for the presence of *B. miyamotoi*. The isolates positive for *B. miyamotoi* were cultivated from the ticks collected in Netolice, Milovice, and Heroltice, the Czech Republic. The presence of *B. miyamotoi* was confirmed with several specific for *B. miyamotoi* sets of primers. A monoclonal culture of *B. miyamotoi* was obtained by plating on a modified 1.5x Modified-Kelly-Pettenkofer with no gelatin, increased rabbit serum to 10 %, and normal 1.7 % agarose, confirmed by PCR, sequencing, and RLB.

1. Introduction

1.1 *Borrelia*

Tick-borne pathogens are among the causative agents of important human diseases (Heyman et al., 2010). The human pathogenic representatives of the genus *Borrelia* consist of two main groups of spirochetes. The first group includes pathogens that cause Lyme disease (LD) that are transmitted by hard ticks from the *Ixodes ricinus* complex. The second includes pathogens that cause relapsing fever (RF) in humans and are transmitted majorly by *argasid* (or soft) ticks (Szekeres et al., 2015). *Borrelia miyamotoi* is similar to the RF species based on DNA sequences but, like the LD bacteria, is transmitted by *ixodid* rather than *argasid* ticks (Barbour et al., 2009; Siński et al., 2016).

The majority of *Borrelia* species are obligate anaerobes, although some species can exhibit aerotolerant traits. *Borrelia* belong to the *Spirochaetaceae* family and to the phylum *Spirochaetes* (Kubiak et al., 2021; Martino et al., 2006). *Borrelia* have a distinctive shape, reflected in a characteristic flat-wavy helical or planar morphology. The outer membrane of most spirochetes consists of a lipid bilayer that does not contain lipopolysaccharide molecules. A thin layer of peptidoglycan (contains L-ornithine) surrounds the inner membrane, strengthening the cell, and is also flexible enough for the motility of spirochetes, which is achieved by axial filaments in the periplasmic space (Adeolu and Gupta, 2014). The periplasmic flagella originate from both ends of the spirochete (endo-flagella), where they are anchored to the cytoplasmic membrane and coil around the protoplasmic cylinder (Fikrig and Narasimhan, 2006; Szekeres et al., 2017). *Borrelia* species vary in the size and number of periplasmic flagella they possess (Motaleb et al., 2015). The length of spirals in spirochetes ranges from 3-180 μm, with a width of only 0.2-3 μm (Adeolu and Gupta, 2014; Barbour and Hayes, 1986; Fikrig and Narasimhan, 2006; Kubiak et al., 2021). The genome includes a linear chromosome along with a large number of linear and circular plasmids, which are unique among prokaryotes (Chaconas and Kobryn, 2010).

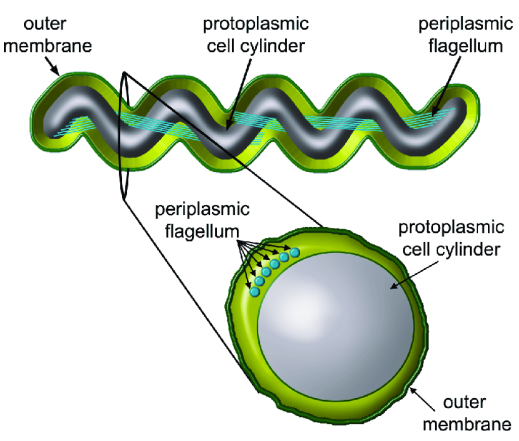


Figure 1: Taken from Kumar et al., shows the structure of *Borrelia* (Kumar et al., 2017)

1.2 Lyme disease

Lyme disease is a multisystem, zoonotic disease that is caused by the spirochetes of the *Borrelia burgdorferi* sensu lato complex and is present in most temperate parts of the Northern hemisphere (Burgdorfer et al., 1982; Rudenko et al., 2011; Rudenko et al., 2019; Seshu and Skare, 2000). In North America and Eurasia, Lyme disease is the most common vector-borne disease (Seifert et al., 2015).

Out of 22 recognized species from the *B. burgdorferi* s.l. complex, a pathogen potential in humans was confirmed for ten species: *B. afzelii, B. bavariensis, B. bissettii, B. burgdorferi* sensu stricto*, B. garinii, B. kurtenbachii, B. lusitaniae, B. mayonii, B. spielmanii and B. valaisiana.* Nevertheless, the most significant contribution to human LD worldwide still belongs to *B. afzelii, B. burgdorferi* sensu stricto and *B. garinii* (Răileanu et al., 2020; Rudenko and Golovchenko, 2021).

The recent worldwide distribution of LD spirochetes is not as strict as it was considered before. Five species from the *B. burgdorferi* s.l. complex (*B. californiensis, B. carolinensis, B. andersonii, B. americana,* and *B. kurtenbachii*) were previously believed to be restricted only to the US, while another 11 species were strictly associated with and identified in Eurasia (*B. turdi, B. valaisiana, B. spielmanii, B. tanukii, B. afzelii, B. bavariensis, B. lusitaniae, B. sinica, B. garinii, B. japonica* and *B. yangtze)* (Rudenko et al., 2011).

In the US, the disease is caused exclusively by *B. burgdorferi* sensu stricto and *B. mayonii*, according to the Centers for Disease Control and Prevention (CDC), while in Eurasia, *B. garinii* and *B. afzelii* are the most common *Borrelia* species infecting humans, followed by *B. burgdorferi* s.s. (Bontemps-Gallo et al., 2018; Marques et al., 2021; Rodino et al., 2020).

Infection with *B. burgdorferi* s.s. is often associated with rheumatological symptoms such as arthritis, while *B. garinii* and *B. bavariensis* are associated with neurological symptoms and *B. afzelii* with dermatological symptoms, erythema migrans (EM) (Feder et al., 2007; Margos et al., 2011; Pal and Fikrig, 2003; Răileanu et al., 2020; Rudenko et al., 2011).

1.3 Relapsing fever

There are two types of relapsing fever caused by *Borrelia*. Louse-borne relapsing fever (LBRF) is an anthroponotic disease caused exclusively by *Borrelia recurrentis*. The other is tick-borne relapsing fever (TBRF), a zoonotic disease caused by various species of *Borrelia* transmitted to humans by soft ticks (*Argas* and *Ornithodoros spp*.), hard ticks (*Ixodidae*) and lice (Jakab et al., 2022; Rodino et al., 2020; Szekeres et al., 2015).

Ticks harboring TBRF *Borrelia* have been reported throughout the world, except Antarctica and Australia. This TBRF *Borrelia* circulate between ticks and various animal hosts and are transmitted to humans by an infectious tick bite (Jakab et al., 2022).

LBRF was always considered more prominent and epidemiologically relevant than TBRF, but this has changed drastically in recent years. Lice-infested populations reduced, and as a result, the disease has become less common in most regions of the world (Jakab et al., 2022).

1.3.1 Clinical picture of relapsing fever

The clinical picture of LBRF is similar to that of TBRF, however, the total number of recurrent febrile flares in LBRF (usually less than two) compared to TBRF (≥2) is lower, while the duration of attacks in LBRF (up to ten days) is longer than in TBRF (≤7 days) (Jakab et al., 2022; Platonov et al., 2011). After an incubation period of 4-18 days, TBRF shows 12 recurrent fever episodes, lasting 2-7 days and are interrupted by afebrile periods of up to 10 days (Jakab et al., 2022; Lopez et al., 2016; Platonov et al., 2011). After the second fever episode, accompanying symptoms such as chills, arthralgia, vomiting, nausea, myalgia, and neurological complications, which include, e.g., radiculopathy, facial paralysis, hemiplegia, encephalitis, and meningitis, may occur more frequently. The antigenic variation of different sequentially expressed versions of the bacterial outer membrane lipoprotein (*Vmp*) is responsible for the characteristic clinical picture of recurrent fever episodes. This modifying property allows the bacterium to temporarily evade the host's humoral immune response (Hamase et al., 1996; Jakab et al., 2022).

1.3.2 Diagnostics

The most common method for detecting RF *Borrelia* is the microscopic examination of blood smears. RF *Borrelia* can be detected in blood from patients during fever episodes (Cutler et al., 2019; Jakab et al., 2022). Microscopy is often supplemented with immunofluorescence (Cutler et al., 2019). Due to cross-reactions with other *Borrelia* and an undiagnosed acute infection, no commercial serological assays have yet been developed to detect TBRF. The PCR method or the detection of anti-*Borrelia* immunoglobulin (*IgM*) in acute or serum samples from the convalescence phase is also often used to detect RF *Borrelia* (Cutler et al., 2019; Jakab et al., 2022).

1.3.3 Treatment

The antibiotics tetracyclines, β-lactams, and macrolides are preferably used to treat TBRF (Jakab et al., 2022). The lethality of untreated TBRF is given as 2-10 % and with antibiotic treatment <2 % (Barbour, 2005; Goddard, 2018; Jakab et al., 2022). After the first dose of antibiotics, the so-called Jarisch-Herxheimer reaction (JHR) can occur (Cutler et al., 2019; Jakab et al., 2022), which causes severe chills and a rise in temperature of about 1–2 hours. JHR is mediated by proinflammatory cytokines (tumor necrosis factor α (TNF-α), interleukin 6 (IL-6)), and symptoms usually resolve within a few hours. Hypotension may be an accompanying symptom. In TBRF, JHR has been reported to occur in up to 54.1 % of all cases and, in rare cases, can end fatally (Jakab et al., 2022).

1.4 *Borrelia miyamotoi*

One of the recently discovered tick-borne pathogens is a spirochete, member of the relapsing fever group, referred to as *Borrelia miyamotoi* (*B. miyamotoi*) (Krause et al., 2015). After a taxonomic description of the first cases in Japan, *B. miyamotoi* was assigned to the genus *Borrelia* and to the phylum *Spirochaetes*. *B. miyamotoi* is transmitted by the same *Ixodid* tick species that transmit the Lyme disease pathogen. It was first discovered in 1995 in *Ixodes persulcatus* ticks in Japan and named after Kenji Miyamoto (Cutler et al., 2019; Krause et al., 2015; Platonov et al., 2011). Fukunaga et al. collected *Ixodes persulcatus* ticks on the island of Hokkaido and analyzed the presence of *Borrelia* in these ticks. Five isolates, including strain type HT31^{T}, were identified to be similar to RF *Borrelia* and different in some characteristics from LD *Borrelia*. Upon analysis, the molecular weight of the protein

for endoflagellin was determined and found to be 38 kDa, which corresponds to that of RF *Borrelia*. The endoflagellar protein of LD *Borrelia* has a mass of 41 kDa. Examination of the rRNA gene revealed only one copy of 23S and 5S RNA, separated from one copy of 16S RNA, clearly defining RF *Borrelia*. LD *Borrelia* has 23S and 5S in two copies repeated in a straight line. DNA-DNA hybridization showed 24-51 % similarity to RF *Borrelia*, while similarity to LD *Borrelia* was only 8-13 %. The limit for belonging to the same species for DNA-DNA hybridization has been set to >70 % (Fukunaga et al., 1995; Moore et al., 1987; Ramasamy et al., 2014; Romana Kejíková, 2018).

B. miyamotoi is consistently prevalent across the Northern temperate regions of North America, Europe, and Asia (Krause et al., 2015; Wagemakers et al., 2015). It has been recorded on the east and west coasts of the United States (US) and in Canada and Japan. It has also been observed in numerous European countries (including the Czech Republic, France, Germany, Norway, Poland, Romania, Denmark, Estonia, Sweden, Switzerland, and the Netherlands) and in Russia (Cutler et al., 2019; Romana Kejíková, 2018).

1.4.1 Tick vectors – *Ixodidae* and *Argasidae*

Members of the *Ixodes ricinus* complex are hard-bodied *ixodid* ticks, while *Argasidae* are soft-bodied ticks (Jakab et al., 2022; Szekeres et al., 2015).

With more than 250 species, *Ixodes* is the genus of ticks with the highest species richness from the family of shield ticks (*Ixodidae*) and is widespread in the temperature zone of the Northern hemisphere (Wagemakers et al., 2015; Yang and Han, 2018). *Ixodes* ticks are three-host ticks. Larva, nymph, and adult females are the three feeding stages, each using, most often, different host species. The larvae are tiny, have three pairs of legs, and feed mainly on small animals (mammals, birds, lizards). Nymphs already have four pairs of legs and feed on larger mammals (squirrels, hares). Adults mainly feed on tall forest game (roe deer, deer, and wild boar) (Romana Kejíková, 2018). Spirochetes of the *Borrelia burgdorferi* s.l. complex are taken up during a blood meal from an infected and infectious host, kept transstadial during the molting process, and are transmitted to other hosts, including humans, during tick feeding (Motaleb et al., 2015; Rodino et al., 2020; Rudenko and Golovchenko, 2021). The *ixodid* tick engorges for 4-8 days on the vertebrate host in order to feed for repletion (Fikrig and Narasimhan, 2006). During the engorgement process, spirochetes multiply significantly in the intestinal tract of the tick (Pal and Fikrig, 2003).

The life cycle stages of soft ticks include egg, larva, several successive nymphs, and the adult. In contrast to hard ticks, soft ticks do not have a hard shell around their mouth parts and do not wait on leaves or grass blades for their prey to pass by. They stay close to their mammalian hosts and, therefore, can infect humans easily. Soft ticks have a short feeding period of 15-90 minutes. Females lay eggs after each blood meal. This reproductive pattern is different from hard ticks, in which adult females reproduce themselves only once in a lifetime. TBRF *Borrelia* can be transmitted after 30 sec of attachment (Estrada-Peña, 2015; Jakab et al., 2022; Romana Kejíková, 2018).

B. miyamotoi has been found in *Ixodes persulcatus* in Japan, *I. scapularis,* and *I. pacificus* in the United States, and *I. ricinus* and *I. persulcatus* in Europe and Asia (Crowder et al., 2014). *B. miyamotoi* can be maintained in the environment due to vertical transmission of the pathogen from a female tick to the offspring, also called transovarial transmission (TOT). This phenomenon is not typical for LD *Borrelia* but typical for RF *Borrelia* (Platonov et al., 2011; Rollend et al., 2013). Due to cross-reactivity and unawareness of the *B. miyamotoi* species, cases of TOT in *B. burgdorferi* s.l. were probably mixed up with *B. miyamotoi* and, therefore, misidentified (Rollend et al., 2013). The fact that the larvae feed only partially (4-8 h) and are therefore not engorged to completion, but already infected after removal, could also explain this occurrence (Romana Kejíková, 2018; van Duijvendijk et al., 2016). At the moment, TOT in *B. burgdorferi* s.l. is still considered unproven (Rollend et al., 2013; Romana Kejíková, 2018; Scoles et al., 2001).

In addition to TOT, *B. miyamotoi* has other established mechanisms, including transstadial transmission and the ability to transmit from infected to uninfected ticks while feeding on one host, which is also known as "co-feeding" (Romana Kejíková, 2018; Scoles et al., 2001; Siński et al., 2016).

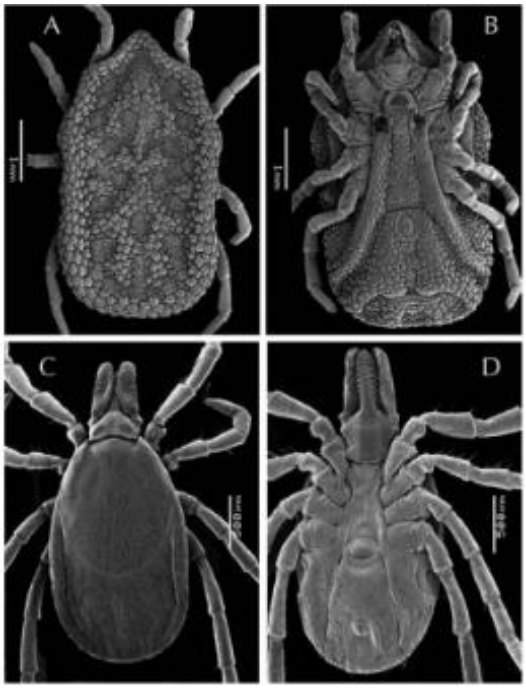


Figure 2: Taken from Estrada-Peña: A and B show the ventral and dorsal views of a female *Ornithodoros puertoricensis*, which belongs to the *argasid* ticks. C and D show the main morphological features of a female *Ixodes ricinus*, which belongs to the *ixodid* ticks (Estrada-Peña, 2015).

1.4.2 Hosts and reservoirs of *Borrelia miyamotoi*

In Europe, *Apodemus flavicollis* (yellow-necked mice) and *Myodes glareolus* (bank vole) are the primary reservoir hosts of *B. miyamotoi* (Burri et al., 2014). In North America, *Peromyscus leucopus* (white footed mouse) is an important reservoir host of *B. miyamotoi* (Romana Kejíková, 2018; Scoles et al., 2001).

B. miyamotoi has also been detected in small rodents, e.g., in *Apodemus argenteus* (small Japanese field mouse) (Fukunaga et al., 1995; Kubiak et al., 2021), *Microtus arvalis* (field vole), *Apodemus sylvaticus* (wood mouse) (Wagemakers et al., 2017), *Myodes rufocanus* (grey red-backed vole), *Apodemus speciosus* (large Japanese field mouse) and in *Myodes rutilus* (Northern red-backed vole) (Taylor et al., 2013). However, the competence to serve as a reservoir for *B. miyamotoi* has not yet been studied in these small rodents (Taylor et al., 2013; Wagemakers et al., 2017). Another mammal in which *B. miyamotoi* has been found is the domestic cat (Romana Kejíková, 2018; Shannon et al., 2017).

Not only mammals can be reservoir hosts since *B. miyamotoi* has also been found in bird spleens of *Carduelis chloris* (European green finch) or *Parus major* (great tit).

Therefore, it is likely that *B. miyamotoi* occurs in a broader range of avian species and birds (Romana Kejíková, 2018; Wagemakers et al., 2017).

It would be reasonable to assume that *B. miyamotoi* and *B. burgdorferi* sensu lato (including *B. afzelii*, *B. burgdorferi* sensu stricto, and *B. garinii*) may behave similarly since they share the same vectors and numerous hosts. However, this is not the case, as many significant distinctions have been illustrated between the spirochetes. For instance, *B. miyamotoi* has been found up to five times more frequently in rodent blood than in the skin, whereas *B. burgdorferi* s.l. has been found up to 40 times more frequently in the skin than in the blood. *B. burgdorferi* s.l. can persist in its hosts, whereas *B. miyamotoi* is probably unable to and therefore applies its TOT ability to survive (Barbour et al., 2009; Cutler et al., 2019; Rollend et al., 2013; Taylor et al., 2013; van Duijvendijk et al., 2016). Scoles et al. showed a progressive decline in the prevalence of *B. miyamotoi* in each subsequent stage and a cumulative effect and an increasing prevalence in the later stages of *B. burgdorferi* s.l. (Scoles et al., 2001; Taylor et al., 2013). There are several reasons why the prevalence of *B. miyamotoi* is not higher than that of *B. burgdorferi* s.l. Its tiny amount in the hosts' skin may play a role because it can reduce the ticks' ability to be infected. Furthermore, the inability of *B. miyamotoi* to cause persistent infection is a possible factor, as the tick can become infected only during a temporary episode of acute spirochetemia in the host blood (Barbour et al., 2009; Hamšíková et al., 2017; Romana Kejíková, 2018; Taylor et al., 2013; Wagemakers et al., 2015).

No variations in the prevalence of *B. miyamotoi* were observed within the sex of rodents or between individuals of different ages (Barbour et al., 2009; Hamšíková et al., 2017; Romana Kejíková, 2018; Taylor et al., 2013).

1.4.3 Co-infection of *Borrelia miyamotoi* and *Borrelia burgdorferi* sensu lato

In certain hosts or reservoirs, *B. miyamotoi and B. burgdorferi* s.l. have been discovered together and shared the same vector (Cutler et al., 2019). However, co-infections in hosts or vectors are not as frequent as one might assume (Barbour et al., 2009; Cutler et al., 2019; Romana Kejíková, 2018).

Some studies report co-infections to a much lower extent than expected, based on the prevalence of both pathogens, while other studies demonstrate a statistically significant number of co-infections that suggest similar transmission and environmental dynamics of both pathogens (Cutler et al., 2019; Dibernardo et al., 2014; Geller et al., 2012; Wagemakers

et al., 2017). According to certain research, the possibility of individual synchronous transmission or the sharing of so-called "amplifying hosts" is more likely to occur (Romana Kejíková, 2018).

Based on their properties and their different distribution in hosts, the dynamics of the two pathogens are apparently different (Barbour et al., 2009). Study of common small rodent hosts points to different seasonal peaks of infection. *B. burgdorferi* s.l. rodent infection has its highest peak in spring and is driven by nymphs, while *B. miyamotoi* rodent infections are predominantly in summer and are caused by tick larvae (Szekeres et al., 2017). This separation could explain the coexistence of both spirochetes in ticks (Cutler et al., 2019; Romana Kejíková, 2018).

1.4.4 Phylogenetic analysis of *Borrelia miyamotoi*

Phylogenetic studies of *B. miyamotoi* are mainly based on Multi-Locus-Sequence analysis that includes housekeeping spirochete genes (MLST analysis). Additional used genetic markers include the gene for the outer membrane protein *p66*, the gene for the 16S rRNA subunit, the gene for flagellin (*fla*), the gene for gyrase (*gyrB*) and the gene for the immunoreactive protein *glpQ* (glycerol phosphodiester phosphodiesterase) or intergenic spacers (IGS) (Mukhacheva et al., 2015; Siński et al., 2016). A necessary discovery is the clonal nature of individual genotypes in *B. miyamotoi* gene markers. This has been discovered mainly in Asia, where identical *B. miyamotoi* genotypes have been found in *I. persulcatus* and *I. pavlovskyi* ticks across the continent (from the Baltic Sea to the Ural Mountains and Central Asia to the Japanese Islands) (Mukhacheva et al., 2015; Siński et al., 2016). As an explanation, an expansion of the origin focus is likely, since both tick species can be found in migratory birds (Mukhacheva et al., 2015). This could be the reason for the spread of the ticks over longer distances. Another similar clonal character can be found in the American type of *B. miyamotoi*, where it is manifested mainly within individual vectors, i.e., *I. pacificus* (west coast of America) and *I. scapularis* (east coast of America) (Mun et al., 2006). Diversity within the European, Asian, and American *B. miyamotoi* genotypes is therefore scarce, which is non-identical to the *Borrelia burgdorferi* sensu lato complex, where differences exist (Iwabu-Itoh et al., 2017; Jiang et al., 2018; Kubiak et al., 2021; Mukhacheva et al., 2015; Romana Kejíková, 2018; Takano et al., 2014).

1.4.5 Transmission mechanisms of *Borrelia miyamotoi* and other relapsing fever *Borrelia*

RF *Borrelia* are present in their vectors in several types of tissues, including salivary glands (Romana Kejíková, 2018). Key proteins that play an essential role in relapsing fever infection are called variable major proteins (*Vmps*). They are divided into variable small proteins (*Vsps*) and variable large proteins (*Vlps*). *Vsps* contain an average of around 210 amino acids, while *Vlps* are about 360 amino acids in size. In RF *Borrelia, Vsps* are orthologues of *ospC* (outer surface protein C), whose production allows spirochetes to exit the gut and infect the host, and *Vlps* of variable major protein-like sequences expressed (*VlsE*) (Barbour et al., 2000; Margolis et al., 1994; Rebaudet and Parola, 2006; Zhang et al., 1997). Genes for these proteins are located on linear plasmids (Barbour et al., 2000). Only one type of *Vmps* is expressed in a given *Borrelia* population, either *Vsps* or *Vlps* (Barbour et al., 2000; Barbour et al., 2006; Romana Kejíková, 2018). During infection, continuous serotype changes occur in the mammalian host. This phenomenon is also called "switching" and is responsible for the typical "relapse" (return) of fever in an infected host. It occurs within the entire *Borrelia* population in a given host, and each subsequent "relapse" is caused by a switch to a new serotype with the following host's immune response (Barbour et al., 2000; Barbour et al., 2006; Romana Kejíková, 2018). Through gene conversion, genomic rearrangement, and point mutations, antigen variability and "switching" occur (Barbour et al., 2000). Complement factor H binding proteins or C4b binding proteins are the main mechanisms causing complement resistance identified in some RF *Borrelia*. The RF *Borrelia* also can induce erythrocytes clustering ('rosseting'), which leads to increased bleeding, formation of micro emboli, and a delayed immune response of the organism. Spirochetes can use these clusters as mechanical protective shields against white blood cells, which then have difficult access to pathogens (Romana Kejíková, 2018). The transmission mechanisms of *B. miyamotoi* are not expected to be similar to those of other RF or LD *Borrelia*. A combination of different mechanisms from both groups or the existence of unique mechanisms seems more reasonable. Studies on the pathogenesis of *B. miyamotoi* are still in progress (Stone and Brissette, 2017). Nineteen *Vlps* and *Vsps* are identified as specific surface antigens of *B. miyamotoi*, which are proteins homologous to *Vmps* in other RF *Borrelia*. These proteins can be used as a strategy of antigenic variability similar to other RF *Borrelia* and correspond to the fact that *B. miyamotoi* is the cause of relapsing fever, or rather a disease similar to relapsing fever – *Borrelia miyamotoi* disease (BMD) (Barbour et al., 2006; Hamase et al., 1996; Romana Kejíková, 2018).

1.4.6 First described cases of *Borrelia miyamotoi* disease in human

The first cases of *B. miyamotoi* infection in humans were reported in 2011 by Platonov et al. in Russia and subsequently in the United States, Japan, and Europe. In the studies by Platonov et al., 302 patients were inspected for Lyme disease and *B. miyamotoi* infection after a tick bite. The assessment of the clinical signs, detection of the pathogen by PCR, serology (ELISA), and possible confirmation by sequencing were included in the diagnosis. Forty-six patients had PCR and serology-confirmed *B. miyamotoi* infection. Fever, headache, fatigue, chills, vomiting, convulsions and muscle aches were the observed clinical signs. Five of those 46 patients experienced a relapse of fever, and one person suffered from two relapses, with a time range of two days to two weeks in between. Compared to Lyme disease, the first stage of *B. miyamotoi* infection appears to be more severe, particularly regarding the number of symptoms. Additionally, compared with *B. burgdorferi* s.l., the disease symptoms (fever, headache) appeared more systemic. Without early initiation of antibiotic treatment, which was effective in all patients, the number of relapses in some instances could have been more significant. After antibiotics (ATB) administration, JHR has been reported in seven patients (Maloy et al., 1998; Platonov et al., 2011; Romana Kejíková, 2018).

Infection with *B. miyamotoi* in humans can also result in serious diseases such as meningoencephalitis in immunocompromised individuals. *B. miyamotoi* can also be detected as a co-infection with other *Ixodes*-borne pathogens (Krause et al., 2015; Page et al., 2018).

Many other human cases of disease caused by *B. miyamotoi* have been described worldwide, e.g., USA, Russia, Netherlands, Canada, China, Japan, and others (Barbour et al., 2009; Cutler et al., 2019; Platonov et al., 2011). Fever reaching 40° C, headache and fatigue, muscle or joint pain, colds, vomiting, nausea, and neck stiffness are the most frequently described symptoms of *B. miyamotoi* infection. Up to a maximum of two relapses of fever were observed, which can increase in number after too late antibiotic treatment (Page et al., 2018; Platonov et al., 2011; Romana Kejíková, 2018; Siński et al., 2016). Leukopenia (decreased white blood cell count), elevated levels of some liver enzymes –aspartate aminotransferase (AST), alanine aminotransferase (ALT), and thrombocytopenia are the most common laboratory findings in *B. miyamotoi* infections (Platonov et al., 2011; Romana Kejíková, 2018). On rare occasions, skin lesions similar to EM can also be caused (Crowder et al., 2014).

1.4.7 Diagnosis of *Borrelia miyamotoi* disease (BMD)

B. miyamotoi may be found using a wide range of diagnostic methods, mainly serological as well as direct techniques such as microscopy, PCR, or sequencing. Blood samples, cerebrospinal fluid samples, especially in individuals with neuroborreliosis, or tissue biopsies are the most commonly examined samples (Cutler et al., 2019; Romana Kejíková, 2018). *B. miyamotoi* can also be detected in ticks or hosts using similar methods. Surveillance of the disease and the level of risk of human infection can be carried out by mapping the occurrence of *B. miyamotoi* in these organisms (Romana Kejíková, 2018).

1.4.8 Cultivation of *Borrelia miyamotoi in vitro*

In 1995, *B. miyamotoi* was first successfully cultured from the blood of the rodent *Apodemus argentus* and gut samples of the tick *I. persulcatus* in BSKII (Barbour-Stoener-Kelly) medium (Fukunaga et al., 1995). Barbour modified the BSKII medium (deletion of glutamine from CMRL 1066 and addition of Yeastolate) and achieved a high spirochete density. He also showed that RF *Borrelia* are more difficult to culture than LD *Borrelia* (Barbour, 1984). Another modified BSKII medium for *B. miyamotoi* was developed by Replogle et al. BSK-R is a diluted BSKII derivative containing a reduced amount of CMRL 1066 and Lebovitz's L-15, mouse, and fetal calf serum. However, in addition to *Borrelia miyamotoi*, the growth of 5 other RF and ten different LD *Borrelia* were detected (Replogle et al., 2021). Raffel et al. researched a modified BSKII medium with a pH of 7.5 and an increased rabbit serum concentration (10 %). In this case, the *Borrelia* were cultured on a solid medium. Bottom plates were poured 1-3 days before the top plates and incubated at 35 °C in a CO_2/O_2 incubator. In addition to the increased rabbit serum concentration, the cover plates contained a 1 % low melting point agarose and were equilibrated to 37 °C (Raffel et al., 2018). Another research by Zückert revealed good results while incubating agar plates in a CO_2-rich atmosphere and excluding gelatin from the agar plates. It was shown that RF *Borrelia* accumulated on the gelatin-free agar plates. The *Borrelia* on the plates were described as white and opaque colonies <1 mm or 2-4 mm in size. The small colonies had a more pointed shape, and the rather larger ones appeared diffuse. *Borrelia* grow within 1 to 4 weeks after plating (Zückert, 2007). *Borrelia miyamotoi* can also be successfully cultivated in MKP (Modified Kelly-Pettenkofer) medium since 2014 (Wagemakers et al., 2014). An original MKP medium contains 7.2 % of heat-inactivated rabbit serum. In addition to rabbit serum, fetal calf serum in a concentration of 10 % was added (Wagemakers et al., 2014).

2. Aim of the study

The aim of this project was to use different molecular biology techniques to detect the presence of pathogenic for humans *Borrelia miyamotoi* in hard ticks from different areas of the Czech Republic. The second goal of the project was to obtain a monoclonal population of *B. miyamotoi*, separating the possibly co-infected cultures on the plates with solid medium, since *B. miyamotoi* is commonly present in hard ticks together with other *Borrelia burgdorferi* s.l. spirochetes.

In addition, the separation of two different *Borrelia* strains via infection of mice with co-infected isolates, followed by tick feeding on these mice, letting the engorged females lay eggs, and further cultivation of *B. miyamotoi* from these eggs, was also tried as an alternative method to obtain a pure *B. miyamotoi* culture, since *B. burgdorferi* s.l. is proved to be not transferred from the female ticks to the offspring.

3. Materials and Methods

The following laboratory techniques include: - preparation of media for *Borrelia* cultivation, cultivation of *Borrelia* spirochetes in liquid and solid media, dark-field microscopy, determination of cultures density by counting spirochetes in the counting chamber, gDNA isolation, DNA quantity and quality determination using a spectrophotometer, spacer, and nested PCR, gel electrophoresis, PCR products purification, direct sequencing of PCR products, cloning, sequencing, sequence analysis using different databases and reverse line blotting.

3.1 Materials

Table 1: Summary of all materials used to perform all experiments of this study

Procedure	Material	Description
gDNA extraction	gDNA isolation kit	DNeasy® Blood & Tissue Kit (250) (Qiagen)
	1x PBS	0.8 g/L NaCl 0.02 g/L KCl 0.144 g/L Na_2HPO_4 0.024 g/L KH_2PO_4 pH = 7.4, sterilized
PCR	2 x Master Mix	Taq DNA polymerase (supplemented with e reaction buffer) (pH 8.5), 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 200 μM dTTP, 3 mM $MgCl_2$ (Promega)
	PCR water	Double-distilled Milli-Q-purified water
Gel electrophoresis	50x TAE buffer	200 mM Tris-HCl, 50 mM EDTA
	Modified TAE buffer	40 mM Tris-acetate, pH 8.0, 0.1 mM Na_2EDTA
	Agarose	0.8 – 2 % agarose (Serva) for DNA ELFO in 1xTAE buffer
	SYBR Gold nucleic acid stain	10 000x concentrate, Invitrogen
	6x sample buffer	Blue/Orange 6x loading dye, (0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 0.4 % orange G, 15 % Ficoll™ 400, 10 mM Tris- HCl (pH 7.5) a 50 mM EDTA (pH 8.0) (MBI Fermentas)
	PCR Marker	Gene Ruller 100 bp Plus DNA Ladder (Thermo Scientific)
PCR product purification	PCR product purification kit	QIAquick Gel Extraction Kit Protocol (Qiagen)
Reverse line blotting (RLB)	Biodyne C blotting membrane	(Pall Biosupport, Ann Arbor, Mich.)
	16 % 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC)	(Sigma, St. Louis, Mo.)
	Miniplotter	(Immunetics, Cambridge, Mass.)

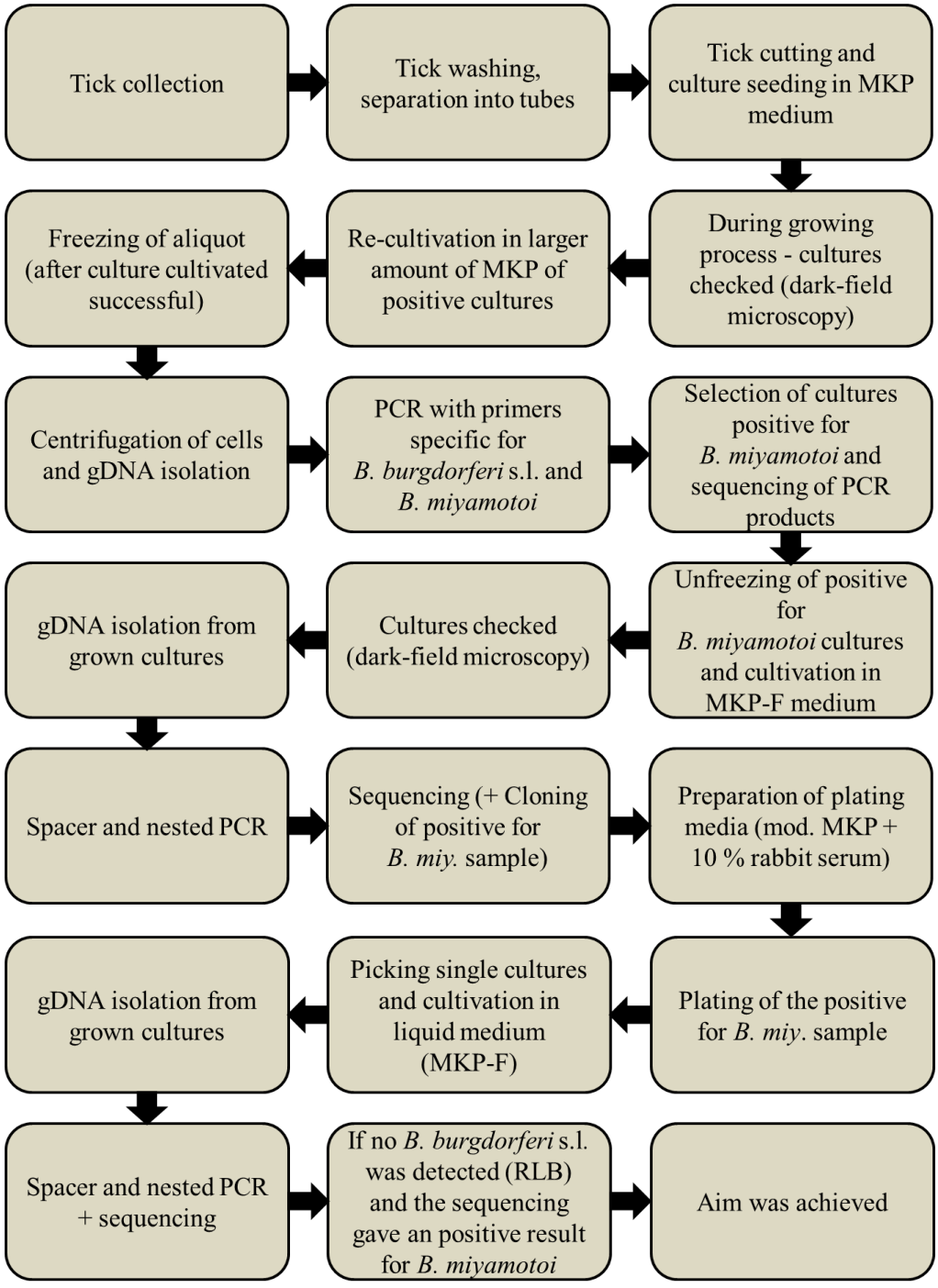
	$NaHCO_3$ (pH 8.4)	(Applichem)
	100 mM NaOH	(Applichem)
	2x SSPE-0.1 % sodium dodecyl sulfate (SDS)	20x SSPE contains: 360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA (pH 7.4) (Sigma)
	2x SSPE-0.5 % sodium dodecyl sulfate (SDS)	(Sigma)
	1:4000 diluted peroxidase-labeled streptavidin	(Merck)
	2xSSPE	(Sigma)
	TMB stabilized substrate for Horseradish Peroxidase (HRP)	(Promega)
	20 mM EDTA (pH 8.0)	-
Molecular Cloning	Molecular cloning kit	TOPOTM TA CloningTM Kit for sequencing (Invitrogen)
	TOPO-TA Cloning Vector	(Invitrogen)
	One Shot TM TOP10 Chemically Competent *E.coli*	(Invitrogen)
	Salt Solution	1.2 M NaCl, 0.06 M $MgCl_2$ (Invitrogen)
	S.O.C medium	2% Tryptone 0.5% Yeast Extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose (Invitrogen)
	Luria-Bertani (LB) medium (Sambrook, J. & Russell, D. W., 2001)	For 100 mL (pH = 7.0): 1 g tryptone 0.5 g yeast extract 1 g NaCl dH_2O
	Ampicilin	50 mg/mL stock solution
	Plasmid DNA isolation kit	(Qiagen)

Table 2: Instruments used in the experiments (Ramzy I., 2019)

Instruments used	Type
Centrifuges	Centrifuge 5415 C (Eppendorf)
	Centrifuge 5415D (Eppendorf)
	Centrifuge 1415 R (Eppendorf)
	Centrifuge Universal 32 R (Hettlich centrifuge)
Electrophoresis	SHU6 (Sigma Aldrich)
	OVL Easycast*TM* B2 (Thermo scientific)
Dark field microscope	Leica DM 1000 LED (Leica)
PCR cycler	Mastercycler *personal* (Eppendorf)
Thermomixer	Thermomixer (Eppendorf)
Petroff - Hausser counting chamber	Cell depth: 0,02 mm, Hausser Scientific, PA, USA
Photosystem for gel documentation	Kodak
Flow box	Gelaire
PCR box	DNA/RNA UV cleaner UVC/T-M-AR (Biosan)
Vortex	Heidolph REAX top
Blotting apparatus	Trans-Blot® SD Semi-Dry Transfer Cell
Protein electrophoresis	Mini Gel Tank Thermo Fisher Scientific

3.2 Methods

3.2.1 Experimental Overview



3.2.2 *Borrelia* isolates cultured from ticks collected in the Czech Republic

Ticks were collected from different areas of the Czech Republic by flagging, washed for surface disinfection, and diced individually in one ml of liquid MKP media (Table 3). Dark-

field microscopy was performed one week later. Cultures that showed growth of spirochetes were further grown in five ml of MKP medium, aliquoted, and frozen at -80 °C. The rest of the cultures were used in further experiments. The cells were centrifuged, the gDNA isolated, and checked by PCR with primers specific for *B. burgdorferi* s.l. (flagellin, *ospC*) and for *B. miyamotoi* (*glpQ*). Samples (cultures) positive for *B. miyamotoi* DNA were used in this project (Table 14). All of them were proved to be the co-infection of different strains of *B. burgdorferi* s.l. and *B. miyamotoi*.

3.2.3 Preparation of Modified Kelly-Pettenkofer (MKP) medium

Modified Kelly-Pettenkofer (MKP) medium, used in this project was prepared according to the protocol by Ružić-Sabljić et al. (Ružić-Sabljić et al., 2014). For the preparation of 200 ml of basic MKP medium for *Borrelia* cultivation, the following ingredients were mixed:

Table 3: Reagents and their amounts for 200 ml complete MKP medium

Reagent	Mass (g)
CMRL-1066	1.94
Neopeptone	0.6
HEPES	1.2
Citric Acid	0.14
D-Glucose	0.6
Pyruvic Acid	0.16
N-acetyl glucosamine	0.08
Sodium bicarbonate	0.4
For preparation of complete MKP medium, the following ingredients were added:	
Reagent	Mass (g)
BSA	2.45
Reagent	Volume (ml)
7 % Gelatin	40
Rabbit serum	14.4

All powdered components needed for basic medium preparation (Table 3) were dissolved in 100 ml of ddH_2O (for about an hour). After complete dissolving, the pH was adjusted with

NaOH to 7.6 and the final volume was adjusted with ddH_2O up to 200 ml. The basic MKP medium can be frozen at -20 °C and kept for up to 3 months.

To prepare the complete MKP medium, BSA, gelatin, and rabbit serum were added to basic MKP medium. The appropriate amount of BSA, heat-inactivated rabbit serum (7.2 %), and freshly autoclaved gelatin (Table 3) were added to 200 ml of basic medium while vortexing and low heating. The gelatin was boiled before in the microwave till complete dissolution. The obtained complete MKP medium was sterilized by filtration, aliquoted into 50 ml tubes, and stored at +4 °C.

3.2.4 Dark-field microscopy and culture density calculation

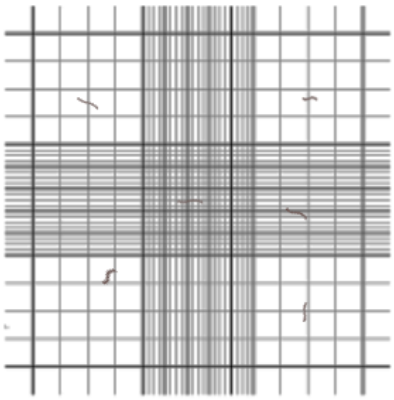
Borrelia cultures were continuously checked by dark-field microscopy to ensure the growth of spirochetes and culture purity. By counting the individual spirochetes under 10 x 40 magnification using a Petroff-Hausser counting chamber (Fig. 3), the concentration of bacteria in the cultures was determined. Ten µl of the *Borrelia*-rich liquid medium were pipetted on the counting chamber, and the average number of *Borrelia* in three larger squares was calculated. The number of spirochetes in one ml culture was then established according to the following formula:

$$A * 1.25 * 10^6$$

Formula 1: to calculate the final concentration of the culture

A… average number of *Borrelia* spirochetes in the three squares of the chamber

Figure 3: Counting chamber with spirochetes



3.2.5 Preparation of the Modified Kelly-Pettenkofer medium with 10 % fetal calf serum (MKP-F) for *Borrelia miyamotoi* cultivation

Modified MKP medium for cultivation of *B. miyamotoi* (MKP-F) was prepared according to Wagemakers et al. (Wagemakers et al., 2014).

For the preparation of 200 ml of basic MKP-F medium for *Borrelia* cultivation, the following ingredients were mixed:

Table 4: Reagents and their amounts for complete 200 ml MKP-F medium

Reagent	Mass (g)
CMRL-1066	1.94
Neopeptone	0.6
HEPES	1.2
Citric Acid	0.14
D-Glucose	0.6
Pyruvic Acid	0.16
N-acetyl glucosamine	0.08
Sodium bicarbonate	0.4
For preparation of complete MKP-F medium, the following ingredients were added:	
Reagent	Mass (g)
BSA	2.45
Reagent	Volume (ml)
7 % Gelatin	40
Rabbit serum	14.4
Fetal calf serum	20

Original MKP medium (200 ml) contains 7.2 % heat-inactivated rabbit serum. MKP-F modified medium contains additional fetal calf serum in a concentration of 10 %. The complete medium was stored at +4 °C.

3.2.6 Isolation of gDNA and checking of its concentration and purity

gDNAs from positive for *B. miyamotoi* local tick isolates (Table 14) were extracted, using a commercial kit (DNeasy® Blood & Tissue Kit (250), Qiagen) according to the manufacturer's instructions (the protocol is presented below). The concentration and quality of the gDNA was determined with NanoDrop spectrophotometer. The measurements were repeated three times for each sample, and the average value was calculated. The presence of *B. miyamotoi* DNA was checked in the samples (see *PCR – detection of Borrelia DNA in the cultures*).

gDNA isolation (according to manufacturer's instructions) shortly:

1. Collect the cells by centrifugation
2. Discard the supernatant and wash the cells with 1 ml of 1xPBS
3. Collect the cells by centrifugation
4. Add 180 µl of ATL buffer to each pellet and resuspend by vortexing
5. Add 20 µl of proteinase K and incubate at 56 °C without mixing for half an hour
6. Vortex each tube 15 sec
7. Add 200 µl of AL buffer and vortex
8. Add 200 µl of 100 % Ethanol and vortex
9. Transfer the mixture into DNeary Mini spin column placed in a 2 ml collection tube
10. Centrifuge for 1 min at full speed
11. Place DNeary Mini spin column in a new 2 ml collection tube
12. Add 500 µl of AW1 washing buffer and centrifuge for 1 min at full speed
13. Place DNeary Mini spin column in a new 2 ml collection tube
14. Add 500 µl of AW2 washing buffer and centrifuge for 2 min at full speed
15. Place DNeary Mini spin column in a new 2 ml collection tube
16. Centrifuge for additional 1 min at full speed
17. Place DNeary Mini spin column in a 1.5 ml Eppendorf tube
18. Add 50 µl of H_2O on the membrane of the column and leave for 1 min at RT
19. Centrifuge for 1 min at full speed
20. Determine the concentration of gDNA with NanoDrop spectrophotometer
21. Store at +4 °C

3.2.7 Polymerase chain reaction (PCR) – detection of *Borrelia* DNA in the cultures

Spacer/nested PCR differs from conventional PCR as it involves two consecutive amplification reactions. Each amplification reaction uses a different primer pair. The second round of PCR (nested) uses the product of the first amplification reaction (spacer) as a template. This template is primed by oligonucleotides placed within the first pair of primers. Nested PCR has a higher sensitivity than conventional PCR (Green and Sambrook, 2019).

glpQ is a gene used for specific PCR detection of *B. miyamotoi*, as it was only found in RF *Borrelia* and the genus *B. miyamotoi*, thereby avoiding cross-reactions with other *Borrelia* strains (Krause et al., 2015). Other genes used to detect *B. miyamotoi* include the gene encoding *fla*, 16S rRNA, and *p66* (Barbour et al., 2009).

In this analysis, nested PCR for detection of *Borrelia miyamotoi* was performed using PCR 2 x Master Mix (Promega), specific primers, gDNA isolated from tick isolates as templates, and cloned *glpQ* gene fragment of *B. miyamotoi* genome (strain HT31) from Russia as a positive control.

Table 5: Primers used for nested PCR (except primers for flagellin gene – normal PCR)

Primer name	Reference	Primer sequence (5' - 3')	Product length (bp) / annealing temperature (°C) / pathogen
SP-Bm-glpQ3(forward)	(Fomenko et al.) (Fomenko et al., 2010)	GCTAGTGGGTATCTTCCAGAAC	722 bp (Fomenko et al., 2010) 54 °C *B. miyamotoi*
SP-Bm-glpQ4(reverse)		CTTGTTGTTTATGCCAGAAGGGT	
N-Bm-glpQ1(forward)	(Fomenko et al.) (Fomenko et al., 2010)	CACCATTGATCATAGCTCACAG	466 bp (Fomenko et al., 2010) 52 °C *B. miyamotoi*
N-Bm-glpQ2(reverse)		CTGTTGGTGCTTCATTCCAGTC	
SP-BM-p66M1(forward)	(Fomenko et al.) (Fomenko et al., 2010)	TTCTATATTTGGACACATGTC	678 bp (Fomenko et al., 2010) 50 °C *B. miyamotoi*
SP-BM-p66M2(reverse)		CAGATTGTTTAGTTCTAATCCG	
N-BM-p66M3(forward)	(Fomenko et al.) (Fomenko et al., 2010)	CTAAATTATTAAATCCAAAATCG	532 bp 52 °C *B. miyamotoi*
N-BM-p66M4(reverse)		GGAAATGAGTACCTACATATG	
FLA120(forward)	(Platonov et al.) (Platonov et al., 2011)	AGAATTAATMGHGCWTCTGATGATG	506 bp (Jiang et al., 2018) 50 °C *B. miyamotoi*
FLA920(reverse)		TGCYACAAYHTCCATCTGTCATT	
SP-glpQ67(forward)	(Bao-Gui Jiang et al.) (Jiang et al., 2018)	ATGGGTTCAAACAAAAAGTCACC	??? bp 52°C *B. miyamotoi*
SP-glpQ789(reverse)		CCAGGGTCCAATTCCATCAGAATATT	
N-glpQ86(forward)	(Bao-Gui Jiang et al.) (Jiang et al., 2018)	CACCATTRATYATAGCTCACAG	461 bp (Jiang et al., 2018) 54°C *B. miyamotoi*
N-glpQ546(reverse)		GATGTCTTTACCTTGTTGTTTATGCCA	
SP-ospC (forward)	(Bunikis et al.) (Bunikis et al., 2004)	ATGAAAAAGAATACATTAAGTGC (position 306-328)	657 bp 52 °C *Borrelia burgdorferi* s.l.
SP-ospC (reverse)		ATTAATCTTATAATATTGATTTTAATTAAGG (position 963-933)	
N-ospC (forward)		TATTAATGACTTTATTTTTATTTATATCT (position 331-359)	617 bp 54 °C *Borrelia burgdorferi* s.l.
N-ospC (reverse)		TTGATTTTAATTAAGGTTTTTTTGG (position 948-924)	

PCR reaction

Reaction mixture (1 reaction – 20 µl total volume):

2x PCR Master Mix (stored at -20 °C)_____10 µl

0.1 mM primer Forward (stored at -20 °C)_____1 µl

0.1 mM primer Reverse (stored at -20 °C) _____1 µl

H_2O (stored at -20 °C) _____variable µl

Template DNA< 200 ng / reaction (stored at +4 °C)_____variable µl

After de-freezing all components of the reaction mixture, the reaction was settled on ice. The primers were diluted to a concentration of 0.1 mM. The first round of the reaction was prepared in a total volume of 20 µl using three µl of template DNA. In the second round, five µl of the reaction from the first round was used as a template. The reaction was conducted under conditions described by Fomenko et al., Platonov et al., and Bao-Gui Jiang et al. The presence of *B. miyamotoi* DNA was confirmed by primers encoding *glpQ*, *p66*, and flagellin (*fla*) genes from *B. miyamotoi* genome. Only one round of PCR (spacer) was performed with primers for flagellin. In the case of *glpQ* gene, the cloned PCR product was used as a positive control. In the case of *fla* and *p66* primers, gDNA of the ticks positive for *B. miyamotoi* was used as a positive control. The negative control was a mix with H_2O as a template. Details of each reaction can be found in Tables 6 and 7 (Fomenko et al., 2010; Jiang et al., 2018; Lesiczka et al., 2022; Platonov et al., 2011; Ramzy I., 2019).

Table 6: PCR conditions for *glpQ*

PCR conditions for PCR reaction with two pairs of primers (spacer and nested) for *glpQ*		
Steps (35x)	Temperature	Time
Initial denaturation	95 °C	5 min
Denaturation	95 °C	30 sec
Annealing (Spacer)	54 °C (Fomenko et al., 2010) / 52 °C (Jiang et al., 2018)	30 sec
Annealing (Nested)	52 °C (Fomenko et al., 2010) / 54 °C (Jiang et al., 2018)	30 sec
Elongation (Spacer)	72 °C	1 min
Elongation (Nested)	72 °C	40 sec
Final elongation	72 °C	10 min
Final hold	12 °C	hold

Table 7: PCR conditions for *p66*

PCR conditions for PCR reaction with two pairs of primers (spacer and nested) for *p66*		
Steps (35x)	Temperature	Time
Initial denaturation	95 °C	5 min
Denaturation	95 °C	10 sec
Annealing (Spacer)	50 °C	15 sec
Annealing (Nested)	52 °C	15 sec
Elongation	72 °C	40 sec
Final elongation	72 °C	10 min
Final hold	12 °C	hold

Conditions for a one-step PCR reaction with primers for *fla* were the same as in Table 7, except for the denaturation time, which was 40 sec, and the annealing time and temperature, which was 40 sec at 50 °C for *fla* primers. Conditions for nested PCR reaction with primers for *ospC* were the same as in Table 6, except for the elongation (nested) time, which was 1 min for 72 °C for *ospC* primers.

3.2.8 Gel electrophoresis

After each PCR reaction the amplicons were separated by gel electrophoresis in agarose gel. Agarose gel (1.5 %) was prepared by heat-dissolving 1.5 g of agarose in 100 ml of 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) (Table 1). The 1x TAE buffer was diluted from a 50x TAE stock. Sample buffer (6x) supplemented with SYBR Gold (Invitrogen) was added to each sample (3.3 µl), and the samples were loaded on the gel. Ten µl of Gene Ruller 100 bp Plus DNA Ladder (Thermo Scientific) was loaded on gel with the samples. The electrophoresis was running for around 20-40 min at 100-150 V. The amplicons were then visualized under UV light. The positive PCR products were extracted from the gel for further sequence analysis. In this case, a modified TAE buffer (40 mM Tris-acetate, pH 8.0, 0.1 mM Na_2EDTA) was used (Lesiczka et al., 2022; Ramzy I., 2019).

3.2.9 PCR product purification and sequencing

PCR products of the expected size (*B. miyamotoi* amplicons) were excised from the agarose gel and purified using a commercial kit (QIAquick Gel Extraction Kit Protocol, Qiagen)

according to the manufacturer's instructions (the protocol is presented below) (Lesiczka et al., 2022).

PCR product purification from the gel (manufacturer's instructions) shortly:

1. Cut DNA fragment from agarose gel (if not immediately used - freeze at -20 °C)
2. Weight gel slice in a 2 ml Eppendorf tube
3. Add 3 volumes of buffer Q6 to 1 volume of gel
4. Incubate at 50 °C for 10 min (vortex in between)
5. Add 1 gel volume of isopropanol and vortex
6. Transfer into a QIAquick spin column placed in a 2 ml collection tube
7. Centrifuge at full speed for 1 min
8. Place QIAquick spin column in a new 2 ml collection tube
9. Add 0.5 ml of buffer Q6
10. Centrifuge for 1 min at full speed
11. Add 0.75 ml of PE buffer
12. Leave tubes for 5 min at RT
13. Centrifuge for 1 min at full speed
14. Discard flow-through
15. Additional centrifugation for 1 min at 13.000 rpm
16. Place Quick spin column in a 1.5 ml collection tube
17. Apply 30 µl of H_2O on the column
18. Leave tubes for 1 min at RT
19. Centrifuge for 1 min at full speed to elute DNA
20. Store at -20 °C - if not immediately used

Purified PCR products were then sequenced in both directions (www.seqme.eu). All reactions were prepared according to the sequencing facility recommendations. Each reaction contained 50 ng (if the PCR product was less than 500 bp) or 100 ng (if PCR product was more than 500 bp) of the template and 25 pMol of primer with a total volume of ten µl. The sequences obtained were then analyzed using DNAStar programs and sent to GenBank™ for comparison with available sequences using Basic Local Alignment Tool (BLAST).

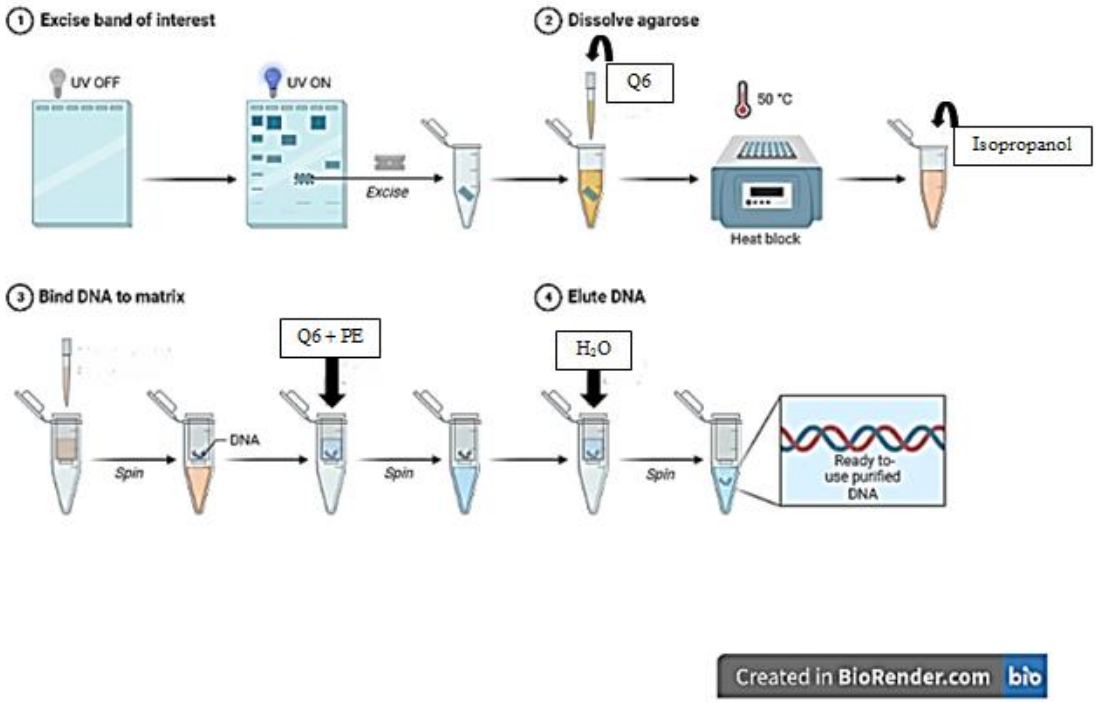


Figure 4: PCR purification as scheme

3.2.10 Plating of confirmed *Borrelia miyamotoi* isolates

To separate *B. miyamotoi* and *B. burgdorferi* s.l. detected and confirmed as co-infection in the identical tick isolates, the obtained cultures were plated on a solid medium.

For plating and successful growing of *B. miyamotoi* on the plates, different solid media were checked. The first plating was done with a BSK-II medium with increased rabbit serum to 10 % and low melting 1.7 % agarose (Table 8) according to Raffel et al.(Rosa and Hogan; Rosa et al.; Samuels) (Raffel et al., 2018; Rosa et al., 1992; Rosa, P.A., Hogan, D., 1992; Samuels, 1995). According to the paper by Zückert, the plating media was changed to 1.5x MKP (Table 9) (Zückert, 2007). Those two media were then combined and modified to a final media, which was a **1.5x Modified-Kelly-Pettenkofer with no gelatin, increased rabbit serum to 10 %, and normal 1.7 % agarose** (Table 10). After plating, the separate colonies were selected, grown in MKP liquid medium, the aliquots of each culture were frozen for further usage, gDNA was purified from the rest of the cells and checked with PCR to detect *B. miyamotoi* DNA.

Solid media and their compositions

Table 8: Reagents and their amounts for 500 ml Barbour-Stonner Kelly II (BSK II) medium with increased rabbit serum to 10 % and low melting 1.7 % agarose

Barbour-Stonner Kelly II (BSK II) medium with increased rabbit serum to 10 % and low melting 1.7 % agarose (500 ml)	
Reagent	**Mass (g)**
10x CMRL	4.85
Neopeptone	2.5
BSA	25
Yeastolate	1
HEPES acid	3
Glucose	2.5
Sodium citrate	0.35
Sodium pyruvate	0.4
N-acetyl glucosamine	0.2
Sodium bicarbonate	1.1
Gelatin	5
Reagent	**Volume (ml)**
Rabbit serum	50
1.7 % low melting agarose	325

Table 9: Reagents and their amounts for 200 ml 1.5x Modified Kelly-Pettenkofer medium (MKP)

1.5x Modified Kelly-Pettenkofer medium (MKP) (200 ml)	
Reagent	**Mass (g)**
CMRL-1066	2.91
Neopeptone	0.9
HEPES	1.8
Citric Acid	0.21
D-Glucose	0.9
Pyruvic Acid	0.24
N-acetyl glucosamine	0.12
Sodium bicarbonate	0.6
BSA	3.675

Reagent	Volume (ml)
7 % gelatin	40
Rabbit serum	14.4
1.7 % agarose	133.33

Table 10: Reagents and their amounts for 200 ml 1.5x Modified-Kelly-Pettenkofer with no gelatin, increased rabbit serum to 10 % and normal 1.7 % agarose

1.5x Modified-Kelly-Pettenkofer with no gelatin, increased rabbit serum to 10 % and normal 1.7 % agarose (200 ml)	
Reagent	**Mass (g)**
CMRL-1066	2.91
Neopeptone	0.9
HEPES	1.8
Citric Acid	0.21
D-Glucose	0.9
Pyruvic Acid	0.24
N-acetyl glucosamine	0.12
Sodium bicarbonate	0.6
BSA	3.675
Reagent	**Volume (ml)**
Rabbit serum	20
1.7 % agarose	133.33

Experimental protocol for plating *Borrelia* cultures

The solid reagents were weight and mixed for around one hour in a beaker filled with 2/3 volume of ddH_2O. After complete dissolution and pH adjustment to 7.6, the mixture was filled up to 200 ml with ddH_2O to obtain the final volume. For preparation of Barbour-Stonner Kelly II (BSK II) medium with increased rabbit serum to 10 % and low melting 1.7 % agarose (Raffel et al., 2018; Rosa et al., 1992; Rosa, P.A., Hogan, D., 1992; Samuels, 1995), rabbit serum and gelatin were dissolved by boiling and added. Preparation of 1.5x Modified Kelly-Pettenkofer medium (MKP) (Zückert, 2007) included the addition of the appropriate amount of BSA, gelatin dissolved by boiling, and rabbit serum (7.2 %). The 1.5x Modified-Kelly-Pettenkofer with no gelatin, increased rabbit serum to 10 %, and normal 1.7 % agarose medium was prepared in the same way as the 1.5x Modified Kelly-Pettenkofer medium (MKP) (Zückert, 2007), except the addition of gelatin and an increased

amount of rabbit serum (10 %). The mixture was sterilized by filtration. Meanwhile, a 1.7 % (low melting) agarose was prepared by boiling in water. Agarose and medium were mixed and sterilized by filtration. The bottom layer of the plates was poured (15 ml). *Borrelia* in cultures were counted and diluted with the media to the desired density. Different concentrations of spirochetes were prepared – 1000, 500, 250, 100 and 50 spirochetes per plate. The top layer on the plates consisted of ten ml of appropriate medium and one ml of the *Borrelia* culture with the desired concentration of spirochetes. The prepared plates were left to solidify and incubated in a CO_2 incubator (2.5 %) at 35 °C. Colonies appeared after 5-7 days.

After growth, the colonies were picked individually from the plates (around 60 colonies), transferred into a sterile Eppendorf tube, and grown in two ml of appropriate medium (MKP-F). Later on, the cultures were separated into two parts, where one part was frozen as a stock and another was used for gDNA isolation and PCR checking for *B. miyamotoi* DNA presence followed by sequencing reaction and RLB analysis.

3.2.11 Reverse line blotting (RLB)

Reverse Line Blotting (RLB) is a molecular biology method used to detect specific DNA sequences in DNA samples. Unlike PCR, RLB allows to analyze several samples for the presence of several DNA simultaneously. This analysis includes the attachment of oligonucleotide probes to a membrane and further hybridizing the probes with biotin labeled PCR products.

RLB was performed to confirm the presence of a monoclonal culture (absence of *B. burgdorferi* s.l. in the plated colonies). The protocol used was described by Gubbels et al. (Gubbels et al., 1999), Gern et al. (Gern et al., 2010), and Springer et al. (Springer et al., 2022).

Experimental protocol for RLB analysis

Samples were subjected to a conventional PCR targeting a fragment of the 5S-23S rRNA intergenic spacer (IGS) by use of biotin-linked forward B5S-Bor (biotin-5'-GAGTTCGCGGGAGAGTAGGTTATT-3'), 23SBor reverse (5'-TCAGGGTACTTAGATG GTTCACTT-3') and biotin-linked forward BMiya-For (biotin-5'-TTAGGATTAATGATRT TKTTACC-3') primers as described by Alekseev et al. (Alekseev et al., 2001) and Blazejak et al. (Blazejak et al., 2018). For the 20 µl reaction set up, ten µl 2x Master Mix (Promega), one µl of each primer, and five µl DNA template (gDNA isolated from re-cultivated picked colonies from plates) were added to the corresponding amount of H_2O. PCR cycling conditions included 45 cycles of 94 °C for 20 s, 52 °C for 30 s, 72 °C for 30 s, and a final elongation step at 72 °C for 7 min. Each run included different *Borrelia* strains corresponding to the used probes as positive controls (Table 11): *B. burgdorferi* sensu lato (-), *B. burgdorferi* sensu stricto (PAbe), *B. garinii* (PWudII), *B. afzelii* (PBas), *B. valaisiana* (VS116), *B. lusitaniae* (Poti B2), *B. spielmanii* (PHap), *B. bissettii* (DN127), RF-like *Borrelia* (-) and *B. miyamotoi* (HT31). PCR products, positive controls, and negative control were hybridized to different oligonucleotide probes linked to the membrane.

Table 11: Probes used for RLB and the *Borrelia* strains used as positive control

Probe	Reference	Sequence (5' - 3')	Target organism
SL	*Gern* et al. (Gern et al., 2010)	CTTTGACCATATTTTTATCTTCCA	*B. burgdorferi* sensu lato
SS		AACACCAATATTTAAAAAACATAA	*B. burgdorferi* sensu stricto
GA		AACATGAACATCTAAAAACATAAA	*B. garinii*
AF		AACATTTAAAAAATAAATTCAAGG	*B. afzelii*
VSNE		TATATCTTTTGTTCAATCCATGT	*B. valaisiana*
LusiNE2		AAATCAAACATTCAAAAAAATAAAC	*B. lusitaniae*
SpiNE2		GAATGGTTTATTCAAATAACATA	*B. spielmanii*
SpiNE3		GAATAAGCCATTTAAATAACATA	*B. spielmanii*
BisNE1		AAACACTAACATTTAAAAAACAT	*B. bissettii*
BisNE2		AACTAACAAACATTTAAAAAACAT	*B. bissettii*
RFLNE		GCATTGCTCAATATGGTTAG	*RF-like*
MIYA	Blazejak et al. (Blazejak et al., 2018); Springer et al. (Springer et al., 2022)	TGAAAAATTATTTAGTGAAAAGTTC	*B. miyamotoi*

Probes	*Borrelia* Strain used as positive control
SL	-
SS	PAbe
GA	PWudII
AF	PBas
VSNE	VS116
LusiNE2	Poti B2
SpiNE2	PHap
SpiNE3	PHap
BisNE1	DN127
BisNE2	DN127
RFLNE	-
MIYA	Pure cloned *B.miyamotoi* DNA (strain HT31) from Russia

RLB experimental procedure

1. Activate membrane in 10 ml 16 % 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) (10 min incubation at RT)
2. Wash for 2 min with distilled H_2O and place into miniblotter
3. Dilute specific oligonucleotides (probes) (1 µl (100 pmol) of the probe + 349 µl of 500 mM $NaHCO_3$ (pH 8.4)
4. Load oligonucleotides on the membrane (also fill empty slots with 500 mM $NaHCO_3$)
5. Incubate for 1 min at RT
6. Aspirate (mark slots with probes with needle)
7. Remove membrane from blotter
8. Inactivate membrane by incubation for 10 min at RT in 100 mM NaOH
9. Rinse membrane with water
10. Block membrane with 125 ml of 2x SSPE-0.1 % sodium dodecyl sulfate (SDS) for 5 min at 60 °C while shaking or block membrane for more than 1 h at 56 °C in 2x SSPE-0.1 % sodium dodecyl sulfate (SDS), 5 % milk while shaking
11. Wash membrane for 5 min at 42 °C with 125 ml 2xSSPE-0.1 % SDS
12. Place membrane into miniblotter with the slots perpendicular on the previously applied specific oligonucleotides (90° rotation of membrane)
13. Add 20 µl of PCR product to 130 µl 2xSSPE-0.1 % SDS, incubate at 100 °C for 10 min
14. Cool on ice immediately
15. Fill the slots
16. Hybridize at 45 °C for 1 h
17. Aspirate PCR products
18. Wash 2x with 2xSSPE-0.5 % SDS for 10 min at 40 °C while shaking
19. Incubate with 1:4000 diluted peroxidase-labeled streptavidin in 2x SSPE-0.5 % sodium dodecyl sulfate (SDS) for 30 min at 40 C in the dark (without shaking)
20. Wash 2x for 10 min with 2x SSPE-0.5 % sodium dodecyl sulfate (SDS) at 40 °C while shaking
21. Rinse with 2xSSPE
22. Add TMB stabilized substrate for HRP to detect the positive signals for 10 min in the dark
23. Stop detection with 20 mM EDTA or rinsing the membrane 3x 5 min with H_2O

3.2.12 Mice infection with proved *Borrelia miyamotoi* cultures

B. burgdorferi s.l. is presumably not transferred from the female ticks to the offspring, but *B. miyamotoi* is (see chapter *Tick vectors – Ixodidae and Argasidae*).

To provide additional control of *B. miyamotoi* cultures, a mice experiment was performed. BALB C mice and ticks (*Ixodes ricinus* females) used in this analysis were provided by the local animal facility.

Experimental procedure for mice experiment

Each mouse was injected with 100 μl of the co-infected culture subcutaneously on the neck and the lower abdomen. Three groups with three mice per group were used – each group was infected with a different isolate. The infection was developed for three weeks. Then blood was taken from the eye of each mouse with a capillary and checked by PCR for the presence of spirochetes. *Ixodes ricinus* female ticks were placed on each mouse and left to feed until full engorgement. The engorged females were collected and left to lay the eggs, which took around three weeks. The eggs were then divided into two parts. One half was used for gDNA isolation, and the other half was used for re-cultivation in MKP–F medium. Total gDNAs of the eggs were used as templates in PCR reactions with *B. miyamotoi*-specific *glpQ* primers (Fomenko et al., 2010) and *B. burgdorferi* s.l. specific *ospC* primers, to confirm the presence of *B. miyamotoi* and the absence of *B. burgdorferi* s.l. The positive PCR products were sequenced.

3.2.13 Molecular Cloning of PCR products of interest

PCR cloning is the capture of a PCR product into a vector of interest (Lessard, 2013).

To serve as a positive control in further PCR reactions, some purified PCR products, which were positively tested for *B. miyamotoi* DNA, were cloned using a commercial kit (TOPO TA Cloning Kit for sequencing, Invitrogen) strictly according to the manufacturer's instructions (the protocol is presented below). The kit is specifically designed to clone Taq DNA polymerase-generated PCR products. The vector used in this cloning reaction has an *ampR* gene, so the obtained transformants are ampicillin resistant.

Table 12: Primers used for PCR screening of the recombinants

Primer name	Reference	Primer sequence (5' - 3')	Annealing temperature (°C)
M13 Forward	TOPO TA Cloning Kit for sequencing, Invitrogen	GTAAAACGACGGCCAG	/ 50 °C /
M13 Reverse		CAGGAAACAGCTATGAC	

Table 13: PCR conditions for *M13* primers

PCR conditions for the primers *M13*		
Steps (30x)	Temperature	Time
Initial denaturation	95 °C	5 min
Denaturation	95 °C	30 sec
Annealing	50 °C	30 sec
Elongation	72 °C	1 min
Final elongation	72 °C	10 min
Final hold	12 °C	hold

Molecular cloning (manufacturer's instructions) (Sambrook, J. & Russell, D. W., 2001; Stine Gangnæs Hammer, 2006) shortly:

Set up for a 6 µl cloning reaction in a 1.5 ml Eppendorf tube

Gel-purified PCR product____ 4 µl

TOPO vector_____ 1 µl

Salt solution_____ 1 µl

1. Mix all above-mentioned reagents in a 1.5 ml Eppendorf tube
2. Incubate for 30 min at room temperature

Transformation of *E.coli* chemically competent cells

3. Add two µl of TOPO cloning reaction into a vial of One Shot TOP10 Chemically Competent *E.coli* cells
4. Mix gently and incubate for 30 min on ice
5. Heat-shock for 30 sec at 42 °C without shaking
6. Incubate on ice
7. Add 250 µl of S.O.C. medium

8. Vigorous shaking for one h at 37 °C
9. Plate on LB agar / ampicillin plates
10. Incubate at 37 °C overnight without shaking

Checking of the obtained transformants for the present of the inserts (screening)

11. Pick several colonies
12. Cultivate colonies overnight in LB containing 50 µl/ml ampicillin
13. Centrifuge at full speed
14. Isolate plasmid DNA

Plasmid DNA isolation (manufacturer's instructions) shortly:

1. Add 250 µl of P1 (resuspension) buffer
2. Vortex till homogenous
3. Add 250 µl of P2 (lysis) buffer, invert (do not vortex)
4. Add 350 µl of N3 (neutralization) buffer, invert (do not vortex)
5. Centrifuge for 10-15 min at full speed
6. Transfer the supernatant into spin column placed in a 2 ml collection tube
7. Centrifuge for 1 min at full speed (discard flow-through)
8. Add 750 µl of PE (washing) buffer
9. Centrifuge for 1 min at full speed (discard flow-through)
10. Centrifuge additionally for 1 min at full speed
11. Place spin column in a 1.5 ml Eppendorf tube
12. Apply 50 µl of H_2O on the membrane of the tubes and incubate for 1 min at RT
13. Centrifuge for 1 min at full speed to elute DNA
14. Determine concentration of plasmid DNA with NanoDrop spectrophotometer
15. Store at -20 °C

All plasmid DNAs were used as templates in PCR reactions with gene-specific (*glpQ* (Fomenko et al., 2010)) and plasmid-specific (*M13* Forward and *M13* Reverse) primers to confirm the presence of the insert of interest in the construct. The reaction conditions for *glpQ* primers are the same as in Table 6 (the first spacer PCR), except for the cycle number, which was 30x in this experiment. The plasmids with the confirmed inserts were sequenced using *M13* primers from the plasmid.

4. Results

4.1 *Borrelia* isolates cultivated from ticks collected in the Czech Republic

The presence of *B. miyamotoi* and *B. burgdorferi* s.l. was confirmed by PCR with the species-specific primers and sequencing in 10 isolates (samples) cultured from ticks collected in the South Bohemia, the Czech Republic.

Table 14: Tick isolates selected for this project (*B. miyamotoi* co-infected with different strains of *B. burgdorferi* s.l.)

Area of tick collection / Isolate number	*Borrelia* strains detected by PCR
Branišov 2 (B2)	*B. valaisiana* + *B. miyamotoi*
Branišov 6 (B6)	*B. afzelii* + *B. miyamotoi*
Divči Hrady 3 (DH3)	*B. afzelii* + *B. miyamotoi*
Heroltice 5 (H5)	*B. afzelii* + *B. miyamotoi*
Heroltice 6 (H6)	*B. afzelii* + *B. miyamotoi*
Milovice 2 (M2)	*B. afzelii* + *B. miyamotoi*
Milovice 7 (M7)	*B. burgdorferi* s.s. + *B. garinii* + *B. miyamotoi*
Milovice 8 (M8)	*B. garinii* + *B. miyamotoi*
Milovice 9 (M9)	*B. afzelii* + *B. miyamotoi*
Netolice 3 (N3)	*B. burgdorferi* s.s. + *B. miyamotoi*

4.2 Polymerase chain reaction (PCR) – confirmation of *Borrelia miyamotoi* DNA in the cultures

The gDNA was isolated from all re-cultivated *Borrelia* isolates (Table 14), and the presence of *B. miyamotoi* was confirmed by PCR with specific primers for *glpQ*, *p66* and *fla* in the following samples: Milovice 8, Netolice 3, Milovice 9, Divči Hrady 3. Those four isolates were selected for further separation of LD spirochetes and *B. miyamotoi*.

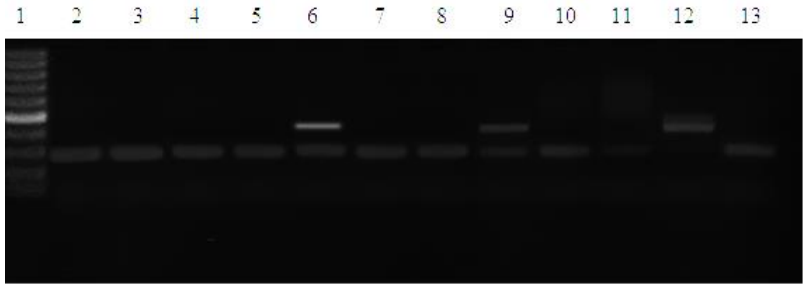


Figure 5: Results of nested-PCR with *glpQ* primers: Lane 1: 100 bp DNA ladder; Lane 2-11: the samples (a positive result was detected in Lane 6 (isolate M9) and Lane 9 (isolate N3) with the 466 bp long PCR product); Lane 12-13: Positive and negative controls

Shown above in Figure 5 is a representative result of the multiple PCR reactions confirming the presence of *B. miyamotoi* DNA in the re-cultivated isolates.

The results of BLAST search of the GenBank database (National Center for Biotechnology Information) with sequenced obtained amplicons revealed 98 % homology of our isolates to *Borrelia miyamotoi* strain CZ-F1E from the Czech Republic (GenBank accession number CP046389). This strain of *Borrelia miyamotoi* was obtained from *Ixodes ricinus* eggs from the Czech Republic by Janeček et al. (Janeček et al., 2020). The BioProject accession number is PRJNA591086, and the BioSample accession number is SAMN13351967.

4.3 Plating results (of confirmed *Borrelia miyamotoi* isolates)

After confirmation of the presence of *B. miyamotoi* DNA in samples by PCR with all sets of primers (Table 5), sample Netolice 3 was selected for plating.

The number of spirochetes plated was 1000, 500, 250, and 100 spirochetes per plate, respectively. After five days of growing (Figure 6), 96 single colonies were picked for further screening, the isolates grown in MKP-F medium (Figure 7), and the gDNA isolated.

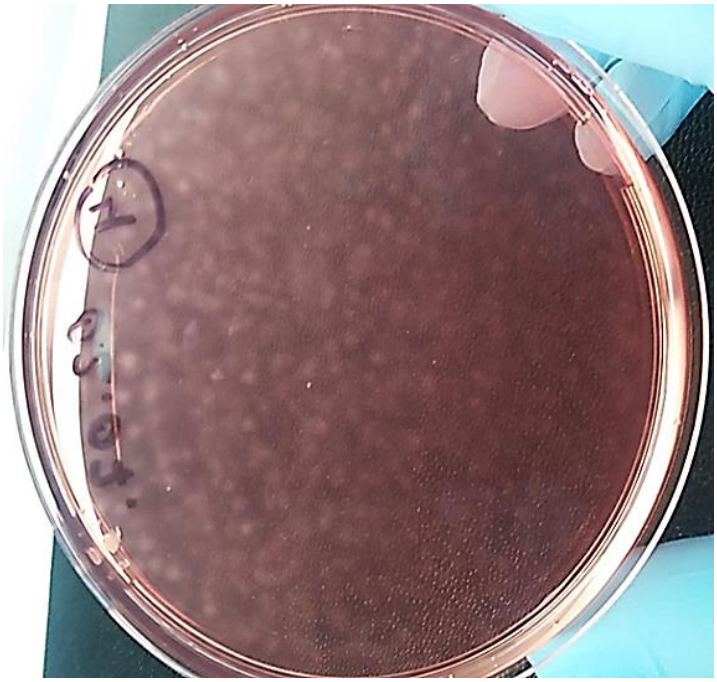


Figure 6: An example of growing culture on solid medium

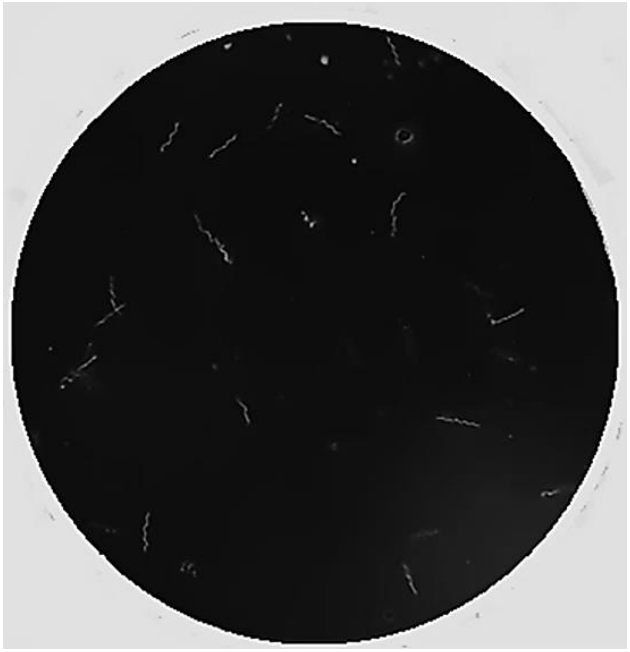


Figure 7: An example of growing culture from a single colony in MKP-F medium (dark-field microscopy)

The presence of *B. miyamotoi* was confirmed by nested PCR with specific primers for *glpQ* and *p66* in the isolates 49 (N3-49) and 60 (N3-60).

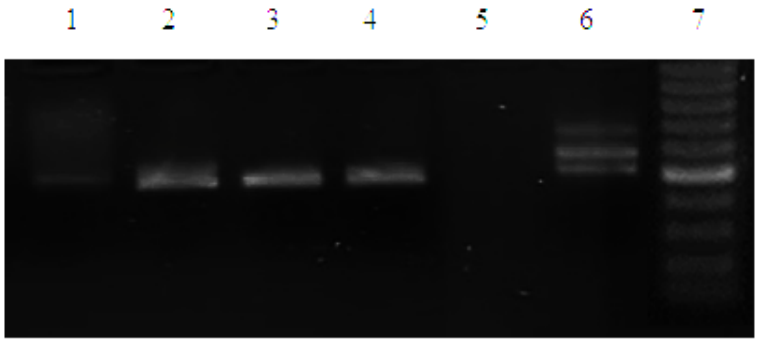


Figure 8: Results of nested-PCR of individual colonies with *glpQ* primers: Lane 1-4: isolate (N3-49) and (N3-60); Lane 5-6: positive and negative controls; Lane 6: 100 bp DNA ladder

Isolate (N3-60) was plated again (to ensure the presence of a monoclonal culture) with 500, 250, 150, and 50 spirochetes per plate, respectively. After five days of growing, 66 single colonies were picked, the isolates grown in MKP-F medium, and the gDNA isolated. All monoclonal cultures positively checked for the presence of *B. miyamotoi* gDNA were further analyzed with three different *B. miyamotoi*-specific sets of primers (*glpQ, p66* and *fla*). The presence of *B. miyamotoi* was confirmed in isolates 8 (N3-60(8)) and 63 (N3-60(63)) (Figure 9).

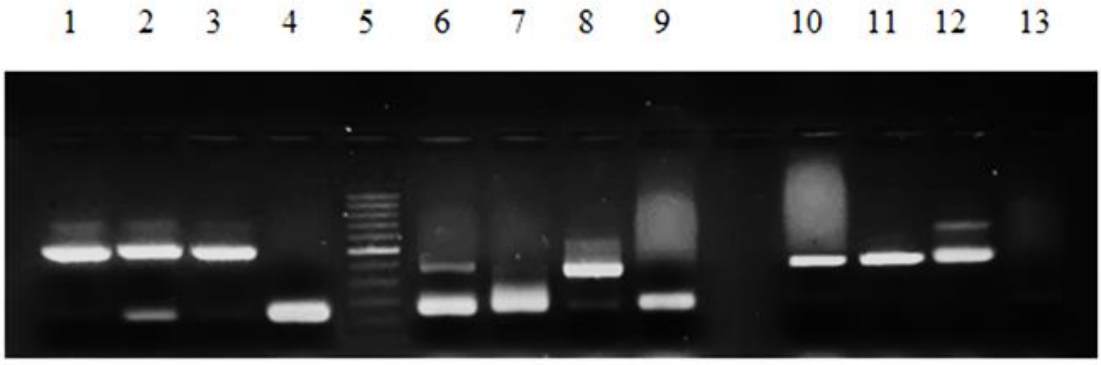


Figure 9: Results of PCR with *glpQ, p66, fla* primers: Lane 1-4: isolates (N3-60(8)) and (N3-60(63)), positive and negative controls with primers for flagellin (506 bp long PCR product); Lane 5: 100 bp DNA ladder; Lane 6-9: isolates (N3-60(8)) and (N3-60(63)), positive and negative controls with primers for *glpQ* (466 bp long PCR product); Lane 10-13: isolates (N3-60(8)) and (N3-60(63)), positive and negative controls with primers for *p66* (532 bp long PCR product)

The obtained PCR products were sequenced. The results confirmed the presence of the DNA of *Borrelia miyamotoi*. All obtained nucleotide sequences are presented in the *Appendix*.

4.4 Reverse line blotting (RLB) results

To re-check the presence of *B. miyamotoi* in a monoclonal culture, reverse line blotting was performed.

All monoclonal cultures positively checked for the presence of *B. miyamotoi* DNA were plated again (250 spirochetes per plate). After eight days of growing, 71 single colonies were picked, the isolates grown in MKP-F medium, gDNA isolated, PCR executed. Reverse line blotting with all PCR products hybridized to six different oligonucleotide probes (Table 11) was performed. Isolate (N3-60(63)) number 6 was positive for *B. miyamotoi* and negative for *Borrelia burgdorferi* s.l. (Figure 10).

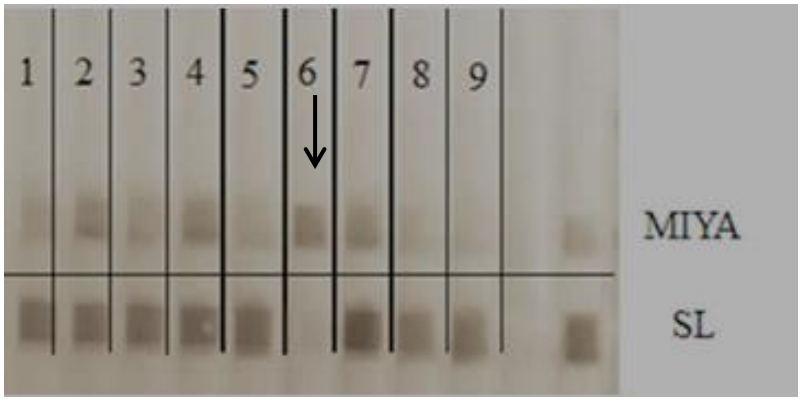


Figure 10: Illustrative image of the RLB analysis of samples of (N3-60(63)) reacting with MIYA and SL probes. The presence of *B. miyamotoi* and the absence of *B. burgdorferi* s.l. in sample 6 was confirmed.

The presence of *B. miyamotoi* was additionally confirmed by nested PCR with specific primers for *glpQ* in sample number 6 from isolate (N3-60(63)).

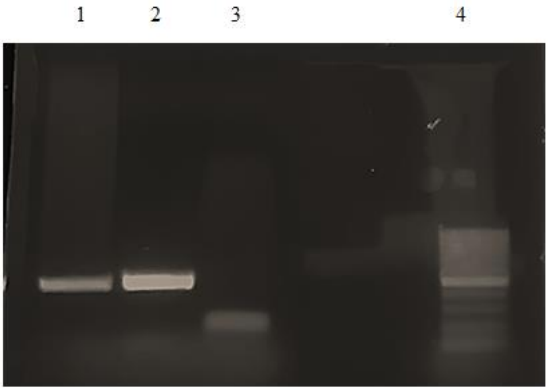


Figure 11: Nested-PCR analysis with *glpQ* primers: Lane 1: Sample 6; Lane 2: positive control with primers for *glpQ* (466 bp long PCR product); Lane 3: negative control; Lane 4: 100 bp DNA ladder

4.5 Results of the first mice experiment

Three mice were infected successfully with Netolice 3 isolate, and two out of three female ticks were successfully engorged and laid eggs. The gDNA from the eggs was checked by PCR with specific primers for *glpQ* (Figure 12) and the presence of *B. miyamotoi* DNA was confirmed.

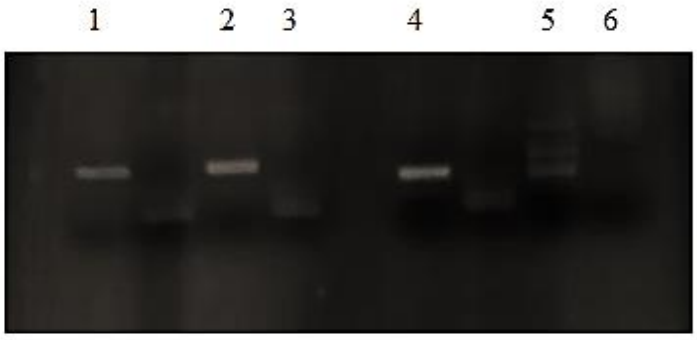


Figure 12: PCR analysis with *glpQ* and *ospC* primers: Lane 1-3: egg sample, positive and negative controls with primers for *ospC* (617 bp long PCR product); Lane 4-6: egg sample, positive and negative controls with primers for *glpQ* (466 bp long PCR product)

4.6 Molecular cloning results

PCR products obtained from *B. miyamotoi* genome were purified and cloned into plasmid with the purpose to obtain control sequences and to use the plasmid as a positive control in the further PCR reactions. *B. miyamotoi p66* partial gene PCR products of Milovice 9 and Netolice 3 isolates were cloned into TOPO vector and *E.coli* chemically competent cells were transformed. The result of the successful transformation is presented in Figure 13.

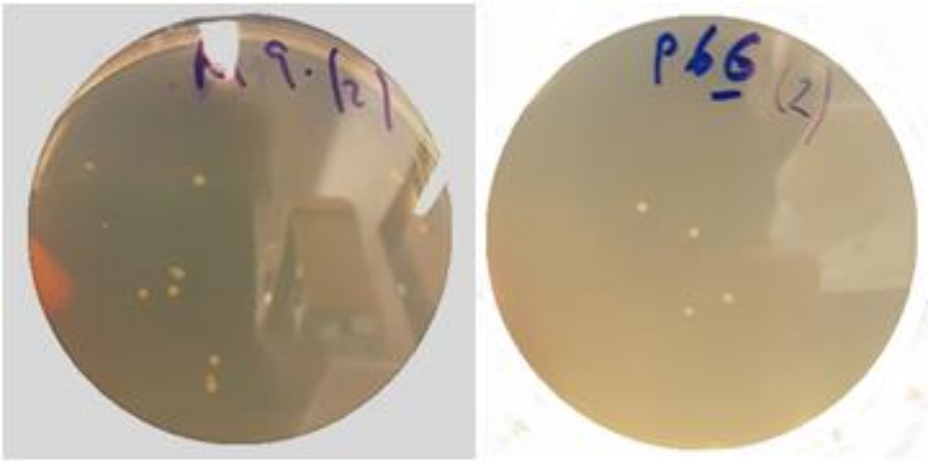


Figure 13: Colonies of *E.coli* one d after plating sample Milovice 9 and Netolice 3

Colonies were picked from the plates, the cells were grown, and the plasmid DNA was isolated. Two recombinants (Netolice 3) were successfully confirmed to have the inserts (Figure 14).

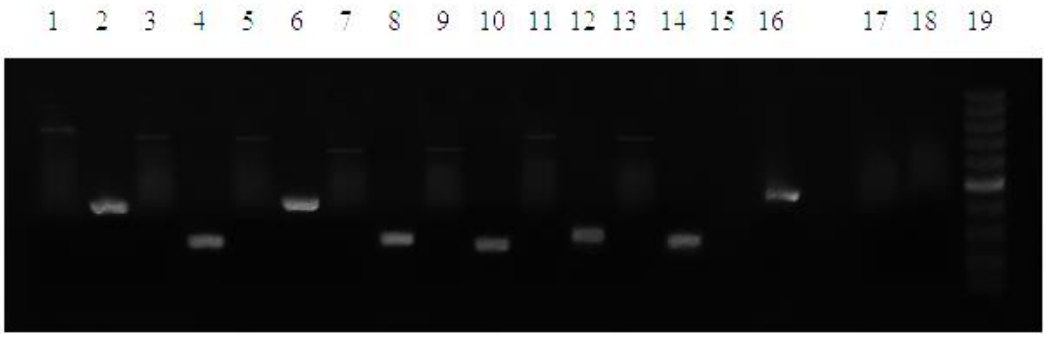


Figure 14: PCR analysis with inner *glpQ* and *M13* primers: Lane 1-14: the recombinants (a positive result was detected in Lane 2 and Lane 6 with the 466 bp long PCR product); Lane 15-18: positive and negative controls; Lane 19: 100 bp DNA ladder

Sequencing of the positive PCR products confirmed the DNA of *Borrelia miyamotoi*.

5. Discussion

The evidence of *B. miyamotoi* in ticks collected in the Czech Republic was already proven and the results obtained in this study agree with previous publications (Bubanová et al., 2022; Crowder et al., 2014).

Already published information confirmed that *B. miyamotoi* is distributed throughout the Northern temperate regions of North America, Europe, and Asia, resulting in three types of *B. miyamotoi* s.l. complex - American (from the ticks *I. scapularis* and *I. pacificus*), Asian (from the ticks *I. persulcatus*, *I. ovatus* and *I. pawlovskyi*) and European (from the *I. ricinus* tick) (Crowder et al., 2014; Hoornstra et al., 2022).

According to Hoornstra et al., the prevalence of *B. miyamotoi* was confirmed in certain questing *Ixodes* ticks at a range of 1.1 % in *I. scapularis*, 0.7 % in *I. pacificus*, 2.8 % in *I. persulcatus* and 1.0 % in *I ricinus* (Hoornstra et al., 2022). *B. miyamotoi* prevalence rates were confirmed as 0.4–6.4 % in the United States, Russia, and Japan and 0.17–5.3 % in Europe (Barbour et al., 2009; Cutler et al., 2019).

In Europe, only a few studies provided data on both the density of foraging nymphs and the prevalence of *B. miyamotoi*. Hansford et al. reported an average of 1.7 (range 0-5.6) *Borrelia*-infected nymphs per 100 m^2 of urban green space and a *B. miyamotoi* prevalence of 1.5 % (Hansford et al., 2022). In Germany, according to Răileanu et al., *B. miyamotoi* was detected in 3.0 % of adults and 0.8 % in nymphs (Răileanu et al., 2020). Crowder et al. confirmed that in the Czech Republic, the incidence of *B. miyamotoi* infection from ticks varied by region, ranging from 0 % to 3.2 %, with an average infection rate of 2 % (Crowder et al., 2014). Bubanová et al. presented an overall *B. miyamotoi* prevalence in questing *Ixodes ricinus* ticks from the Czech Republic of 2 % (Bubanová et al., 2022).

The presence of *B. miyamotoi* in ticks and hosts seems to increase as time passes. The latest publications confirm the higher prevalence of *B. miyamotoi* in samples compared to *B. burgdorferi* s.l. For example, according to Lagunova et al., the spirochete loads of *B. miyamotoi* in taiga ticks *I. persulcatus* studied in Mongolia, were higher than those of *B. burgdorferi* s.l. (mean: 5.2 vs. 4.0 log10 genome copies/tick, respectively) (Lagunova et al., 2022).

The original isolate we worked with was a co-infection of *Borrelia burgdorferi* s.s. and *B. miyamotoi* from *I. ricinus* tick collected in Netolice (South Bohemia, the Czech Republic) by flagging. The co-infection of *B. burgdorferi* s.l. and *B. miyamotoi* was proved by us in several tick isolates.

Such kind of co-infection was previously discussed in the literature as neither more nor less common than random expectations since *B. burgdorferi* s.l. and *B. miyamotoi* use different strategies for maintenance and dissemination in the same reservoir host and vector species (Romana Kejíková, 2018; Scoles et al., 2001; Siński et al., 2016). However, further investigations proved that this co-infection exists, and it appeared to be not such a rare phenomenon.

In certain reservoirs hosts, *B. miyamotoi* and *B. burgdorferi* s.l. were detected together at a relatively high rate. Therefore, the study of co-infection frequency was expanded to include samples from a broad survey of field sites in the Northeast, Mid-Atlantic, and Northern United States (Barbour et al., 2009). According to Barbour et al., out of 7205 investigated nymphs, 139 were infected with *B. miyamotoi* and 20 with both species, *B. burgdorferi* s.l. and *B. miyamotoi* (Barbour et al., 2009). Also, co-infections of *P. leucopus* (host) or *I. scapularis* (vector) with both *B. burgdorferi* s.l. and *B. miyamotoi* were neither more frequent nor less frequent than expected (Barbour et al., 2009) which led to a null hypothesis, that *B. burgdorferi* sensu lato and *B. miyamotoi* maintain independently in environments where they coexist (Barbour et al., 2009). Dibernado et al. performed analyzes where out of 23 *I. scapularis* ticks, one-third of the *B. miyamotoi*-infected ticks were co-infected with *B. burgdorferi* s.l., which confirms a higher co-infection rate than someone would have expected (Dibernardo et al., 2014).

European studies also confirm co-infection rates of *B. burgdorferi* s.l. and *B. miyamotoi*. For example, the researchers from Poland proved that 14 % of all ticks investigated, were infected with *B. burgdorferi* s.l. and 2 % were infected with *B. miyamotoi*, while *B. afzelii* was the dominant genospecies (68.5 %). In addition, four co-infections in single adult ticks were found: *B. miyamotoi/B. afzelii*, *B. miyamotoi/B. burdorferi* s.s., *B.miyamotoi/B. garinii* and *B. afzelii/B. burgdorferi* s.s. (Kiewra et al., 2014).

According to Hamšíková et al., in Slovakia, co-infections of *B. miyamotoi* and *B. burgdorferi* s.l. were found in 24.1 and 9.3 % of the questing ticks and rodents, respectively, whereas the proportion of ticks and rodents co-infected with *B. miyamotoi* and *B. afzelii* was 6.9 and 7.0 %, respectively. The results suggest that *B. miyamotoi* and *B. afzelii* share amplifying hosts (Hamšíková et al., 2017).

In the Netherlands, according to Wagemakers et al., out of 3360 questing nymphs, 2.1 % were infected with *B. miyamotoi*, 9.3 % with *B. burgdorferi* s.l., and 0.4 % were co-infected with both *B. miyamotoi* and *B. burgdorferi* s.l. Co-infection occurred more often than expected from single infection prevalence (p=0.03) (Wagemakers et al., 2017).

In Mongolia, genetic analysis of *Borrelia spp*. in 11 *I. persulcatus* ticks infected with *Borrelia miyamotoi*, six ticks were found to be co-infected with *Borrelia burgdorferi* sensu lato (s.l.), which completely corresponds to our discoveries (Lagunova et al., 2022).

Since the aim of this study was to obtain a monoclonal *B. miyamotoi* culture by separating the co-infected isolates on the solid medium, several cultivation media (both liquid and solid) have been evaluated.

The knowledge about the cultivation of *B. miyamotoi in vitro* is rather limited. As *in vitro* cultivation is an essential prerequisite for a detailed characterization of pathogens or the production of specific antibodies (Margos et al., 2015), the question of the successful cultivation of *B. miyamotoi* is of great importance. As mentioned previously, a first-time increased *B. miyamotoi* spirochete density was obtained by Barbour through *in vitro* cultivation in a modified BSKII liquid medium (Barbour, 1984). Another modified BSKII medium for *B. miyamotoi* was developed by Replogle et al. BSK-R is a diluted BSKII derivative containing a reduced amount of CMRL 1066 and Lebovitz's L-15, mouse, and fetal calf serum. This medium aided in the *in vitro* culture recovery of RF and LB spirochetes according to the authors (Replogle et al., 2021).

In the studies by Margos et al., it was acknowledged that rabbit serum inhibits and human serum supports the growth of *B. miyamotoi*, resulting in 75 % and even 100 % higher spirochete concentrations. Similar to human serum, fetal calf serum is also intended to promote the growth of the spirochetes (Margos et al., 2015). Therefore, the liquid culture medium we used in this study was a modified Kelly-Pettenkofer medium (described in *Materials and Methods*) supplemented with 10 % fetal calf serum (MKP-F), firstly described by Wagemakers et al. They inoculated *B. miyamotoi* strains LB-2001 and HT31 in MKP-F medium and confirmed the increased occurrence of *B. miyamotoi* spirochetes (Wagemakers et al., 2014).

As aforementioned, the first attempt to cultivate *B. miyamotoi* on a solid medium was made by Raffel et al., who used BSKII plating medium for *B. burgdorferi* s.l. *(*Rosa and Hogan; Rosa et al.; Samuels*)* with increased rabbit serum concentration (10 %) in bottom agar (Raffel et al., 2018; Rosa et al., 1992; Rosa, P.A., Hogan, D., 1992; Samuels, 1995). The top agar additionally contained low melting point agarose instead of standard agarose, as in the protocol by Rosa and Hogan (Rosa, P.A., Hogan, D., 1992). The increased rabbit serum concentration should promote the growth of *Borrelia*.

Another modification was proposed by Zückert et al., who described a 1.5x concentrated MKP plating medium without gelatin (Zückert, 2007). According to Stone et al., gelatin is a growth inhibitor for *B. miyamotoi* (Stone and Brissette, 2016). After checking

the effectiveness of all the above-mentioned media modifications, we combined selected modifications and developed our own plating media for *B. miyamotoi* - a 1.5x Modified-Kelly-Pettenkofer with no gelatin, increased rabbit serum to 10 % and normal 1.7 % agarose.

This plating medium resulted in successful growth of monoclonal populations of *B. miyamotoi* in the presented project.

Two criteria seem to be necessary for the successful cultivation of *B. miyamotoi*, namely the atmospheric conditions of the culture and the type and quantity of the serum used. Margos et al. reported a high concentration of *B. miyamotoi* spirochetes at 6 % CO_2 (Margos et al., 2015). This information could lead to a potentially more suitable plating medium for *B. miyamotoi*, increasing the CO_2 concentrations from 2.5 % (in our case) to 6 %. Further research, in this case, would be necessary.

To our knowledge, no other previous data regarding the existence of a pure *B. miyamotoi* isolate from the tick *Ixodes ricinus* obtained by the separation of co-infected cultures exist in the literature. Therefore, our results seem hereby to be the first in this case. The PCR with the specific primer sets for *glpQ*, sequencing, and the additional reverse line blotting confirmed the presence of a monoclonal culture of *B. miyamotoi*.

6. Conclusion

As a conclusion, we obtained the monoclonal culture of *B. miyamotoi* using the separation of the LD spirochetes and *B. miyamotoi* presented as co-infection in tick-originated culture, by cultivation on the solid medium, optimized for the purposes of this project. We confirmed the presence of single *B. miyamotoi* species in obtained monoclonal isolate by different techniques of molecular biology.

Further research on genomic sequencing and phylogenetic analysis of this particular isolate is necessary, and further experiments on the transovarial transmission of *B. miyamotoi* should be continued, as *B. miyamotoi* is one of the tick-borne pathogens with a significant impact on human health.

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8. Appendix

Sequence of isolate (N3-60(8)) with *fla B. miy*. specific primers

GGTGTTGCTGGTAAGCTTAATTCACAAATTAGAGGATTGTCTCAAGCTTCTAGAA
ATACCTCAAAGGCTATAAATTTTATTCAAACAACAGAAGGAAATTTGAACGAGG
TAGAGAAAGTATTAGTAAGAATGAAAGAACTTGCTGTTCAGTCTGGTAATGGTA
CATACTCAGATTCAGATAGAGGGTCTATTCAGATTGAAATTGAACAACTTACAG
ATGAAATAAACAGAATTGCTGATCAGGCTCAATACAACCAAATGCATATGTTAT
CTAATAAGTCAGCTGCTCAAAATGTAAAAACTGCTGAAGAGCTTGGAATGCAAC
CTGCAAAAATTAACACACCAGCATCATTGGCTGGATCACAAGCTTCATGGACAT
TGAGAGTGCATGTAGGTGCAAATCAGGATGAAGCAATTGCTGTCAATATTTATG
CAGCTAATGTTGCAAATCTTTTTAATGGAGAAGGTGCTCAAGCAGCTCCAGCTC
AAGAGGGAGCACAACAGGAGGGAGTTCAAGCAGTTCCAGCTCCAGCAGCCGCT
CCAGTGCAAGGTGGAGTTAATTCTCCAATTAATGTTACAACTGCTATTGATGCTA
ATATGTCACTTTCAAAGATCGAAGATGCTATTAGAATGGTAACTGATCAAAGAG
CAAATCTTGGTGCTTTTCAAAATAGACTTGAGTCTGTTAAGGCTAGCACAGACTA
TGCTATTGAAAACTTGAAAGCATCTTATGCTCAAGTTAAAGATGCAATAATGAC
AGATGAGGTTGTGGCATCTACAACTAACAGCATTTTGACACAATCCGCAATGGC
TAT

99.75 % similarity to *Borrelia miyamotoi* strain Yekat-18 chromosome, complete genome

GenBank # CP037471.1

Sequence of isolate (N3-60(63)) with *fla B. miy*. specific primers

TGGTAAGCTTAATTCACAAATTAGAGGATTGTCTCAAGCTTCTAGAAATACCTCA
AAGGCTATAAATTTTATTCAAACAACAGAAGGAAATTTGAACGAGGTAGAGAA
AGTATTAGTAAGAATGAAAGAACTTGCTGTTCAGTCTGGTAATGGTACATACTC
AGATTCAGATAGAGGGTCTATTCAGATTGAAATTGAACAACTTACAGATGAAAT
AAACAGAATTGCTGATCAGGCTCAATACAACCAAATGCATATGTTATCTAATAA
GTCAGCTGCTCAAAATGTAAAAACTGCTGAAGAGCTTGGAATGCAACCTGCAAA
AATTAACACACCAGCATCATTGGCTGGATCACAAGCTTCATGGACATTGAGAGT
GCATGTAGGTGCAAATCAGGATGAAGCAATTGCTGTCAATATTTATGCAGCTAA
TGTTGCAAATCTTTTTAATGGAGAAGGTGCTCAAGCAGCTCCAGCTCAAGAGGG

AGCACAACAGGAGGGAGTTCAAGCAGTTCCAGCTCCAGCAGCCGCTCCAGTGCA
AGGTGGAGTTAATTCTCCAATTAATGTTACAACTGCTATTGATGCTAATATGTCA
CTTTCAAAGATCGAAGATGCTATTAGAATGGTAACTGATCAAAGAGCAAATCTT
GGTGCTTTTCAAAATAGACTTGAGTCTGTTAAGGCTAGCACAGACTATGCTATTG
AAAACT

100 % similarity to *Borrelia miyamotoi* strain Yekat-18 chromosome, complete genome

GenBank # CP037471.1

Sequence of isolate (N3-60(8)) with *glpQ B. miy*. specific primers

CACCAGTGATCATAGCTcaCAGGGGTGtTAGtGGGTATcTTCCAGAACATACCTTA
GAAGCTAAAGCATATGCTTATGCATTAGGAGCTGATTATCTAGAACAAGACATA
GTTCTAACAAAGGACAATATTCCTGTTATAATGCACGACCCAGAAATTGACACA
ACCACAAATGTTGCACAATTATTTCCCAATCGAGCTAGAGAAAACGGACGATAT
TACGCCACTGACTTCACACTAACTGAACTTAAATCACTAAGTCTCAGTGAAAGA
TTTGATCCTGAAAACAAAAAACCAATATACCCTAATCGTTTCCCCTTAAATGAAT
ATAATTTTAAAATTCCAACTTTAGAAGAAGAAATAaAATTCATACAAGGACTAAa
TAAAAGCACAGGAAGAAATGtTGGGATTA

99.51 % similarity to *Borrelia miyamotoi* isolate 373-723 glycerophosphodiester phosphodiesterase (glpQ) gene, partial cds

GenBank # MK674171.1

Sequence of isolate (N3-60(8)) with *p66 B. miy*. specific primers

TTAATACAAATCAAACCACTAATTTTGCATTTTCTGCAGGAATAGGTATTGGTTT
CGCTTGGAATAAAGATGAAGGGGAAAAAGAATCATGGGCAATTAGAGGGAGTA
ATTCTTTCAGTAAAAAAATATTTGGTGAACAAGATAAAAAATCTGGAGTTGGAA
TTGGAATTAGCTATGGACAAAACTTATATAATACTCAATCTTCCAACATACCACT
ACTAAAGAAAATCTCTGAAAAATCATTTAAAACTCTCAATGCAGAGCTTTCAAC
TTATGAAGATAATAAGAAAGGTCTTATTCCTGGTCTTGGATGGATAGCTTCAATT
GGGGTTTATGACCTATTAAAAGAGTATCCTGAATCAGATAATATAATGAATGCT

CTTGACACAAATTTTAAGAACAATAATAATAGTAAAGCAAATTCATTTTATAAG
TCAACTAAGCTTGGAGGTGCTTTATACATCGATTATGCAATACCTGTAGAATCTA
TATCAAAAAACACATATAT

100 % similarity to *Borrelia miyamotoi* isolate Kem117-2019 outer membrane protein (omp66) gene, partial cds

GenBank # MT185688.1

Sequence of isolate (N3-60(63)) with *p66 B. miy*. specific primers

TCTTAAATTATTAAATCCAAAATCGAATAATCTTAACTTTAATACAAATCAAACC
ACTAATTTTGCATTTTCTGCAGGAATAGGTATTGGTTTCGCTTGGAATAAAGATG
AAGGGGAAAAAGAATCATGGGCAATTAGAGGGAGTAATTCTTTCAGTAAAAAA
ATATTTGGTGAACAAGATAAAAAATCTGGAGTTGGAATTGGAATTAGCTATGGA
CAAAACTTATATAATACTCAATCTTCCAACATACCACTACTAAAGAAAATCTCTG
AAAAATCATTTAAAACTCTCAATGCAGAGCTTTCAACTTATGAAGATAATAAGA
AAGGTCTTATTCCTGGTCTTGGATGGATAGCTTCAATTGGGGTTTATGACCTATT
AAAAGAGTATCCTGAATCAGATAATATAATGAATGCTCTTGACACAAATTTTAA
GAACAATAATAATAGTAAAGCAAATTCATTTTATAAGTCAACTAAGCTTGGAGG
TGCTTTATACATCGATTATGCAATACCTGTAGAATC

100 % similarity to *Borrelia miyamotoi* isolate Tomsk 9(6) outer membrane protein (omp66) gene, partial cds

GenBank # MN689813.1