The University of South Bohemia in České Budějovice
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Detection of Lyme disease spirochetes and *Borrelia miyamotoi* in samples of Czech patients

Bachelor Thesis

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Annotation

Lyme borreliosis is a disease caused by infection of selected members of the *Borrelia burgdorferi* sensu lato complex, a genus that keeps growing. With time, more and more members of this complex were identified as pathogenic for humans. This work aims to detect the spectrum of *Borrelia* spirochetes present in samples of Czech patients diagnosed with Lyme disease. Analysis will be used to suggest a tendency between the presence of the spirochetes‘ DNA found and the diagnosis of the patients.

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.

České Budějovice …15.12.2021…….. ……

Helena Kapurani

Acknowledgments

I want to thank my supervisor, Dr. Rudenko, for the unique opportunity she has given me to work with her sharing her knowledge and guidance.

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Abbreviations

Abbreviation	*Meaning*
ACA	Acrodermatitis chronica atrophicans
Bb s.l.	*Borrelia burgdorferi* sensu lato
bp	Base pairs
cat. #	Catalog number
CSF	Cerebrospinal fluid
dNTPs	Deoxynucleotide triphosphates
ds	Double-stranded
EM	Erythema migrans
FP	Forward primer
gDNA	Genomic DNA
LB	Lyme borreliosis
LD	Lyme disease
LNB	Lyme neuroborreliosis
MM	Master mix
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RLB	Reverse line blotting
RP	Reverse primer
RT	Room temperature
qPCR	Quantitative PCR
SDS	Sodium dodecyl sulfate
s.l.	sensu lato
s.s.	sensu stricto

Abstract

Lyme disease is an imminent vector-borne infectious disease of the Holarctic region, with a surging number of cases reported annually in endemic areas and new regions. The disease is caused by the spirochetes from *Borrelia burgdorferi* sensu lato complex. The three main species that were recognised as etiological agents of LB worldwide, *B. burgdorferi* s.s. (US), *B. garinii*, and *B. afzelii* (Eurasia), appear to be crossing the borders of their endemic areas and expanding beyond them.

Thus far, nine spirochete species from *B. burgdorferi* s.l. complex have been confirmed as human pathogens. Recently, the relapsing fever spirochete, *B. miyamotoi*, attracted attention as the pathogen that accompanies LB spirochetes and might cause unexpected manifestation of the disease in humans. Therefore, this study aimed to analyze a cluster of "problematic" samples of Czech LB patients and detect the *Borrelia* DNA to provide more information about the spectrum of pathogens distributed in the southern part of the country. A total of 67 samples were analyzed by PCR, sequencing, and RLB. It was found that *B. burgdorferi* s.s. is abundantly present as an LB etiological agent in the Czech Republic, followed by *B. garinii* and *B. afzelii*, with few cases of infection with *B. bisettii* and *B. carolinensis*. The disease appeared to be more prevalent in males than females and was primarily observed in children 0-9 years and adults or 40-69 years. A significant discovery was detecting a *B. miyamotoi* infection in a patient, co-circulating with *B. garinii*. Co-infections were also recognized as a frequent occurrence during the analysis of *Borrelia* infections.

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1. Introduction

1.1. Lyme Disease

Lyme Disease, also known as Lyme borreliosis (LB), is a globally spread tick-borne infectious disease predominantly within the Northern hemisphere. The disease is caused by spirochetes of the *Borrelia burgdorferi* s.l. (*Bb* s.l.) complex, which are principally transmitted during the bites of hard ticks of the *Ixodes* genus [34].

The disease was first described in 1976 due to significant emerging cases of children with arthritis in Lyme, Connecticut, in the USA. Childrens' arthritis was later related to other symptoms, such as erythema migrans (EM), Bannwarth syndrome, and acrodermatitis chronica atrophicans (ACA), a condition previously reported in Europe [63]. After further research, a relationship between the symptoms and bacteria was made when the first spirochete of *Borrelia burgdorferi* was isolated from an *Ixodes scapularis* tick. The same spirochete was retrieved from patients (1983), showcasing the already mentioned symptoms alongside other skin, heart, nervous system manifestations, and more [49]. Thus, Lyme disease was described as a multisystem condition, affecting and causing inflammation of numerous body parts [34] [64].

The *Borrelia burgdorferi* s.l. complex currently comprises 22 recognised species and several proposed candidates [71]. Three genospecies of the complex are repeatedly reported as responsible for human LB: *B. burgdorferi s.s.*, *B. afzelii,* and *B. garinii,* with the first being a primary source of LB in the USA, all of them as causative agents in Europe, and *B. afzelii* and *B. garinii* present in Asia as well [34]. The vast intraspecies heterogeneity is attributed to geographical differences of clinical manifestations of the disease, i.e., *B. burgdorferi* s.s. in the northeastern US is correlated to arthritogenic manifestation, *B. afzelii* mainly incites skin infections, and *B. garinii* is notably neurotropic [34] [64].

As for epidemiology, in the US, the infection age distribution is presented as bimodal, where a higher prevalence is observed in children of age range 5-15 and adults of age range 45-55, and a gendered tendency of higher infection among men compared to women in the ages of <60. This tendency diminishes for the older population, creating an almost equal sex ratio, slightly leaning towards women [64]. In European countries, LB is more common amongst adult women than men, fixing a 55:45 ratio. Furthermore, a strong correlation is established between the infection prevalence and tick abundance and exposure, where a farming occupation showcases high-risk for infection. Other leisure ventures, such as hunting or mushroom collecting, pose a high infection risk. Europe equally confirms a bimodal infection age distribution [52]. Generally, the disease onset culminates in the summer months of July and June, a characteristic attributed to the feeding habits of nymphal ticks,

which are mainly responsible for transmission. This timescale is also accompanied by increased tick quest in higher humidity environments and mixed woodlands with high ecotonal indices [52].

1.2. Tick vectors and animal hosts

As previously mentioned, spirochetes infect humans with the help of hard ticks of the genus *Ixodes*, a well-known disease vector. Four particular *Ixodes* species are established as vectors worldwide: *I. scapularis* and *I. pacificus* in eastern and western North America correspondingly, *I. ricinus* in Europe, and *I. persulcatus* in Asia. This relationship was confirmed early in the description of the disease, as the appearance of the bulls-eye rash EM was closely associated with tick bites. However, aside from ticks, spirochetes are also present in different animals (hosts), including small mammals, birds, or mice, all of which function as reservoirs for the maintenance and reproduction of the bacteria. This way, an enzootic cycle of the spirochete is modeled, with the four life cycles of ticks centering as protagonists. The longevity of the enzootic cycle depends on the time needed by the tick to go through the course of life (egg, larvae, nymph, and adult), which averagely results in approximately 2- 3 years [50].

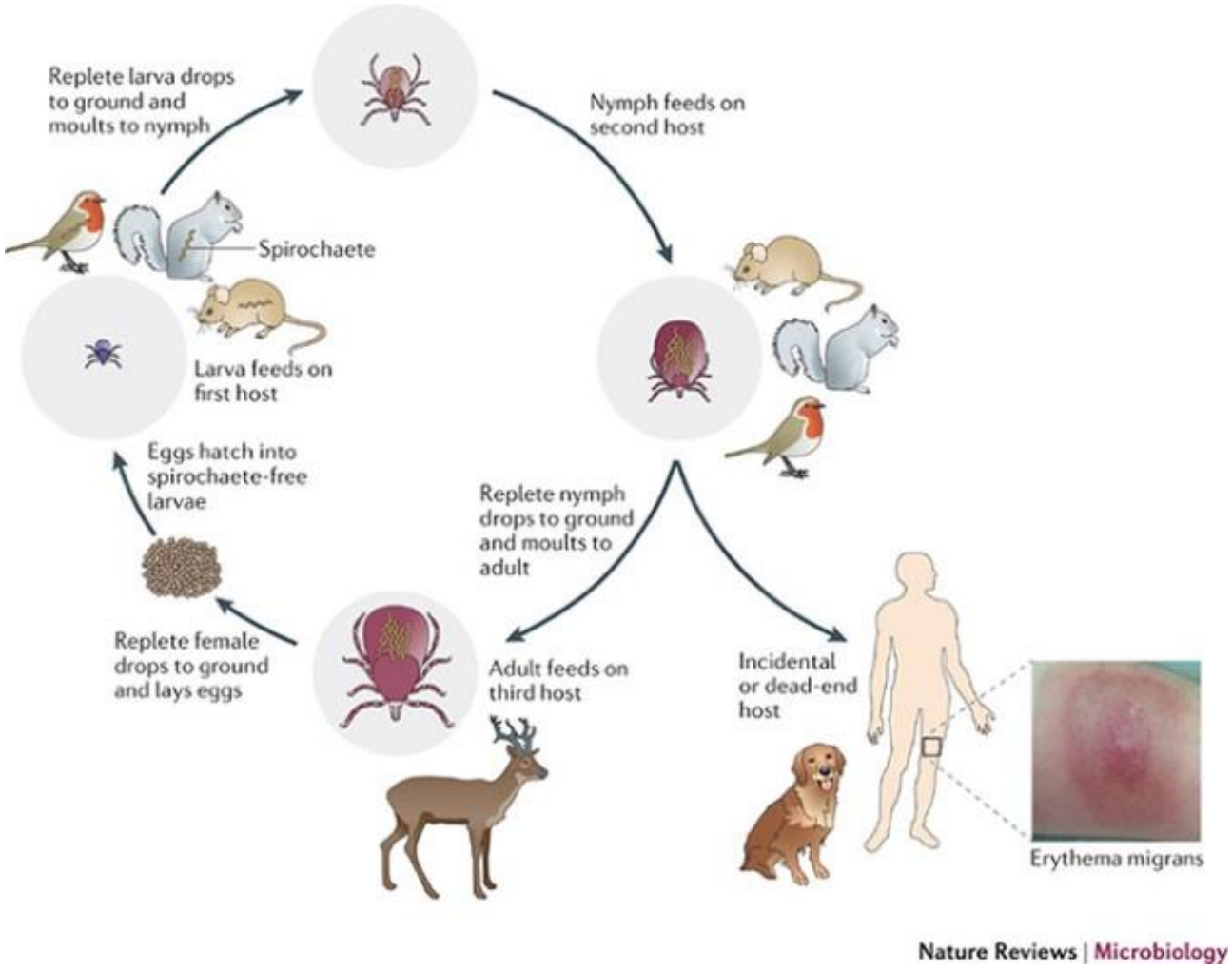

Figure 1: Enzootic cycle of LD spirochetes alongside life cycle of Ixodes ticks. Picture from Nature Reviews Microbiology [50].

For *Borrelia burgdorferi* s.l. spirochetes, contrary to relapsing fever *Borrelia*, the transovarial transmission, where the bacterium pass from adult tick to egg, was not confirmed. All tick generations must acquire the LB spirochete anew from an infected reservoir host during the blood feeding. The cycle starts when a larval tick acquires the spirochetes with a blood meal. The bacterium is retained within the tick trans-stadially (through each blood meal and molt). Nymphs then feed on a similar range of animals as larvae, thus transmitting the spirochetes and maintaining the enzootic cycle for the forthcoming generation of larval ticks. Lastly, the adult ticks play little to no role in the cycle continuance in the wild as they feed on predominantly larger animals that are inadequate hosts for the bacterium. The relevance of larger mammals is in supporting the reproduction of adult ticks. When it comes to human infection, although all three stages of *Ixodes* ticks can bite humans, nymphs carry primary liability for spirochete transmission. Their time of action is mainly in the spring, although infection can occur year-long. Humans are considered a dead-end host of the cycle, as it is still unproven if infected humans can transmit spirochetes to feeding larvae. On the other hand, dogs are viewed as incidental hosts and do not play a role in the enzootic cycle [50].

Ixodes ticks have an extensive feeding range in different mammals, lizards or birds, which accounts for the geographical extent of disease spread and the incidental infection of humans altogether. Different study methods have been applied to establish cases with solid experimental support of competence of reservoirs hosts of the pathogen. The most prominent method used is xenodiagnosis, which is essentially the detection of a parasite by feeding of a suitable tick vector on presumably infected host and later examining the presence of parasite in vector. Based on this method, multiple species of birds have been confirmed as competent reservoirs for *Bb* s.l., for example: *Phasanius colchicus* for *B. burgdorferi* s.s., *Turdus merula* for *B. garinii*, *B. turdi*, and *B. valaisiana*, and *Parus major* for *B. afzelii* in Eurasia, and *Turdus migratorius* for *B. burgdorferi* s.s. in North America [72]. Among 62 species of rodents that have been thoroughly studied, seven of them resulted as competent reservoirs for several Eurasian *Bb* s.l. complex species transmitted by *I. ricinus* (*Apodemus flavicollis, Apodemus sylvaticus, Eliomys quercinus, Mus musculus, Muscardinus avellinarius, Myodes glareolus, Tamias sibericus barberi*) [72]. Five rodent species (*Mesocricetus auratus, Microtus pennsylvanicus, Peromyscus leucopus, Peromyscus maniculatus, and Neotoma fuscipes*) were identified as reservoir hosts of multiple North American *Bb* s.l. complex species (*B. americana*, *B. bissettiae*, *B. burgdorferi* s.s., and *B. mayonii*). The European hedgehog *Erinaceus europaeus* has been confirmed as a competent reservoir for *B. spielmanii*, while the reptile species, *Psammodromus algirus*, is confirmed as a host for *B. lusitaniae* [72].

Although the material provided here stands correct, it is also incomplete as there is an enhanced bias in the studies performed for host association for *Borrelia* species. The majority of studies focus on

investigating hosts for a certain *Borrelia* species (46% of studies focus on *B. burgdorferi* s.s.), while another pronounced imbalance is the study of the host taxa, where the survey of rodents of terrestrial birds take up the vast majority. Ideally, potential host species tested should be all species that could play a potential ecological role as reservoir hosts for all *Bb* s.l. complex species circulating in a specific area [72].

1.3. Clinical manifestation of the disease

The clinical manifestation of LB can range from acute to chronic illness, where the variation is highly credited to the different *Borrelia* genospecies implicated in the infection. The most common symptoms are etiologically and geographically separated. Whereas in Europe *B. afzelii* is correlated to dermatological manifestation, and *B. garinii* is correlated to neuroborreliosis, in the USA, the presence of *B. burgdorferi s.s.* is believed to be involved in carditis and arthritis [50] [52].

Following a tick bite, spirochetes are deposited into the skin through the tick saliva during the feeding process. If caught within the first 24 hours of the ticks' attachment to the body, infection likelihood is low, but transmission of the pathogen becomes higher with time closing to 48 hours or longer. The genome of *B. burgdorferi* does not encode any notorious toxins or secreting mechanisms, so the tissue damage is proclaimed to be mediated by the inflammatory response evoked in the mammalian host [50]. The tick saliva contains a mixture of immunomodulatory molecules, which enable protection of spirochete against the innate immune response during the early phase of host colonization/ invasion. Following a favorable host intrusion, *Borrelia* spirochetes apt for residence in immune-privileged sites in the host. Another suggested decoy mechanism is the transformation of spirochetes from motile infective to dormant forms, capable of withstanding unfavorable environmental conditions, including antibiotic treatment [57].

The clinical manifestation of LB may be divided into three stages to facilitate diagnosis. The first stage, also known as the early localized stage, develops in the first weeks after infection following an incubation period of 3 to 32 days. In some cases at this stage of the disease a rash called erythema migrains appears at the place of the tick bite. Other early symptoms include: fever, fatigue, headaches, and muscle or joint pain. The second stage, known as the early disseminated stage, may occur weeks to months following exposure and may consist of multiple erythema lesions, borrelial lymphocytoma, Lyme neuroborreliosis (LNB), carditis, or seldom, Lyme arthritis [7]. A frequent phenomenon contributing to the second and third stages of the disease in an untreated patient is low-level spirochetamia. It refers to the circulation of spirochetes via the bloodstream, occasionally affecting the peripheral or central nervous system, joints, or even the heart [50]. The third stage, also known as the late disseminated stage, manifests itself with acrodermatitis chronica atrophicans, Lyme arthritis, and neurological symptoms. The symptoms of LB can be collectively organized into four

categories, based on the body part that is affected, namely: dermatological manifestations, Lyme arthritis (joints), Lyme carditis (heart), and Lyme neuroborreliosis [7]. Nevertheless, there have also been frequent reports of asymptomatic or subclinical infections in endemic areas [14].

When speaking of dermatological manifestations, the most relevant one is EM, which is present in 70-95% of affected patients within the first three weeks of infection, and it is more common among children than adults. It looks like a red to bluish-red round or oval patch (Fig. 2a) with a central clearing that expands 5 to 10 cm. It can also have a target-like morphology (Fig. 2b), where sometimes a burning sensation persists. This symptom may be associated with fever or swelling of the lymph nodes, and when left untreated, it can persist up to a few months and multiply throughout the body. Secondary EM occurs when multiple lesions occur, with characteristics similar to the original lesion but with a reduced redness or swelling. Expansion usually occurs in the face or limbs [68].

Another significant manifestation is ACA, a rare skin disease that occurs in the late stage of LB, most commonly in Europe, where many cases are attributed to infection with *B. afzelii*. The condition develops more often in older women, where lesions initiate as bluish-red plaques with edema that slowly progress within years to atrophic and hyperpigmented plaques with translucent skin that allows visibility of underlying blood vessels (Fig. 2e, 2f). In time the lesions can become bilateral or affect other extremities. More than half of the patients also report neurological pain of the extremities, also known as allodynia [31].

Another encountered cutaneous symptom of LB is borrelial lymphocytoma, which debuts weeks to months after EM or infection. The lesion consists of a single red/bluish bulgy nodule that measures 1 to 5 cm, which mainly affects children on the earlobe (Fig. 2c), and adults on the breast or nipple area (Fig. 2d). The manifestation has been reported due to infections with *B. afzelii*, although appearance of lymphocytoma was reported with infection of the human with other strains *B. garinii*, *B. burgdorferi s.s.*, and *B. bissettii* as well. Other dermatological symptoms have also been reported for LB, such as morphea, Parry-Romberg syndrome, lichen sclerosus, and lymphoma, although their association with the disease is controversial. The existence of many *Borrelia* species, the diverse spectrum of manifestations they cause, and the usage of various diagnosis techniques can lead to a poor identification of different skin lesions with Lyme disease [7].

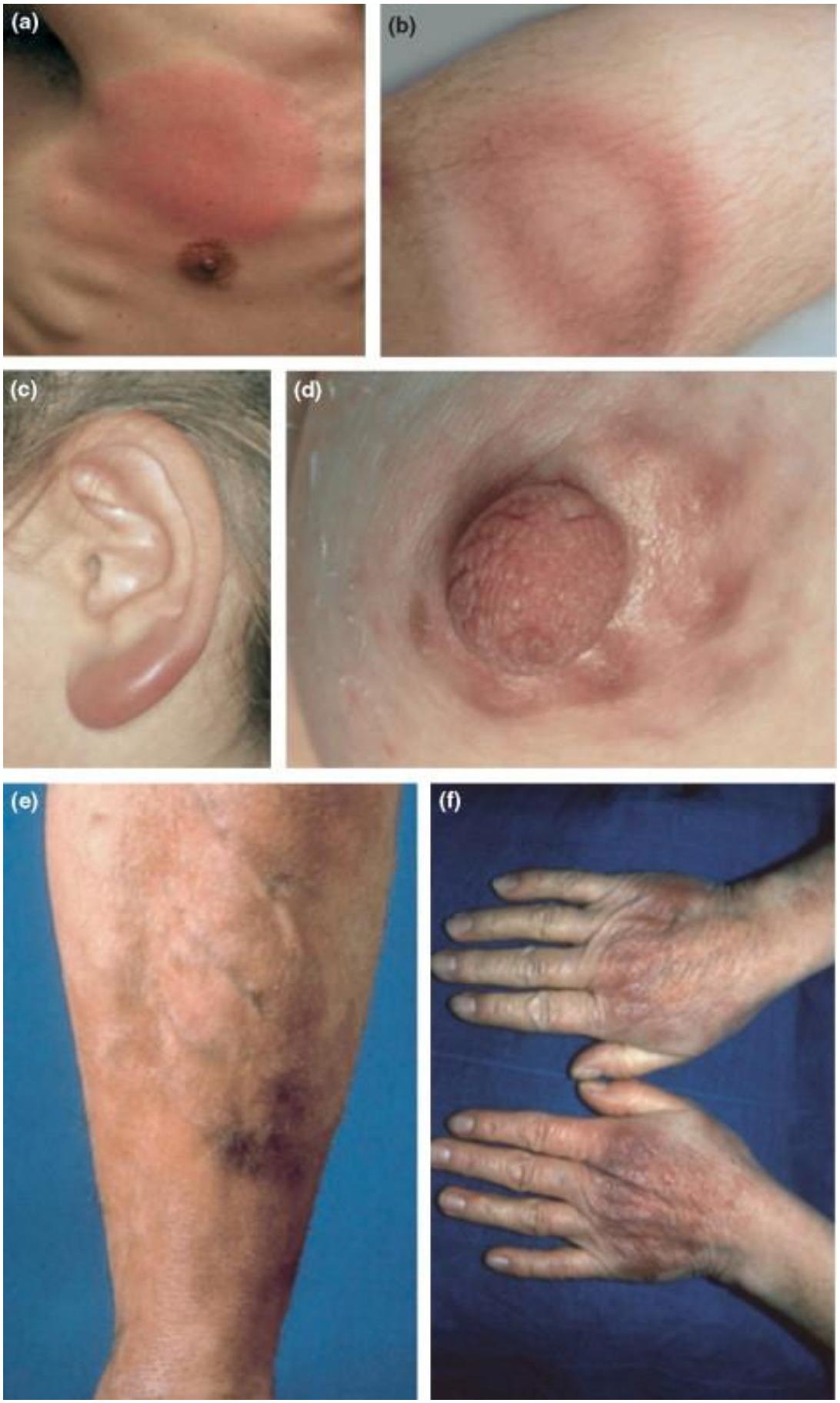

Figure 2: Dermatological manifestations of LB (pictures by Hasel Druck und Verlag GmbH, 1090 Vienna, Austria). (a) Erythema migrans on the left breast; 4 weeks after a tick bite and three weeks after onset of the lesion. (b) Erythema migrans with central clearing on the lower leg. (c) Borrelial lymphocytoma on the ear lobe. (d) Borrelial lymphocytoma on the nipple. (e) Acrodermatitis chronica atrophicans on the left leg. (f) Acrodermatitis chronica atrophicans on the dorsal side of the hands. Pictures by [62].

Aside from dermatological manifestations, Lyme arthritis is the second most common clinical finding among American patients, while in Europe, it is encountered a bit more seldom. In up to 60% of cases in the USA, untreated patients with EM develop arthritis within six months. The arthritis is usually asymmetrical, occurs in one or many joints, and can occur at irregular or persistent episodes. The most frequently affected joint is the knee, but synovitis of up to five joints in one episode can occur. The swelling is usually severe, with moderate to mild pain. Typically, adequate antibiotic treatment can help alleviate the pain and subside arthritis, but there are persistent cases where chronic or erosive arthritis may develop. In such cases, the symptoms should be treated similarly to chronic inflammatory arthritis with methotrexate and hydroxychloroquine [1] [7].

Lyme carditis is another symptom group that refers to the cardiovascular manifestations of LB. Although such symptoms were somewhat recurrent in the 1980s, their prevalence has steadily dropped in Europe and North America due to reporting and inspection methodology changes. Nowadays, cardiac involvement is reported in 1 to 2% of LB cases, where most affected are adult males. These manifestations usually present in the early dissemination stage, two to five weeks after the appearance of EM, but there have been reports of earlier appearances as well. The symptoms include palpitations, syncope or pre-syncope, dyspnea, and chest pain, all of which should be carefully read by medical professionals due to their potential mortality. Severe manifestations of Lyme carditis include cardiac conduction abnormalities such as atrioventricular (AV) block, which may rapidly progress to a third-degree one, as well as myocarditis, pericarditis, and acute heart failure [7] [53].

Lyme neuroborreliosis is the fourth group of neurological manifestations caused by infection with *Borrelia* spirochetes. It is mostly present in the second stage, in 10 to 15% of untreated patients with EM. The prevalence of LNB is 12-14% in the USA, and it increases to 16-23% in European patients, while having a higher preponderance in males and a bimodal trend affecting children, teenagers, and adults ≥ 50 years old. LNB follows different manifestation courses in North America and Europe. In North America, patients usually experience acute or subacute lymphocytis meningitis, associated with headache and neck stiffness. The headache might be referred to in the frontal or occipital lobe, with intermittent periods of intense pain, followed by mild pain, which might be accompanied by cranial neuritis, radiculoneuritis, or encephalitis. Recently, there have also been reported cases of facial palsy, which are presented as acute, often unilateral, most commonly occurring between summer and fall. The facial palsy can often be misconstrued with Bell's palsy, but a history of tick bite and other symptoms must be considered during diagnosis. In Europe, a ubiquitous manifestation of LNB is a painful meningoradiculitis known as the Bannwarth syndrome, which is often associated with *B. afzelii*. The condition may be accompanied by other features such as a sharp pain that is

exacerbated during the night, which in turn causes sleep disturbances, CSF lymphocytosis, headache, fatigue, paresthesia/ paresia, facial palsy, and meningeal signs [7] [23] [41].

Prevention of tick bites is suggested to avoid Lyme borreliosis, which can be achieved by using protective clothing, skin and clothing inspection, tick and insect repellent use, and swift removal of attached ticks upon discovery. Depending on the stage of the disease or the advancement of the symptoms, the treatment can be varying. The usual treatment scheme consists of antibiotics, but that is applied only when specific criteria are fulfilled regarding the tick bite and *Borrelia* infection of the tick. Another recent feature is the co-infection of *Ixodes* tick species with different genospecies of *Borrelia* or even other tick-borne pathogens. This eventually leads to a higher likelihood of human infection with different strains, and therefore there is a probable alteration in clinical presentation and response to treatment. The frequency in which this phenomenon occurs is not yet fully understood, and the variance in clinical manifestation is not yet fully characterized [7] [26].

1.4. *Borrelia burgdorferi* sensu lato complex

B. burgdorferi sensu lato complex is a part of the phylum Spirochaetes and class Spirochaetae, including several families such as Spirochaetaceae and Leptospiraceae. The class Spirochaetae is notorious for housing many human pathogens such as *Treponema pallidum*, *Leptospira interrogans*, and *Borrelia recurrentis*, etiological agents of syphilis, leptospirosis, and relapsing fever borreliosis, correspondingly [33]. Morphologically, *B. burgdorferi* s.l. spirochetes are bacteria with outer and inner membranes with a thin layer of peptidoglycan in between; the outer membrane lacks lipopolysaccharides. It measures 20 to 30 microns in length and 0.2 to 0.3 microns in width, where the motility is ensured by seven to eleven bundled periplasmic flagella at each end that provide motility in low- and high- viscosity media alike, a feature contributing to the high virulence (Fig. 3) [37]. Furthermore, it can be cultivated in the Barbour-Stoenner-Kelly (BSK) medium with a doubling time of 24 to 48h, but it cannot be recovered reliably from human samples [33] [58].

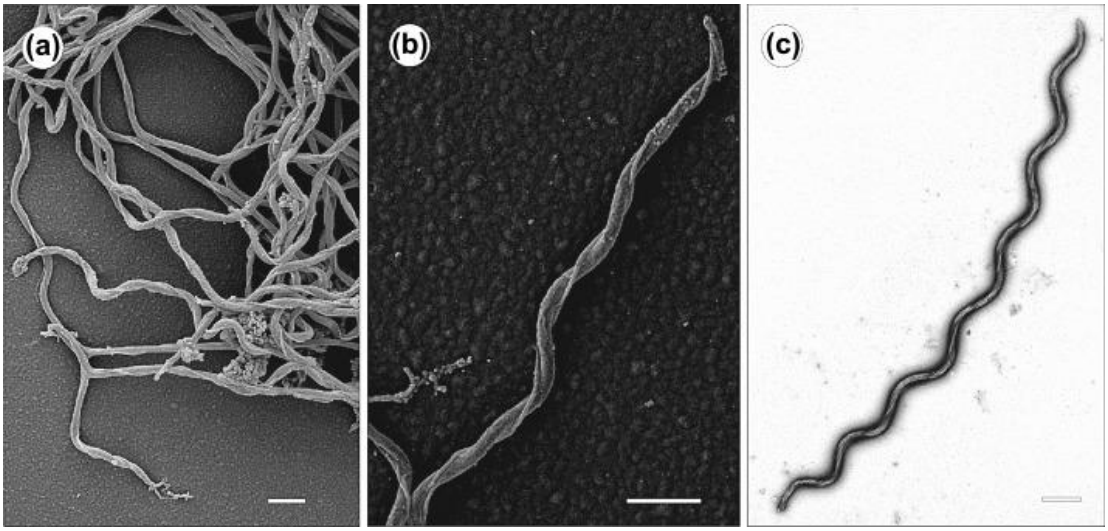

Figure 3: Morphology of Borrelia spirochete M6p strain imaged in SEM (a,b) and TEM (c). Picture by Vancova M. [57].

B. burgdorferi sensu lato (*Bb* s.l.) species complex refers to a heterogeneous group that, as of 2021, contains 22 accepted and several proposed genospecies (henceforward referred to as species) (Table 1) [72]. Of all the species, three have consistently and extensively associated with Lyme borreliosis: *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*, with the first one being a major pathogenic agent in Eurasia and the USA. Other than the previously mentioned, the six following species have been suggested to have pathogenic potential to human as well: *B. bavariensis*, *B. bissettiiae*, *B. kurtenbachii*, *B. lusitaniae*, *B. spielmanii*, and *B. valaisiana* [56].

Table 1: Summary of all LD Borrelia species known so far, the vector, host/ reservoir, geographical distribution, and human pathogenicity according to certain studies.

Borrelia species	*Vector*	*Hosts/ reservoirs*	*Geographical distribution*	*Human Pathogenicity*	*Reference*
B. afzelii	*I. ricinus, I.persulcatus*	Rodents	Asia, Europe	+	Canica et al. 1993
B. americana	*I. pacificus, I. minor*	Birds	America	-	Rudenko et al. 2009
B. andersonii	*I. dentatus*	Cotton tail rabbit	America	-	Marconi et al. 1995
B. bavariensis	*I. ricinus*	Rodents	Europe	+	Margos et al. 2009; Margos et al. 2013
B. bissettii	*I. ricinus, I. scapularis, I. pacificus, I. minor*	Rodents	Europe, America	+	Postic et al. 1998; Margos et al. 2010; Margos et al. 2016
B. burgdorferi s.s.	*I. ricinus, I. scapularis, I. pacificus*	Rodents, birds, lizards, big mammals	America, Europe	+	Johnson et al. 1984
B. californiensis	*I. pacificus, I. jellisonii, I. spinipalpis*	Kangaroo rat, mule deer	America	-	Postic et al. 1998; Margos et al. 2016
B. carolinensis	*I. minor*	Rodents, birds	America	-	Rudenko et al. 2009; Rudenko et al. 2011
B. chilensis	*I. stilesi*	Deer	America	-	Ivanova et al. 2014
B. finlandesis	?	?	Europe	-	Casjens et al. 2011
B. garinii	*I. ricinus, I. persulcatus, I. hexagonus, I. nipponensis*	Birds, lizards, rodents	Asia, Europe	+	Baranton et al. 1992
B. japonica	*I. ovatus*	Rodents	Japan	-	Postic et al. 1993; Kawabata et al. 1993
B. kurtenbachii	*I. scapularis*	Rodents	America, Europe	-	Margos et al. 2010; Margos et al. 2014
B. lanei	?	?	America	-	Schwan et al. 1993; Postic et al. 2007
B. lusitaniae	*I. ricinus*	Rodents, lizards	Europe, North Africa	+	Le Fleche et al. 1997
B. maritima	?	?	America	-	Margos et al. 2019
B. mayonii	*I. scapularis*	Rodents	America	+	Pritt et al. 2016

B. sinica	*I. ovatus*	Rodents	China	-	Masuzawa et al. 2011
B. spielmanii	*I. ricinus*	Rodents	Europe	+	Richter et al. 2006
B. tanukii	*I. tanuki*	Rodents, dogs	Japan	-	Fukunaga et al. 1996a; Fukunaga et al. 1996b
B. turdi	*I. turdus*	Birds	Japan	-	Fukunaga et al. 1996a; Fukunaga et al. 1996b
B. valaisiana	*I. ricinus, I. granulatus*	Birds, lizards	Asia, Europe	+	Wang et al. 1997
B. yangtzensis	*Haemaphysalis longicornis, I. granulatus*	Rodents	China	-	Chu et al. 2008; Margos et al. 2015

1.5. Current association of spirochetes with LB symptoms

Aside from the previously mentioned notorious trio of *B. afzelii*, *B. garinii*, and *B. burgdorferi* s.s., whose suggested typical clinical manifestations were already explained, other spirochetes are considered to have pathogenic potential. *B. bavariensis* has not yet been related to a particular clinical manifestation; it can invade different tissues, including the nervous system, the joints, and the skin. *B. spielmanii* has been isolated frequently from skin lesions of patients manifesting EM. Two other proposed etiological agents, *B. bissettii* and *B. valaisiana*, are detected in specimens of patients of LB, where a possible correlation could be made to LNB. The role of *B. lusitaniae* and *B. kurtenbachi*, related to any possible association to clinical manifestations, is not fully understood [61]. The information concerning this topic is minimal, and studies of a bigger sample size are necessary to determine a more defined relationship, if there is any. A principal issue that might arise during this study is the frequent presence of two or more Borrelia spirochetes in samples of human origins, which makes it harder to form an association between the symptoms and species present.

1.6. *Borrelia miyamotoi*

Borrelia miyamotoi is a causative agent of elapsing fever. While the common vectors of relapsing fever spirochetes are soft-bodied ticks, or lice, B.miyamotoi is transmitted by hard ticks from *Ixodes ricinus* complex. [59]. The same ticks transmit the LB spirochetes. Many previous studies have stated that *B. miyamotoi* spirochete is also an etiological agent for LB, as they share a common vector, and there have been multiple cases in patients diagnosed with LB infected with *B. miyamotoi* as well [45] [58].

The spirochetes were firstly observed in 1985 in the United States. However, they were mistaken for *B. burgdorferi* spirochetes in *I. pacificus* and *I. scapularis* adult ovarial tissue, eggs, and/or larvae,

which eventually led to a false conviction that *B. burgdorferi* was transmitted transovarially in hard ticks [44]. This was later experimentally proven untrue for *B. burgdorferi* spirochetes while holding for *B. miyamotoi* in *I. scapularis* ticks [54]. Later in 1994, the spirochetes identified as *B. miyamotoi* were isolated from field-collected *I. persulcatus* ticks and field mice in Japan. The sequenced 16S ribosomal gene was used to identify *B. miyamotoi* found in laboratory-bred *I. scapularis* ticks, which tested negative for *B. bugdorferi* infection. The two species showed close relation [5]. Although the presence of the spirochete was recognized for a relatively long time, it was not until its 'pathogenic potential was materialized in many cases of human infections of relapsing fever-like disease in Russia in 2011 [45]. Many more cases were reported throughout the United States, Europe, and Japan.

Phylogenetically, *B. miyamotoi* and other relapsing fever spirochetes such as *B. lonestari* or *B. theileri* cluster together [2]. The classification includes both Old and New World species, such as *B. crocidurae* and *B. hermsii*, respectively. Real-time qPCR based on primers and probes of the 16S ribosomal RNA gene distinguishes between the relapsing fever group and the LB group [38]. There are phylogenetic differences between the various *B. miyamotoi* isolates, depending on their endemic areas; however, they are still genetically heterogeneous. It is suggested that the species *B. miyamotoi* exists through the Asian, European, North American - West and East Coast genotypes [29]. Aside from the genetic distance from *B. burgdorferi* and other LD species, *B. miyamotoi* and other relapsing fever species share the unique feature of expression of a glycerophosphodiester phosphodiesterase (glpQ) biosynthetic gene. The antibody response to *B. miyamotoi* glpQ antigen is a characteristic of relapsing fever, separating it from the LB spirochetes, which is also used as a diagnostic method in this study [60].

B. miyamotoi can be acquired by larval ticks either horizontally from an infected vertebrate host or transovarially from an infected female tick. The bacterium is transmitted by the same tick vectors as LB spirochetes: *I. scapularis* in northeastern and north-central United States up to Canada, *I. pacificus* in the far-western United States and British Columbia, *I. ricinus* in Europe, and *I. persulcatus* in Europe and Asia [5] [45] [58]. Furthermore, similar to LD spirochetes, the enzootic cycle of *B. miyamotoi* consists of multiple vertebrate reservoir hosts. According to recent findings, the white-footed mouse (*Peromyscus leucopus*) is a qualified reservoir in the northeastern US [59]; other species, such as field mice, voles, and birds, are confirmed reservoirs in Europe, and field mice are validated as a host in Japan [9] [32] [66]. In comparison to LB, as *B. miyamotoi* can be transmitted by unfed larvae due to transovarial acquisition, the peak transmission season by certain species, such as *I. scapularis*, occurs mainly in the later summer, when larval activity is at its highest [61].

Albeit the knowledge of the geographical distribution of *B. miyamotoi* is relatively truncated, it is believed that the epidemic areas overlap with those of LB spirochetes. There have been multiple

reports of human infections with *B. miyamotoi* in Russia, the US, the Netherlands, and Japan, but the geographic spread of the infections in ticks is much broader than in the areas where human cases are reported. It has been found in ticks from Asia (Japan and central Russia), North America (the United States and Canada), and Europe (Czech Republic, Denmark, England, Estonia, France, Netherlands, Poland, Germany, Switzerland, and Sweden) (Fig. 4) [3] [5] [9] [11] [32] [35] [59].

Figure 4: Approximate geographic distributions of the principal tick vectors of B. miyamotoi as of 2015. Picture by [24].

Besides infection by tick bite, *B. miyamotoi* infection in humans may also occur through blood transfusion, which results in broadening of the epidemic area. High-density spirochetamia is also observed in *B. miyamotoi*, similar to other relapsing fever spirochetes, thus increasing the risk of transfusion transmission. There have been reported cases of transfusion-transmitted infection with *B. recurrentis* and *B. duttonii* in humans, whereas *B. miyamotoi* transfusion-transmitted infection has been observed experimentally in mouse models (Fig. 5) [25].

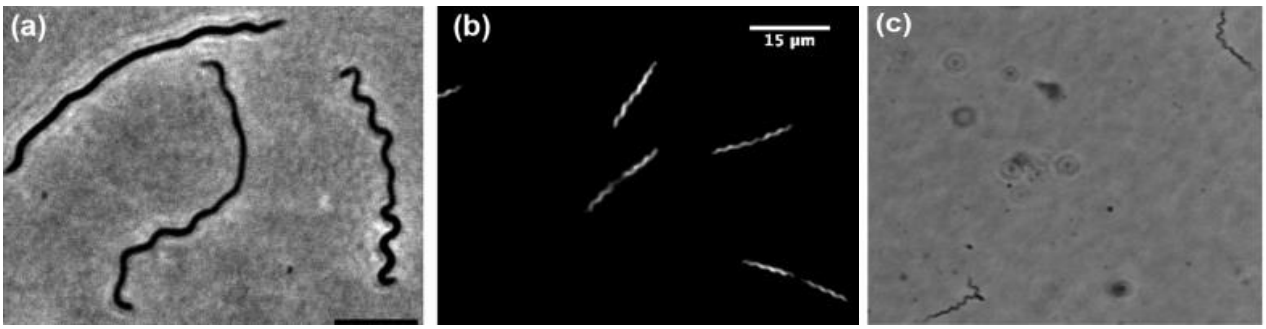

Figure 5: (a)/(b) B. miyamotoi grown in vitro. (c) B. miyamotoi in infected mouse blood. Picture by [24].

As a relapsing fever spirochete, *B. miyamotoi* infection is a febrile illness consisting of a fever that may surpass 40°C, headache, chills, fatigue, arthralgia, myalgia, and nausea. A decisive factor in diagnosing relapsing fever by *B. miyamotoi* infection is the presence of a tick bite [45]. In some reported cases, the patients experienced up to 2-3 episodes of fever, each lasting 2-5 days and a mean interval of 9 days between each episode (minimum two days; maximum two weeks). The occurrence of the relapsing of the illness could have been higher if the patients had not been given antibiotics. Generally, the fever associated with *B. miyamotoi* infection lasts less than a week, but other

symptoms, such as fever, might persist even longer [45]. Other than the previously mentioned symptoms, there have been two reports of *B. miyamotoi* infected patients that experienced symptoms in the central nervous system and signs of meningoencephalitis. Both patients experienced an accelerating decline in cognition and unsteady gait over several months, but neither experienced fever [19] [22]. Another case was reported in Germany of an immunocompromised 74-year-old patient who showed symptoms of neuroborreliosis. The patient showed no signs of *B. burgdorferi* infection after having experienced two tick bites but instead resulted in infection with the European strain of *B. miyamotoi* [4]. Since there are insufficient studies on different antibiotic treatments for *B. miyamotoi*, the prescribed medication is a 14-day course of tetracyclines or amoxicillin/cefuroxime for young children or pregnant women. These treatments have been chosen based on the susceptibility of the LD or other relapsing fever spirochetes towards the antibiotics [45].

A typical event documented in reports from various countries is the co-infection of *Bb* s.l. spirochetes and *B. miyamotoi*, which was detected by the presence of erythema migrains rash. The presence of *B. burgdorferi* was not confirmed, but it was assumed to be the only explanation as EM rash is not associated with soft-bodied tick-transmitted relapsing fever spirochetes [45]. Further studies with large sample sizes are necessary to determine whether *B. miyamotoi* and *B. burgdorferi* co-infection influences disease outcomes in humans.

1.7. Infection situation in the Czech Republic and worldwide

This study aims to provide further insight into the infection situation of patients in the Czech Republic and potentially suggest a tendency between the diagnosis of the patients and the spirochetes‘ DNA detected in the samples. Therefore, it is important to recall some data regarding different studies that have assessed the infection situation in the Czech Republic in particular and further in Western Europe and worldwide. The latest survey of the epidemiology of LB conducted in the Czech Republic is that of 2018 and 2019, which recorded the disease incidence and trend. Compared to the rest of the region of Western Europe, the Czech Republic appears to have a higher LB incidence. However, hotspots of high incidence (>100 cases/ 100,000 population) are also established in parts of Slovenia, Austria, Germany, southern Sweden, some Finnish and Estonian islands, and Lithuania [42].

From 2018-2019, a total of 8,826 LB cases were reported to the Czech reporting system ISIN: 4,724 and 4,102 cases in each year, respectively, leading to an overall incidence of 44.5 and 38.5 cases per 100,000 population. The female-to-male ratios were 1.19 and 1.12 in 2018 and 2019, representing a slight gender difference, 46.3% males to 53.7% females. When speaking of the age of patients, in 2018, the mean age was 45.5 years, while the median was 50; in 2019, the mean age was 44.7, and the median was 49. Again the difference is slight, and the younger mean age is observed in males (43.0; 41.5) compared to females (47.7; 47.5) for both years. During this period, the most affected

age group is that of 60-69 years, with 1,742 LB cases, or 19.74% of the overall cases. The group ages with the highest morbidity with over 50 cases per 100,000 population are adults 50-75 years and children 5-9 years old. The LB incidences were spread throughout the Czech Republic, but the most affected regions according to the place of residence were Vysočina, Olomoucký, and Zlínský regions. The occurrence of LB was mainly concentrated from April to November in both years, with an abrupt increase in May, more pronounced in 2018 than 2019. In both spring and autumn, about 60% of the patients experienced a tick bite or had direct contact with the tick during removal, while for the rest, the exposure was unknown or incomplete. For 20% of the patients over two years, hospitalization was required, while 29% were treated on an outpatient basis. Lastly, the most common clinical manifestation was EM, reported in 67.2% of the patients yearly, while LNB was recorded in 12.6% of the patients in 2018 and 13.8% in 2019 [42].

Zooming out the picture of LB incidence in Western Europe and North America, an estimated number of around 85,000 cases/ year, and 30,000 cases/ year, respectively, were observed until 2013 [43] [65]. The year 2013 was a pivotal moment of LB, as it was precisely during this time that the Centers for Disease Control and Prevention (CDC) released the statement that about 10-fold of the initially-thought number (329,000 cases/ year) was the actual incidence of LB in the US between 2005 and 2010 [27] [39]. During 2010-2018, the numbers increased even further, where an average of 476,000 patients were diagnosed and treated annually in the US [28]. The situation in Europe was reevaluated; a resolution of the European Parliament in 2018 estimated that the incidence of LB in the region was almost 850,000 cases/ year [55].

2. Materials

Table 2: Summary of all materials used to perform all experiments of this study.

Procedure	*Material*	*Description*
gDNA extraction	Genomic DNA isolation kit	DNeasy® Blood & Tissue Kit (250) **Qiagen**, cat. # 69504
	1x PBS	$0.8\ g \cdot L^{-1}$ NaCl, $0.02\ g \cdot L^{-1}$ KCl, $0.144\ g \cdot L^{-1}$ Na_2HPO_4, $0.024\ g \cdot L^{-1}$ KH_2PO_4, pH = 7.4, sterilized.
PCR	Master Mix (2x)	HotStarTaq® Plus Master Mix Kit (1000) **Qiagen**, cat. # 203445
DNA gel electrophoresis and extraction	Agarose	Agarose I^{TM} **Amresco**, cat. # 0710-500G
	50x TAE buffer	200 mM Tris-HCl, 50 mM EDTA
	50x Modified TAE buffer	Modified TAE buffer concentrate (50x) **Millipore**, cat. # LSKMTAE50
	Loading dye	GelRed Prestain Plus 6x DNA Loading Dye **Biotium**, cat. # 41011
	DNA Ladder	GeneRuler 100 bp DNA Ladder **Thermoscientific**, cat. # SM0241
PCR product purification	Purification columns	Ultrafree®-DA Centrifugal Filter Units **Millipore**, cat. # 42600
Reverse line blotting (RLB)	Nitrocellulose Transfer membranes	BioTrace^{TM} NT Nitrocellulose Transfer Membrane **PALL**, prod. ID 66593
	EDC	*N*-(3-Dimethylaminopropyl)-*N*′-ethyl-carbodiimid **Sigma-Aldrich**, cat. # 39391
	$NaHCO_3$	Sodium Hydrogen Carbonate **Applichem**, code 131638
	NaOH	Sodium hydroxide - pellets **Applichem**, code 131687
	Milk	Difco^{TM} Skim Milk **BD Biosciences**, cat. # 90002-594
	SSPE	SSPE (20x), RNase-free **Invitrogen^{TM}**, cat. # AM9767
	SDS	Lauryl Sulfate SDS **Sigma**, No. L3771
	Streptavidin-peroxidase	Streptavidin-Peroxidase Polymer, Ultrasensitive **Merck**, S2438
	TMB stabilized substrate for HRP	TMB Stabilized Substrate for Horseradish Peroxidase **Promega**, cat. # W4121

Table 3: Summary of laboratory devices used in experimental procedures.

Device	*Type*
Centrifuge	Centrifuge 5415 C, **Eppendorf**
	Centrifuge 5415D, **Eppendorf**
	Centrifuge 1415 R, **Eppendorf**
Electrophoresis	SHU6, **Sigma Aldrich**
	OVL Easycast*TM* B2, **Thermo scientific**
PCR cycler	Mastercycler *personal*, **Eppendorf**
	Thermomixer, **Eppendorf**
Flow box	**Gelaire**
PCR box	DNA/RNA UV cleaner UVC/T-M-AR, **Biosan**
Vortex	REAX-top, **Heidolph**
Blotter	Miniblotter 25, **Immunetics**
Spectrophotometer	**Nanodrop**

3. Methods

3.1. Genomic DNA (gDNA) extraction

Analysis of the human samples begins with genomic DNA extraction and purification. A ready-to-use kit is employed to avoid using phenol or chloroform extraction. The samples usually consisted of human tissue (brain), blood/ serum, cerebrospinal fluid, or knee punctate, and the kit is designed to purify total DNA. It makes use of the binding properties of the silica-based membrane present in the spin-column, which absorbs the DNA in the presence of specific salts of the buffer (ATL), filtering all other contaminants through the membrane following centrifugation and two washing steps. The purified samples are stored at $-20℃$ (long-term storage) or $+4℃$ (short-term storage).

gDNA was isolated strictly according to the protocol provided [47]. Shortly:

1. Each sample was washed with 1 mL 1x PBS solution. Then, the cells were collected by centrifugation at 16,100 x g for ~ 20 min. *
2. 180 µL of ATL buffer were added to the cells and thoroughly vortexed until complete resuspension.
3. 20 µL of proteinase K were added to the mix to lyse the cells, followed by thorough vortexing and incubation at 56℃ for 45 min - 1 h until the complete lysis of the cells. Vortex thoroughly a few times during the incubation to disperse the sample.
4. After incubation, 200 µL of buffer AL were added, followed by 200 µL ethanol ($96-100\%$) and thorough vortexing in between the steps. A white precipitate may form, and it is essential to resuspend it by vortexing, pipetting up and down, or reheating the mixture at 70℃.
5. The mixture of Step 4 was pipetted to a spin-column placed in a 2 mL collection tube (provided in the kit) and centrifuged for 1 min at $\geq$ 6,000 x g. The flow-through was discarded together with the collection tube. The spin column was placed in a new collection tube.
6. 500 µL of washing buffer AW1 were added to the spin-column, and the tube was centrifuged for 1 min at $\geq$ 6000 x g. The flow-through was discarded, and the collection tube was replaced.
7. 500 µL of washing buffer AW2 were added, followed by centrifugation for 3 min at $\geq$ 20,000 x g to dry the membrane.
8. The spin column was placed in a sterile 1.5 mL Eppendorf tube, where the gDNA was eluted with $30-100$ µL of nuclease-free water.

* 1x PBS buffer, which maintains pH and osmotic balance, was used in washing to facilitate the lysis of cells by declustering them and allowing the solutions to penetrate better [20].

3.2. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is an omnipotent tool in molecular biology, based on the constructive ability of Taq DNA polymerase, an enzyme that uses nucleotides as building blocks in synthesizing complementary strands to the offered DNA templates. The method uses low quantities of DNA to yield millions of copies of the targeted sequence that can later be used in further analysis [69].

PCR is based on preparing a master mix (MM), where the DNA template is later added. The master mix comprises the enzyme Taq DNA polymerase, the primers, and the nucleotides, alongside some reaction buffers and nuclease-free water. The *HotStarTaq Master Mix Kit* was used for MM preparation, including everything but the primers and DNA template, avoiding excessive pipetting that can introduce contamination [48]. The preparation of the MM for each sample was done on ice, in sterile conditions, following the recipe (Table 4). The primers used in this study are adopted from previous publications. Primers were diluted to a concentration of 0.01 mM from the 0.1 mM stocks.

Table 4: MM preparation recipe.

Compound	*Amount needed for 20 µL*
HotStarTaq Master Mix (2x)	10 µL
Forward primer (FP) (0.01 mM)	1 µL
Reverse primer (RP) (0.01 mM)	1 µL
DNA template (30-100 ng/µL)	3 µL
Nuclease-free water	5 µL

Aliquotes of MM were pipetted to small 200 µL Eppendorf tubes subjected to PCR via a thermocycling machine. On average, the PCR program used is as described in Table 5; the options that vary are the annealing temperatures of primers and the number of cycles. A positive (pure gDNA from *Borrelia* culture as template) and negative control (no DNA, but water as template) is included in each PCR analysis to screen contamination or false-negative amplification.

Table 5: PCR cycling conditions.

Step	*Temperature [°C]*	*Time [s]*
Polymerase activation	95	60-120
25-35 cycles (denaturation, annealing, extension)	95	30-60
	It depends on the primers*	30-60
	72	30-60
Elongation	72	600
Infinite Hold	4	∞

3.2.1. Nested PCR

All samples analyzed originate from humans, so the analysis requires increased sensitivity and specificity. Nested PCR ensures that and prevents the inhibition of the reaction by non-target DNA (human). This process involves using two sets of primers (spacer and nested) and two subsequent PCR amplifications. The first set of spacer primers is used in an initial PCR reaction, and they bind on the outer part of the target sequence. The amplicons from the first PCR reaction are then used as the template for the second PCR amplification, where the second set of nested primers is used. Nested primers anneal with the target sequence downstream from the spacer primers, ensuring an enhanced sensitivity and specificity for the amplification. All works are conducted in a sterilized PCR chamber to decrease contamination, and filtered tips are used [16]. The initial PCR mix is prepared as described in Table 4, and the 3-step cycling is run for 30-35 cycles. 5 µL from the first reaction is used as a template in the second PCR run. The number of cycles is reduced to 25-30 cycles. The primers used for all spacer and nested PCRs are listed in the following table.

Table 6: Data for (spacer and nested) primers for nested PCR.

Target gene	*Primer*	*Sequences: 5` to 3` (Position)*	*T_m [°C]*	*Product size [bp]*	*Ref.*
flaB	Out F	AARGAATTGGCAGTTCAATC (271-290)	52	465	[8]
	Out R	GCATTTTCWATTTTAGCAAGTGATG (743-767)			
	In F	ACATATTCAGATGCAGACAGAGGTTCTA (301-328)	55	388	
	In R	GAAGGTGCTGTAGCAGGTGCTGGCTGT (663-689)			
flaB	Out F	GCATCACTTTCAGGGTCTCA	55	503	[70]
	Out R	TGGGGAACTTGATTAGCCTG			
	In F	CTTTAAGAGTTCATGTTGGAG	58	447	
	In R	TCATTGCCATTGCAGATTGT			
ospC	Out F	ATGAAAAAGAATACATTAAGTGC (306-328)	52	657	[6]
	Out R	ATTAATCTTATAATATTGATTTTAATTAAGG (963-933)			
	In F	TATTAATGACTTTATTTTTATTTATATCT (331-359)	55	617	
	In R	TTGATTTTAATTAAGGTTTTTTTGG (948-924)			

These primers are specific for the whole *Borrelia burgdorferi* s.l. complex and further sequencing is required to determine *Borrelia* species present in the sample.

3.2.2. Species-specific PCR

Species-specific PCR amplification employed in this study uses the primers of the ospA gene specific to the three most dominant *Borrelia* species in Europe, *B. burgdorferi* s.s. (GI), *B. garinii* (GII), and *B. afzelii* (GIII). Aside from the *B. burgdorferi* s.l. complex, species-specific primers (glpQ and p66) were also used to detect *B. miyamotoi*. Re-amplification is used to eliminate the inhibition in the first reaction.

Table 7: Species-specific primers used in PCR assay.

Target gene	*Primer*	*Sequences: 5` to 3` (Position)*	*T_m [°C]*	*Product size [bp]*	*Ref.*
ospA GI	F	AACAAAGACGGCAAGTACGATCTAATT (139-165)	55	543	[13]
	R	TTACAGTAATTGTTAAAGTTGAAGTGCC (655-682)			
ospA GII	F	TGATAAAAACAACGGTTCTGGAAC (201-224)	55	344	
	R	GTAACTTTCAATGTTGTTTTGCCG (522-545)			
ospA GIII	F	TAAAGACAAAACATCAACAGATGAAATG (347-374)	55	188	
	R	TTCCAATGTTACTTTATCATTAGCTACTT (508-536)			
glpQ	Out F	CACCATTGATCATAGCTCACAG	50	-	[15]
	Out R	CTGTTGGTGCTTCATTCCAGTC			
	In F	GCTAGTGGGTATCTTCCAGAAC	52	633	
	In R	CTTGTTGTTTATGCCAGAAGGGT			
p66	Out F	CTAAATTATTAAATCCAAAATCG	50	-	[15]
	Out R	GGAAATGAGTACCTACATATG			
	In F	TTCTATATTTGGACACATGTC	50	-	
	In R	CAGATTGTTTAGTTCTAATCCG			

3.2.3. Gel electrophoresis and gel extraction

The PCR products are visualized using gel electrophoresis, which separates the negatively-charged DNA fragments that travel towards the positive cathode at different speeds due to the size [30]. The concentration of agarose in the gel can be different. In case of the need for further sequencing, the gel concentration is $\leq 0.8\%$ and is prepared in $1x$ modified TAE buffer. If the gel is needed solely for the visualization of products, 1.5% agarose in 1x TAE buffer is prepared. The running buffer used in the electrophoresis chamber should be the same as the one used for the gel preparation. Before loading into the gel slots, $20\ \mu L$ of each sample are mixed with GelRed loading dye (6x) to a 1x final concentration, which allows the immediate loading and visualization of the products against UV light. A DNA ladder is used to estimate the size of the PCR product in the gel.

3.3. PCR product purification and sequencing

To extract DNA from agarose, minicolumns were used. PCR products of the appropriate sizes were cut from the gel, transferred to the columns, and centrifuged at 6,000 x g for 10 min [36].

The concentration of the purified PCR products was measured at **NanoDrop**. The sequencing reaction was collected according to the recommendation of the sequencing facility used (**SeqMe**). One reaction consists of 50 to 100 ng of PCR product (< 500 bp or $500 - 1000$ bp, respectively) and 25 pM of the primer, with the total volume of the reaction being 10 µL. Each PCR product was sequenced with both forward and reverse primers.

3.3.1. Sequence analysis

All sequences were analyzed using **DNASTAR** set of programs. The sequences were then sent to **GenBank** to be compared to the already published sequences. Generally, the result for one sample is confirmed by sequencing amplicons from at least two different primers (Table 4) together with PCR assays with species-specific primers or RLB.

3.4. Reverse Line Blotting (RLB)

Reverse line blotting (RLB) is a subsequent diagnostic step to nested PCR amplification. In this case, the amplification of 5S-23S rRNA intergenic spacer polymorphic region was used. The method integrates up to 25 samples (using a 25-lane membrane format), amplified with the 5S-23S biotin-labeled primer pairs that provide labeled products that hybridize with specific, membrane-bound, amine labeled oligonucleotide probes by RLB. The probes are 5` amine labeled, which allows fixation to the membrane, while the primers are 5` biotin modified to detect the hybridized PCR products by streptavidin peroxidase and a chemiluminescent substrate. The method allows the simultaneous detection and differentiation of *B. burgdorferi* s.l subspecies in human samples [40].

In this study, the RLB assay was conducted using probes (**Generi Biotech**) for *B. burgdorferi* s.l., *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, and *B. bissetii*, (5S-23S) as well as for the glpQ gene for *B. miyamotoi*, using primers mentioned in Table 5, with the difference of nested FP being 5` biotin-labeled. In addition, a panel of species-specific positive controls and negative control are included to detect contamination or false-negative hybridization.

The protocol for the RLB assay follows the steps:

1. Nested PCR amplification using primers specific for 5S-23S intergenic region in case of *Bb* s.l. and glpQ in case of *B. miyamotoi*. The volume of the second reaction was increased up to 50 µL. Data for the primers are given in the following table:

Table 8: Data for (spacer and nested) primers of 5S-23S intergenic spacer region and glpQ gene.

Target gene	*Primer*	*Sequences: 5` to 3` (Position)*	*T_m [°C]*	*Product size [bp]*	*Ref.*
5S-23S	23SN1	ACCATAGACTCTTATTACTTTGAC (469-446)	52	377	[40]
	23SC	TAAGCTGACTAATACTAATTACCC (92-115)			
	5SCB (biotin)	ACCATAGACTCTTATTACTTTGACCA (243-263)	55	266	
	In R	GAGAGTAGGTTATTGCCAGGG (469-444)			
glpQ	Out F	CACCATTGATCATAGCTCACAG	50	-	[40]
	Out R	CTGTTGGTGCTTCATTCCAGTC			
	In F (biotin)	GCTAGTGGGTATCTTCCAGAAC	52	633	
	In R	CTTGTTGTTTATGCCAGAAGGGT			

2. The membrane (14x14cm) was activated with 10% EDC in dH_2O for 30 min at RT.
3. After activation, the membrane was rinsed with dH_2O and placed in the blotter.
4. Probes ($12.5 - 100$ pM) in 500 mM $NaHCO_3$ were loaded on the slots and incubated for 2 min at RT. The empty slots were filled with $NaHCO_3$. The data for the used probes follows:

Table 9: Data for the probes used for the RLB reactions.

Target spirochete	*Sequence*	*Reference*
s.l. (general)	CTTTGACCATATTTTTATCTTCCA	[51]
B. burgdorferi s.s.	AACACCAATATTTAAAAAACATAA	[51]
B. garinii 1	AACATGAACATCTAAAAACATAAA	[51]
B. garinii 2	CAAAAACATAAATATCTAAAAACATAA	[46]
B. afzelii	AACATTTAAAAAATAAATTCAAGG	[51]
B. bissettiiae	AAACACTAACATTTAAAAAACAT	[16]
B. miyamotoi glpQ	GCACGACCCAGAAATTGACAC	[15]

5. The solutions were removed, the order and position were marked, and the membrane was deactivated with 100 mM NaOH for 10 min at RT.
6. The membrane was rinsed with dH_2O and blocked for ≤ 1 h at 56°C (shaking) using 100 mL of 2x SSPE, 0.1% SDS, 5% milk.
7. The washed membrane was placed in the blotter rotated counterclockwise by 90^0.
8. 50 µL of the PCR products were dissolved in 450 µL 2x SSPE 0.1% SDS, heated at 99^0 for 10 min to denature, and chilled on ice. The samples were then loaded in the slots.
9. The samples were hybridized at 45°C for 1 h.
10. After hybridization, the membrane was washed twice for 10 min each with 2xSSPE, 0.5% SDS at 40°C (shaking).
11. The washed membrane was then incubated with streptavidin-peroxidase (diluted 1:40000 in 2xSSPE, 0.5% SDS) for 30 min, at 40°C, in the dark.
12. Another washing step followed (2x) for 10 min each with 2xSSPE, 0.5% SDS at 40°C (shaking).
13. The membrane was rinsed twice with 2xSSPE, and detection was done with 30 mL TMB stabilized substrate for HRP for 10 min in the dark. Lastly, the detection was stopped by rinsing three times with dH_2O.

3.5. Data analysis

All results retrieved from the sample analysis are used to build plots that give a better insight into how the disease affects the population. The plots are created using Excel by **Microsoft Office**. The constructed plots aim to survey what spirochete DNA are mainly present, how the disease affects genders or different age groups, and what trends can be observed between the clinical manifestations of the patients and the *Borrelia* species, the DNA of which was detected in the samples.

4. Results

All samples were investigated for *Borrelia* presence by PCR, sequencing, and RLB. Analysis was performed on three different sample groups:

A. Brain samples (post-mortem) originating from one middle-aged patient XY three days after his death; all samples were analyzed for the presence of *Bb* s.l. spirochetes and *B. miyamotoi*. Necropsy was taken from the following parts of the brain: Occipital lobe, Temporal lobe, Parietal lobe, Frontal lobe, Basal ganglia, Cerebellum, Choroid plexus, Varoli, Spinal cord, Medulla oblongata. Each necropsy was divided into two parts. One part was fixed with 4% formaldehyde for further immunohistochemical processing, and the other part was frozen for subsequent DNA isolation. A detailed diagnosis of a patient is subject to medical confidentiality. It can only be stated that the patient had been suffering from unexplained headaches for a long time, accompanied by depressive moods and memory disorders. The patient developed Lyme disease during school age, which was treated with antibiotics. Although Lyme disease was not confirmed in the patient prior to his death, the patient took high concentrations of various antibiotics for the disease.

B. Human samples (blood, CSF, knee punctate) deemed as problematic according to Czech hospital protocols; there is complete information on the patient's gender, year of birth, age at the time of diagnosis, as well as the respective diagnosis. The samples were analyzed for the presence of *Bb* s.l. spirochetes and *B. miyamotoi*. This analysis was used to suggest a tendency between the diagnosis and spirochete DNA present if possible.

C. Human samples (blood, CSF, knee punctate) deemed as problematic according to Czech hospital protocols, but with insufficient or no information on the samples. The samples were analyzed for the presence of *Bb* s.l. spirochetes and *B. miyamotoi*.

The following images are illustrative results of many performed gel electrophoresis and RLB reactions.

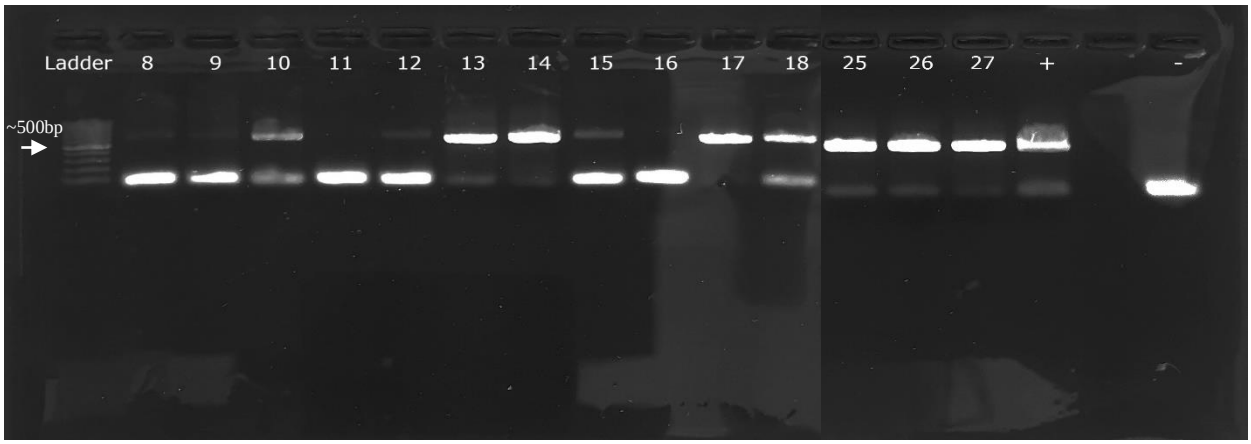

Figure 6: Illustrative gel image of the analysis of samples from group C that underwent PCR amplification of ospA gene with GI primers. The bands showing B. burgdorferi s.s. presence are ~500bp (ladder). Samples: 8, 9, 10, 12, 13, 14, 15, 17, 18, 25, 26, 27, are positive for B. burgdorferi s.s. Negative samples: 11, 16. The second bands showing are remnants of the primers.

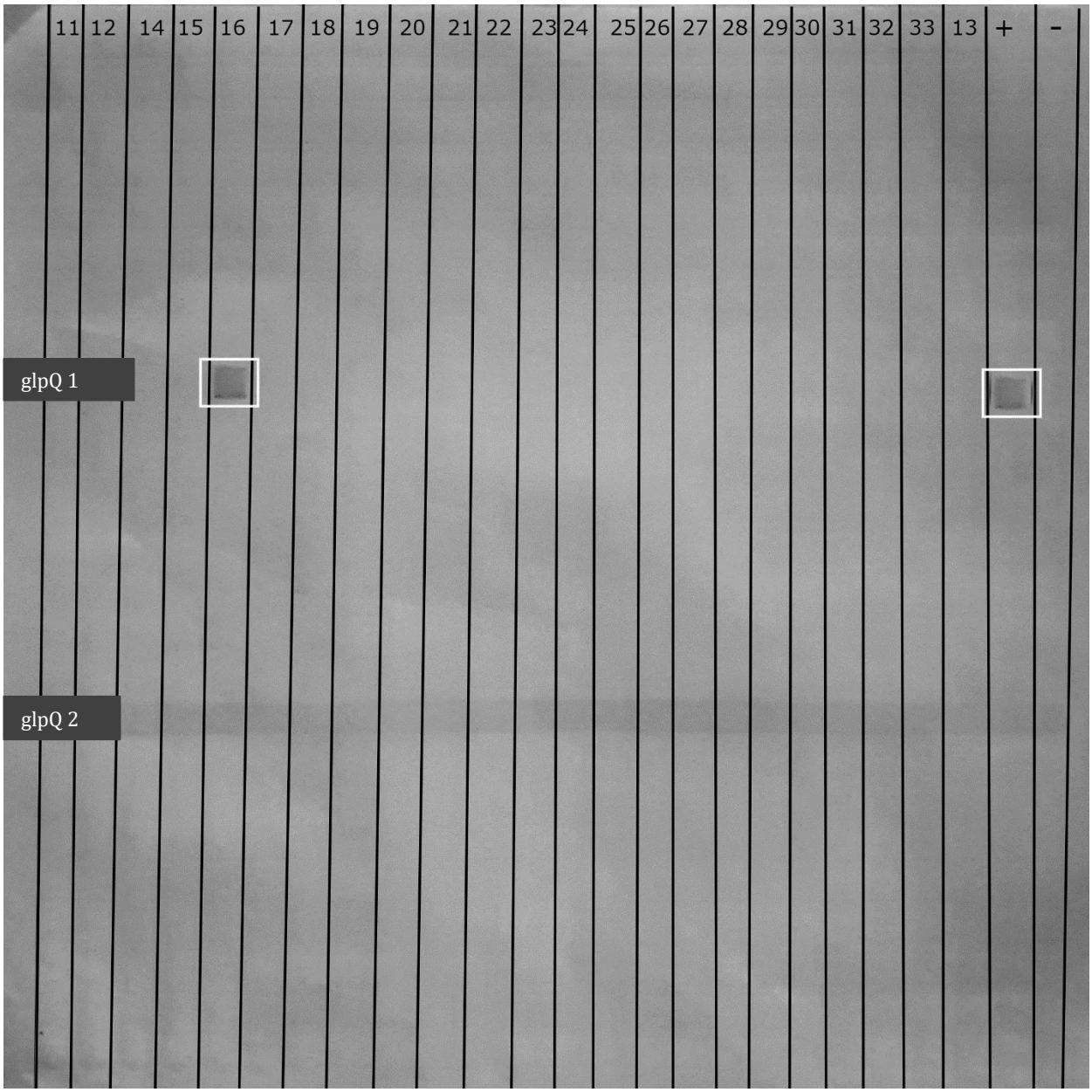

Figure 7: Illustrative image of the RLB analysis of samples of group B reacting with glpQ probes. The first probe was later used for further analysis; the second probe was unsuccessful. The presence of B. miyamotoi in sample 16 was confirmed.

The first sample group was analyzed using species-specific PCR with GI, GII, and GIII primers amplifying ospA gene, nested PCR using OspC and flaB primers (sequencing), and lastly by RLB. They underwent PCR with p66 and glpQ primers that are species-specific for *B. miyamotoi*, and all samples came back negative for its presence. The strains DNA detected in the samples are concluded in the following table.

Table 10: Summary of sequencing results of brain samples (group A).

Sample no.	*Result of ospC gene amplification*	*Result of flaB gene amplification*
1	*B. burgdorferi* s.s.	*B. burgdorferi* s.s.
2	No result	*B. burgdorferi* s.s.
3	*B. burgdorferi* s.s.	No result
4	*B. burgdorferi* s.s.	*B. burgdorferi* s.s.
5	*B. burgdorferi* s.s.	*B. burgdorferi* s.s.
6	*B. garinii*	*B. garinii*
7	*B. afzelii*	*B. garinii*

The next group of patient samples was also analyzed in the same way as the first one, and the sequencing results are summarized in the following table.

Table 11: Summary of data and sequencing results of human samples (group B).

Sample no.	*Age*	*Year of birth*	*Gender*	*Diag. code*	*Material*	*Borrelia detected*
1	3	2015	F	G519	CSF	*B. burgdorferi* s.s. *B. garinii, B. afzelii*
2	4	2014	F	A510	CSF	*B. burgdorferi* s.s. *B. carolinensis*
3	75	1944	F	G510	CSF	negative
4	6	2012	M	R291	CSF	*B. burgdorferi* s.s. *B. garinii*
5	6	2013	M	A692	CSF	*B. garinii*
6	4	2015	M	M175	Punctate	*B. garinii, B. afzelii*
7	67	1951	M	G519	CSF	*B. burgdorferi* s.s. *B. garinii*
8	10	2007	M	G510	CSF	*B. burgdorferi* s.s. *B. garinii*
9	42	1978	M	G519	CSF	negative
10	69	1950	M	R42	CSF	*B. burgdorferi* s.s. *B. garinii*
11	43	1975	M	G98	CSF	*B. burgdorferi* s.s.
12	11	2003	F	A879	CSF	negative
13	9	2005	F	G510	CSF	*B. garinii*
14	68	1948	M	M5440	CSF	*B. bissettii*
15	49	1966	M	A692	CSF	*B. garinii*
16	6	2009	M	R51	CSF	*B. garinii* *B. miyamotoi*
17	7	2010	F	R291	CSF	*B. garinii*
18	8	2009	M	G510	CSF	*B. garinii*
19	9	2008	M	G510	CSF	*B. burgdorferi* s.s.
20	16	1998	M	A692	Punctate	*B. burgdorferi* s.s.
21	38	1975	F	M358	CSF	*B. burgdorferi* s.s.
22	5	2009	F	G510	CSF	inconclusive
23	40	1972	F	G039	CSF	*B. burgdorferi* s.s.
24	9	2005	M	A692	CSF	*B. burgdorferi* s.s.
25	69	1944	M	M2556	Punctate	*B. afzelii*
26	63	1950	M	A692	CSF	*B. afzelii*
27	49	1963	F	G98	CSF	*B. burgdorferi* s.s.
28	9	2005	F	G510	CSF	*B. burgdorferi* s.s.
29	59	1954	M	M5413	CSF	*B. burgdorferi* s.s.
30	7	2008	M	G519	CSF	*B. burgdorferi* s.s.
31	49	1964	M	A692	CSF	*B. garinii*
32	5	2007	M	G519	CSF	*B. burgdorferi* s.s.
33	24	1989	M	R51	CSF	*B. burgdorferi* s.s.

Lastly, the same analysis method was employed for the last sample group. The results are summarized in the following table.

Table 12: Summary of sequencing results of human samples (group C).

Sample no.	*Borrelia spirochete DNA detected*
1	*B. bissettii*
2	*B. burgdorferi* s.s.
3	inconclusive
4	negative
5	negative
6	*B. burgdorferi* s.s.
7	negative
8	*B. burgdorferi* s.s., *B. garinii, B. afzelii*
9	*B. burgdorferi* s.s., *B. garinii*
10	*B. burgdorferi* s.s., *B. garinii*
11	*B. garinii*
12	*B. burgdorferi* s.s., *B. afzelii*
13	*B. burgdorferi* s.s., *B. garinii*
14	*B. burgdorferi* s.s., *B. garinii*
15	*B. burgdorferi* s.s., *B. garinii*
16	*B. garinii*
17	*B. burgdorferi* s.s., *B. garinii, B. afzelii*
18	*B. burgdorferi* s.s., *B. garinii*
19	*B. burgdorferi* s.s., *B. garinii*
20	*B. burgdorferi* s.s., *B. garinii*
21	*B. burgdorferi* s.s., *B. afzelii*
22	*B. burgdorferi* s.s., *B. garinii, B. afzelii*
23	*B. burgdorferi* s.s., *B. garinii, B. afzelii*
24	*B. burgdorferi* s.s., *B. garinii*
25	*B. burgdorferi* s.s., *B. garinii, B. afzelii*
26	*B. burgdorferi* s.s., *B. garinii, B. afzelii*
27	*B. burgdorferi* s.s., *B. garinii*

After analyzing all samples (groups B and C), a plot was constructed to survey the abundance of spirochete DNA found in the samples (Fig. 8), the frequency of co-infection compared to infection with only one strain (Fig 9).

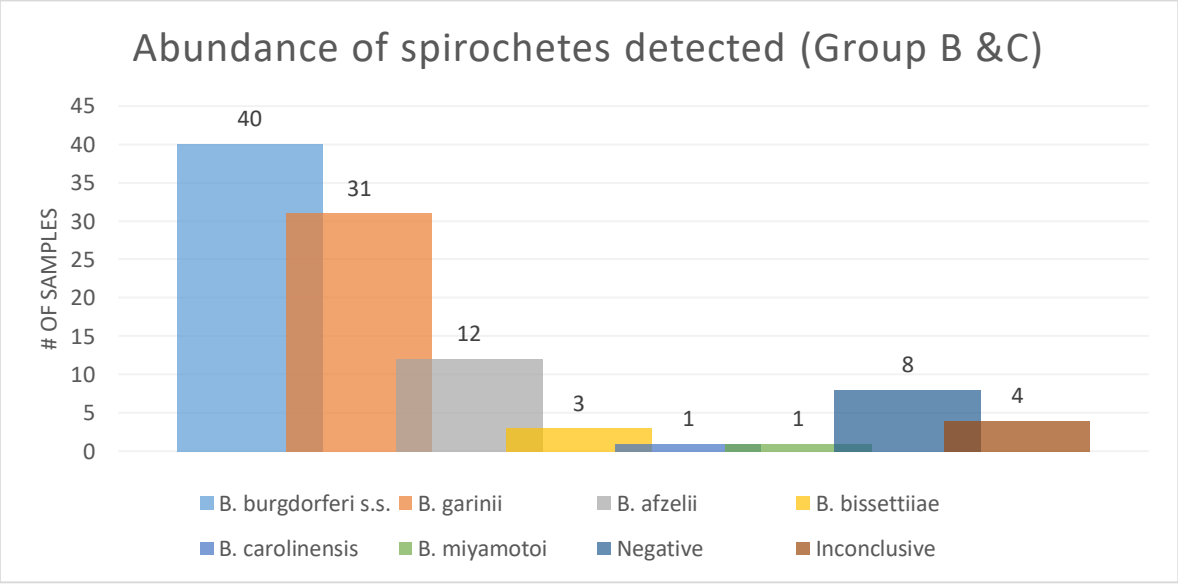

Figure 8: Abundance of spirochetes detected after analysis of human samples, where B. burgdorferi s.s. is the most encountered species.

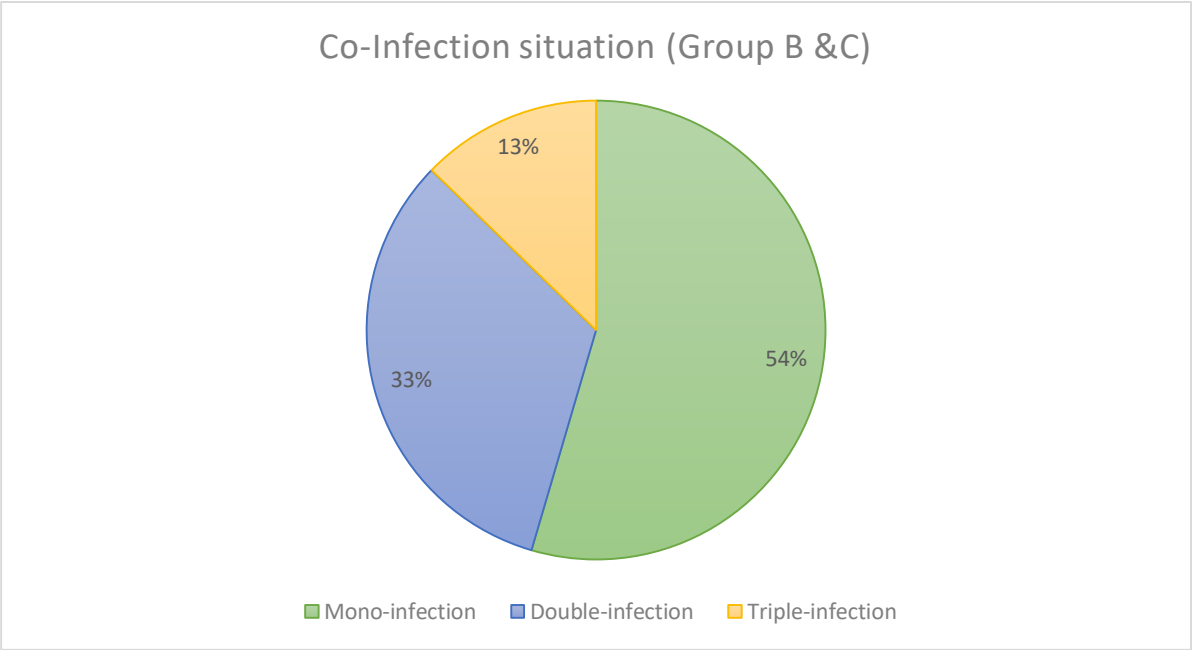

Figure 9: Plot that investigates the presence of co-infection in the analyzed human samples. Even though infection with only one species is more common, the risk of co-infection is also very high and encountered in about 46% of the cases.

In addition, for group B that has more information on the patients, more plots were constructed to observe if gender (Fig. 10) or age (Fig. 11) play a role and suggest a tendency between the spirochetes and the clinical manifestations of the patients (Fig 12).

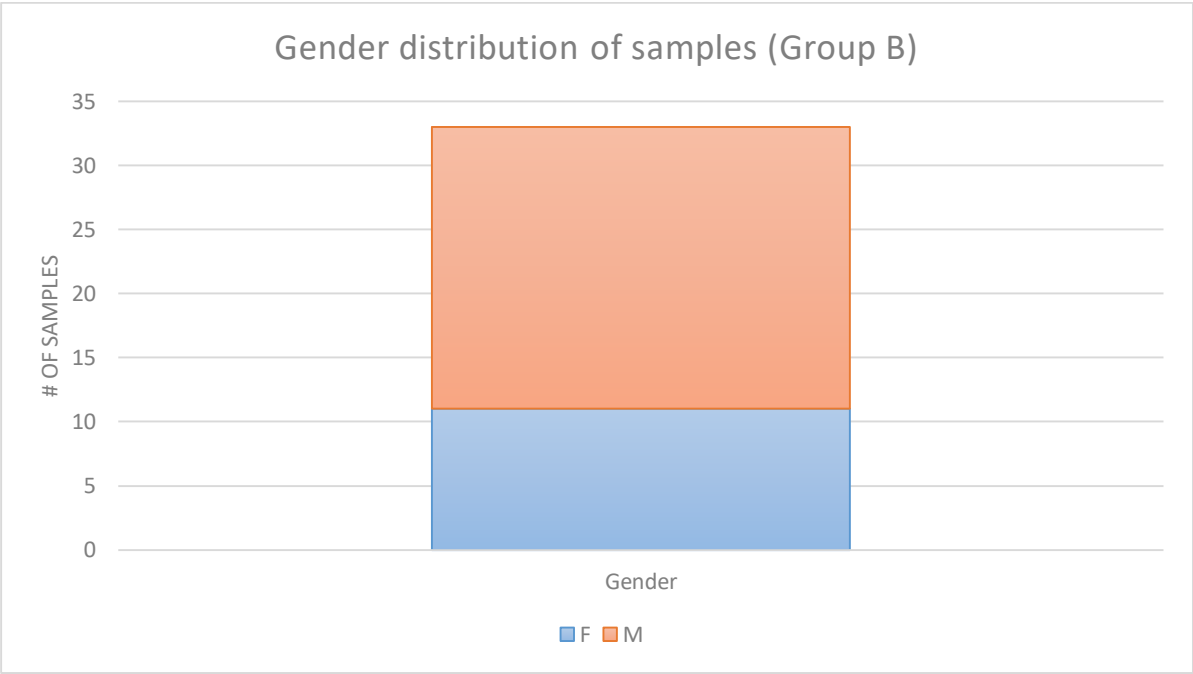

Figure 10: Gender distribution of the patients infected by Borrelia spirochetes. The frequency of infection in males is twice as high as that of females.

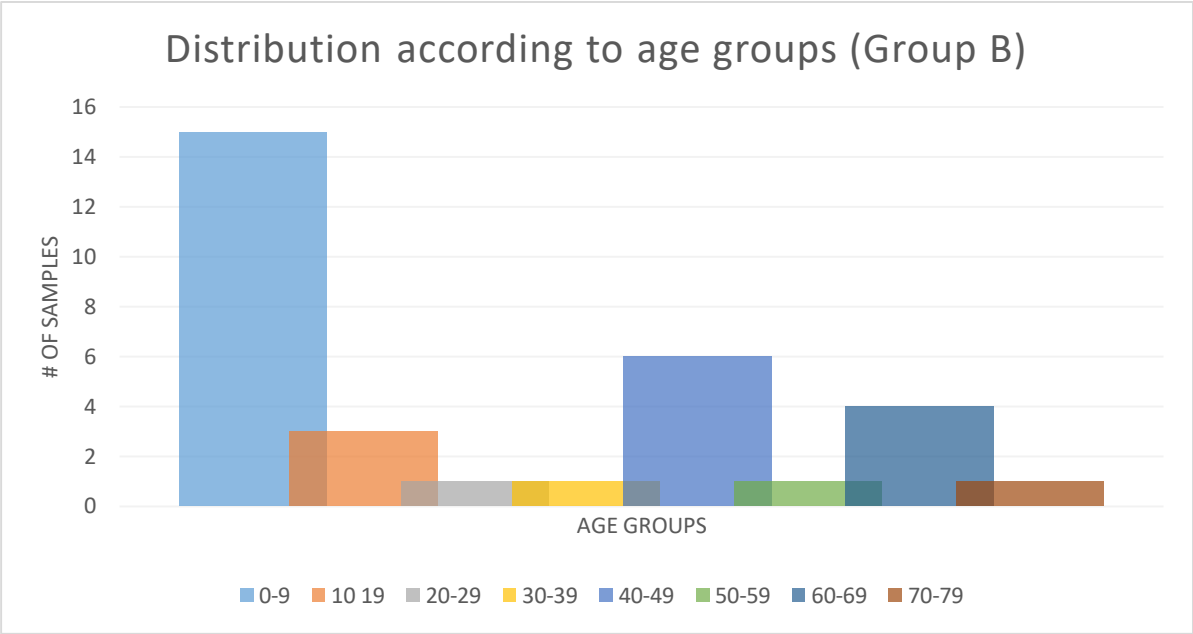

Figure 11: Distribution of sample abundancy according to age groups. The most affected age group is children 0-9 years followed by adults 40-49 years.

The samples of group B are associated with a diagnosis code for each patient, all of which are explained in Table 13 (Appendix), and according to it, four main groups of clinical manifestations were formed, namely:

- Lyme Disease: A692
- Neurological manifestations: A879, G069, G510, G519, G98, R291, R42, R51
- Bone, muscle, joint manifestations: M175, M2556, M358, M5413, M5440
- Other manifestations: A510

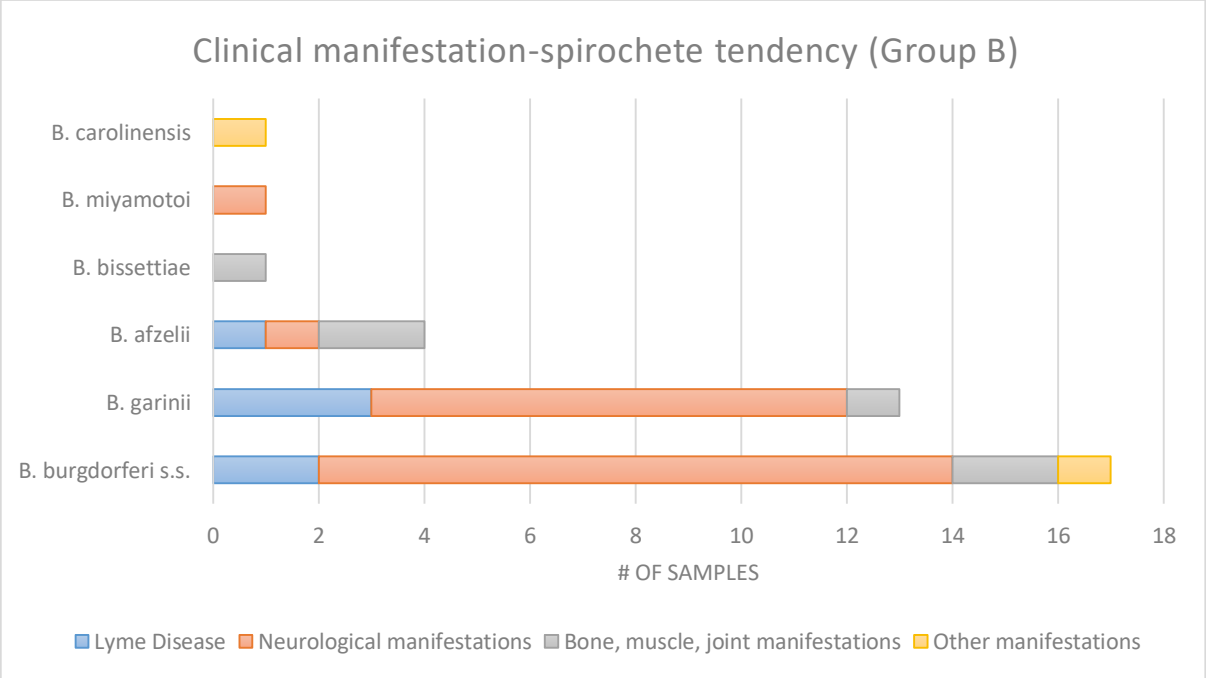

Figure 12: Tendency of spirochetes to cause specific clinical manifestations. A higher tendency is observed when associating the presence of B. burgdorferi s.s. with neurological manifestations.

5. Discussion

Since 1976, Lyme borreliosis has rapidly become the most prevalent vector-borne infectious disease predominantly in the Northern hemisphere, caused by infection with spirochetes of *Borrelia burgdorferi* sensu lato complex [34]. The disease is a multisystem disorder that affects and causes inflammation in various body parts, including skin, muscles, joints, heart, or the nervous system [34]. As the number of *Borrelia* species continues to increase, more and more spirochetes are discovered to have pathogenic potential to humans, increasing the infection risk. Correspondent to different endemic parts of the world, three main spirochetes are continuously described as etiological agents: *B. burgdorferi* s.s. in North America, *B. garinii,* and *B. afzelii* in Eurasia [34] [64]. This study aimed to analyze samples of human origin obtained from patients diagnosed with LB in the Czech Republic. The patients were treated by antibiotics, but their samples were classified as problematic by hospitals. Therefore, the samples were checked for the presence of different species to investigate further into the growing pathogenic group of *Borrelia* spirochetes.

Due to medical confidentiality, not all samples are accompanied by sufficient information about the patients, which would make this study more complete. Samples of human origin tend to be harder to analyze for different reasons; the main reason would be the presence of human DNA or infection by more than one spirochete, which might interfere with the used diagnostic methods.

The first sample group included brain samples of a patient post-mortem, whose information is minimal due to medical confidentiality. However, the patient was said to have suffered from unexplained neurological problems that could potentially be attributed to chronic Lyme borreliosis. Since the patient was reported to have been infected by LB in his young years, which was followed by extensive antibiotic treatment, a possible explanation would be the persistence of the bacterium through the treatment, and potential spirochetamia would explain the presence of spirochetes in the brain. A thorough analysis of the samples exhibited a triple infection by *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii,* where *B. burgdorferi* s.s. was predominant. Although traditionally, *B. garinii* is mainly associated with the neurological manifestations of the disease, the other two spirochetes could also be etiological agents. Another exciting point observed after this analysis is the strong presence of *B. burgdorferi* s.s as a causative agent of LB in Europe, more specifically in the Czech Republic. This phenomenon was previously noticed also in surveys of ticks or different borrelia animal hosts [12] [21], where *B. burdgorferi* s.s. is suggested as a present pathogen in Europe and that additional analysis is required to establish it further.

The rest of the samples analyzed were analyzed by PCR with different primers, the products of which were sequenced. Species-specific PCR and RLB aided the sequencing results to a conclusion. A critical remark on the sequencing results is that it was often noticed that in certain chromatogram positions, more than one high-quality peak was present, hinting that probably more than one *Borrelia*

species was present in the sample. If this was the case, sequencing was repeated a few times until the result matched species-specific PCR or RLB.

Furthermore, the chromatograms obtained were generally not of the highest quality due to possible sample degradation. Another observation made was the presence of inconclusive or negative results, although the patients were already diagnosed. It is curious to see that borrelia DNA is not detected in a patient with suspected LB, and suggested explanations for this would be:

a) It is unknown if the sample collection was done before or after treatment with antibiotics, which might have affected the presence of borrelia DNA.
b) Possible degradation of the samples in time due to improper handling.
c) Incorrect diagnosis by the medical institutions.

The results of groups B and C were collectively plotted to study the spirochete abundancy, and it is noticeable that the most frequent spirochete DNA detected is that of *B. burgdorferi* s.s., followed by *B. garinii* and *B. afzelii* in third place. The presence of *B. burgdorferi* s.s. could potentially explain why these samples were considered problematic by the hospital, being that the typical analysis is aimed at detecting *B. garinii* or *B. afzelii*, which are major LB spirochetes in Europe. As previously mentioned, *B. burgdorferi* s.s. is not a commonly encountered LB pathogen in Europe, suggesting once more that this bacterium could be more present in Europe than formerly thought. Furthermore, the confirmation of *B. bissetti* DNA in the human sample is of great interest. The less frequent spirochete *B. bissetti* has been isolated in Europe from humans [61], and this result confirms this data. In previous cases, *B. bissettii* had a higher manifesting tendency of LNB, but in this study, the patient showed symptoms of back problems.*B. carolinensis* was yet to be confirmed as pathogenic to humans. Unfortunately, this finding can not add any information to its pathogenic potential as it was part of a co-infection with *B. burgdorferi* s.s. that is known to be pathogenic. A fascinating find in this study was the presence of *B. miyamotoi* spirochete in a human, co-circulating with *B. garinii*. No *B. miyamotoi* human infection has been recorded in the Czech Republic this far, but the closest report was the case of a 51-year-old woman in Austria [67] that showed symptoms of relapsing fever, rather than LB, as well as a 74-year-old patient in Germany who showed symptoms of neuroborreliosis [4]. The infection in both cases was with *B. miyamotoi* only. However, in the Czech Republic, *B. miyamotoi* has been found in ticks [10], hinting that there might be more human infections present than the reported numbers portray.

As shown in Fig. 9, almost half of the samples showed a simple infection with only one spirochete species, but the rest showed either double or triple infection with different spirochetes species. The ones that usually made up the duo/ trio were either *B. burgdorferi* s.s., *B. garinii*, or *B. afzelii*, but there were also the previously mentioned cases. It is unknown if the double or triple infections

somehow affect the clinical manifestations, but it can be proposed that the presence of multiple infections could make the proper diagnosis statement difficult.

The Fig. 10 presents the gender distribution of samples (group B), where the presence of LB in males was twice the number of female patients, which is surprising because previous studies in the Czech Republic [36] have stated a higher distribution of cases in females rather than males. This fact can be explained by the limited number of samples obtained for the analysis, most probably with the preference of the samples from the male gender. Additionally, a bimodal trend can be observed in Fig. 11, where a distribution of cases according to the age groups is represented. The majority of cases are children 0-9 years and adults of 40-69 years. These data correlate with the already published information as the trend was also noticed in the latest survey conducted in the Czech Republic [42] and other European surveys [64]. These age groups could mainly be affected due to everyday life activities that require presence in parks, wooded areas, or forests, such as recreational purposes and/or collection of plants or foods. More so, the negligence towards wearing protective clothing, bug repellents, or inspection of the body for ticks could be higher in young children or older adults.

Lastly, the data was used to plot a chart displaying the tendency of spirochetes to cause specific clinical manifestations (Fig. 12). *B. burgdorferi* s.s. was the most abundantly detected spirochete DNA; it also shows a higher tendency to cause specific symptoms, based on the number of samples analyzed. In $\sim 71\%$ of the cases, its 'infection resulted in neurological manifestations, either due to the spirochete itself or co-infection with other spirochetes. The second most prominent spirochete DNA detected was *B. garinii*, which also showed a higher tendency in causing neurological manifestations, an association already suggested by many studies previously. In $\sim 69\%$ of the cases, *B. garinii*'s presence was associated with neurological manifestations, while in $\sim 23\%$ of the cases, the patients were diagnosed with classical Lyme borreliosis, according to hospital protocols. The other detected spirochetal DNA, *B. afzelii*, had a tendency of 25% to be associated with Lyme borreliosis, while in 50% of the cases, the pathogen caused manifestations in bones, muscles, or joins, but it is unclear if that occurred due to infection with *B. afzelii* or co-infection with other spirochetes. An interesting find is that infection with *B. miyamotoi* has led to the appearance of neurological symptoms, but it could also be due to co-infection with *B. garinii*.

A statistical approach was considered in finding a mathematical correlation between the diagnosis of the patients and the spirochetal DNAs detected in their samples, but the number of samples was too low to construct a correlogram. Other than that, it was impossible to establish whether the diagnosis-spirochete correlation was truly significant ($p < 0.05$) or was obtained due to the minimal sample size. Nevertheless, this study provided some important findings on the topic, but further studies of a much larger scale are necessary to provide proper insight into the infection situation in the Czech Republic, where Lyme disease is becoming more and more problematic in endemic areas.

6. Conclusion

Lyme borreliosis has become an emerging infectious disease predominantly in the Northern hemisphere, with more and more cases reported in endemic areas, alongside new regions, annually. The disease is caused by spirochetes of the expanding *Borrelia burgdorferi* sensu lato complex. To date, nine spirochetes have been confirmed to be pathogenic to humans, but nowadays, more are suspected of joining the list. Recently, relapsing fever spirochete, *B. miyamotoi*, attracted much of attention as possible cause of LB as well. Therefore, this study aimed to analyze samples of Czech LB patients, whose samples were classified as problematic by hospital protocols.

The main finding of this study was the abundant presence of *B. burgdorferi* s.s. in patients‘ samples from the Czech Republic, followed by *B. garinii* and *B. afzelii*, with sparse cases of infection with *B. bissettii* and *B. carolinensis*. Another significant finding was detecting of *B. miyamotoi* presence in a human patient, as co-infection with *B. garinii*. Co-infections were also recognized as a frequent phenomenon when observing *Borrelia* infections. All these factors could most probably explain the difficulties in evaluation of these samples accorinding to the standard hospital protocols and therewhy their classification as "problematic".

It is noteworthy to recognize that this study was of a microscopic scale, and much bigger sample sizes are needed to properly assess the wide spectrum of *Borrelia* species that cause LB in the Czech Republic and potentially give more insight into the tendencies of certain spirochetes to cause specific clinical manifestations.

7. Appendix

Table 13: Explanations of all diagnosis codes mentioned in this text, according to ICD-10-CM.

Code of Diagnosis	*Diagnosis*
A510	Primary genital syphilis
A692	Lyme disease
A879	Viral meningitis
G039	Meningitis, unspecified
G510	Bell's palsy
G519	Disorder of facial nerve, unspecified
G98	Other disorders of nervous system not elsewhere classified
M175	Other unilateral secondary osteoarthritis of knee
M2556	Pain in knee
M358	Other specified systemic involvement of connective tissue
M5413	Radiculopathy, cervicothoracic region
M5440	Lumbago with sciatica, unspecified side
R291	Meningismus
R42	Dizziness and giddiness
R51	Headache

8. References

[1] Arvikar, Sheila L., and Allen C. Steere. "Diagnosis and treatment of Lyme arthritis." *Infectious Disease Clinics* 29, no. 2 (2015): 269-280.

[2] Barbour, Alan G. "Phylogeny of a relapsing fever Borrelia species transmitted by the hard tick Ixodes scapularis." *Infection, Genetics and Evolution* 27 (2014): 551-558.

[3] Barbour, Alan G., Jonas Bunikis, Bridgit Travinsky, Anne Gatewood Hoen, Maria A. Diuk-Wasser, Durland Fish, and Jean I. Tsao. "Niche partitioning of Borrelia burgdorferi and Borrelia miyamotoi in the same tick vector and mammalian reservoir species." *The American journal of tropical medicine and hygiene* 81, no. 6 (2009): 1120.

[4] Boden, Katharina, Sabine Lobenstein, Beate Hermann, Gabriele Margos, and Volker Fingerle. "Borrelia miyamotoi–associated neuroborreliosis in immunocompromised person." *Emerging infectious diseases* 22, no. 9 (2016): 1617.

[5] Bunikis, Jonas, Jean Tsao, Ulf Garpmo, Johan Berglund, Durland Fish, and Alan G. Barbour. "Typing of Borrelia relapsing fever group strains." *Emerging infectious diseases* 10, no. 9 (2004): 1661.

[6] Bunikis, Jonas, Ulf Garpmo, Jean Tsao, Johan Berglund, Durland Fish, and Alan G. Barbour. "Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents Borrelia burgdorferi in North America and Borrelia afzelii in Europe." *Microbiology* 150, no. 6 (2004): 1741-1755.

[7] Cardenas-de la Garza, Jesus Alberto, Estephania De la Cruz-Valadez, Jorge Ocampo-Candiani, and Oliverio Welsh. "Clinical spectrum of Lyme disease." *European Journal of Clinical Microbiology & Infectious Diseases* 38, no. 2 (2019): 201-208.

[8] Clark, Kerry, Amanda Hendricks, and David Burge. "Molecular identification and analysis of Borrelia burgdorferi sensu lato in lizards in the southeastern United States." *Applied and Environmental Microbiology* 71, no. 5 (2005): 2616-2625.

[9] Cosson, Jean-François, Lorraine Michelet, Julien Chotte, Evelyne Le Naour, Martine Cote, Elodie Devillers, Marie-Lazarine Poulle et al. "Genetic characterization of the human relapsing fever spirochete Borrelia miyamotoi in vectors and animal reservoirs of Lyme disease spirochetes in France." *Parasites & vectors* 7, no. 1 (2014): 1-5.

[10] Crowder, Chris D., Heather E. Carolan, Megan A. Rounds, Vaclav Honig, Benedikt Mothes, Heike Haag, Oliver Nolte et al. "Prevalence of Borrelia miyamotoi in Ixodes ticks in Europe and the United States." *Emerging Infectious Diseases* 20, no. 10 (2014): 1678.

[11] Cutler, Sally, Muriel Vayssier-Taussat, Agustín Estrada-Peña, Aleksandar Potkonjak, Andrei Daniel Mihalca, and Hervé Zeller. "A new Borrelia on the block: Borrelia miyamotoi–a human health risk?." *Eurosurveillance* 24, no. 18 (2019): 1800170.

[12] Danielová, V., M. Daniel, N. Rudenko, and M. Golovchenko. "Prevalence of Borrelia burgdorferi sensu lato genospecies in host-seeking Ixodes ricinus ticks in selected South Bohemian locations (Czech Republic)." *Central European journal of public health* 12, no. 3 (2004): 151-156.

[13] Demaerschalck, Isabelle, Abdelouahid Ben Messaoud, M. Y. R. I. A. M. De Kesel, Bernard Hoyois, Yves Lobet, Philippe Hoet, Geoffroy Bigaignon, Alex Bollen, and Edmond Godfroid. "Simultaneous presence of different Borrelia burgdorferi genospecies in biological fluids of Lyme disease patients." *Journal of clinical microbiology* 33, no. 3 (1995): 602-608.

[14] Fahrer, Heinz, Sjef M. Van Der Linden, Marie-Josephe Sauvain, Lise Gern, Elyes Zhioua, and Andre Aeschlimann. "The prevalence and incidence of clinical and asymptomatic Lyme borreliosis in a population at risk." *Journal of infectious diseases* 163, no. 2 (1991): 305-310.

[15] Fomenko, N. V., N. N. Livanova, V. Yu Borgoyakov, I. V. Kozlova, I. V. Shulaykina, N. M. Pukhovskaya, N. K. Tokarevich, S. G. Livanov, E. K. Doroschenko, and L. I. Ivanov. "Detection of Borrelia miyamotoi in Ixodes persulcatus ticks in Russia." *Entomological Review* 90, no. 8 (2010): 1088-1094.

[16] Gern, Lise, Véronique Douet, Zully López, Olivier Rais, and Francisca Morán Cadenas. "Diversity of Borrelia genospecies in Ixodes ricinus ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting." *Ticks and tick-borne diseases* 1, no. 1 (2010): 23-29.

[17] Gomes, A., and Br Korf. "Chapter 5-Genetic testing techniques." *Pediatric Cancer Genetics. Amsterdam: Elsevier* (2018): 47-64.

[18] Green, Michael R., and Joseph Sambrook. "Nested polymerase chain reaction (PCR)." *Cold Spring Harbor Protocols* 2019, no. 2 (2019): pdb-prot095182.

[19] Gugliotta, Joseph L., Heidi K. Goethert, Victor P. Berardi, and Sam R. Telford III. "Meningoencephalitis from Borrelia miyamotoi in an immunocompromised patient." *New England Journal of Medicine* 368, no. 3 (2013): 240-245.

[20] Hallmaier-Wacker, Luisa K., Simone Lueert, Christian Roos, and Sascha Knauf. "The impact of storage buffer, DNA extraction method, and polymerase on microbial analysis." *Scientific reports* 8, no. 1 (2018): 1-9.

[21] Hönig, Václav, P. Svec, Petr Halas, Z. Vavruskova, Hana Tykalova, Patrik Kilian, V. Vetiskova et al. "Ticks and tick-borne pathogens in South Bohemia (Czech Republic)–Spatial variability in Ixodes ricinus abundance, Borrelia burgdorferi and tick-borne encephalitis virus prevalence." *Ticks and tick-borne diseases* 6, no. 5 (2015): 559-567.

[22] Hovius, Joppe WR, Bob de Wever, Maaike Sohne, Matthijs C. Brouwer, Jeroen Coumou, Alex Wagemakers, Anneke Oei et al. "A case of meningoencephalitis by the relapsing fever spirochaete Borrelia miyamotoi in Europe." *Lancet* 382, no. 9892 (2013): 658.

[23] Koedel, Uwe, Volker Fingerle, and Hans-Walter Pfister. "Lyme neuroborreliosis—epidemiology, diagnosis and management." *Nature Reviews Neurology* 11, no. 8 (2015): 446-456.

[24] Krause, Peter J., Durland Fish, Sukanya Narasimhan, and Alan G. Barbour. "Borrelia miyamotoi infection in nature and in humans." *Clinical Microbiology and Infection* 21, no. 7 (2015): 631-639.

[25] Krause, Peter J., Jeanne E. Hendrickson, Tanner K. Steeves, and Durland Fish. "Blood transfusion transmission of the tick-borne relapsing fever spirochete B orrelia miyamotoi in mice." *Transfusion* 55, no. 3 (2015): 593-597.

[26] Krishna, Bandari Vamshi, Thogaru Sandeep Reddy, and K. Rohith Kumar. "A Review On Lyme Disease."

[27] Kuehn, Bridget M. "CDC estimates 300 000 US cases of Lyme disease annually." *Jama* 310, no. 11 (2013): 1110-1110.

[28] Kugeler, Kiersten J., Amy M. Schwartz, Mark J. Delorey, Paul S. Mead, and Alison F. Hinckley. "Estimating the frequency of Lyme disease diagnoses, United States, 2010–2018." *Emerging Infectious Diseases* 27, no. 2 (2021): 616.

[29] Kuleshov, Konstantin V., Gabriele Margos, Volker Fingerle, Joris Koetsveld, Irina A. Goptar, Mikhail L. Markelov, Nadezhda M. Kolyasnikova et al. "Whole genome sequencing of Borrelia miyamotoi isolate Izh-4: reference for a complex bacterial genome." *BMC genomics* 21, no. 1 (2020): 1-18.

[30] Lee, Pei Yun, John Costumbrado, Chih-Yuan Hsu, and Yong Hoon Kim. "Agarose gel electrophoresis for the separation of DNA fragments." *JoVE (Journal of Visualized Experiments)* 62 (2012): e3923.

[31] Lenormand, Cédric, Benoît Jaulhac, Sébastien Debarbieux, Nicolas Dupin, Florence Granel-Brocard, Henri Adamski, Cathy Barthel, Bernard Cribier, and Dan Lipsker. "Expanding the clinicopathological spectrum of late cutaneous Lyme borreliosis (acrodermatitis chronica atrophicans [ACA]): a prospective study of 20 culture-and/or polymerase chain reaction (PCR)-documented cases." *Journal of the American Academy of Dermatology* 74, no. 4 (2016): 685-692.

[32] Lommano, Elena, Charles Dvořák, Laurent Vallotton, Lukas Jenni, and Lise Gern. "Tick-borne pathogens in ticks collected from breeding and migratory birds in Switzerland." *Ticks and tick-borne diseases* 5, no. 6 (2014): 871-882.

[33] Margos, Gabriele, Stephanie A. Vollmer, Nicholas H. Ogden, and Durland Fish. "Population genetics, taxonomy, phylogeny and evolution of Borrelia burgdorferi sensu lato." *Infection, Genetics and Evolution* 11, no. 7 (2011): 1545-1563.

[34] Marques, Adriana R. "Lyme disease: a review." *Current allergy and asthma reports* 10, no. 1 (2010): 13-20.

[35] Michelet, Lorraine, Sabine Delannoy, Elodie Devillers, Gérald Umhang, Anna Aspan, Mikael Juremalm, Jan Chirico et al. "High-throughput screening of tick-borne pathogens in Europe." *Frontiers in cellular and infection microbiology* 4 (2014): 103.

[36] Millipore, Ultrafree-DA Centrifugal Filter Units. Cat. # 42600. Site accessed on Aug. 9th, 2021. https://www.merckmillipore.com/INTL/en/product/Ultrafree-DA-Centrifugal-Filter-Unit,MM_NF-42600?ReferrerURL=https%3A%2F%2Fwww.google.com%2F&bd=1

[37] Motaleb, Mohammed Abdul, Linda Corum, James L. Bono, Abdallah F. Elias, Patricia Rosa, D. Scott Samuels, and Nyles W. Charon. "Borrelia burgdorferi periplasmic flagella have both skeletal and motility functions." *Proceedings of the National Academy of Sciences* 97, no. 20 (2000): 10899-10904.

[38] Mukhacheva, T. A., and S. Y. Kovalev. "Borrelia spirochetes in Russia: genospecies differentiation by real-time PCR." *Ticks and tick-borne diseases* 5, no. 6 (2014): 722-726.

[39] Nelson, Christina A., Shubhayu Saha, Kiersten J. Kugeler, Mark J. Delorey, Manjunath B. Shankar, Alison F. Hinckley, and Paul S. Mead. "Incidence of clinician-diagnosed Lyme disease, United States, 2005–2010." *Emerging infectious diseases* 21, no. 9 (2015): 1625.

[40] Niu, Qingli, Guiquan Guan, Jifei Yang, Yuguang Fu, Zongke Xu, Youquan Li, Miling Ma et al. "Detection and differentiation of Borrelia burgdorferi sensu lato in ticks collected from sheep and cattle in China." *BMC veterinary research* 7, no. 1 (2011): 1-9.

[41] Ogrinc, Katarina, Lara Lusa, Stanka Lotrič-Furlan, Petra Bogovič, Daša Stupica, Tjaša Cerar, Eva Ružić-Sabljić, and Franc Strle. "Course and outcome of early European Lyme neuroborreliosis (Bannwarth syndrome): clinical and laboratory findings." *Reviews of Infectious Diseases* 63, no. 3 (2016): 346-353.

[42] Orlíková, Hana, Kateřina Kybicová, Marek Malý, and Jan Kynčl. "Surveillance and epidemiology of Lyme borreliosis in the Czech Republic in 2018 and 2019." *Biologia* (2021): 1-10.

[43] Petrulionienė, Agnė, Daiva Radzišauskienė, Arvydas Ambrozaitis, Saulius Čaplinskas, Algimantas Paulauskas, and Algirdas Venalis. "Epidemiology of Lyme disease in a highly endemic European zone." *Medicina* 56, no. 3 (2020): 115.

[44] Piesman, J., J. G. Donahue, T. N. Mather, and A. Spielman. "Transovarially acquired Lyme disease spirochetes." *Borrelia burgdorferi* (1986): 446-447.

[45] Platonov, Alexander E., Ludmila S. Karan, Nadezhda M. Kolyasnikova, Natalya A. Makhneva, Marina G. Toporkova, Victor V. Maleev, Durland Fish, and Peter J. Krause. "Humans infected with relapsing fever spirochete Borrelia miyamotoi, Russia." *Emerging infectious diseases* 17, no. 10 (2011): 1816.

[46] Poupon, Marie-Angèle, Elena Lommano, Pierre-François Humair, Véronique Douet, Olivier Rais, Michael Schaad, Lukas Jenni, and Lise Gern. "Prevalence of Borrelia burgdorferi sensu lato in ticks collected from migratory birds in Switzerland." *Applied and Environmental Microbiology* 72, no. 1 (2006): 976-979.

[47] Qiagen, DNeasy Blood & Tissue Kit, cat. # 69504. Site accessed on Aug. 6th, 2021. https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit/

[48] Qiagen, HotStarTaq Master Mix Kit (1000U), cat. # 203445. Site accessed on Aug. 6th, 2021. https://www.qiagen.com/us/products/discovery-and-translational-research/pcr-qpcr-dpcr/pcr-enzymes-and-kits/end-point-pcr/hotstartaq-master-mix-kit/?catno=203445

[49] Radolf, Justin D., Klemen Strle, Jacob E. Lemieux, and Franc Strle. "Lyme Disease in Humans." *Current issues in molecular biology* 42, no. 1 (2021): 333-384.

[50] Radolf, Justin D., Melissa J. Caimano, Brian Stevenson, and Linden T. Hu. "Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes." *Nature reviews microbiology* 10, no. 2 (2012): 87-99.

[51] Rijpkema, S. G., M. J. Molkenboer, Leo M. Schouls, Frans Jongejan, and J. F. Schellekens. "Simultaneous detection and genotyping of three genomic groups of Borrelia burgdorferi sensu lato in Dutch Ixodes ricinus ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes." *Journal of clinical microbiology* 33, no. 12 (1995): 3091-3095.

[52] Rizzoli, A. N. N. A. P. A. O. L. A., HEIDI CHRISTINE Hauffe, G. I. O. V. A. N. N. A. Carpi, G. I. Vourc'h, M. Neteler, and R. Rosa. "Lyme borreliosis in Europe." *Eurosurveillance* 16, no. 27 (2011): 19906.

[53] Robinson, Matthew L., Takaaki Kobayashi, Yvonne Higgins, Hugh Calkins, and Michael T. Melia. "Lyme carditis." *Infectious Disease Clinics* 29, no. 2 (2015): 255-268.

[54] Rollend, Lindsay, Durland Fish, and James E. Childs. "Transovarial transmission of Borrelia spirochetes by Ixodes scapularis: a summary of the literature and recent observations." *Ticks and tick-borne diseases* 4, no. 1-2 (2013): 46-51.

[55] Rudenko, Natalie, and Maryna Golovchenko. "Sexual Transmission of Lyme Borreliosis? The Question That Calls for an Answer." *Tropical Medicine and Infectious Disease* 6, no. 2 (2021): 87.

[56] Rudenko, Nataliia, Maryna Golovchenko, Libor Grubhoffer, and James H. Oliver Jr. "Updates on Borrelia burgdorferi sensu lato complex with respect to public health." *Ticks and tick-borne diseases* 2, no. 3 (2011): 123-128.

[57] Rudenko, Natasha, M. Golovchenko, M. Vancova, K. Clark, L. Grubhoffer, and J. H. Oliver Jr. "Isolation of live Borrelia burgdorferi sensu lato spirochaetes from patients with undefined disorders and symptoms not typical for Lyme borreliosis." *Clinical microbiology and infection* 22, no. 3 (2016): 267-e9

[58] Sato, Kozue, Ai Takano, Satoru Konnai, Minoru Nakao, Takuya Ito, Kojiro Koyama, Minoru Kaneko, Makoto Ohnishi, and Hiroki Kawabata. "Human infections with Borrelia miyamotoi, Japan." *Emerging infectious diseases* 20, no. 8 (2014): 1391

[59] Scoles, Glen A., Michele Papero, Lorenza Beati, and Durland Fish. "A relapsing fever group spirochete transmitted by Ixodes scapularis ticks." *Vector borne and zoonotic diseases* 1, no. 1 (2001): 21-34.

[60] Schwan, Tom G., Merry E. Schrumpf, B. Joseph Hinnebusch, Donald E. Anderson Jr, and Michael E. Konkel. "GlpQ: an antigen for serological discrimination between relapsing fever and Lyme borreliosis." *Journal of clinical microbiology* 34, no. 10 (1996): 2483-2492.

[61] Stanek, G., and M. Reiter. "The expanding Lyme Borrelia complex—clinical significance of genomic species?." *Clinical Microbiology and Infection* 17, no. 4 (2011): 487-493.

[62] Stanek, G., V. Fingerle, K-P. Hunfeld, B. Jaulhac, R. Kaiser, A. Krause, W. Kristoferitsch et al. "Lyme borreliosis: clinical case definitions for diagnosis and management in Europe." *Clinical Microbiology and Infection* 17, no. 1 (2011): 69-79.

[63] Steere, A. C. Lyme disease. *N. Engl. J. Med.* **321**, 586–596 (1989).

[64] Steere, Allen C., Franc Strle, Gary P. Wormser, Linden T. Hu, John A. Branda, Joppe WR Hovius, Xin Li, and Paul S. Mead. "Lyme borreliosis." *Nature reviews Disease primers* 2, no. 1 (2016): 1-19.

[65] Stone, Brandee L., Yvonne Tourand, and Catherine A. Brissette. "Brave new worlds: the expanding universe of Lyme disease." *Vector-Borne and Zoonotic Diseases* 17, no. 9 (2017): 619-629.

[66] Taylor, Kyle R., Ai Takano, Satoru Konnai, Michito Shimozuru, Hiroki Kawabata, and Toshio Tsubota. "Borrelia miyamotoi infections among wild rodents show age and month independence and correlation with Ixodes persulcatus larval attachment in Hokkaido, Japan." *Vector-Borne and Zoonotic Diseases* 13, no. 2 (2013): 92-97.

[67] Tobudic, Selma, Heinz Burgmann, Gerold Stanek, Stefan Winkler, Anna-Margarita Schötta, Markus Obermüller, Mateusz Markowicz, and Heimo Lagler. "Human Borrelia miyamotoi infection, Austria." *Emerging infectious diseases* 26, no. 9 (2020): 2201

[68] Vasudevan, Biju, and Manas Chatterjee. "Lyme borreliosis and skin." *Indian journal of dermatology* 58, no. 3 (2013): 167.

[69] Waters, Daniel LE, and Frances M. Shapter. "The polymerase chain reaction (PCR): general methods." In *Cereal Genomics*, pp. 65-75. Humana Press, Totowa, NJ, 2014.

[70] Wills, Melanie KB, Andrea M. Kirby, and Vett K. Lloyd. "Detecting the Lyme disease spirochete, Borrelia burgdorferi, in ticks using nested PCR." *JoVE (Journal of Visualized Experiments)* 132 (2018): e56471.

[71] Wolcott, Katherine A., Gabriele Margos, Volker Fingerle, and Noémie S. Becker. "Host association of Borrelia burgdorferi sensu lato: a review." *Ticks and Tick-borne Diseases* (2021): 101766.

[72] Wolcott, Katherine A., Gabriele Margos, Volker Fingerle, and Noémie S. Becker. "Host association of Borrelia burgdorferi sensu lato: a review." *Ticks and Tick-borne Diseases* (2021): 101766.

[73] Zückert, Wolfram R. "Laboratory maintenance of Borrelia burgdorferi." *Current protocols in microbiology* 4, no. 1 (2007): 12C-1.