University of South Bohemia in České Budějovice

Faculty of Science

Detection of persistent forms of *Borrelia burgdorferi* sensu stricto in infected mice after antibiotic treatment

Bachelor Thesis

Ida Ramzy

Supervisor: Marina Golovchenko, MSc.

Co-supervisor: Natasha Rudenko MSc., PhD.

Biology Centre CAS, Institute of Parasitology

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Annotation

Antibiotic treatment of Lyme disease is a challenging procedure with unexpected outcomes that may arise sometime. According to standard protocols recommended by the Centers for Disease Control and Prevention, USA (CDC) for early Lyme disease, a short course of oral antibiotics cures the majority of cases. This thesis deals with the analysis of the efficiency of 2 majorly used antibiotics, doxycycline and amoxicillin, in elimination of *Borrelia* infection on laboratory mice model after 2 weeks of treatment. Our results confirmed the presence of persistent forms of spirochetes in mice tissues after antibiotic treatment using the spirochete cultivation method in liquid MKP medium.

Declaration

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List of abbreviation

Amox	amoxicillin
ATB	antibiotic
AV	atrioventricular
bp	base pairs
BSK	Barbour-Stonenner-Kelly
CDC	Centers for Disease Control and Prevention
DNA	deoxyribonucleic acid
dNTP's	deoxynucleotide triphosphates
Doxy	doxycycline
ECL	enhanced luminol-based chemiluminescent
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPS	exopolysaccharide
GBP	glucan-binding protein
HEPES	N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
LD	Lyme disease
MKP	modified Kelly-Pettenkofer medium
ospC	outer surface protein
PCR	polymerase chain reaction
PTLD	post-treatment Lyme disease
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electro-phoresis
TAE	tris-acetate-EDTA
TBS	tris-buffered saline

Abstract

Lyme disease is a tick borne illness caused by the spirochetes of *Borrelia burgdorferi* sensu lato complex. This tick borne disease generates controversy among medical providers and researchers for decades. According to Centers for Disease Control and Prevention (CDC), people treated with antibiotics in the early stage of Lyme disease using CDC protocol are usually able to recover rapidly and completely. The most commonly prescribed antibiotics for early Lyme disease treatment are doxycycline and amoxicillin and combination of both. One of the key topics of the debate about Lyme disease is the existence of persistent infection with the Lyme spirochete in patients who have been treated with recommended doses of antibiotics.

The aims of this thesis are to analyze the efficiency of both antibiotics in elimination of the Lyme disease spirochetes, applying the CDC protocol on infected mice model and to examine the ability of replicating spirochetes to survive antibiotic treatment in host tissues in persistent forms. In this study we present the evidence of persistent *Borrelia* infection despite antibiotic therapy in mice.

Finally, we opt to analyze the reversibility of the process of atypical spirochetal forms formation, i.e. the ability of dormant/persistent forms of spirochetes to transform to the replicative spiral forms under the favorable conditions.

Summary

The efficiency of two antibiotics used for the treatment of *Borrelia burgdorferi* sensu stricto, doxycycline and amoxicillin, was evaluated on mouse model. Firstly, the mice were injected with NE-5264 (*B.b.* s.s.) strain of *Borrelia*. Ten days after inoculation, we confirmed the infection by Western blotting and polymerase chain reaction (PCR). Antibiotics were then administered to the infected mice, using the adjusted CDC protocol for human treatment to the laboratory mice model. The antibiotics (doxycycline and amoxicillin) were used in treating the mice both separately and in combination. Following the antibiotic treatments, spirochetes were re-cultivated from the tissue samples of the mice. Using multiple detection methods, we were able to prove that mice had ongoing infection despite antibiotic treatments. The optimum treatment for *Borrelia* infection remains to be determined.

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1 Introduction

1.1 Lyme disease (LD)

Lyme disease is a multisystem disorder caused by the spirochete from the *Borrelia burgdorferi* sensu lato complex, which is transmitted primarily through the bite of *Ixodes* ticks (1). According to the Centers of Disease Control and Prevention, Lyme disease accounts for more than 80% of vector borne diseases and if untreated can cause long lasting debilitating symptoms (2). The CDC estimates approximately more than 300,000 US citizens diagnosed with Lyme disease each year (3).

The causative agent of LD, *Borrelia burgdorferi* sensu lato complex is composed of 22 named species, including three, that are recognized as a definite causative agent of Lyme disease in humans and 5 other as a highly probable ones. *B. burgdorferi* sensu stricto*, B. garinii,* and *B. afzelii* are amongst those species that definitely infect humans (4). In the US, according to CDC the disease is caused exclusively by *B. burgdorferi* sensu stricto and *B. mayonii*, whereas in Eurasia, *B. garinii, B. bavariensis* and *B. afzelii* are the most common species of borrelia that infect humans, followed by *B. burgdorferi* ss. Other strains found in human samples in Eurasia are *B. spielmanii, B. bissettii, B. lusitaniae,* and *B. valaisiana* (5).

B. garinii and *B. afzelii* are antigenically different from *B. burgdorferi* sensu stricto, and it is widely accepted that each species is responsible for a different Lyme disease manifestation. All genospecies can trigger appearance of erythema migrans, the skin manifestation of the early disease. The most severe chronic diseases appear to be caused by *Borrelia burgdorferi* sensu stricto, with skeletal, neurological and intermittent cardiovascular manifestations arising from hematogenic transmission. *B. garinii* is specifically associated with neuroborreliosis, whereas *B. afzelii* is affiliated in causing late skin manifestations, such as borrelial lymphocytoma, and acrodermatitis chronica atrophicans (6).

1.1.1 Stages of Lyme disease

Three stages are recognized in human Lyme disease: 1) the early localized (acute) stage, 2) the early disseminated stage and 3) the late disseminated stage. The manifestations of the acute stage are characterized sometime by a solid red or bull's eye rash (also known as erythema migrans) at the site of the tick bite. It appears days to weeks after the initial tick bite. The rash starts to expand and ends up forming an even larger lesion of around 70 cm in diameter (7). Erythema migrans is the only manifestation of Lyme disease that is sufficiently distinctive to allow clinical diagnosis in the absence of laboratory confirmation but unfortunately it appears only in very small number of the LD cases. In 2007, the study showed that 53.8% of patients who were diagnosed with Lyme disease revealed no signs of erythema migrans and were therefore misdiagnosed (8). Other symptoms of the early stage of LD include swelling of the lymph glands near the bite, fever, fatigue, myalgia and arthralgia (9).

The early disseminated stage which occurs weeks to months later after the initial infection is manifested by smaller, but multiple erythema migrans all around the body regardless of where the initial bite was. This stage occurs when the bacteria begins to overtake and spread throughout the body and migrate within the bloodstream. Other common manifestations of the early disseminated stage could be cardiac, ophthalmic symptoms, cranial and facial nerve paralysis and signs of meningitis (when the bacteria spread to the linings of the brain). Additionally, if the bacteria infect the heart tissue, carditis or inflammation of the heart could occur. Although heart tissue inflammation can affect all sorts of functions, it often occurs as an atrioventricular (AV) heart block, which means that the signal from the upper chambers to the lower chambers is blocked or held up and thus affects the timing of the heartbeat (10). In 1980 Steere et al. tested 20 patients diagnosed with Lyme disease. According to their results, 18 patients had inconsistent changes of AV heart block, while 8 of the patients' hearts were entirely blocked (11). In another study done on Rhesus monkeys, one monkey out of the five infected animals developed swelling in multiple joints 14 days after inoculation of *B. burgdorferi*. On the other hand, neurological symptoms such as oculomotor paresis and facial paralysis were not noted (12).

The final stage of Lyme disease, which is known as the late disseminated stage, occurs generally up to a year after infection. This is when the infection has fully spread throughout the whole body. It is characterized by chronic arthritis of two or more joints, including knees in particular. Occasionally, neurological disorders develop as well as numbness in the extremities. Usually this is generally linked to with insomnia, impaired intellection, and infrequently behavioral changes (13).

1.1.2 Chronic Lyme disease

As it was mentioned above, the existence of persistent infection with the Lyme spirochete in patients who have been treated with recommended doses of antibiotics, is the topic of the ongoing debate between medical doctors, researchers and medical providers.

"Post-Lyme disease treatment syndromes" or Chronic Lyme diagnoses, is proposed to describe patients who experience continuous symptoms following Lyme disease antibiotic treatment. The US Center for Disease Control and Prevention (CDC) estimates that approximately "10 to 20% of patients treated for Lyme disease with a recommended 2-4 week course of antibiotics will have persisting symptoms of fatigue, pain, or joint and muscle aches" (14). People with persistent symptoms often experience episodes of fatigue, restless sleep, aching joints, pain or swelling in the knees, decreased short-term memory or ability to concentrate and speech problems.

The opposite view suggests that the chronic signs of Lyme disease could be attributed to spirochetal "debris" without active infection. Borrelial DNA or antigens in human tissues was observed in a range of reports in Europe and the United States. However, studies that have shown the culture of live *Borrelia* spirochetes, which is the most efficient evidence for the persistent infection in patients with chronic Lyme disease, are rather limited (15).

1.1.3 Different methods of LD diagnosis

The diagnosis of Lyme disease can be sometimes tricky, especially in the absence of erythema migrans. Furthermore, Lyme disease can mimic the

symptoms of fibromyalgia, chronic fatigue syndrome, multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's and Alzheimer's, and more than 350 other diseases, resulting in significant difficulty in diagnosis (16). Accurate diagnosis of early Lyme disease is especially essential, as delayed or missed diagnosis may have severe complications in the latter. Sometimes even diagnosing erythema migrans can be challenging as the lesion may originally be mistaken with other infections such as nummular eczema, granuloma annulare, ringworm, or even spider bites (18).

The first and foremost direct way of spirochete detection is by microscope. There are two microscopic techniques, dark field and phase contrast microscopy. It is important to note however, that negative findings of *Borrelia burgdorferi* identification in biological fluids and tissues should not be disregarded due to the low concentrations that occur as single spirochetes and the apparent lack of colony formation in tissues (19). Most of the studies use microscopes to check cultures that are cultivated in a nutrient rich media, like the BSK II medium (34°C), for positive bacterial growth.

The most common way to detect spirochete in the bloodstream is through standard serological tests such as western blotting, Enzyme-Linked Immunosorbent Assay (ELISA), and immunofluorescence assay. These indirect detection tests are susceptible to false negative and false positive outcomes and, particularly during the course of the disease, may be inaccurate. Henceforth, it is important to use other methods of detection (20).

With the high technology developed today, the polymerase chain reaction (PCR) has been the most useful to identify *B. burgdorferi* DNA. Although studies in research highly praise PCR testing, contamination is a requiring issue in laboratories and hence it is exceedingly essential to acquire suitable testing material. PCR is a method that enables a particular fragment of DNA to be amplified from a diverse DNA (21). However, the positive detection of borrelia DNA in the tissue does not indicate the presence of live spirochetes there.

1.2 Typical and atypical forms of *Borrelia*

The dormant *B. burgdorferi sensu lato* is considered to be pleomorphic, meaning that it is capable of changing its morphology in response to opposing environmental conditions. Researchers during the 19th century were the first to propose that *Borrelia* spirochetes had multiple atypical forms. Presently, it is known for a fact that various bacteria are able to alter their forms both *in vivo* and *in vitro* (22). The morphology of *Borrelia* spirochete is primarily the spiral form during its growth phase, however it may transform into other forms including (but not limited to) granules, L-forms, cyst forms and biofilm communities. This happens when the bacteria are subjected the presence of a strong immune response or appear in adverse conditions (23). All these forms of *Borrelia* are able to reverse back into their original form under favorable conditions.

1.2.1 Spirochetal form of *Borrelia*

Spirochetes are bacteria that are long, thin and spiral-shaped. They spin or twist in order to move around (24). The length of the spirochete ranges from 8-30 μm, with width as small as 0.2-0.5 μm (25). *Borrelia* can be easily distinguished from other types of bacteria due to their feature of linear chromosomes and 21 distinct circular and linear plasmids or micro chromosomes. A complete genome sequence of *B. burgdorferi* sensu stricto (B31) was published in 1997, with 853 genes on linear chromosome of 910,725 base pairs with around 29% of G and C content, and 21 plasmids with a size of 613,000 base pairs. Among all, no genes synthesizing amino acids, nucleotides, and fatty acids were discovered. Instead, DNA synthesis (including replication, translation, transcription) as well as, recombination, storage, uptake of nutrients, metabolism, and gene expression regulation code for their genes. The spirochete can therefore only develop within nutrients, amino acids,

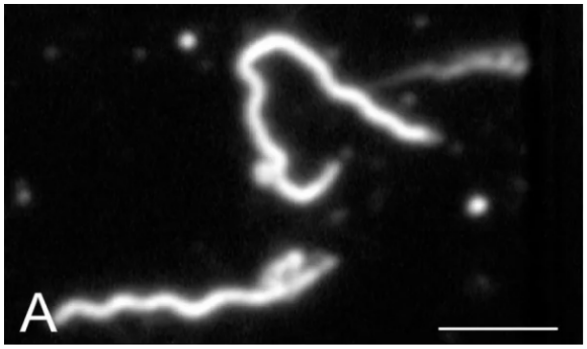

Figure 1: Taken from Miklossy, J. et al., is the characteristic morphology of *Borrelia burgdorferi* under a dark field microscope showing the usual spiral form of spirochetes (28).

antioxidants, bovine serum albumin and rabbit serum in a BSK medium (26). Many researchers have observed that the Lyme disease spirochete attaches to the human cell's tip, wiggles and squirms until it enters the cell. This causes the cell to release digestive enzymes that would eventually dissolve the cell, allowing the spirochete to go move in its desired locations (27).

1.2.2 Granules or blebs

Lyme disease spirochetes have been observed vigorously shaking and breaking into pieces, producing small particles known as blebs or granules. *In vitro* studies have shown that *B. burgdorferi* transforms into membrane blebs sizing from small vesicles to sizes surpassing spirochetal cylinders (25). According to Meriläinen et al., blebs are found to be an "intermediate stage" between the spirochete and round bodies, with an outer membrane and a protoplasmic cylinder inside (22). It has been found that both linear and circular DNA molecules could be identified on the "pinched-off" bits of the cell wall or purified membrane blebs (29). Treating with antibiotics like penicillin and ceftriaxone increased the formation of blebs after bacteria incubation for 24 hours. Similarly, membrane blebbing was developed by exposure of doxycycline after 96 hours of incubation (30).

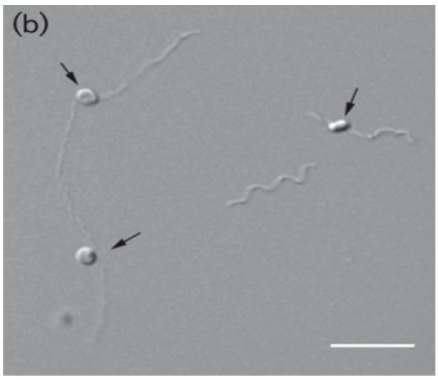

Figure 2: Meriläinen et al., observes blebs on spirochetes (black arrows) (22).

It has also been argued that granules are the remnants of previous outer surface proteins which have been shed in an attempt to confuse and evade the immune system. Blebs or granules is another way that *B. burgdorferi* survive the action of bacterial agents.

1.2.3 Cell wall deficient form (L-forms)

Another atypical form of borrelia formed in response to unfavorable conditions is so called L-form or cell wall deficient form. The spirochete becomes unable of maintaining its spiral shape, when it loses its cell wall. When the spirochete no longer carries its cell wall, it

also loses proteins that are markers or signals to the human immune system. Consequently, the immune system has difficulty tracing this new form of bacteria. L-forms can be reverting back to its regular cell wall form in the absence of antibiotics or after coming back to the favorable environment (31).

1.2.4 Cyst forms

B. burgdorferi develops cyst forms when a single Lyme disease spirochete curls up into a ball and forms a sheath around itself, becoming highly resistant to most antibiotics. Referring to Brorson and Brorson, the presence of cysts were seen due to unfavorable environment conditions where the spirochete was incubated for 2 weeks in BSK II medium without the addition of rabbit serum (which is an important source of fatty acids) (32). Gunter et al. were able to successfully determine the change of *B. burgdorferi* sensu lato complex's cystic forms back into their normal spirochete forms. They also showed the ability of *B. garinii* to convert back to its motile spirochete forms in both *in vitro* and *in vivo* even with an unexpected environment of freeze/ thawing the cysts (33). Moreover, studies have also observed that the antibiotics such as penicillin, doxycycline and ceftriaxone, triggered the formation of cysts when cultivated in a nutrient-rich liquid Barbour-Stoenner-Kelly (BSK) medium (30, 34).

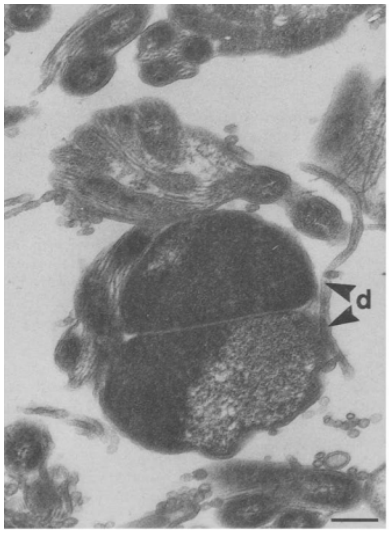

Figure 3: Taken from Brorson and Brorson, the cyst (d) is in the process of dividing (32).

1.2.5 Biofilm communities

Spirochetes may also often develop biofilm communities. Biofilm communities are protein barrier or shells that protect the bacteria from antibiotics. K.K. Jefferson discussed the four driving forces behind biofilm formation (35). Those include, biofilm formation in response to defense, colonization to remain a favorable habitat, communal behavior and as a default mode of growth.

Biofilms are resilient against physical forces which are produced naturally in organisms. They are able to endure changes in pH, deficiency of nutrients, build ups of oxygen radicals and most important are resistant to antibiotics (35). Phagocytes that attempt to engulf a biofilm may actually cause more harm to the tissues surrounding it than the biofilm itself, thus making biofilms resistant to phagocytes. An important feature that biofilms acquire and which plays a role in resistance is their hydrated matrix of polysaccharide and protein. Together they form a sticky, slimly layer which contains DNA and polymers, including exopolysaccharides (EPS). Biofilms are able to resist forces and phagocytes by the help of EPS (36). Moreover, it was shown that surface components, such as glucan-binding protein (GBP), in *Streptococcus mutans*, for example, plays a role in polysaccharide production and thus biofilm formation (37).

Multicellular organisms have dedicated tissues or cells primarily for dealing with the threat of an infection due to the critical reason of microorganisms constantly trying to invade or inhabit into their bodies. Therefore, they develop a strong immune system to fight against those microorganisms. Because the body is filled with rich nutrients, there is a constant ongoing race between bacterial strategies trying to invade the body and the development of an intricate immune system against it. Occasionally, at some point a compromise between the body and bacteria must be made and that is where biofilms inhabit in the body. Given that the body is clearly a favorable location for bacteria to reside, the main motive for changing to biofilm communities may be to stay fixed in the body. It was found that the carbon catabolite induced gene regulation plays a vital role in biofilm formation (38). The production of EPS and biofilms are increased significantly in some bacteria when there is an abundance of glucose or other useable carbon source. The bacteria becomes planktonic when nutrients are reduced, and therefore indicating that the lack of nutrients causes the bacteria to migrate in search of a better niche. A further supporting hypothesis that K.K. Jefferson proposes is that bacteria could be able to perceive high concentrations of glucose as a signal that they are in the bloodstream. As a result, indicating that they need to create biofilms in order to flee from the blood and guard themselves from the incoming antibodies (35).

Although bacteria are single celled microorganisms, one could argue that biofilms exhibit unselfish, cooperative behavior which could be greatly compared to multicellular organisms.

The first evidence of bacterial transition from unicellular to multicellular was seen on fossils of prokaryotes and cyanobacteria like organisms indicating that the first multicellular organisms could have been bacteria that have transformed into a biofilm environment (39). Biofilms are greatly compared to multicellular organism due to the similarities they share in the sense that biofilms will have abilities and properties that are much greater than single cell organisms. For example, biofilms are able to influence gene expression and communicate between cells by secreting specific substances that helps them do so. Similarly to multicellular organisms, biofilms are able to sense theirs near surroundings which help them to regulate metabolism, optimize their availability of nutrients and protect their environments. Additionally, the same way as cellular differentiation occurs in multicellular organisms, biofilms also experience a similar process. When bacteria is growing inside a biofilm, changes in gene expression naturally befalls, which results in phenotypic heterogeneity that can be regarded as cellular division. However, bacterial cells do not enduringly differentiate. Instead they react and modify to their environmental surrounding for their own mode of survival. Therefore, it is more valid to refer to biofilms as colonial organisms rather than multicellular organisms. Working in a community benefits the bacteria much greater than working separately and for this reason, they manifest selfless properties and gene transfer (35).

The fourth and final theory proposed for bacteria to transform into biofilm is simply it being part of their natural biological cycle. Bacteria are likely to grow as biofilm for most of their life. Even the development of a biofilm can easily be triggered by the existence of an appropriate substrate. Biofilms can be developed even while growing cells planktonically in laboratory conditions (40). Just by the interaction between a surface and bacteria, changes in gene regulation may begin to arise. This implies that cells detect the surface they are attached to. This processing mechanism causes a signaling pathway that could lead to premature gene expression essential for the development of biofilm. A study shows that *Staphylococcus. epidermidis* cells begin to form some sort of outgrowth from their body just by slight contact with a surface (41). Such research indicates that bacterial cells have sensing mechanisms that stimulate strong signals to cause changes genetically and morphologically. As new nutrients are supplied, biofilms will proceed to evolve. Only when there is a lack of nutrients, biofilms start to detach themselves from the surface and switch to their planktonic growth pattern (38).

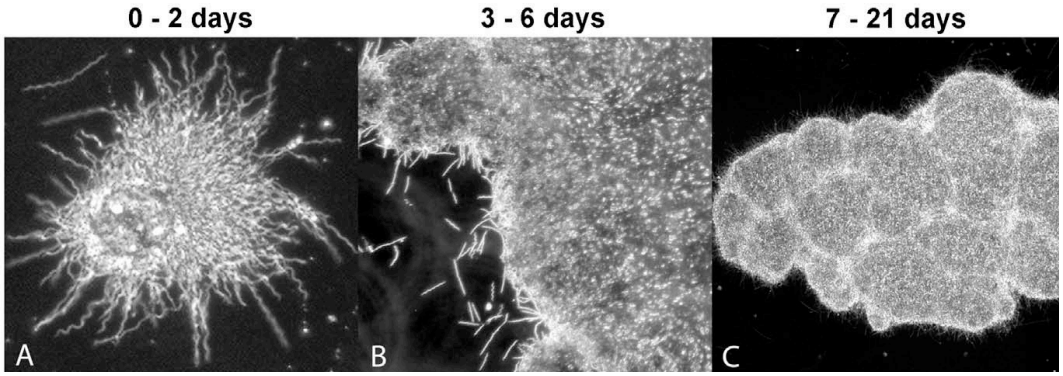

Figure 4: Characterization of biofilm communities shown by Sapi et al., in 2012 (42).

1.3 Antibiotic treatment and chronic Lyme disease

Antibiotic treatment for Lyme disease can be challenging as unexpected outcomes can arise.

A 2-4 week course of antibiotics such as doxycycline, amoxicillin, or cefuroxime is currently the ongoing treatment for Lyme disease (43). Amoxicillin interferes in specific steps to inhibit or disrupt the cell wall of the bacteria which leads to the death of the bacteria, while doxycycline enters the bacteria to stop the replication and multiplication of the bacteria. Nevertheless, even though some patients with Lyme disease who receive this treatment for around 6 months, still continue to have chronic symptoms (14).

In some situations, excessive or insufficient treatment procedures may exert an undue pressure on bacteria (44), which therefore, leads to an antibiotic resistance of some tolerant bacteria that find themselves in a less competitive and favorable environment (45). The transformation of spirochetal *Borrelia* into atypical persistent forms is sometime triggered by antibiotics used to cure early Lyme disease (46). *Borrelia* thus becomes tolerant to the drugs which activated spiral change. Doxycycline and amoxicillin are good examples of antibiotics that can kill 98% of replicating *Borrelia* when administered at high dosages, but still show no activity against an immobile culture with high concentrations of persistent forms (47). In a study done by Sapi et al., treatment with higher concentrations of doxycycline showed around 90% of spirochetal structure reduction, but a twofold increase of round body forms. On the other hand, treatment with higher concentrations of amoxicillin reduced spirochetal structures by around

70% and round body forms by approximately 68% (48), which can conclude that both drugs are ineffective against round body forms. Comparisons of gene expression changes between both drugs indicate that they share certain characteristics that are related to spirochete survival within or after antibiotic treatment. For example, it was found that a number of ribosomal genes were down-regulated in persistent *B. burgdorferi,* which decreases the demand for metabolism and energy, allowing cells to transition into persistent cells (43).

The failure of antibiotic treatment and the recurrence of bacterial infections or illnesses is due to the tolerance of dormant forms to antibiotics (49). Cystic forms of *Borrelia* were discovered in the brains of three patients after ATB treatment with confirmed chronic Lyme neuroborreliosis. The location of this more resistant form of *Borrelia* may explain the dormant, ongoing chronic infection (28). Studies have found that biofilms are likely to cause chronic diseases due to antibiotic tolerance and the resistance to host serum (50). It was confirmed that the biofilm's resistance to antibiotics has not been influenced even after the degradation of the cell wall of the biofilm's surface. This implies that alginate from biofilm might not be the only reason for antibiotic resistance, but other components may play a role (51).

Contrary to resistant cells that develop because of antibiotics, persister cells do not develop when antibiotics are present. Instead they arise due to the state of dormancy, when the cells are inactive (52). Persister cells usually compromise about 1% in the immobile state and in biofilms (53, 54). Hobby et al. discovered that in *Staphylococcus aureus*, 1% of cells that were not destructed by penicillin became persister cells (52). Persister cells in biofilms seem to be the cause of chronic infections since antibiotics destroy the majority of cells, yet persister cells still remain thriving and reproductive when the level of antibiotic reduces (55).

2 Materials

Table 1: Materials used in this study.

Borrelia cultivation	
BSK – II medium	complete BSK – II medium 6% rabbit serum
Persteril – solution of acids, hydrogen peroxide and acetic acid (Acidum peraceticum)	0,5 % solution
DNA isolation	
Genomic DNA isolation kit	DNeasy® Blood & Tissue Kit (250), (Qiagen)
PCR products purification (columns)	Centrifugal Filter Units DNA extraction from agarose (Millipore)
PCR	
PCR 2x Master Mix	Taq DNA polymerase (supplemented with e reaction buffer) (pH 8.5), 200µM dATP, 200µM dGTP, 200µM dCTP, 200µM dTTP, 3mM MgCl2 (Promega)
DNA electrophoresis	
50x TAE buffer	200mM Tris-HCl, 50mM EDTA
Agarose	0,8 – 2 % agarose (Serva) for DNA ELFO in 1xTAE buffer
6x sample buffer	Blue/Orange 6x loading dye, (0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 15% Ficoll™ 400, 10mM Tris-HCl (pH 7.5) a 50mM EDTA (pH 8.0) (MBI Fermentas)
PCR Marker	Gene Ruller 100 bp Plus DNA Ladder (Thermo Scientific)

Western Blotting	
Preparation of protein lysate	Borrelia culture 10^9/ml, HN- Buffer (50mM NaCl, 50 mM HEPES, pH 7.6), B-PER- protein extraction solution (Thermo Scientific),
Solutions for SDS-PAGE	30% acrylamide/0.8% bis-acrylamide (Roth) 2.5 resolving gel buffer: 1.875M TrisCl, pH 8.9, 0.25% SDS 5x stacking gel buffer: 0.3M Tris-phosphate, pH 6.7, 0. 5% SDS
5x Running buffer	0.5M Tris base, 1.92M glycine, 0.5% SDS (pH 8.8)
2x Laemmli loading buffer	0.125M Tris-HCl (pH 6,8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, dH_2O
Transfer (blotting) buffer	25mM Tris base, 150 mM glycine, 10% methanol (pH 8.3)
Blocking buffer	5% milk in TBS, pH 7.4
TBS TBS-Tween buffer	10 mM Tris-Cl, pH 7.5, 150 mM NaCl 20 mM Tris-Cl, pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween 20
Staining and detection solutions	Immobilon Western HRP Substrate (Millipore) Pierce ECL Western Blotting Substrate (Thermo Scientific) Detection Reagent A (Peroxide Solution) and Detection Reagent B (Luminol Enhancer Solution)

Table 2: Laboratory supplies used in this study.

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

3 Methods

3.1 Genomic DNA isolation, preparation of positive control template by pretreatment of bacterial cells.

To serve as a template for positive controls during the experiments, the genomic DNA was isolated from the *Borrelia* cultures of different species and strains originated from local ticks:

Borrelia afzelii strain CB 43 – (Ceske Budejovice, Czech Republic)
Borrelia garinii strain K 112 – (Košice, Slovakia)

Genomic DNA was purified by Qiagen DNeasy blood and tissue kit strictly according to the provided protocol. Briefly:

1. Centrifuge pure culture at 9,000 rpm at 4°C.
2. Remove supernatant.
3. Wash with PBS (2x) and move culture into 1.5 ml Eppendorf tube.
4. Centrifuge again for 10 minutes at 7,500 rpm and discard supernatant.
5. Add 180 µl of ATL buffer and resuspend by vortexing.
6. Add 20 µl of proteinase K and mix thoroughly by vortexing.
7. Incubate at 56°C until tissue is completely lysed (15-30 minutes).
8. Remove from incubator and vortex for 15 seconds.
9. Add 200 µl of AL buffer to the sample, and mix thoroughly.
10. Add 200 µl of 100% ethanol and vortex thoroughly, then incubate at 70°C for 10 minutes.
11. After 10 minutes, pipet all the mixture into a DNeasy mini spin column placed in a 2ml collection tube.
12. Centrifuge at 8,000 rpm for 1 minute and discard flow-through and collection tube.
13. Place a new collection tube and add 500 µl of AW1 buffer while centrifuging again at 8,000 rpm for 1 minute.
14. Discard flow-through and collection tube.

15. Place the mini column in a new collection tube, add 500 μl of AW2 buffer and centrifuge at 8,000 rpm for 1 minute (3x).
16. Discard flow through and collection tube.
17. Place the mini column in a clean 1.5 ml Eppendorf tube, add 100 μl of H_2O to elute and incubate for 1 minute at room temperature.
18. Centrifuge for 1 min at 8,000 rpm.
19. Place DNA at 4°C if not used immediately.

Afterwards, the concentration of the genomic DNA was measured using NanoDrop spectrophotometer. The measurements were repeated for three times in each sample and the average value was calculated.

3.2 PCR (polymerase chain reaction) analysis

Polymerase chain reaction (PCR) is a technique that enables the amplification of a particular region of DNA between two regions of a specific sequence (primers). PCR reaction includes the PCR water, a buffer mix, Taq DNA polymerase, primers, nucleotides, and DNA templates in appropriate concentrations.

PCR water:

Double-distilled Milli-Q-purified water was used for dilution of each reagent.

2x PCR pre-mix which contains:

1. DNA polymerase 10x Buffer mix: PCR is carried out in a buffer that provides a suitable chemical environment for activity of DNA polymerase. The buffer ensures a proper pH of around 8.0 so that the reaction can proceed in optimal conditions.
2. Taq DNA Polymerase: The Taq DNA polymerase is the key enzyme that links the individual nucleotides together in order to form a PCR product. It is a major tool of

DNA extension while the primers and deoxynucleotide triphosphates (dNTP's) are present.

3. dNTP's: deoxynucleotide triphosphates consist of four basic nucleotides which build up the blocks of the new DNA strands- dATP, dCTP, dGTP, and dTTP.

Table 3: PCR primers used in this work.

Primers	**Target species**	**Oligonucleotides Sequence**	**Size of PCR product (bp)/annealing temperature**
G1 forward	*B. burgdorferi* sensu stricto	AACAAAGACGGCAAGTACGATCTAATT	543 55°C annealing
G1 reverse		TTACAGTAATTGTTAAAGTTGAAGTGCC	
G2 forward	*B. garinii*	TGATAAAAAC AACGGTTCTG GAAC	344 55°C annealing
G2 reverse		GTAACTTTCAATGTTGTTTTGCC	
G3 forward	*B. afzelii*	TTCCAATGTTACTTTATCATTAGCTAC	189 55°C annealing
G3 reverse		TAAAGACAAAACATCAACAGATGAAAG	
Flagellin forward (out)	*Borrelia burgdorferi*	AARGAATTGGCAGTTCAATC	497 52°C annealing
Flagellin reverse (out)		GCATTTTCWATTTTAGCAAGTGATG	
Flagellin forward (in)		ACATATTCAGATGCAGACAGAGGTTCTA	388 55°C annealing
Flagellin reverse (in)		GAAGGTGCTGTAGCAGGTGCTGGCTGT	

Taken from Demaerschalck et al., 1995 (56) and Clark et al., 2005 (57).

Template:

Amplification depends on the amount of DNA template used and quality of it. The template used in our PCR reactions was the genomic DNA isolated from borrelia spirochetes (positive control) and different animal tissues that were under investigation:

Reaction mixture (1 reaction - 20µl):

2x PCR Master Mix……....10 µl

0,1mM primer Forward……...1 µl

0,1 mM primer Reverse……...1 µl

H_2O……..…variable µl

Template DNA< 200 ng/reaction ……variable

PCR reaction cycles:

The above mentioned components were mixed in 0.2 ml PCR tubes and then placed in a machine that allows repeated cycles of DNA amplification to occur in the following steps.

Table 4: PCR steps, temperature and time needed for each step.

Steps (30x)	Temperature	Time
Initial denaturation	95 °C	5 mins.
Denaturation	95 °C	30 secs.
Annealing	52 - 55°C depending on primers used	30 secs.
Elongation	72 °C	1 min.
Final elongation	72 °C	10 min.
Final hold	4 °C	hold

Steps 2-4 were repeated 30 times. Each reaction included positive (borrelia gDNA as a template) and negative (H_2O instead of DNA) controls.

3.3 Gel electrophoresis

The results of every PCR reaction were checked by gel electrophoresis. Agarose gel (1%) was prepared by dissolving (boiling) of 2 g of agarose in 200 ml of 1x TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Loading dye (6x concentrated) was added to each sample. The dye contained SYBR green for DNA visualization under UV light. The samples were then loaded on the gel, and run for around 20-40 minutes at 100-150 V. The gel is then removed and viewed under UV light. The results of each PCR were documented.

3.4 *Borrelia* cultivation

3.4.1 Preparation of the modified Kelly-Pettenkofer medium for borrelia cultivation from mouse organs.

200 ml of basic MKP medium was prepared.

Table 5: The amount of each reagent used for preparation of basic medium (200 mL).

Reagent	**Mass (g)**
CMRL-1066 (10x) without Glutamine	1.94
Neopeptone	0.6
HEPES	1.2
Citric Acid	0.14
Glucose	0.6
Pyruvic Acid	0.16
N-acteylglucoseamine	0.08
Sodium biocarbonate	0.4

All powdered components were added to 100 ml ddH2O and mixed until completely dissolved. The pH was then adjusted with 5N NAOH to 7.6. A final volume was adjusted to 200 ml by adding dd H_2O. The solution was sterilized by filtration through 0.22 microne filter, aliquoted into 50 mL tubes and stored at -20°C freezer.

Preparation of complete MKP medium:

Table 6: The amount of each reagent used for the preparation of the complete MKP medium.

Reagent	Volume (mL)
Basic MKP medium	100
7% Gelatin	20
Rabbit serum	7.2
35% BSA	1.225

The basic medium should not be kept for more than 3 months at -20 °C. All the ingredients were mixed together while heating at 33°C until completely dissolved. The solution was sterilized by filtration through a 0.22 microne filter and aliquoted. Modified MKP should never be frozen but stored at +4 C°.

3.4.2 Preparation of Barbour-Stonner Kelly II (BSK II) medium

Table 7: For preparation of BSK II media (500 ml) the following reagents were used:

Reagent	Mass (g)
10x CMRL	4.85
Neopeptone	2.5
BSA	25
Yeastolate	1
HEPES acid	3
Glucose	2.5
Sodium citrate	0.35
Sodium pyruvate	0.4
N-acetyl glucosamine	0.2
Sodium bicarbonate	1.1
Gelatin	5

The first 3 ingredients were mixed and stirred until dissolved. Other ingredients were added and dissolved. Gelatin should be dissolved bit by bit in boiling water while constantly stirring. pH was adjusted to 7.6, then flask was placed at 55°C until it was ready to be filtered through 0.22 microne filter. The medium was aliquoted into 100 ml bottle. Thirty ml of rabbit serum was added to each aliquot and stored frozen (-20°C).

3.4.3 *Borrelia burgdorferi* cultivation, dark-field microscopy, density calculation

Three different strains of *Borrelia* were grown in liquid media from the frozen bacterial stocks:

Borrelia burgdorferi sensu stricto SLV-1 (Slovenia- cultured from human, *ospC* type L)
Borrelia burgdorferi sensu stricto NE-5264 (Switzerland - cultured form from *I. ricinus, ospC* type B)
Borrelia burgdorferi sensu stricto SCW-53 (USA- cultured from *I. affiinis*, *ospC* type B)

During the cultivation of *Borrelia* in liquid medium, the cultures were continuously checked under a dark field microscope. Controls monitored the growth of spirochetes and culture purity. The number of spirochetes in cultures was monitored in the dark field microscope under a magnification of 40x and was determined by counting individual spirochetes using a Petroff - Hausser counting chamber. To determine the exact numbers of cells, the average number of *Borrelia* in the 5 larger squares of the Petroff-Hausser counting chamber was calculated. The final density was calculated using the formula A x 1.25 x 10^6, where A is the average number of borrelia spirochetes in the 5 squares of the chamber.

According to density and absence of contamination strain NE-5264, *Borrelia burgdorferi* sensu stricto, was chosen for further experiments.

3.5 Mice infection with *B. burgdorferi*

The group of mice in the experiment included 10 pathogen free C3H stain females, 6 week old, originated from a local animal facility. For spirochete inoculation, 9 mice were injected

with 150 µl of 10^5 of NE-5264 culture abdominally and under the skin. One mouse (a negative control) was injected with pure BSK II medium. After 4 weeks, the infectivity of mice was checked. Mice were put under methoxyflurane anesthesia and infection status was assisted by ear punch biopsies and blood sample which were collected from the eyes of the mice using a capillary tube. Mouse ear biopsy and blood samples were checked by PCR for the presence of borrelia DNA and by Western blotting.

3.5.1 Mice serum preparation

After collecting of the whole blood from mice, blood was allowed to clot for around 30 minutes by leaving at room temperature. The clot was separated from serum by centrifugation at 1,000-2,000 x g for 10 minutes at room temperature. The resulting supernatant (serum) was collected into clean 1.5 mL Eppendorf tubes. If not analyzed immediately, serum was stored at -20°C or lower.

3.5.2 Preparation of protein lysate

For the preparation of the protein lysate, the growing culture of *Borrelia burgdorferi* sensu stricto NE-5264 strain (that was injected into mice) was used. Culture was first checked under microscope for high density.

The following protocol for protein lysate preparation was used:

1. Centrifuge culture for 10 minutes at 20°C (8,000 rpm), remove the supernatant.
2. Add 1 ml of HN-buffer and mix well.
3. Transfer into 1.5 ml Eppendorf tube.
4. Centrifuge for 10 mins at 20°C (8,000 rpm).
5. Remove supernatant. Repeat steps 2 and 4-5.
6. Add 200 µl of B-PER-Bacterial protein extraction and mix well.
7. Leave for 10 mins at room temperature.
8. Measure concentration of protein on Nano drop.

9. Add 190 µl 2x Laemmli sample buffer and 10µl of β-mE Capto together in a 1.5 ml Eppendorf tube and mix well.
10. Add that sample in the protein lysate and mix.
11. Freeze at -20° C.

3.5.3 Western blotting (immunoblotting)

1. Prepare 12% SDS gel.
2. Heat prepared protein lysate with appropriate amount of loading dye for 15 mins at 100°C for denaturation and immediately after place on ice.
3. Add 8 µl of protein marker into first well of the gel.
4. Add 15 µl of protein lysate into each well.
5. Run the gel at 120 V for 60 minutes –> until the bands reach the bottom of the gel.
6. Cut the piece of nitrocellulose membrane the size of the gel and activate it in methanol for 10 min. Rinse the membrane in water.
7. Wet 2 papers of blotting paper and 1 piece of nitrocellulose membrane in blotting buffer for 15 mins.
8. Wet the surface of blotting apparatus with blotting buffer.
9. Add the blotting sandwich in the following way:
    a. Blotting paper.
    b. Nitrocellulose paper.
    c. SDS page gel (remove it from glass plate carefully to avoid breakage and mark the positions of the wells).
    d. Blotting paper.
10. Wet the sandwich with blotting buffer and smooth the sandwich with a roller to remove the air bubbles.
11. Close apparatus and let it run for 30 mins at 25 V.
12. In the meantime, prepare blocking solution by mixing 100 ml of 1xTBS + Tween 20 with 5 g of milk powder.
13. Let the blocking solution stir for approximately 30 minutes.

14. Open apparatus, discard gel and shake the nitrocellulose paper for 2 hours in blocking solution.
15. After 2 hours, cut and label the nitrocellulose paper in pieces.
16. In meantime, prepare the mice serum with blocking buffer:
    a. Mix 995 µl of blocking solution with 5 µl of serum into a new 1.5 ml Eppendorf tube (dilution 1:200).
17. Each stripe is covered with 1 ml of different sample (mixture of blocking solution and sera) in a plastic bag.
18. Plastic bag is closed, labelled and left overnight at +4 C°.
19. The next day, the stripes of membranes were left shaking for 1 hour.
20. Remove the mixture from the plastic bags (membrane).
21. Wash the membranes for 15 mins with 1x TBS + tween buffer.
22. Repeat this step 2x again -> so in total 3x washing with 1x TBS + tween buffer for 15 mins.
23. In between, prepare secondary antibodies by adding 5 µl of "Antimouse IgG" antibodies into 15ml of blocking solution (1: 10000dilution).
24. After the 3x washing with 1x TBS + Tween buffer, add the blocking solution with the secondary antibodies onto the membranes and shake for 1 hour.
    a. NOTE: make sure every once in a while that the membranes do not stick to each other otherwise the secondary antibodies will not be able to bind.
25. Remove the blocking solution and secondary antibody and wash membrane 3x for 15 minutes with 1x TBS + Tween 20 by shaking.
26. Place the membrane protein-side up in a container and add 6 ml of HRP substrate onto the blot.
27. Incubate the blot for 2 to 5 minutes at room temperature. Rinse with water.
28. In the meantime, add 1 ml of Detection Reagent A and 1 ml of Detection Reagent B from the kit Pierce® ECL Western Blotting Substrate (Thermo Scientific) in an Eppendorf tube (2 ml in total) and vortex well (this is per 1 gel).
29. Add the reagent mixtures from step 28 onto the membranes and shake for 5 minutes. Stop the reaction with water.

30. Arrange the membranes in between transparent foil in the proper order and remove the bubbles.
31. Take an image of the samples.

The second part of each sample was used for PCR.

3.6 Mice antibiotic treatment

Four weeks after infection, the antibiotic treatment was started. CDC protocol for a course of 14 days adjusted to mouse weight was used:

- 3 mice -> Treated with 71.5 μg of doxycycline dose per mouse per day.
- 3 mice ->Treated with 625 μg of amoxicillin dose per mouse per day.
- 1 mouse -> Double treatment (1st day dox, 2nd day amox, 3rd day dox…etc.).
- 1 healthy mouse (uninfected).
- 1 infected mouse (untreated).

3.7 Post antibiotic treatment

- Two weeks after mice were injected with antibiotics, they were killed with carbon dioxide gas followed by cardiac exsanguination.
- All mice were dissected; blood and tissue samples from each mouse were collected.
- Organs examined: bladder, spleen, heart, brain and joint. Blood was used for serum preparation and further Western blotting analysis (as described above).
- All organs were divided into two parts – one part was used for *Borrelia* cultivation in modified Kelly-Pettenkofer (MKP) medium supplemented with *Borrelia* antibiotic (1:100 dilution) and another part was checked by PCR for presence of borrelia DNA.
- All mice cultures were kept to grow at 34ºC for about 4 weeks. All cultures were checked weekly for the presence of spirochetes; positive cultures were re-cultivated into a larger volume of MKP.

3.8 Purification of total DNA from animal tissues

The collected mice tissues were frozen by the addition nitrogen and crushed into tiny pieces. Genomic DNA was then purified according to protocol for gDNA preparation from Qiagene DNeasy Blood and Tissue kit (described above).

To eliminate the inhibition of PCR reaction by foreign DNA (mouse), two-steps PCR (spacer and nested) was implemented. The result of the first (spacer) PCR was used as a template in the second (nested) PCR. The PCR was conducted with two pairs of flagellin primers **described in the table above.** The annealing temperature of the first round of PCR was 52 °C, and the second round of PCR - 55°C. The primers and the conditions of PCR amplification were taken from Clark et al., 2005. All the reactions included positive and negative controls. The results of the PCR were checked on 1 % agarose gel electrophoresis.

PCR products were then purified from the gel and sent for sequencing to confirm the infection with NE-5264 strain of borrelia (the one that was inoculated into mice).

3.9 Purification of the PCR products for sequencing

For the purification of the PCR products from the gel, UltaFree-DA centrifugal filter units for DNA extraction from agarose (Merck) were used. The frozen gel fragments were transferred into a column and centrifuged for 10 minutes at 5000xg. The DNA concentrations were then measured using a Nano-drop. Purified PCR products were then mixed with the primers and sent for sequencing. The samples were prepared strictly according to the recommendation of the sequencing facility (Seqme.eu). Finally, the obtained sequencing data were analyzed using DNAStar programs and sent to GenBank for the comparison.

4 Results

4.1 *Borrelia* cultivation

Borrelia burgdorferi sensu stricto strain NE-5264 was cultivated in MKP medium till a total density of 2 x 10^7 and after an appropriate dilution (1 x 10^5) was used for infection of 9 female mice by injection of 150 µl of NE-5264 culture abdominally and under the skin.

4.2 Confirmation of infection

Four weeks after infecting mice with borrelia culture, blood and skin samples were taken from 8 mice (accidental death of 1 mouse) to analyze mice infection by PCR and Western blotting. Sera were analyzed by Western blotting. The results of the Western blotting are shown on figure 5. Establishment of infection in all mice was confirmed (sera positive).

The control used: *Borrelia duttonii* 1220K3 lysate and the serum of 1120K3 infected mouse.

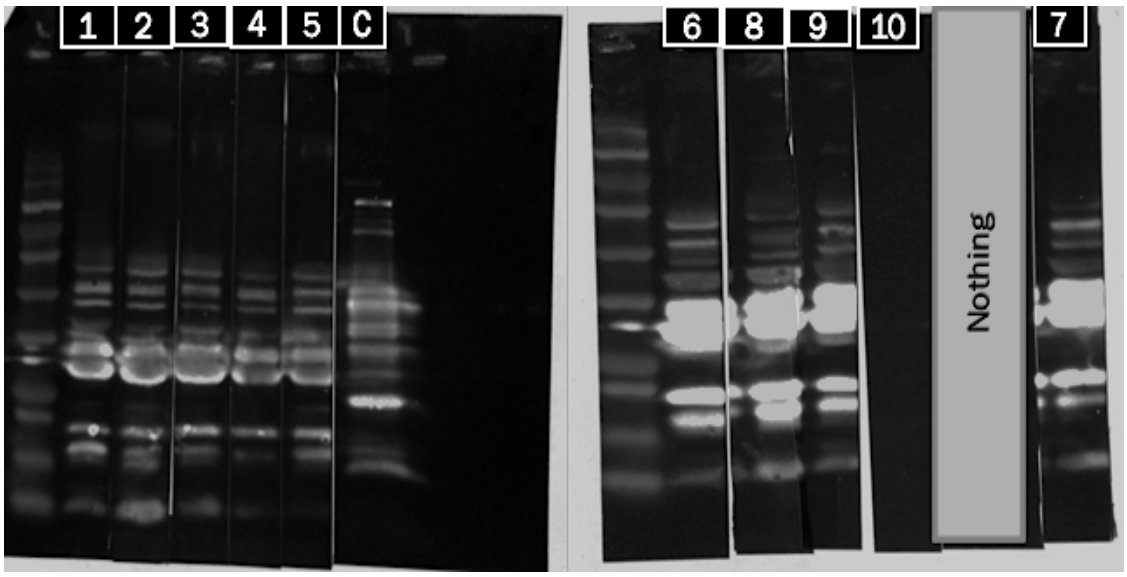

Figure 5: Results of western blotting seen under UV light. 1-10 - mice sera samples, C – control.

gDNA was isolated from ear biopsy of all mice and the presence of borrelia DNA was confirmed by nested PCR with primers for flagellin (picture of the gel not shown).

4.3 Antibiotic treatment

Four weeks after infection, 14 days course of antibiotic treatment was started on 9 mice.

Table 8: ATB treatment of the experimental group of mice.

Mouse	Un-infected (Negative control)	Non-treated	Double treated	Amoxicillin treated	Doxycycline treated
1					✓
2					✓
3					✓
4			✓		
5		✓			
6					
7				✓	
8				✓	
9				✓	
10	✓				

After the ATB treatment was completed (two weeks), mice were killed and mouse tissues (blood, bladder, spleen, heart, brain and joint) were checked by culture and PCR.

- All mice cultures were kept grown at 34ºC for 4 weeks.
- Cultures were checked weekly for 4 weeks for the presence of spirochetes and contamination under a dark filed microscope.

Table 9: Overview of tissues and organs taken from mice for cultivation in MKP medium.

Mouse 10 (Un-infected)		**Mouse 5 (Non-Treated)**		**Mouse 4 (Double Treated)**	
1	Blood	7	Blood	13	Blood
2	Bladder	8	Bladder	14	Bladder
3	Spleen	9	Spleen	15	Spleen
4	Heart	10	Heart	16	Heart
5	Brain	11	Brain	17	Brain
6	Joint	12	Joint	18	Joint
Mouse 3 (Doxycycline Treated)		**Mouse 2 (Doxycycline Treated)**		**Mouse 1 (Doxycycline Treated)**	
19	Blood	25	Blood	31	Blood
20	Bladder	26	Bladder	32	Bladder
21	Spleen	27	Spleen	33	Spleen
22	Heart	28	Heart	34	Heart
23	Brain	29	Brain	35	Brain
24	Joint	30	Joint	36	Joint
Mouse 7 (Amoxicillin Treated)		**Mouse 8 (Amoxicillin Treated)**		**Mouse 9 (Amoxicillin Treated)**	
37	Blood	43	Blood	49	Blood
38	Bladder	44	Bladder	50	Bladder
39	Spleen	45	Spleen	51	Spleen
40	Heart	46	Heart	52	Heart
41	Brain	47	Brain	53	Brain
42	Joint	48	Joint	54	Joint

4.4 Spirochetal detection post ATB treatment

After 1 week of cultivation, the following results were observed:

Table 10: Number of spirochetes that were found in mice cultures (1 week old).

Mouse culture	Number of alive Spirochetes	Number of dead spirochetes
7 (Non-treated: spleen)	13	0
8 (Non-treated: bladder)	0	Several
15 (Double-treated: spleen)	1	0
18 (Double-treated: joint)	0	3
20 (Doxy-treated: bladder)	0	2
32 (Doxy-treated: bladder)	0	3
34 (Doxy-treated: heart)	1	0
36 (Doxy-treated: joint)	5	3
38 (Amox-treated: bladder)	0	Several
42 (Amox-treated: joint)	0	Several
44 (Amox-treated: bladder)	0	Several
45 (Amox-treated: spleen)	0	Several
47 (Amox-treated: brain)	2	0
48 (Amox-treated: joint)	0	Several
49 (Amox-treated: blood)	0	Several
50 (Amox-treated: bladder)	3	0
53 (Amox-treated: brain)	5	0
54 (Amox-treated: joint)	0	7

After the first week of checking all positive cultures were taken and re-cultivated into a larger volume of MKP medium. While the negative cultures and contaminated cultures were either discarded or re-checked a week later.

Table 11: The following cultures (positive) were re-cultivated into a larger volume of MKP.

Culture	Organ
7	Non-treated: spleen
8	Non-treated: bladder
32	Doxy-treated: bladder
36	Doxy-treated: joint
38	Amox-treated: bladder
42	Amox-treated: joint
44	Amox-treated: bladder
45	Amox-treated: spleen
47	Amox-treated: brain
48	Amox-treated: joint
49	Amox-treated: blood
50	Amox-treated: bladder
53	Amox-treated: brain
54	Amox-treated: joint

Both the new (re-cultured) and the old cultures were checked for the presence of spirochetes during week 2. The cultures that had a **high** density of spirochetes were then centrifuged for 15 mins at maximum speed and their pellet was frozen at -80 °C.

Table 12: Presence of *Borrelia* spirochetes found in both the old and re-cultivated cultures after 2-4 weeks.

Week 2		
Mouse culture	**Old Culture**	**New Culture**
7 (Non-treated: spleen)	✓	✓
8 (Non-treated: bladder)	✓	✓
32 (Doxy-treated: bladder)	✓	✓
34 (Doxy-treated: heart)	×	×
36 (Doxy-treated: joint)	✓	✓

38 (Amox-treated: bladder)	✓	✓
42 (Amox-treated: joint)	×	✓
44 (Amox-treated: bladder)	✓	✓
45 (Amox-treated: spleen)	×	×
47 (Amox-treated: brain)	contaminated	
48 (Amox-treated: joint)	×	✓
49 (Amox-treated: blood)	×	×
50 (Amox-treated: bladder)	✓	✓
53 (Amox-treated: brain)	×	✓
54 (Amox-treated: joint)	×	×
Week 3		
34 (Doxy-treated: heart)	×	×
36 (Doxy-treated: joint)	✓	✓
42 (Amox-treated: joint)	×	×
45 (Amox-treated: spleen)	contaminated	
48 (Amox-treated: brain)	✓	✓
49 (Amox-treated: blood)	×	×
53 (Amox-treated: brain)	contaminated	
54 (Amox-treated: joint)	×	×
Week 4		
12 (Non-treated: joint)	✓	✓
16 (Double-treated: heart)	×	×
18 (Double-treated: brain)	✓	✓
19 (Doxy-treated: blood)	×	×
42 (Amox-treated: joint)	✓	×
46 (Amox-treated: heart)	×	×
48 (Amox-treated: joint)	✓	✓
49 (Amox-treated: blood)	×	×
54 (Amox-treated: joint)	✓	✓

Table 13: Overview of positive cultures from mouse tissues.

Mouse 10 (Un-infected)		**Mouse 5 (Non-Treated)**		**Mouse 4 (Double Treated)**	
1	Blood	7	Blood	13	Blood
2	Bladder	8	Bladder	14	Bladder
3	Spleen	9	Spleen	15	Spleen
4	Heart	10	Heart	16	Heart
5	Brain	11	Brain	17	Brain
6	Joint	12	Joint	18	Joint
Mouse 3 (Doxycycline Treated)		**Mouse 2 (Doxycycline Treated)**		**Mouse 1 (Doxycycline Treated)**	
19	Blood	25	Blood	31	Blood
20	Bladder	26	Bladder	32	Bladder
21	Spleen	27	Spleen	33	Spleen
22	Heart	28	Heart	34	Heart
23	Brain	29	Brain	35	Brain
24	Joint	30	Joint	36	Joint
Mouse 7 (Amoxicillin Treated)		**Mouse 8 (Amoxicillin Treated)**		**Mouse 9 (Amoxicillin Treated)**	
37	Blood	43	Blood	49	Blood
38	Bladder	44	Bladder	50	Bladder
39	Spleen	45	Spleen	51	Spleen
40	Heart	46	Heart	52	Heart
41	Brain	47	Brain	53	Brain
42	Joint	48	Joint	54	Joint

Highlighted in yellow are the positive cultures.

In the double-treated mouse, the density of the spirochetes was very low even after re-cultivation and the spirochetes were all moveless. Moreover, the bladder of mice, treated with doxycycline and amoxicillin separately, showed to have the highest density of spirochetes compared to all other cultures. No spirochetes were found in the blood of treated mice.

4.5 PCR results

Genomic DNA was isolated from all the samples and tested for the presence of borrelia DNA by PCR.

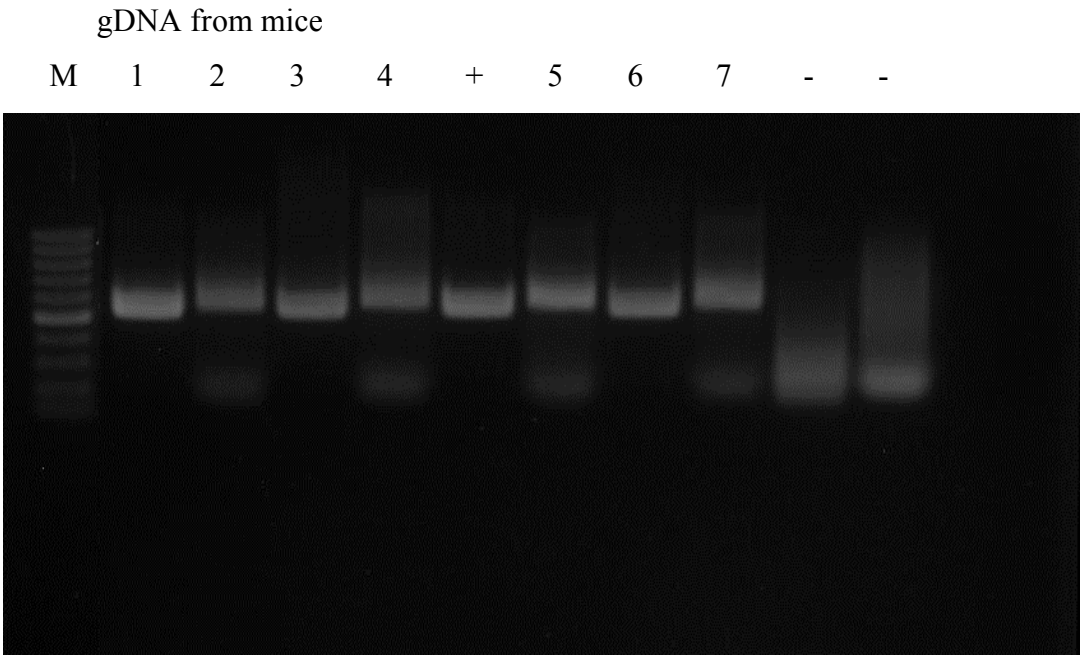

Figure 6: PCR with Fla out primers (497 bp), 1-4: infected mouse gDNA samples, +: positive control, 5-7: infected mouse gDNA samples, -: negative controls (water and gDNA of uninfected mouse)

Shown above in figure 2 is one of the results of the multiple PCR reactions confirming the presence of *Borrelia* DNA post ATB treatment from mouse organs (joint, bladder, and brain).

4.6 Sequencing results

After confirmation of infection of different mice organs by PCR, selected PCR products were sequenced. The obtained results confirmed the presence of the DNA of *Borrelia* sensu stricto strain NE-5264 in them.

To conclude our results: out of the 9 infected and antibiotic treated mice, we were able to culture live replication spirochetes from 6 mice. The most effective in elimination of live spirochetes was double-antibiotic treatment, following by the doxycycline treatment. On the other hand, the amoxicillin treatment proved to be ineffective, as live spirochetes were cultured from all amoxicillin treated mice.

5 Discussion

As it was mentioned above in some small group of patients after the recommended antibiotic treatment for acute LD the symptoms of the disease persist. They last for months and even years after treatment. These symptoms are usually referred to as chronic Lyme disease or post-treatment Lyme disease syndrome (PTLDS). This topic was and still is the controversial subject of arguments and discussion within medical doctors and scientists. Some physicians discount the likelihood that post-treatment symptoms are directly related to the earlier Lyme infection. They suggest that patients are simply reporting symptoms commonly seen in the general population or those of a secondary condition such as chronic fatigue syndrome or fibromyalgia. Other physicians think that such symptoms are most likely directly connected to the previous infection and represent treatment failure. These physicians note that most of the reported post-treatment Lyme disease symptoms began before treatment and that the original Lyme diagnoses were, in part, based on their very existence. To date evidences generally support the second group. However, the discussion continues as there are only a few case studies in the medical literature describing people who continued to have evidence of infection despite adequate treatment, but in most cases there is no laboratory proof that the bacteria is still present.

The blood test the CDC (both US and European) recommends for diagnosing Lyme disease checks for an immune response to the bacteria, not for the presence of live *Borrelia* itself. That's why the test can be negative if the disease is present for less than a month. It takes at least a couple of weeks to mount an immune response that would turn the test positive. On the other hand, some atypical forms of borrelia avoid the human immune system so there is no immune response at all.

Logically, if making a diagnosis can be complex, the controversy about the treatment appears. Nowadays this controversy is so intense that it is sometimes called "Lyme wars." One of the main points of dispute is the choice of the proper ATB as well as the duration of antibiotic treatment — not only for acute Lyme but also for PTLDS. As for today, various researches of Lyme disease have suggested an indefinite treatment. Although current antibiotics such as

erythromycin, amoxicillin, doxycycline and azithromycin are mainly used for early treatment of Lyme disease, at least 50% of patients undergo treatment failure (58).

According to standard protocols recommended by CDC for early Lyme disease, a short course of oral antibiotics (two- to-four-weeks), such as doxycycline or amoxicillin, cures the majority of cases. That is why in the present study, these two antibiotics separately and in combination were used on mice models infected with *Borrelia burgodorferi* sensu stricto. From the results seen above, two weeks course of antibiotic treatment failed to completely wipe out the spirochetes. These results totally correspond with the results obtained in a recent study by Middelveen et al. conducted in 2018 where they showed the presence of live spirochetes found in tissues and blood samples despite antibiotic short-term therapy among the 12 subjects with Lyme disease symptoms. All subjects were treated for 2-4 weeks with antibiotics prescribed by the centers of Disease Control and Prevention (CDC). The results of their cultures proved positive when both spiral forms and spherical forms were visible (15). On the other hand, Hodzic et al. conducted a series of experiments comparing a short-term treatment with ceftriaxone and with saline in C3H/HeN mice infected with an N40 strain of *Borrelia burgdorferi*. Regardless of whether the subject was treated early or late, they concluded the animal cultures of numerous tissues negative, even though some single spirochetes were observed by the culture methodology (60). Some studies investigated the effect of the extended course of ATB treatment. In 2001, a well-documented study described the treatment of 78 patients with persistent symptoms of LD who were properly treated with different antibiotics, including intravenous ceftriaxone, and/or oral doxycycline for 90 days. However, patients continued having persistent fatigue, musculoskeletal pain, neurocognitive symptoms, and dysesthesia. Concluding their research, they found that there was no significant effect in long lasting treatment of Lyme disease patients with ATBs (61). Afterwards, multiple prospective trials have revealed that prolonged courses of antibiotics neither prevent nor alleviate post treatment Lyme disease syndromes. Bockenstedt et al., for example, documented several spirochetes that were found both in a portion of mice who were treated for two weeks or for 30 days with doxycycline (60). Therefore, it is safe to assume that treatment periods do not appear to alter the results as spirochetes were still found.

Our results confirmed that doxycycline treatment worked more efficiently than amoxicillin on the infected mice. These results are in contrast to the results obtained by Moody et al. (1994), where the efficacy of various dosages of eight antibiotics used for Lyme disease treatment was evaluated for C3H/HeNCrIBR mice, which reproducibly develop persistent infection, arthritis, and carditis when inoculated with *Borrelia burgdorferi*. According to their results, amoxicillin, ceftriaxone, and high-dose penicillin G effectively eliminated infection and disease, while oxytetracycline, doxycycline, chloramphenicol, erythromycin, and azithromycin failed to cure infected mice (62).

The combination of two antibiotics worked more effectively than the use of single ones. As standard monotherapy treatment appeared to be ineffective, a lot of physicians have been prepared to do clinical trials of antibiotic combinations in treatment Lyme disease patients. Now we present the results in animals that support the reasonability of such trials. In the latest study by Zhang and colleagues (2019), they showed that mice infected with dormant forms of borrelia were resistant to mono therapy of standard antibiotics such as doxycycline (63). However, treating these mice with the combination of ATBs eradicated the infection efficiently.

In our experiments we found out that the majority of spirochetes were found in bladder of the infected mice after ATB treatment. That was confirmed both by PCR and culture. These results correlate with the results obtained by Schwan et al., 1988, where they conducted the similar experiment on white footed mice. White-footed mice, *Peromyscus leucopus*, were experimentally infected in the laboratory with *Borrelia burgdorferi*, the causative agent of Lyme disease. After infection, attempts were made to culture spirochetes from the urinary bladder, spleen, kidney, blood, and urine. According to the authors, spirochetes were most frequently isolated from the bladder (94%) (64). These results demonstrate that the concentration of the spirochetes in bladder of the infected mice is very high and that the cultivation from the bladder could be very effective, which therefore could explain that *B. burgdorferi* ss may have tropism towards the urinary bladder rather than other organs. In another study by Callister et al. the efficacy of culturing of *Borrelia burgdorferi* from urinary bladder tissue of naturally infected, wild *Peromyscus leucopus* mice was also determined. The urinary bladders were very productive in cultivation of live spirochetes. The rapid *B. burgdorferi* isolation (mean, 6 days)

from mouse urinary bladders should aid in defining new Lyme disease foci (65). Joints were the second organ in our experiments with the high concentration of the spirochetes. These results correlate with those described by Yrjänäinen et al., 2007. In their experiments, mice were infected with *B. garinii* A218 or *B. burgdorferi* sensu stricto N40 followed by ATB treatment. The infection status was assessed by spirochete cultivation. Spirochetes grew from the tissue samples in 30% of the treated mice. Living spirochetes were detected only in the joints of mice 4 weeks after antibiotic treatment, and not in any other tissue (66).

Barely any spirochetes during this study were found in the spleen and heart of mice. Yet, laboratory studies with Syrian hamsters have proven otherwise. Sixty two percent of *B. burgdorferi* were mainly detected and isolated from the spleen after 2 weeks of inoculation of over 100 organisms (67). Suggesting that the spleen is in fact an appropriate organ to isolate *Borrelia* from, which opposes to the results above where no spirochetes were found in the spleen of both treated and non-treated mice. Another study conducted in 2007, found that the vessels at the base of the heart are a preferred collagen-rich place for the persistent spirochetes (68). These findings however, do not match our results as no spirochetes were found in the heart in our experiments under selected conditions. This controversy could be explained by the difference in *B. burgdorferi* strain used for the infection meaning the different organotropism of different strains or by the different laboratory model.

To conclude, our experiments confirmed that the monotherapy of established infection in experimental laboratory mice model either with doxycycline or amoxicillin failed in complete eradication of the pathogen under conditions that we selected (adjusted CDC protocol). The possible explanation of our results could be either in an inadequate dosage or duration of antibiotic treatment or in the necessity of application the complex ATB treatment involving antibiotics that are effective against replicative forms as well as forms in stationary stage or in dormant/persistent forms as well. Novel protocol for treatment Lyme disease, including the choice of antibiotics, the combination of more than one or even two ATBs and the proper time for their application, that could be different for acute or persistent infection should be introduced.

6 Conclusion

For years, the spirochetes from *Borrelia burgdorferi* sensu lato complex has been recognized as the causative agents of Lyme disease. Lyme disease is a tick borne illness which generates controversy amongst medical providers and researchers.

In this study we tested the efficiency of two antibiotics, doxycycline and amoxicillin, that are widely used for the elimination of *Borrelia* spirochetes. Following ATB treatment conducted according adjusted to the laboratory mice model CDC protocol, we were able to cultivate the live spirochetes from multiple tissues of infected mice. Using multiple methods of detection, we proved the existence of an ongoing infection despite treatment. The better treatment of Lyme disease is still an ongoing question and debate. However, based on the already published results from multiple laboratories and on our results, it is safe to state that monotherapy is not sufficient enough in complete elimination of spirochetes from infected hosts. Most probably effective protocol of treatment of Lyme disease might be the complex procedure that will include antibiotics with strong ability to eliminate as typical so as atypical, dormant or persistent forms of spirochetes. Further research and experiments in this field are highly needed.

7 Literature

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