University of South Bohemia in České Budějovice

Faculty of Science

Master thesis

2019 **Veronika Morávková**

University of South Bohemia in České Budějovice
Faculty of Science

Metamorphoses of *Borrelia burgdorferi* sensu lato spirochetes: from dormant to motile forms

Master thesis

BSc. Veronika Morávková

Supervisor: Nataliia Rudenko, Ph.D.
Specialist: MSc. Maryna Golovchenko

České Budějovice 2019

Morávková V. 2019: Metamorphoses of *Borrelia burgdorferi* sensu lato spirochetes: from dormant to motile forms. MSc. Thesis, in English – 76 pp., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

The aim of the study was to obtain and elaborate information focused on metamorphoses of *Borrelia burgdorferi* sensu lato (s.l.) spirochetes. The research included detection of various stress conditions for production of dormant forms of *Borrelia burgdorferi* s.l. spirochetes *in vitro*. Subsequently, metamorphoses from dormant to motile stages was observed under favourable conditions. Proper PCR method for primers aimed to detect dormant forms of *Borrelia* was delevoped. The infectious potential of dormant spirochetes of *Borrelia burgdorferi* sensu stricto (s.s.) complex was observed *in vivo*. Transformations of spirochetes have also been observed in real time and individual stages have been recorded.

Declaration

I hereby declare that I have worked on my master thesis independently and used only the sources listed in the bibliography.

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Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Natasha Rudenko, Ph.D. for her selection of very interesting thesis.

I wish to express my sincere thanks to specialist of my research MSc. Maryna Golovchenko for her helpful assistance and patience.

I am also very grateful to Ing. Gabriela Loosová, Mgr. Zuzana Vavrušková, for their help, assistance and pleasant working environment.

I am very thankful to RNDr. Marie Vancová, Ph.D. and Mgr. Martin Strnad for cooperation and opportunity to do part of my research in Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University. I am also very thankful to Mgr. Aleš Benda, Ph.D who provided us with the necessary and very valuable laboratory equipment.

Last but not the least, I would like to thank my family and love of my life for their enormous encouragement.

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1. INTRODUCTION

Vector-borne diseases are caused by pathogens such as bacteria, viruses and parasites transmitted by arthropods and molluscs among human populations and are resulting in over one billion cases of infections with almost one million deaths per year worldwide (WHO 2017[1]).

Bacteria of the *Borrelia burgdorferi* sensu lato (s.l.) complex maintain an enzootic life cycle between vertebrates and ixodid ticks and consist of 22 confirmed species (Margos et al. 2010; Rudenko et al. 2009 a, b; Rudenko et al. 2011). Several species of this group are known to cause tick-borne disease in humans, called Lyme disease, also known as Lyme borreliosis (LB). The Lyme disease is recognized worldwide, including 1 species known to cause human infection in both USA and Europe (*B. burgdorferi* sensu stricto (s.s.)). Two infectious species are considered to occur only in Eurasia (*B. afzelii, B. garinii*) (Baranton et al. 1992; Canica et al. 1993; Rudenko et al. 2008, 2009a; Girard et al. 2010). A number of infrequent positive cases of various *Borrelia burgdorferi* species as *Borrelia lusitaniae* or *B. valaisiana* from humans in Europe occurred, nevertheless the high prevalence of these species in their specific regions in compare to rare isolates from patients does not yet indicate an association with the human disease (Collares-Pereira et al. 2004; Diza et al. 2004).

The Lyme disease is the most prevalent disorder caused by tick-vector interaction among Holarctic region. The epidemiological data of diagnosed cases of LB show dramatic increase in the both Eurasia and USA due to the development of progressive diagnostic methods during the last decades (Hubálek, 2009). LB is associated with various symptoms depending on specific species of *Borrelia burgdorferi* s.l. complex from skin manifestations to invasion of the central nervous system (Steere et al 1986; Canica et al. 1993; Rijpkema et al. 1997; Ornstein et al 2001; Dennis & Hayes, 2002; van Dam 2002; Randolph 2008; Stanek and Strle 2009).

In spite of the fact that about 80% of LB patients may be cured with antibiotic treatment, there are patients suffering from prolonged multi-organ symptoms, also called post-treatment Lyme disease syndrome (PTLDS). The cause of PTLDS is uncertain and its existence is questioned. Nevertheless, there are morphological variants, also called dormant forms (round bodies, biofilms), of *B. burgdorferi* developing under stress conditions which are associated with the disease. Previous studies suggest that the atypical forms of *Borrelia* may occur in

nerve tissues, joints and eyes. It is considered that progressive deterioration of patients health status is caused by re-proliferation of *Borrelia* cells transformed from dormant forms in the organism and by subsequent inflammatory of body tissues by their products of metabolism and immunopathological reactions (Kurtti et al. 1987; Gruntar et al. 2001; Pícha 2006; Miklossy et al. 2008; Sharma et al. 2015). Therefore, it is necessary to continue in research of this pathogen, its morphological diversity and functions of these variants, and possible resistance to antibiotic treatment.

This study is focused on metamorphoses of *Borrelia burgdorferi* s.l. spirochetes caused by various stress conditions *in vitro*. Subsequently, reversible process of the spirochetes from dormant back to motile form was observed by maintaining favourable conditions. Knowing the optimal parameters to create different forms of *Borrelia* spirochetes may be helpful in future studies on atypical forms of borreliae. Morphotypes of *Borrelia* have been observed in real time, aiming to record their metamorphoses from motile to atypical form and back to vital form.

The infectious potential of dormant spirochetes after antibiotic treatment of *Borrelia burgdorferi* s.s. complex was studied *in vivo*. Detection of Borrelia in inoculated laboratory model was performed with properly set PCR method for primers aimed to detect dormant forms of *Borrelia*.

2. LITERATURE SURVEY

2.1. *Borrelia burgdorferi* sensu lato, geographical distribution

Borrelia burgdorferi s.l. complex belongs to the genus *Borrelia* (Spirochaetales: Spirochaetacea).

Borrelia burgdorferi complex includes 22 valid genospecies, while the current number is probably not final considering the fact that an identification of new species and variants continue to be recognized. *Borrelia* spirochetes vary in geographic distribution, host-specifity and ability to cause human disease. Out of 22 species, 11 species were recognized in the Old World (*B. afzelii, B. bavariensis. B. garinii. B. japonica, B. lusitaniae, B. sinica, B. spielmanii, B. tanukii, B. turdi, B. valaisiana* and *B. yangtze*). Another 5 species from the *B. burgdorferi* s.l. complex were previously strictly associated with the New World only (*B. americana, B. andersonii, B. californiensis, B. carolinensis* and *B. kurtenbachii*). Nowadays, *B.burgdorferi* s.s., *B. bissetti* and *B. carolinensis* are identified from both Eurasia and the USA (Rudenko et al. 2011). Geographic distribution, diversity of vectors and host specificity of different *Borrelia* species is shown in Appendicces (Apendix 1).

2.1.1. General characteristics

Borrelia is a genus of spiral-shaped, unicellular bacterium, considered to has a dual-membrane (diderm) cell envelope with a layer of peptidoglycan situated between the outer and inner membrane. The bacterium does not possess a rigid cellular wall and lipopolysaccharide (LPS) , the major component of the outer membrane of Gram-negative bacteria, mainly responsible for structural integrity and protection (Zhang et al. 2013). Instead of LPS, *Borrelia* spirochetes contains numerous lipoproteins at the outer surface, essential for host adaptation (Radolf et al. 2012; Caimano et al. 2016).

The spirochete may reach up to 10–40 μm in length, and 0.2–0.5 μm in diameter. The movement of bacterium is caused by 7–14 periplasmic flagella, longitudinally coiled around the helical protoplasmic cylinder between the peptidoglycan layer and the outer membrane. The bundled flagella provide a screw-like motion and allow the cell to move in media with various viscosity level. Therefore, a link is considered between the features of flagella mentioned above and a high virulence factor of the pathogen (Motaleb et al. 2000). These appendages consist predominantly of two types of outer layer flagellin proteins, major FlaB (41kDA) and minor FlaA (38kDa) (Ge et al. 1998). Flagellin B can reduce motility and change the shape of spirochete to rod-like form when targeted inactivation of the protein is performed (Motaleb et al. 2000).

At the molecular level, *B. burgdorferi* cells possess segmented genome represented by approximately 911 kb linear chromosome and numerous circular and linear plasmids. The stability of plasmids varies, resulting in possible loss of these DNA molecules. Specific *Borrelia* plasmids encode functions influencing the infectious potential of the pathogen (Rosa et al. 2005). Variants in genomes of *B. burgdorferi* s. l. complex provide differing intensity of infection and dissemination in host. Each genome is characterized by different antigens specific to the host infection on its membrane receptor. One of the membrane receptors, the outer surface protein C (OspC), as the one of the major lipoproteins localized at the outer membrane of the bacteria is a significant indicator of the degree of dissemination in addition to identification of genomic classification according to specific number of OspC loci (Theisen et al. 1995). It is the most studied virulence factor of *Borrelia* pathogen due to its importance for transmission of the bacteria from its specific vector to vertebrate host and early infection establishment. The OspC is induced in the spirochete during nymphal tick feeding and during early phase of mammalian infection (Pal et al. 2004; Carrasco et al. 2015).

In general, the doubling time of borreliae spirochete takes from 24 to 48 hours, when optimal conditions achieved (Zückert 2007). The bacteria is microaerophilic and capable to survive without iron using manganese to substitute iron-sulfur cluster enzymes in compare to many other pathogenic bacteria acquiring iron to persist (Gladwin & Trattler 2009).

Life cycle of *Borrelia* spirochetes is characterized by dynamic interaction between the pathogen, vector and reservoir host (Fig. 1.). The sylvatic (enzootic) cycle of the bacteria is closely linked to landscape and climatic factors of their invertebrate and vertebrate hosts, which broadly determine the spirochete ecological niches (Kurtenbach et al. 2006).

All recognized vectors of *B. burgdorferi* s. l. complex are known to be three host ticks of the genus *Ixodes*. Ixodid life cycle includes three female feeding stages (larvae, nymph, adult) performed at a different individual host for each stage, with the exception of nest-living (nidicolous) adult tick females such as *I. dammini* with preferrence of large vertebrates hosts (such as deer). Although a larval infestastion of deer by *I. dammini* was proven, *Borrelia* spirochetes were detected only in about 1% of examined developed nymphs, showing that deer appear not to become infected by Lyme disease spirochetes (Telford et al. 1988).

The infection to tick is transmitted through the bloodmeal from an infected animal. Bacteria persist in the nutrient-poor midgut of the tick transstadially from larval stage through moulting process to a nymph, transmitted to other vertebrate hosts during the

subsequent bloodmeal of the next life stage. Other known possibilities of transmission include transovarial infection (Gern & Humair 2002) of specific ticks species, such as *Borrelia miyamotoi* and co-feeding between ticks in close location, feeding on a susceptible or non-susceptible vertebrate (Ogden et al. 1997; Scoles et al. 2001; Piesman 2002).

The preference of larval and nymphal stages for small to medium sized reservoir hosts (mammals, birds or lizards) is crucial for maintaining persistent infection of the bacteria in their natural transmission cycles (Gern & Humair 2002).

Another moulting results in adult formation of tick. Adult ticks usually feed on larger mammals, considered not be able to support the survival of the spirochetes, as noted above (Telford et al. 1988; Gern & Humair 2002; Brisson et al. 2012).

The enzootic cycle is complete when infection to vertebrate host occurs. *B. burgdorferi* is mostly transmitted to the mammalian host through a nymphal stage of an *Ixodes* tick. (Brisson et al. 2009). Depending on the species of *Borrelia*, transmission of the pathogen to humans is possible, while it is considered to be a dead-end host, unlikely to continue the cycle of the bacteria (Radolf et al. 2012).

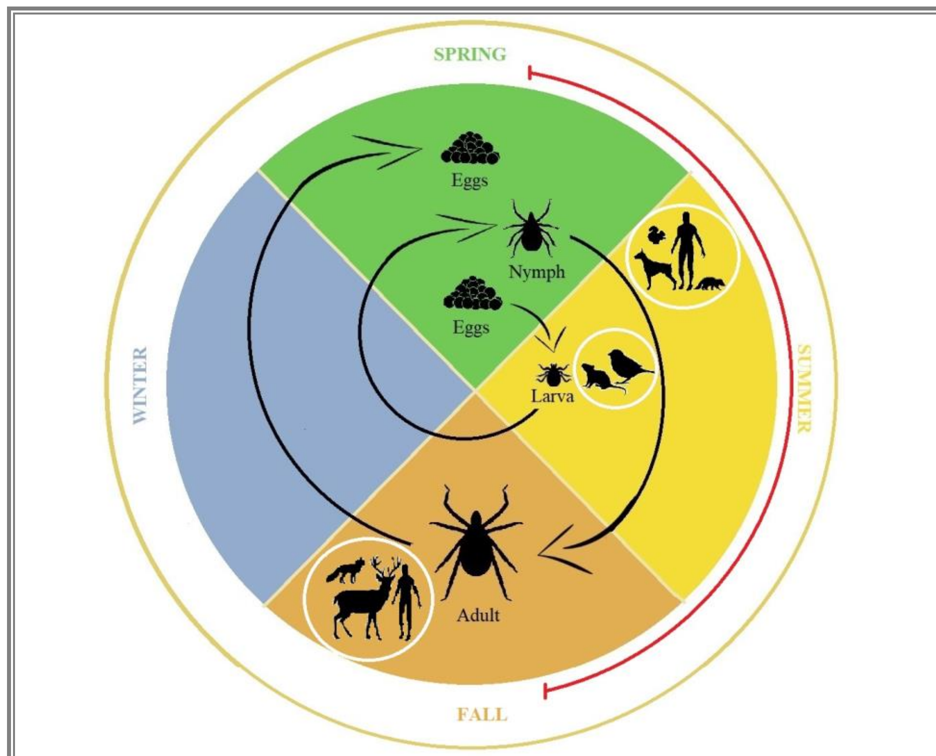

Fig. 1. Brief scheme of life cycle of *Ixodes* ticks with their hosts and appearance during seasons of the year. White circles include hosts of individual stages of the ticks. Red line represents the greatest risk of human infection. *Note: The real size ratio of vertebrates and invertebrates shown in the scheme was not applied.*

2.1.2. Metamorphoses of *Borrelia* caused by various environmental conditions

Spirochetes of *B. burgdorferi* s.l. complex are pleomorphic, occuring in various distinct shapes and forms as a response to environmental conditions (see Fig. 2.). Pleomorphism is observed among many Gram-negative and Gram-positive bacterial species both *in vitro* and *in vivo* for over a century (Winkler 1899; Domingue & Woody 1997; Mattman 2001). Morphological variations of bacterial cells are commonly induced in *vitro* using compounds such as lytic enzymes, which lyse the cellular wall. Another chemical substances may interfere with the cell wall synthesis, such as antibiotics (Briers et al., 2012). Antibiotics result in complete or partial loss of peptidoglycan wall of the bacteria. Such cells are called "cell wall deficient" (CWD), L-forms or spheroplasts (Glover et al., 2009; Ranjit & Young 2013). Generally, the spirochaetal form of *Borrelia* cells mainly occur during log phase growth, while the atypical forms are present during older, stationary phase or when subjected to stress conditions in bacterial cultivations.

In addition to CWD forms, *B. burgdorferi* cells are also seen such as non-motile round bodies (RBs) (also called coccoid bodies, globular bodies, granules, reproductive propagules, spherical bodies, or vesicles). The round body develops by forming into a ball and maintaining a cocoon around itself. These non-motile forms may vary in size and organization. Borreliae RBs may occur isolated as well as congregated into larger colonies, also called biofilms (Sapi et al., 2012; Srivastava & de Silva, 2009). Another morphological type of spirochete is observed as 'bleb' and is characterized as a spirochete with an irregular membrane bulge (Kersten et al. 1995).

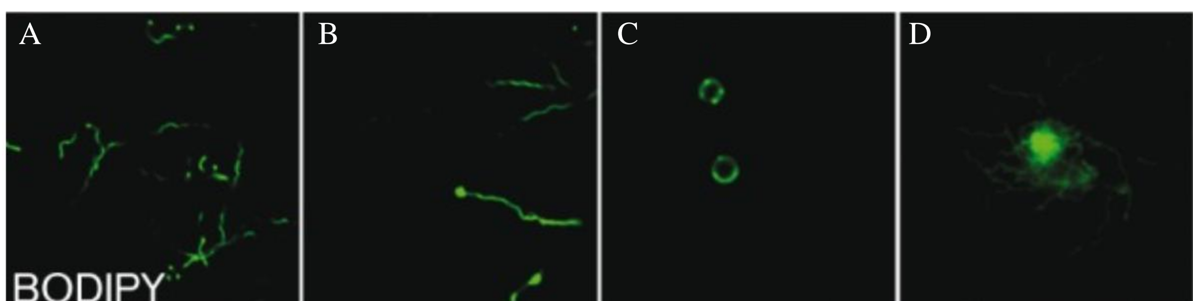

Fig. 2. Different pleomorphic forms of *Borrelia* spirochetes. (A) live spirocheatal cells, (B) blebs, (C) round bodies, (D) biofilms. Cells were BODIPY stained and observed with an Olympus confocal microscope IX81, set-up with ×60 objective using 488 nm laser and DIC. (Adapted from Meriläinen et al. 2015)

RBs colony formation (biofilms) of *Borrelia* covered by collagen may escape immune response, resulting in seronegativity. It has been shown that ceftriaxone antibiotic caused decrease of antibody titers even though spirochetes presence persisted. This phenomenon was leading to falsely negative results of the ELISA and Western blot methods of blood testing (Hodzic et al. 2008).

Stress conditions leading to aberrations of *Borrelia* standard morphology are induced by non-optimal pH (acidity-alkalinity), salts, gas composition (oxygen, hydrogen sulfide, i.e.) absence of serum, aging of cultures, antibodies, carbohydrates, amino acids and vitamins. Other environmental changes include varieties in viscosity or temperature. Presence of antibiotics or contamination may also lead to atypical forms of spirochetes (Barbour & Hayes 1986; MacDonald 2006a, b).

Previous studies demonstrated the borreliae ability to tranform to globular body form when incubated in BSK-II medium lacking rabbit serum or medium being close to nutrient exhaustion (BSKII-S) (Brorson & Brorson 1997; Brorson & Brorson 1999; de Oliveira et al., 2010).

It has been proved that RBs forms were able to reconvert to vegetative spirochetes both *in vitro* and *in vivo*. One study has shown that the RBs prepared in distilled water were able to reconvert into motile spirochetes, even after freeze-thawing (Fig. 3.). Successfull infection with RBs forms of spirochetes was achieved *in vivo* on experimental model. Vital spirochaetes were successfully isolated from 2 out of 15 mice inoculated intraperitoneally with granular bodies (Gruntar et al. 2001).

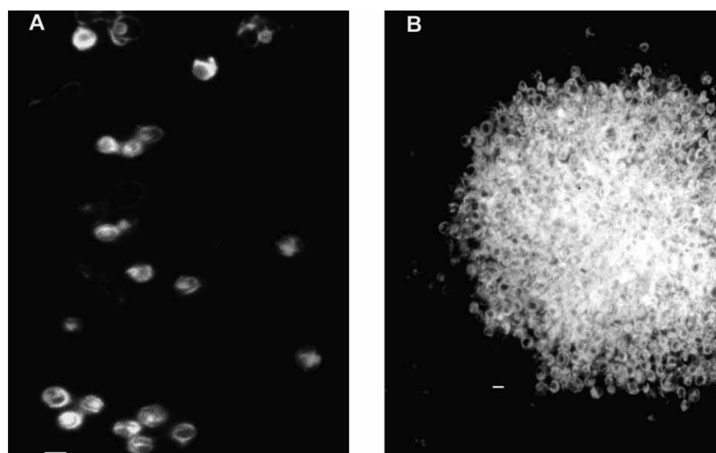

Fig. 3. (A): *B. garinii* RBs forms in distilled water, 24 h after their formation: the RBs are singular and globularly shaped. (B): *B. garinii* granular forms in distilled water, 7 days after formation: a typical aggregate of RBs. Spirochetal structures are visible inside the biofilms

in both A and B. Darkfield microscopy, original magnification 400 À; bar½1 mm. (Reproduced from Gruntar et al., 2001).

Results of Alban and colleagues (2000) have shown the ability of RBs to reverse to standard spiral-shaped spirochete when rabbit serum was added to 48 h serum-starved cell culture. Nevertheless, the motility of cells was observed after 12-15 hours from emerging from the RBs.

It was proven that RBs of borreliae may appear during antibiotic treatment. Common antibiotics used in human medicine such as ceftriaxone and penicillin induce granular formation (Murgia et al. 2002, Kersten et al. 1995). In addition, decrease of penicillin concentration supports transformation of granules back to active spirochetes (Belichenko, 2006).

On contrary, a low level of RBs was observed during macrolide antibiotics, metronizadole and tetracycline treatment (Alban et al. 2000; Murgia et al. 2002). In addition, a temperature-dependent influence of metronidazole on RBs was observed . Granules were rather eliminated at 37-38ºC than at 30ºC. The specific temperature has a similar effect on the response of other antibiotics and may play an important role in case of RBs located in the dermis (Margulis et al. 2009).

Such structurally modified cells of *Borrelia burgdorferi* complex may represent a low metabolic activity related to possible survival of spirochetes in a hostile environment until conditions are favourable to multiply again (Domingue & Woody 1997; Justice et al. 2008). Similar morphological formations called cysts found in different types of bacteria are not yet proved to be found in *Borrelia burgdorferi* (Lantos et al. 2014). Nonetheless, the protoplasmatic cylinder is folded inside the external membrane during the formation of cyst and they are known to express less lipoproteins of external membrane and periplasmic flagella. It may result in resistance to the action of antibodies and antibiotics. It is considered, that such features may possess round body forms of *Borrelia* spirochetes. The structural modification of RBs may also lead to decrease of immune response of the host (Domingue & Woody 1997; Justice et al. 2008).

In spite of the antibiotic treatment, the presence of *Borrelia* coccoid form was observed in cerebrospinal fluid (CSF), skin, iris, heart and joint biopsies (Brorson & Brorson 1998a).

Inflammated skin of patients suffering from Acrodermatitis Chronica (ACA), a degenerative skin condition associated with Lyme disease, revealed presence of several forms of *Borrelia* cells. The inflammation may be induced, in part, to a conglomeration of

granular form of borreliae cells in the affected skin. Additionally, the RBs forms have been observed to form spontaneously in human lymphoid tissue *in vivo* (Duray et al. 2005).

Brazilian Lyme disease-like illness, also known as Baggio-Yoshinari syndrome (BYS), was proven to be caused by *Borrelia* granular spirochetes. As it was shown, BYS is a disease transmitted by *Amblyomma* and/or *Rhipicephalus* ticks causing systemic and relapsing complications. The BYS clinical picture is similar to LD. In the acute phase, around 50% of patients develop papular lesion, also called erythema migrans (EM) (de Oliveira et al., 2010).

It is known that *Treponema pallidum* causing syphilis may occur in atypical cystic and granular forms These variations of bacteria cells lead to the latent stage of the disease, followed by chronic central nervous system infection and inflammation (Warthin & Olsen 1930; Warthin & Olsen 1931).

A typical syphilis cells possess transparent mucoid capsule resisting the penetration of drugs and antibodies. It was investigated whether a similar situation may occur in Lyme neuroborreliosis. Atypical (RBs) forms of *Borrelia burgdorferi* cells (strains B31 and ADB1) were induced to stress conditions such as osmotic and heat shock, and exposure to the binding agents Thioflavin S and Congo red. Morphotypes of *Borrelia* were analysed *in vitro*, when infected of primary chicken and rat neurons, as well as rat and human astrocytes. Furthemore, the morphotypes of spirochetes found in the brains of three patients suffering from Lyme neuroborreliosis were identical to those induced *in vitro* (Miklossy et al. 2008).

The intracellular location of these more resistant spirochaetal forms in neurons and glial cells may explain the long latent stage and persistence of the infection. The detection of atypical forms of *Borrelia* cells in infected tissues is essential for the diagnosis and the treatment due to their possible occurence in the absence of the typical spiral *Borrelia* form (Miklossy et al. 2008).

2.2. Lyme borreliosis

2.2.1. General characteristics

As mentioned before, Lyme disease is a multisystem disorder caused by 7 some genospecies of *Borrelia burgdorferi* s.l. complex (Rudenko et al. 2011). A typical sign of infection is an expanding skin rash reaching up to 30 cm, also known as EM. The redness of skin usually occurs approximately a week after the tick bite. It may appear at the site of the tick bite, nevertheless there are regular cases of EM on any area of the body. Other early signs (up to 30 days) include nonspecific symptoms such as fatigue, fever and headache. Symptoms mentioned above are characteristic for an early Lyme disease. If left untreated, late Lyme disease may occur, including neck stiffness, additional EM skin rashes, facial palsy at one or both sides and swelling of joints (mostly the knees). Othere severe symptoms include heart palpitations, intermittent and shooting paints, nerve pain and inflammation of the brain and spinal cord. Despite treatment, up to 20% of patients develop joint pains, problems with short-term memory and tiredness. Repeated periods of joint pain and swelling of large joints may appear even years after the first signs of LB (Aucott 2015; Schwartz et al. 2017). Asymptomatic infections also exists, although the prevalence of these cases is very low. For instance in the USA is prevalence of asymptomatic cases less than 7% (Steere et al. 2003; Biesiada et al. 2012).

The clinical features of LB in both continents are very similar, although differing in various aspects due to the greater variety of genospecies in Europe. From 5 species of *Borrelia burgdorferi* s.l. known to cause LB in Europe, *B. spielmanii* is associated only with EM, while *B. afzelii* is the only known pathogen causing the chronic dermatological condition leading to atrophy of the skin, called acrodermatitis chronica atrophicans. Studies suggest that the *B. burgdorferi* s.s. can apparently give rise to all of the clinical manifestations of borreliosis, while *B. garinii* and *B. bavariensis* are found more often in cases of neuroborreliosis (Ohlenbusch et al. 1996; Wilske et al. 1996). Another species causing neuroborreliosis is *B. afzelii* mentioned above. Nonetheless, neurological manifestations of *B. afzelii* result in different and often less specific symptoms in compare to clinical symptoms of *B. garinii* (Busch et al. 1996). B. garinii patients show a distinct clinical manifestations of Bannwarth syndrome, while neurological diagnoses of *B. afzelii* infections are generally nonspecific and more difficult to diagnose (Strle et al. 2006).

Clinical features of U.S patients with *Borrelia burgdorferi* s.s. have shown briefer duration of EM in compare to *B. afzelii* in European patients, although systemic manifestations were more likely to occur in the U.S. patients (Strle et al. 1999).

Infections usually occur from late spring to early autumn due to highest activity of the vector of LB during these seasons. The nymphal stage of the tick is considered to be responsible for most human cases of LB, owing to the fact that the size of nymphs causes difficult and almost impossible detection on the skin of the human host (Stańczak et al. 1999). If the tick attached to the skin is found within 24 hours and carefully removed, there is a high chance that the infection will not occur. The risk of infection increases up to 100% within 72 hours of tick attachment to the human skin (Flisiak & Prokopowicz 2000; Tilly et al. 2008).

Generally, transmission of LB to humans mostly occurs from specific mammals and birds by ticks. Nevertheless, there are cases of transmission of LB spirochetes through placenta during pregnancy. There is no evidence of sexual transmission of LB spirochetes. (Moody & Barthold 1991; Schmidt et al. 1995; Woodrum & Oliver 1999; Bach 2001; Porcella & Schwan 2001).

As mentioned before, the existence of chronic form of Lyme disease (PTLDS) is intensively discussed. Up to 20% of patients treated from LB suffer from persistent symptoms, acting as multi-organ disease. The permanent symptoms include pain, fatigue, muscle and joint aches. It is considered that dormant forms of *Borrelia*, such as RBs, biofilms and blebs occur in body during ATB treatment and they are resistant to the drugs and the host immune system. It is discussed that RBs may be an escape form of overcoming stressful environmental conditions (Murgia & Cinco 2004; Brorson et al. 2009; Feng et al. 2016).

2.2.2. Treatment

As mentioned above, antibiotic drugs are the primary choice of LB treatment. When used properly, recurrences are considered to be rare. Nevertheless, reinfections are possible after another tick bites due to no lasting imunity after previous infection (Wright et al. 2012; Kaiser et al. 2015).

The choice of the antibiotic, dosage, and the duration of treatment depend, among other reasons, on the age and health condition (for example pregnacy, severe illness, allergy, combination with other antibiotics) of the patient. Moreover, phase of the disease and the clinical manifestations of LB are other important factors of the choice of treatment.

Recommended antibiotics for LB therapy (see the Tab. I) are mostly used up to 21 days and they are designed for early Lyme disease with occurence of clinical manifestations approximately from 7 to 30 days after a tick bite. Prolongation of the treatment is mostly not required and it is rather considered to cause side effects, such as pseudomembranous colitis and the accumulation of ceftriaxone calcium salts in the gall bladder. In case of late Lyme disease, further treatment by a medical specialist should be suggested (Wright et al. 2012; Kaiser et al. 2015; Berende et al. 2016).

Tab. I. List of specific antibiotic drugs used in LB treatment with recommended dosage and duration of therapy ((Wormser et al. 2006; Hu 2016; Sanchez et al. 2016).

Age category	Antibiotic	Dosage	Duration [days]
Adults	Doxycycline	100 mg, twice per day	10-21
	Cefuroxime axetil	500 mg, twice per day	14-21
	Amoxicillin	500 mg, 3x per day	14-21
Children	Amoxicillin	50mg/kg, divided into 3 doses	14-21
	Doxycycline	4mg/kg divided into 2 doses	10-21

There are 2 types of usage of antibiotics for LB treatment. Patients with specific cardiac or neurological complications may use intravenously ceftriaxone or penicilin. Otherwise, doxycycline, amoxicillin or cefuroxime axetil are taken orally. Doxycycline is recommended to be applied in treatment for less than 21 days. In case of intolerance of generally used drugs, doxycycline, amoxicillin or cefuroxime axetil are replaced by one of the following: clarithromycin, erythromycin or macrolides azithromycin. It is noted, that alternative

antibiotics mentioned above are less reliable in treatment of LB (Wormser et al. 2006; Kowalski et al. 2010; Stupica et al. 2012; Hu 2016; Sanchez et al. 2016).

Most frequently, doxycline is the first choice of treatment. It is contraindicated for children up to 9 years, whereas it may cause yellow discoloration of the teeth. Therefore, amoxicillin is usually given instead (Wormser et al. 2006).

Symptoms may persist or reemerge weeks after adequate treatment, causing fatigue, impaired concentration, or chronic pain (Krupp et al. 1991). As mentioned above, this symptom complex is often defined as chronic Lyme disease (or post-Lyme syndrome, PTLDS), indicating the possibility of a persistent infection (Feder et al. 2007). Reintroduction or prolongation of antibiotic treatment was not yet fully successful. Several experimental data have shown that the *Borrelia* causing LB in such patients could no longer be detected, while they were still suffering from clinical manifestations (Auwaerter 2007). For instance, a reintroduction of antibiotic treatment including a new 28-day course of ceftriaxone (2 g/day) in one study has shown improvement of exhaustion. Nevertheless the cognitive impairment was simultaneously present (Krupp et al. 2003). Few studies suggest that T lymphocyte clones activated during an infection causing LB may react with *Borrelia* antigens along with endogenous proteins such as heat shock protein 90 (HSP90). It is assumed that the cross-reaction to exogenous and inner antigens may result in an autoimmune reaction, explaining antibiotic-resistant cases after LB infection (Eiffert et al. 2005; Lünemann et al. 2007). This hypothesis brings up a question whether a borrelial infection can induce an autoimmune disease of rheumatic type that persists after the pathogen has been eliminated.

It has been shown, that several currently recommended ATB against Lyme disease may induce round body forms of *Borrelia burgdorferi*. Stationary phase cultures enriched with atypical forms such as RBs and biofilms are showing resistence to amoxicillin and doxycycline (Kersten et al. 1995; Brorson et al. 2009; Barthold et al. 2010; Sapi et al. 2011; Feng et al. 2015). Metronidazole has shown significant activity agains the RBs. Nevertheless, metronidazole was unable to eradicate 100% of RBs of *Borrelia* (Sapi et al. 2011).

Research of Feng and colleagues (2016) indicates that amoxicillin-treated borreliae (RBs) *in vitro* are viable and they may revert to spirochetes after 5 days of subcultures with fresh BSK-H medium. Culture of 6 days of incubation treated with even 100 μg/ml of amoxicillin could not be induced to round body form exlusively. Nevertheless, culture with density of $1x10^5$ cells/ml after 3 days of 50 μg/ml amoxicillin has shown 96% round body

forms. Another antibiotics such as doxycycline, cefuroxime and ceftriaxone were tested against *Borrelia* and results showed that these ATBs had higher activity against spirochetes in compare to RBs.

Due to the cost and questionable effectiveness of specific antibiotic treatment, an alternative therapy is studied. Results from the Cowden Condensed Support Program are demonstrating that various natural antimicrobial herbal agents, namely Samento and Banderol, are able to significantly reduce different morphological forms of *B. burgdorferi*. When used in combination, these herbal extracts may provide an effective therapeutic approach for patients suffering from LB (Datar et al. 2010).

3. AIMS OF STUDY

1. To evaluate parameters of atypical (dormant) forms of *Borrelia* using different types of media for cultivation and exposing *Borrelia* cultures to selected stress conditions such as pH changes, temperature changes and antibiotic treatments used in human medicine against Lyme disease.

2. Demonstrating the ability of borreliae spirochetes *in vitro* to return from the dormant to the active form under favourable conditions.

3. Development of a suitable pcr methodology for primers aimed to detect dormant forms of *Borrelia*.

4. Monitoring of infectious potential of atypical forms of spirochetes *in vivo* on a laboratory model.

5. Real time observation of the transformation of spirochete from active to dormant form and subsequently back to the vital form.

4. MATERIALS AND METHODS

4.1. Media used for cultivation of *Borrelia burgdorferi* s. l. spirochetes

The cultivation media used in the following experiments of *Borrelia burgdorferi* s.l. complex are widely used and appropriate for the cell propagation, containing specific volume of individual ingredients (Tab. II.). Previous studies have shown, that the contents of media for cultivation of *Borrelia* may impact the cell culture features (Preac-Mursic et al. 1986; Barbour 1984; Pollack 1993; Ružić-Sabljić et al. 2014). Therefore, borreliae cultures were observed for changes during cultivation in media mentioned below based on the following criteria: growth time, presence of atypical forms and biofilms.

Tab. II. Components of media used for cultivation of *Borrelia burgdorferi* s. l. in our experiments.

N°	Component	BSK II [per liter]	MKP [per liter]	MKP-F [per liter]
1	10x CMRL	9.7 g	9.7 g	9.7 g
2	Neopeptone	5 g	3 g	2 g
3	BSA	50 g	12,25 g	65.6 g
4	Yeastolate	2 g	-	-
5	HEPES acid	6 g	6 g	3.9 g
6	Glucose	5 g	3 g	3.3 g
7	Sodium citrate	0.7 g	0.7 g	0,5 g
8	Sodium pyruvate	0.8 g	0.8 g	0.5 g
9	N-Acetyl-D-glucosamine	0.4 g	0.4 g	0.3 g
10	Sodium bicarbonate	2.2 g	2 g	1.4 g
11	Gelatin	10 g	10 g	7 g
12	Rabbit serum	64 ml	55.1 ml	44.8 ml
13	Mili-Q water	-	-	162.8 ml
14	Fetal calf serum	-	-	100 ml

Volume differences between specific components of the media are highlighted (in orange).

4.1.1. Preparation of BSK II medium

Medium Barbour-Stoenner-Kelly II (BSK II) was prepared according to protocol of Dr. P. Rosa (NIAID 2017[2]). The first three ingredients in the list were stirred until dissolved in ¾ of final volume of MilliQ water. Subsequently, following ingredients were added. After the whole solution was dissolved, gelatin and heat-inactivated rabbit serum were added. When all components dissolved, the pH was adjusted to 7.6 with 5N NaOH. The final

volume was heated up to 55°C until ready to filter. The medium was filtered through 0.2 µm filter. The medium was stored at -20°C before use.

4.1.2. Preparation of MKP medium

Modified Kelly-Pettenkofer (MKP) medium was prepared according to protocol of Ružić-Sabljić (Ružić-Sabljić et al. 2014)

Basic medium was prepared as follows: Components 1-10 (except n°3) from the Tab II. were stirred until dissolved in ¾ of final volume of MilliQ water. The pH of medium was adjusted to 7.6 with 5N NaOH. Basic medium may be stored after filtration through 0.2 µm filter at -20°C.

MKP medium was made by adding gelatin, heat-inactivated rabbit serum and the BSA solution to the basic medium (Tab. II) at 33°C. When mixed well, MKP was sterilized by filtration (0.2 µm). Complete MKP medium was stored at +4°C.

4.1.3. Preparation of MKP-F medium

Modified Kelly-Pettenkofer (MKP) medium containing 10% fetal calf serum (MKP-F) was prepared according to protocol of Wagenmakers and collective (Wagenmakers et al. 2014).

Medium was prepared by dissolving ingredients from point 1-10, including point 13 (Tab. II) in ¾ of final volume of MilliQ water. Subsequently, 500 ml of 65.57 g/L BSA dissolved in Milli-Q water was added. Finally, 127.3 ml of 7% gelatin and heat-inactivated fetal calf serum were added. When all components dissolved, the pH was adjusted to 7.7 with 5N NaOH. The final volume was heated up to 55°C until ready to filter. The medium was filtered through 0.2 µm filter. The medium was stored at +4°C before use.

The media must be heated up to 55°C until the time of filtration due to the room temperature of 21°C, causing cooling down and solidification of gelatin. This feature results in blocking of the filter pores and causing incorrect concentration of gelatin in the media. The temperature of the media during heating must not exceed 55°C, while overheating the media has a negative impact on the multiplying of spirochetes.

Experiments were generally prepared in aliquots of 200 ml. After filtration, the medium was divided into 50 ml falcon tubes in the fume hood (Esco Airstream® Class II BSC) and stored at +4 °C / -20 °C (depending on storage conditions of specific medium, see Tab. III) to avoid frequent temperature changes (such as thawing) of the entire volume and the risk of contamination and degradation of the content.

Tab. III. Storage conditions of media used in our study.

Type of medium	Temperature [°C]	Maximum time of storage [months]
BSK II	-20	3
MKP- basic medium	-20	3
MKP-complete	+4	1
MKP-F	-20/+4	3/1

Cultivation media BSK II and MKP were used for up to 3 months. It is known that exceeding of limit of the above-mentioned maximum storage length (+4°C) for MKP medium does not affect any of the above observation criteria for *Borrelia burgdorferi* s. s. strain, which was mostly used in our study (Veinovic et al. 2013).

In order to prevent contamination of the culture, 2 ml of fresh filtered media was collected from the falcon tubes to Eppendorf tubes (2 ml) and stored at 33.5 ° C within 3 days. In case of contamination, the yellow colour of the medium begins to emerge within 24 hours of propagation. If no colour change of the Eppendorf tube appeared, the sample was considered negative for contaminatom. The result was additionally confirmed by microscopic observation using dark field (DarkField LED microscope Leica DM1000). In the presence of contamination, the stored falcon tube was thawed, filtered again through the 0,2 μm filter and the test for contamination was repeated. For all experiments, only media with no contamination were used to avoid undesirable changes during cultivation of *Borrelia* spirochetes.

4.2. Strains of *Borrelia burgdorferi* s. l. complex used in our study

Borrelia types used in our study included strains SCW53, GFP and CB43 (*B. afzelii*). Both SCW53 and GFP belong to genospecies *Borrelia burgdorferi* s. s., while GFP (*Bbss 297*) has been genetically modified to express green fluorescent protein "GFP" (strain provided by MSc. Martin Strnad). This strain was used in most experiments to obtain the necessary photo documentation of the results in the highest quality.

4.3. Observation of *Borrelia* cultures *in vitro*

Culture samples of GFP strain were examined with a fluorescence microscope Olympus BX-60 equipped with an Olympus C-3030 ZOOM camera. Strains SCW53 and CB 43 were observed with dark field microscope Leica DM 1000 LED.

4.4. Production of atypical forms of *Borrelia burgdorferi* s. l. spirochetes

For the experiment of observing various metamorphoses of borreliae spirochetes were used following stress conditions: changes of pH of cultivation media, changes of temperature of cultivation, and antibiotic treatment used in human medicine against LB.

The cultures used for the production of atypical forms were clear from contamination and well grown (spirochet density: 10^7 cells / ml) in the initial phase of the experiments, stored at 33.5 ° C. Handling of samples was always performed in a flow box ((Esco Airstream® Class II BSC). Visual check of the cultures was performed with dark-field microscopy at 200-400 magnification and GFP fluorescence microscopy at 200-1000 magnification. Cultures were monitored for changes in following parameters: density of motile spirochetes, number of RBs, blebs and biofilms. The spirochete concentration was evaluated by means of an average of 10 fields of view multiplied by x 3.9 x 10^5. This resulted in number of spirochetes per ml. Least-square fitting analysis of Petroff-Hauser counting chamber (Counting Chamber w/2 cover glass Hausser Scientific) was applied too.

4.4.1. Changes of pH.

The pH values used during cultivation of *Borrelia* under stress conditions were inspired by pH of human blood and organs and their possible pH changes, characteristic for various health problems. Bacteremia of *Borrelia* generally occurs in the bloodstream with a typical pH of 7.4. Depending on the health condition, pH may fluctuate and pontential acidosis of organism (acidemia) may occur when pH of blood falls below 7.35 (kidney or lung failure, etc.) (Atkinson & Burke 1995; Boron & Boulpaep 2016; Pizzorno 2015). On the other hand, alkalemia occurs at pH over 7.45 (Atkinson & Burke 1995).

Optimal growth of spirochetes in medium is observed at pH range of 7.4-8. Therefore, we decided to investigate the state of *Borrelia* in cultivation below pH 7.4, while these pH values may also occur in human organism (see Tab. IV) (Atkinson & Burke 1995; Barbour 1984).

Tab. IV. pH values of various human tissues and blood.

Organ	pH	Refference
blood	7.3-7.4	Boron & Boulpaep 2016; Pizzorno 2015
saliva	6.4-7.0	Satyanarayana & Chakrapani 2013
skin	4.0-7.0	Lambers et al. 2006

This study included pH values of 7.06, 7.14, and 7.3 to create conditions, which *Borrelia* spirochetes might possibly occur in. Media used in the study were following: BSK II, MKP, and MKP-F. Strains used in the study included CB43, SCW53 and GFP. The media were freshly made and the pH was adjusted with 35% hydrochloric acid solution (11 M). Well-grown spirochetes (1 x 10^5 cells/ml) in a particular medium with optimal pH conditions for growth were put in the given pH-reduced medium at a ratio of 1:10 to a total volume of 5500 µl. Samples were observed 5 days after transfer to pH-reduced medium. After 5 days, samples were used for reverse process (see details of reverse procedure in chapter 4.5.). Samples were observed for another 20 days. The experiment was repeated 15 times.

4.4.2. Changes of cultivation temperatures

Three strains of *Borrelia* (CB43, SWC 53, and GFP) were used for the experiment. Well-grown spirochetes (1 x 10^7 cells/ml) in a particular medium (BSK II, MKP, MKP-F) were transferred to a new cultivation tube at a ratio of 1:10 (well-grown culture: fresh medium) to a total volume of 5500 µl. Samples were placed in the thermostat at 33.5 ° C and room temperature, while 33- 34°C is known to be an optimal temperature for cultivation of borreliae. Therefore, samples in 33.5 °C were considered as control tubes. See the procedure in the detailed scheme below (Tab. V). Cultures were observed 5 days after the beginning of the experiment. Following controls were done at least 2 times per week. The experiment was monitored for 50 days with repetition of 30 times.

Tab. V. Media and temperatures used for individual strains to observe their behaviour under possible stress conditions.

Strain	Medium	Temperature [°C]	
CB43	BSK II	21	33.5
	MKP	21	33.5
	MKP-F	21	33.5
SCW53	BSK II	21	33.5
	MKP	21	33.5
	MKP-F	21	33.5
GFP	BSK II	21	33.5
	MKP	21	33.5
	MKP-F	21	33.5

For one experiment, 6 sample tubes were made for each strain. Each strain was grown in BSK II, MKP and MKP-F medium with impact of 2 cultivation temperatures (21°C, 33.5°C)

4.5. Reverse process of atypical forms to motile spirochetes

When atypical forms of borreliae under above mentioned stress conditions occured, the samples were observed for reverse process to motile forms of spirochetes. Only samples with above 80% of atypical forms of spirochetes (blebs, biofilms, RBs) were used. Such cultures were transferred to new cultivation tubes with medium at a ratio of 1:10 to a total volume of 5500 µl. The medium used to cultivate an individual strain of *Borrelia* under reverse process has always been the same as from the initial growth of culture and subsequent atypical formation experiment samples were placed in the thermostat at 33.5 °. New cultivation tubes used for reverse process were checked 1 time per week.

4.6. Development of a new polymerase chain reaction (PCR) method to detect atypical forms of *Borrelia* cultivated *in vitro*

4.6.1. Genomic DNA (gDNA) isolation from *Borrelia* cultures

Purification of total DNA of well-grown ($1x10^7$ cells/ml) culture and 90% dormant-form culture (biofilms, blebs, RBs) of GFP strain (density $1x10^7$ cells/ ml) was prepared

according to protocol designed for Gram-negative Bacteria in DNeasy® Blood & Tissue Handbook 07/2006 (Qiagen).

Firstly, each cell culture was centrifuged in a microcentrifuge tube for 10 min at 5000 x g (Eppendorf 5415D). Subsequently, the purification of DNA was performed. Proteinase K and all buffers used for this method were provided by DNeasy® Blood & Tissue Kit (Qiagen). DNA isolates were stored at +4 °C.

4.6.2. PCR settings

Various PCR programs using several sets of primers have been tested for the amplification of *Borrelia* genes from cultures of 90% dormant forms (biofilms), due to the fact that persistent forms of *Borrelia* are known to express other sets of genes (*hipA, relA, glpA, glpB*) in compare to motile spirochetes (Zhang 2014).

Annealing temperatures of individual sets of primers were calculated (Tm Calculator v 1. 10.2 NEBtools^{TM}) and tested for evaluation of optimal annealing temperature (Tab. VI). The annealing temperature gradients of the primers were tested for gDNA of dormant-type GFP culture with gDNA concentration= 2 ng. Subsequently, the annealing temperatures showing the most significant responses were applied in nested-PCR with following dilution series of gDNA: 1, 1:10, 1:100, 1:1000, and 1:2000. DNA was diluted with Milli-Q water.

The highest sensitivity for DNA recognition at the lowest possible concentrations in the nested-PCR reaction was performed with sets of primers 3, 4 and subsequently their combination (Tab. VII) at the their previously detected optimum temperature. Therefore, this primer combination was used in further studies of this work.

PCR for testing of annealing temperatures was performed in a 20 µl reaction volume containing 10 µl 2x Taq Hot Start Plus Polymerase (Qiagen), 1 µl of each primer (GeneriBiotech), 3 µl of purified DNA and the remaining volume was adjusted with RNase-Free H_2O (Qiagen). Same values of individual ingredients have been used for the first PCR program of nested-PCR. For the second phase of nested PCR were used following ingredients: 10 µl 2x Taq Hot Start Plus Polymerase (Qiagen), 1 µl of each primer (GeneriBiotech), 5 µl RNase-Free H_2O (Qiagen) and 5 µl of the PCR product of the first reaction.

The most successful settings of the amplification, transformation and cloning parameters are listed in the Table VIII.

Combination of 3rd a 4th set of primers (as shown in Tab. VII) was applied in nested-PCR reaction for gDNA of well-grown culture with no presence of persistent forms and culture of 98% of dormant forms (95% biofilms) to determine efficacy of primers to individual morphotypes of spirochetes. In addition, gDNa of reverse culture from dormant to motile forms was tested, while the response in PCR product showed similar results as for the well-grown culture without dormant types of spirochetes. Concentration of gDNA of individual *Borrelia* culture was equalized to 2 ng/ μl. Following dilution series were performed for each type of culture gDNA: 1, 1:10, 1:100, 1:1000, and 1:2000. DNA was diluted with Milli-Q water. To compare the efficacy of individual primers in detection of specific forms of *Borrelia* spirochetes, primers set of *flagellin* inn/out (Tab. IX) was used in nested-PCR for gDNA of well-grown culture, persistent-form culture and reverse culture in the same way as for *GlpA* primers. The annealing temperatures and other sets of PCR for *flagellin* are known (Tab. X).

Tab. VI. Calculated temperatures of annealing of individual sets of *GlpA* primers.

Set of primers	Description	Primer	Primer sequence (5 ' → 3')	Suggested annealing temperature [°C]	Size of product [bp]
1	1598A1	*glpA1*	TAATTGGAGGAGGCGCAACA	52	508
	1598A2	*glpA2*	CTCCGTGTAGTTGAGGGCAA		
2	1598A3	*glpA3*	GCCTTACACGAAAAAGCCGT	53	323
	1598A4	*glpA4*	GTTGAGGGCAATACCCCCTT		
3	1598A7	*glpA7*	AGGGGGTATTGCCCTCAACT	53	332
	1598A8	*glpA8*	TCGGTGCTTCCACAAACAAC		
4	1598B1	*glpB1*	ATTGCCCTCAACTACACGGA	52	328
	1598B2	*glpB2*	ATGTCGGTGCTTCCACAAAC		

Tab. VII: Combination of primers used in nested-PCR showing the best results.

PCR	Primer	Primer sequence (5 ' → 3')	Annealing temperature [°C]	Product size [bp]

1st run	*glpA7*	AGGGGGTATTGCCCTCAACT	56	335
	glpB2	ATGTCGGTGCTTCCACAAAC		
2nd run	*glpA8*	ATTGCCCTCAACTACACGGA	55	325
	glpB1	TCGGTGCTTCCACAAACAAC		

Tab. VIII. Master Mix and PCR settings used for amplification of GFP strain-atypical form.

PCR program 1			
Step	Temperature [°C]	Time [min.]	
Initialization	95	5:00	
Denaturation	95	0:30	35x
Annealing	*	0:30	
Elongation	72	1:00	
Final extension	72	5:00	
Hold	12	∞	

PCR program 2			
Step	Temperature [°C]	Time [min.]	
Initialization	95	5:00	
Denaturation	95	0:30	25x
Annealing	*	0:30	
Elongation	72	1:00	
Final extension	72	5:00	
Hold	12	∞	

* The annealing temperature of specific set of primers is shown in Results, Tab XVI.

Tab. IX. Description of *flagellin* primers used in the study.

Primer	Primer sequence (5 ' → 3') F/R	Annealing temperature [°C]	Size of PCR product [bp]	Refference
Flagellin out	AARGAATTGGCAGTTCAATC	52	497	Clark et al. 2005
	GCATTTTCWATTTTAGCAAGTG			
Flagellin inn	ACATATTCAGATGCAGACAGAG	55	389	Clark et al. 2005
	GAAGGTGCTGTAGCAGGTGCTG			

Tab. X. Nested-PCR process for testing detection of GFP gDNA dilutions using *flagellin* primers.

PCR program 1			
Step	Temperature [°C]	Time [min.]	
Initialization	95	5:00	
Denaturation	95	0:30	35x
Annealing	52	0:30	
Elongation	72	1:00	
Final extension	72	5:00	
Hold	12	∞	

PCR program 2			
Step	Temperature [°C]	Time [min.]	
Initialization	95	5:00	
Denaturation	95	0:30	25x
Annealing	55	0:30	
Elongation	72	1:00	
Final extension	72	5:00	
Hold	12	∞	

4.7. Gel electrophoresis

The results of PCR reactions were visualized in 1.5% agarose gels (Serva) stained with SYBR Green (6x Orange DNA Loading Dye, 50x SYBR Green, Thermo Scientific). Total volume of each sample was loaded on the gel in 1x TAE buffer using 105 V for 40 minutes (Thermo Scientific Owl EasyCast B2 Mini Gel Electrophoresis Systems). PCR product size was determined according to the 100 bp DNA ladder (Thermo Scientific). Visualisation of DNA fragments was performed with UV light (Gel Logic 112).

4.8. Cultivation with antibiotics used for treatment for Lyme borreliosis *in vitro*

Borrelia spirochetes were exposed to antibiotics used in human medicine against LB to observe the ability of *Borrelia* spirochetes to modify into atypical form and subsequently create a culture of motile cells.

Borrelia culture (strain GFP, stored at -80 °C) was grown in 2 x 15 ml falcon tubes filled with MKP medium, kept in dark at 33.5 °C. After 14 days the the concentration of spirochaetes reached 1x 10^7 sp/ml. Cultivation tubes without contamination were centrifuged

at 3200g/30min at room temperature. Sediment of each falcon tube was re-suspended in 3 ml of supernatant. Suspensions of 500μl of the first falcon tube were placed to 5x 15 ml falcon tubes filled with 12 ml of MKP medium with doxycycline concentration of 25, 50, 100, 200 μg/ml and to 12 ml of Milli-Q water (Tab. XI). Another new falcon tube contained only 12 ml fresh MKP medium with 500 μl of GFP suspension, as a positive control. All antibiotic stocks were filter-sterilized using a 0.2 μm filter.

Suspensions of 500 μl of the second falcon tube were placed to 5x 15 ml falcon tubes filled with 12 ml of MKP medium with amoxicillin concentration of 25, 50, 100, 200 μg/ml and (Tab. XII). Another falcon tube contained only 12 ml fresh MKP medium with 500 μl of GFP suspension, as a positive control.

All samples were kept in dark at 33.5 °C and examined by darkfield microscopy at 250x and 400x magnification. Spirochete density was counted with Petroff-Hausser chamber.

Tab. XI. Ratio of antibiotic treatment (amoxicillin, doxycycline) and Milli-Q water used in cultivation of GFP strain in MKP medium.

N°	ATB		MKP [μl]	GFP culture [μl]
	Concentration [μg/ml]	Actual volume [μl]		
1	25	325	11675	500
2	50	650	11350	500
3	100	1300	10700	500
4	200	2600	9400	500
	H_2O			
5		13000	-	130

4.8.1. Reverse process of spirochetes from atypical to vital form

Antibiotic-treated and Milli-Q water-treated samples reached 98% presence of RBs after 72 hours.

After 2, 7, 14 and 21 days of emerging 98% RBs in cultures, 240 μl of each sample was transferred into a new glass cultivation tube with 6 ml of MKP medium. Another 240 μl of each culture was filtered through 0.45 μm filter to a new cultivation tube with 12 ml of MKP after 2, 7, 14 and 21 days of RBs emerging. A previous study confirms that this process prevents the passage of RBs and other atypical forms of spirochetes through the 0.45 μm filter, while vital individual spirochetes are capable of passing through the membrane (Gruntar et al. 2001). Filter-treated cultures in our experiment should not show any

proliferation of spirochetes, since these cultures contained almost 100% RBs forms of *Borrelia* cells.

Samples were kept at 33.5 °C and examined by darkfield microscopy at least 1 per week for the presence of motile spirochetes. Cultures were centrifuged every 2 weeks at 3200 g/ 30 min, at room temperature. The supernatant was removed and replaced with 12 ml fresh MKP medium.

4.9. Infection of laboratory model with atypical form of GFP strain

4.9.1. Preparation of material used for *in vivo* experiment.

Borrelia culture (strain GFP, stored at -80 °C) was grown in 15 ml falcon tube filled with MKP medium, kept in dark at 33.5 °C. After 14 days the the concentration of spirochaetes reached 1x 10^7 cells/ml. Cultivation tube without contamination was centrifuged at 3200g/30min at room temperature. Sediment was re-suspended in 3 ml of supernatant. Suspensions of 500 µl were placed to 15 ml falcon tubes filled with 12 ml of MKP medium with doxycycline concentration of 25, 50, 100, 200 µg/ml and to 12 ml of Milli-Q H_2O (Tab. XII). Another falcon tube contained only 12 ml fresh MKP medium with 500 µl of GFP suspension. *Borrelia* cultures treated with doxycycline were kept in dark at 33.5 °C for 72 hours until reached 98% of presence of RBs. All antibiotic stocks were filter-sterilized using a 0.2 µm filter.

Cultures were examined by darkfield microscopy at 250x and 400x magnification and counted with Petroff-Hausser chamber.

Tab. XII. Ratio of doxycycline treatment and Milli-Q water used in cultivation of GFP strain in MKP medium.

N°	Doxycycline		MKP [µl]	GFP culture [µl]
	Concentration [µg/ml]	Actual volume [µl]		
1	25	325	11675	500
2	50	650	11350	500
3	100	1300	10700	500
4	200	2600	9400	500
	H_2O			
5		13000	-	130

4.9.2. Process of infection of laboratory model

Naive C3H laboratory mice from the Institute of Parasitology were used for inoculation with various experimental material. Laboratory models were males, 6-8 weeks old with weight of 25 g each. A total of 20 mice divided into 7 groups were provided. The process of infection of individual groups of mice is shown in Tab. XIII. Each mouse was infected with specific material in two ways, subcutaneously (100 μl) and intraperitoneally (150 μl).

Tab. XIII. Detailed scheme of spirochaetal material exposed to different types of 'anti-spirochete- treatment' before inoculation to individual groups of mice.

Group	Inoculation material		Number of mice
	Doxycycline [μg/ml]	Other material	
1	25	-	2
2	50	-	4
3	100	-	4
4	200	-	4
5	-	Milli-Q H_2O	4
6	-	fresh MKP medium	1
7	-	GFP culture (c= $1x10^7$ sp/ml)	1

Individual groups of mice were inoculated with specific material based on considered infectious potential of borreliae. The individual inoculation was ranged from negative control to the highest probability of infection in order to avoid potential contamination by the more vital spirochetes based on observations from the previous study (3.3.1.). The first group contained one mouse that was inoculated with fresh MKP medium, without *Borrelia*, serving as a negative control (n ° 6). Following group of mice was infected with *Borrelia* spirochetes cultivated in Milli-Q H_2O. Other groups of mice were injected with doxycycline-treated borreliae, in the order of ATB concentration of 200 μg/ml, 100 μg/ml, 50 μg/ml and 25 μg/ml, respectively. To inoculate laboratory mouse of the positive control was used uncontaminated well-grown spirocheatal culture.

4.9.3. Tissue collection of laboratory model for further analyses

Dissection of mice was performed 40 days post infection in the following order: from group of the negative control (7), subsequently groups 5-1 and finally, group 6 to avoid contamination of potentially negative samples by positive cases (see the Tab. XIII). Firstly, mice were anesthetized intraperitoneally with 150 µl of anesthesia (8mL 5% Narkamon (Spofa) + 2ml 2% Rometar (Spofa) + 10 ml PBS). Blood was collected under anesthesia, followed by termination of animal and organ harvesting – urinary bladder, joint of right hind limb. Tissues were harvested in 2ml microcentrifuge tubes. Tissues were cut up to 25 mg amounts with sterile tools (scalpel, scissors, and tweezers) in the fume hood. Subsequently, they were divided into two halves. One half of the organs were collected in sterile 2ml eppendorf tubes for cultivation of spirochetes from tissues of infected mice. 1.5ml MKP medium was added into each eppendorf used for this experiment. Blood of mice was centrifuged at 1400 x g for 15 min, at room temperature (Eppendorf 5415D). Centrifugation separated the blood serum from erythrocytes. Supernatant (blood serum) was placed into a new microcentrifuge tube 1.5 ml and the elution was repeated. Another half of tissues and blood sera were stored at -20°C for further analyses.

4.9.4. Control experiment *in vitro* observing spirochaetal changes after re-establishment of optimal conditions after antibiotic treatment.

Volume about 240 µl of 98% RBs suspension of GFP cultures from experiment mentioned above was transfered to 12 ml MKP medium: 2, 7, 14, 21 days after doxycycline treatment of various concentrations and Milli-Q water treatment. Control tubes of 240 µl with 0.45 µm filtered 98% RBs suspension of each experiment (2, 7, 14, 21 day) were made, using 12 ml MKP medium. All samples were kept at 33.5 °C and examined by darkfield microscopy at least 1 per week for the presence of motile spirochetes. Spirochetes were counted with Petroff-Hauser chamber. Cultures were centrifuged every 2 weeks at 3200 g/ 30 min at room temperature. Sediment was resuspended in 1 ml of supernatant and 11 ml of fresh MKP was added. Samples were observed for 2 months.

4.10. Detection of *Borrelia burgdorferi* s. s. from tissues and blood sera of infected laboratory models by atypical form of GFP strain.

Laboratory mice were injected GFP RBs produced by various stress conditions for the growth of *Borrelia* spirochetes. Organs and blood of mice were observed for the presence of *Borrelia* DNA by various diagnostic methods.

4.10.1. Rapid immunochromatographic test

Fresh blood serum of 1 mouse of each experimental group was tested for the detection of specific antibodies of *Borrelia burgdorferi* s. l. with BORRELIA LYMETOP® Rapid Test. Blood serum of 20 µl was dispensed in the sample well (LYMETOP®). After 30 seconds, 80 µl of the developer solution from the kit was added to the sample well "S" (LYMETOP®). Results were ready at 10 minutes exactly.

4.10.2. PCR detection of spirochetes from blood and tissues (nested-PCR)

The infectious potential of *Borrelia* for various tissues and blood of laboratory mice was analysed with PCR method, based on previously designed settings of specific *GlpA/GlpB* primers (see chapter 4.6. for more details) and *flagellin* out/in primers.

4.10.2.1. Isolation and purification of gDNA from tissues and blood

Tissue collections for PCR analysis were cut to small pieces with sterile scalpel immersed in 180 µl of ATL Buffer (DNeasy® Blood & Tissue Kit Qiagen) in flow-through box. Isolation of genomic DNA (gDNA) was performed according to the kit manufacturer's protocol (DNeasy® Blood & Tissue Handbook 07/2006 Qiagen).

Subsequently, gDNA purification was done with 1 parameter modified in the proposed procedure, point 7. This part of the protocol proposes the elution of gDNA with 200 µl of Buffer AE. In our experiment, 50 µl of Milli-Q H_2O was used to obtain sufficient DNA concentration for PCR analysis, since using the originally designed buffer may lead to undesirable dilution of samples gDNA used in this study.

All substances used for gDNA preparation for PCR analysis were provided by the manufacturer mentioned above. The isolated and subsequently purified gDNA was stored at + 4 ° C.

4.10.2.2. Nested-PCR settings

Nested polymerase chains reaction (Nested-PCR) has been applied to reduce a non-specific binding in product (mouse gDNA) and simultaneously increase the amplification of targeted DNA (gDNA of *Borrelia*). This analyses involves two sets of primers, used in two successive runs of PCR (Master cycler personal Eppendorf). Sets of primers were targeting *GlpA7,8* and *GlpB*1,2 genes. In the first step, a set of *GlpA*7 and *GlpB*2 primers was used, while *GlpA*8 and *GlpB*1 primers were used in the second run (Tab. XIV). Master Mix and settings of PCR reaction are described in the scheme below (Tab. XV). Master Mix was prepared in UV-cabinet for PCR (BioSan UVC / T-M-AR). DNA was added to MasterMix outside the UV-cabinet. RNase-Free HO was used as a negative control. Confirmed gDNA of GFP strain was used as a positive control.

Tab. XIV. List of primers used for nested-PCR.

PCR	Primer	Sequence (5 ' → 3')	Annealing temperature [°C]	Size of PCR product [bp]
1st run	*glpA*7	AGGGGGTATTGCCCTCAACT	56	335
	*glpB*2	ATGTCGGTGCTTCCACAAAC		
2nd run	*glpA*8	ATTGCCCTCAACTACACGGA	55	325
	*glpB*1	TCGGTGCTTCCACAAACAAC		

Tab. XV. Master Mix and settings of the first PCR program used for amplification of GFP strain-dormant form.

Master Mix		
Product	20 µl reaction	
2x Taq Hot Start Plus Polymerase	10	Qiagen
Primer F	1	GeneriBiotech
Primer R	1	Generibiotech
RNase-Free H_2O	5	Qiagen
gDNA	3	

PCR program			
Step	Temperature [°C]	Time [min.]	
Initialization	95	5:00	
Denaturation	95	0:30	35x
Annealing	56	0:30	
Elongation	72	1:00	
Final	72	5:00	
Hold	12	∞	

The second PCR procedure was performed in total volume of 20 µl including 10 µl of 2x Taq Hot Start Plus Polymerase (Qiagen), 1 µl of each primer (GeneriBiotech), 3 µl of RNase-Free H_2O (Qiagen) and 5 µl of the previous reaction as a template. The annealing temperature was 55 °C and the amplification was repeated 25x. Other settings were the same as for the first run.

4.11. Real time observation of the transformation of spirochete from active to dormant form and subsequently back to the vital form

Monitoring conditions were designed to possibility of observation of spirochetes on the microscope slide from several minutes to 1 hour. At the same time, it was necessary to expose the observed spirochetes to various conditions with the aim of RBs formation and subsequently back to the moving vital form in a fixed position.

For microscopy observation, µ-Dish 35 mm, low Grid-500 slides were used (Ibidi, glass bottom). Cells of borrelia culture were fixed in 1.7 % agar prepared in PBS. The agar gel was kept at 50 °C to prevent solidifacion before use. Next, 0.1 % lecithin in chloroform was prepared in a 2 ml Eppendorf tube. The Eppendorf tube was sealed with parafilm to prevent leakage of chloroform. Other components included BSK II medium, well-grown GFP culture in log phase without presence of atypical forms of spirochetes ($1x10^7$ cells/ml). Observation was performed with an optical microscope Leica TCS SP8 (using fluorescence widefield, 3

dimensional axis-x, y, z), kindly provided by Biocev. Z-series images were acquired in green channel.

Eppendorf tubes of GFP culture, BSK II medium of 2 ml, microscopic slide and cover slip were heated to 35 °C. Cover slip was cleaned with 70% ethanol and dried. Lecithin was spread over the entire surface of the coverslip and dried. A 10 µl GFP culture was added to the heated microscopic slide, followed by 10 µl of heated agarose. The entire volume was gently mixed by pipetting on the slide. The slide was covered with a lecithin-treated coverslip due to feature of lecithin to create a uniform layer of GFP in agarose mixture. Therefore, it was easier to observe the individual spirochetes in the Z-axis. The corners of the cover slide were coated with BSK II medium within 30 seconds of the slide covering the GFP-agarose mixture. After a few minutes, removing of the coverslip and adding required amount of anti-borreliae serum (rabbit serum/ ATB) or additional BSK II medium was possible. Lecithin has also been used to facilitate removal of the coveslip from viscous agarose consistency.

Concentrations and types of sera for induction of dormant forms of spirochetes varied. Rabbit serum was used for the experiment, known for its reduction of spirochetes and their formation to dormant morhotypes up to 90% of culture in a short time. Agarose and lecithin were freshly made before the experiment. Rabbit serum was store at -20°C during the transport to the external laboratory of Biocev.

Firstly, the control experiment of rabbit serum against GFP was tested in our laboratory. Experiment was observed for 1 hour. The rabbit serum was diluted 5:1 with Borrelia culture in 2ml Eppendorf tube. The mixture was observed on a microscopic slide (BP microscope slides 76.2 x 25.4 app, 1.0 MM) with a darkfield microsope every 10 minutes (magnification 200 x, 400 x). The culture was fixed on the slide as in the above mentioned procedure. Afer 10 minutes, the culture contained 60% biofilms, 10% RBs and only 30 % individual and mobile spirochetes. After another 10 minutes, 80% of dormant forms was observed (70% biofilms, 10% RBs). Remaining spirochetes were mostly immobile or slightly moving. 30 minutes after the initiation phase no motile spirochetes and no significant increase of dormant forms was observed. This control experiment included only metamorphoses from vital to dormant form.

Another control experiment was done without using agarose and lecithin. Vital GFP culture without dormant forms (density $1x10^5$ cells/ ml) was grown in 2 ml Eppendorf tube, with rabbit serum as mentioned before. Samples were observed every 10 minutes using darkfield microscopy with magnification of 200 x and 400 x. When 80% of dormant forms

were present, mixture of GFP and rabbit serum was transferred to a new cultivation tube with BSK II, in ratio of 1:10 with total volume of 1 ml. The presence of motile spirochetes appeared in such culture after 15 minutes.

Another control experiments were applied for amoxicillin and doxycycline, where GFP culture of density $1x10^7$ cells/ ml was grown in Eppendorf tube, mixed with 500 µl/ ml amoxicillin in ratio of 1:1. Another Eppendorf tube was mixture of vital GFP with the same density as mentioned above, and 500 µl/ ml doxycycline in the same ratio (1:1). Cultures were grown in BSK II medium. Samples were observed every 10 minutes, while a new sample on microscopic slide from the eppendorf tube was prepared for each observation (darkfield microscopy, magnification 200 x, 400 x). During amoxicillin treatment, even 1 hour after initiation, 50% spirochetes were still motile and spirochaetal. The rest of the culture contained RBs, blebs and biofilms. Nevertheless, the concentration of dormant forms was still low for our experiment. Doxycycline was able to immobilize 90% of the cells within 20 minutes of treatment. GFP was cultivated with doxycycline for another 20 minutes to reach 98% dormant morhpotypes. After that, the culture was transferred to BSK II medium in 1:1 ratio of a total volume 1.5 ml. After 15 minutes, 50% of spirochetes in BSK II were able to move and perform in spirochaetal shape. The motility and presence of spirochaetal cells no longer increased exponentially.

Experiment using confocal microscope Leica TCS SP8 was performed with following instructions: 10 µl agarose (1.7%) and 10 µl well-grown GFP ($1x10^7$ cells/ml) were mixed together by pipetting on the microscopice slide. The sample was covered with a lecithin-treated cover slip. The corners of cover slide were coated with BSK II medium. In several minutes, the coverslip was removed and 80 µl rabbit serum was added to the mixture on the slide. A video of the spirochete response to rabbit serum was produced. After dormant formations, 1 ml BSK II was added and the culture was further observed.

In addition to rabbit serum, amoxicillin, doxycycline were tested in our laboratory for RBs and reverse process formation. However, our lab's experimental results could not be repeated on a confocal microscope. Fresh BSK II medium, which was used to restore spirochetal motility, was in such a high volume in the ratio of the slide on the agarose gel that the spirochetes were moved from their original position by the applied medium.

5. RESULTS

5.1. Production of atypical forms of *Borrelia burgdorferi* s.l.

5.1.1. Changes of pH

Strains SCW53, GFP and CB43 have been observed for morhotype changes caused by non-optimal pH reduction of various cultivation media. The lowest pH (7.0) used in the study resulted in a rapid decrease in spirochetes in each medium and for each strain. This condition was observed 5 days after well grown culture have been transferred into an inappropriate culture environment. At this stage, the cells were moved into fresh medium at a 1:10 ratio. New culture tubes were observed 5, 10 and 20 from the initial phase of the reverse process. Following acid pH treatment, spirochetes have been forming following morphotypes: RBs, blebs, and biofilms. However, they were capable of re-proliferation under optimal conditions. The following figures describe parameters for the formation of different spirochetal morphotypes related to pH changes and individual media for SCW53 strain (Fig. IV, V, VI, VII). The values in the graphs are the average of 15 observations of the same parameters for individual strains.

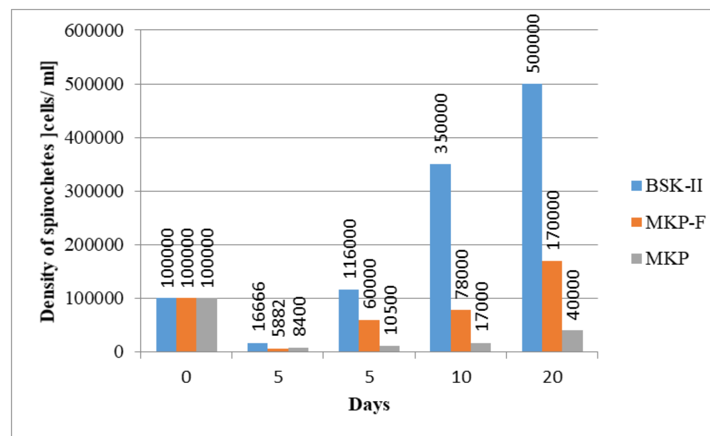

Fig. IV. The growth rate of strain SCW53 at pH= 7.0 cultivated in BSK II, MKP-F and MKP medium.

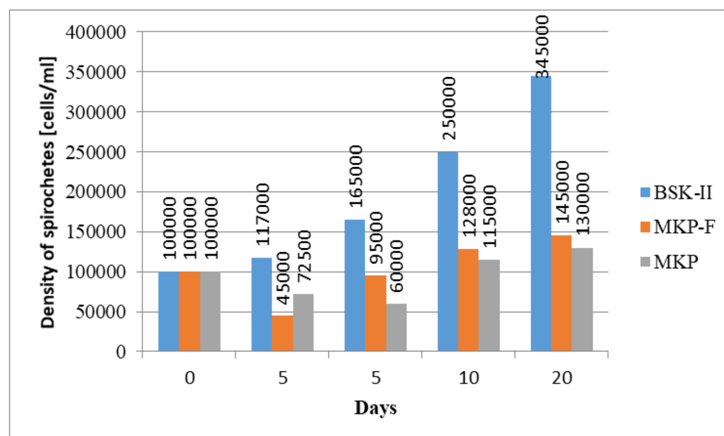

Fig. V. The growth rate of strain SCW53 at pH= 7.1 cultivated in BSK II, MKP-F and MKP medium.

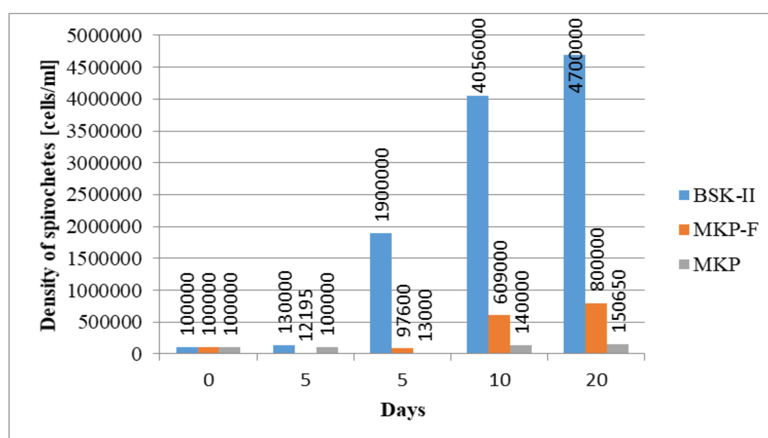

Fig. VI. The growth rate of strain SCW53 at pH= 7.3 cultivated in BSK II, MKP-F and MKP medium.

The ratio of formation of atypical forms in the treatment of different media with a specific pH is shown in the following figure (day 5). Graphs show appearance of atypical form of spirochetes from pH 7.0 to pH 7.3.

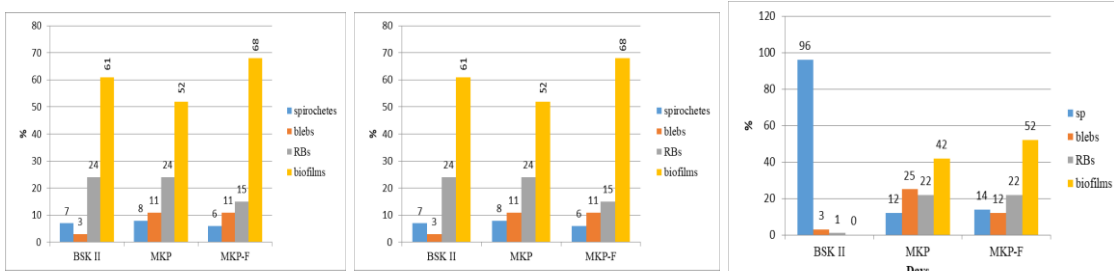

Fig. VII. Ratio of morhotypes induced by pH changes 7.0, 7.1, 7.3 after 5 days of low-pH exposure.

Typical morhotypes of SCW53 after 5 days of low-pH exposure (7.3) using MKP as a cultivation medium, are shown in the following figure (Fig. VIII). Typical morphotypes of SCW53 exposed to 7.0 pH are shown in figure VIX.

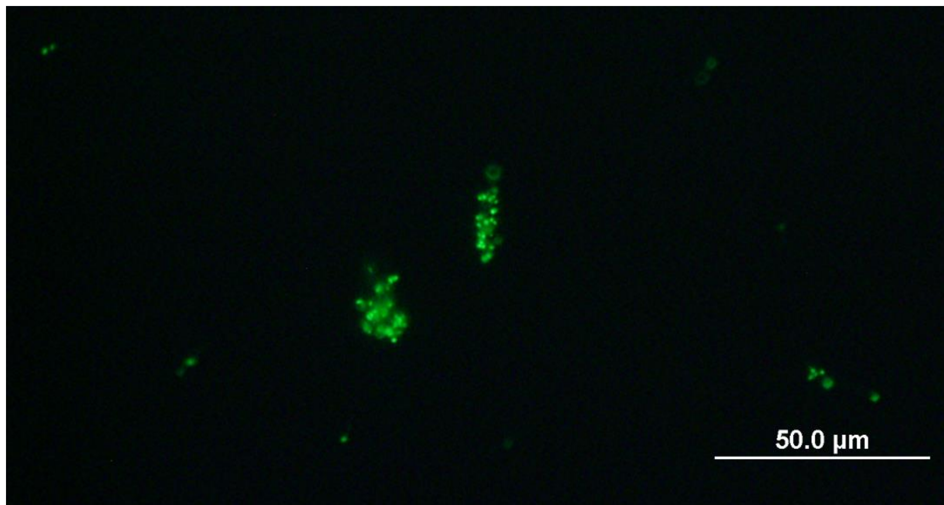

Fig. VIII. Round body morhotypes and biofilm-like forms of *Borrelia burgdorferi* s. s. (SCW 53) exposed to pH changes for 5 days. (Fluorescence microscope Olympus BX-60, magnification= 1000x)

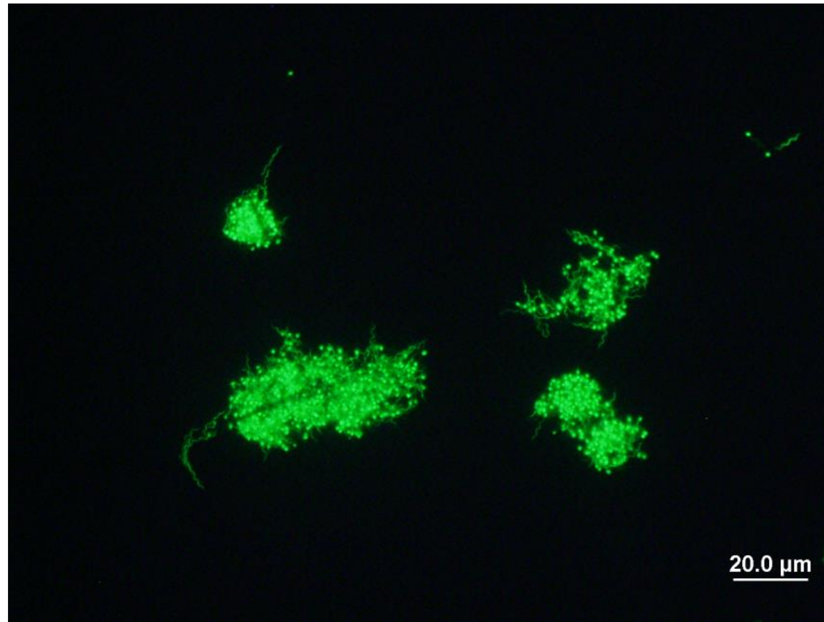

Fig. VIX: Blebs and biofilms produced in any type of cultivation media in our experiments, exposed to pH 7.0 for 5 days. (Fluorescence microscope Olympus BX-60, magnification= 400x).

The scheme describes that the pH decrease of 7.1 and 7.0 induced production of biofilms in higher numbers in compare to 7.3 pH value. Furthermore, RBs were present in approximately 25% of each culture, followed by blebs and moving spirochetes in the lowest number. For BSK II at pH 7.3, the spirochetes did not decrease their numbers at all. pH 7.3 in BSK II medium did not appear to cause stress conditions, while cells were able to proliferate. Other media with pH 7.3 induced decline of spirocheteal concentration and production of atypical morphotypes though at a lower rate than at pH 7.1 and 7.0.

Similar result have been observed for the strain GFP and CB43. GFP was capable of faster proliferation, when transferred to fresh medium with recommended pH (according to protocols of individual media). On the other hand, strain CB43 performed as least adaptive to changes of its environment. Nevertheless, CB43 was capable of proliferation after exposing to optimal medium, too.

Graphs and schemes showing the results of CB43 and GFP strains and detailed schemes of GFP graphs are attached in Appendices.

5.1.2. Changes of cultivation temperature

The results of exposing different strains of *Borrelia* to the non-optimal temperature of the cultivation showed the formation of biofilms and blebs but almost no RBs occured. Their presence in the observed culture would be possible but it was not visually detectable. MKP- has been shown to be the medium of the fastest spirochaetal profliferation.There was almost no difference between behaviour of GFP, SCW53 and CB43 strains during this experiment. Cultures exposed to temperatures of 21 ° C grew more slowly but were able to grow up to 40 days from initial phase, reaching 9.2 x 10^6 spirochetes / ml. At 33.5 ° C, cultures reached the highest concentration at 20th from initial phase with approximately 1.5 x 10^8 cells / ml, while they were exposed to MKP-F medium. See the graphs in Appendices.

5.2. New PCR method for detection of dormant forms of *Borrelia burgdorferi* s. l.

Optimal temperatures of annealing of primers were calculated and tested. Recommended annealing temperatures differed from the actual optimum temperatures of about 3 °C.

Tab. XVI. Identified temperatures of annealing of individual sets of *GlpA* primers.

Set of primers	Primer	Primer sequence (5 ' → 3')	Calculated temperature [°C]	Optimal temperature [°C]	Size of product [bp]
1	*glpA1*	TAATTGGAGGAGGCGCAACA	53	56°	508
	glpA2	CTCCGTGTAGTTGAGGGCAA			
2	*glpA3*	GCCTTACACGAAAAAGCCGT	52	55	323
	glpA4	GTTGAGGGCAATACCCCCTT			
3	*glpA7*	AGGGGGTATTGCCCTCAACT	53	56	332
	glpA8	TCGGTGCTTCCACAAACAAC			
4	*glpB1*	ATTGCCCTCAACTACACGGA	52	55	328
	glpB2	ATGTCGGTGCTTCCACAAAC			

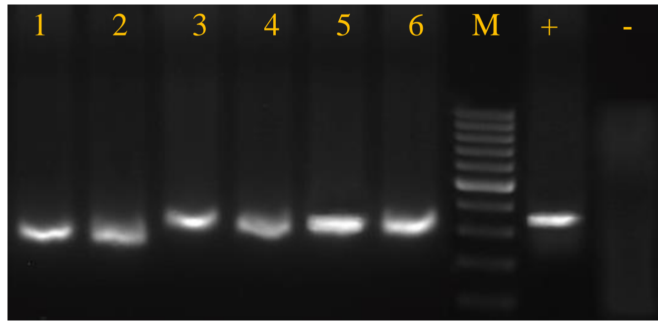

Fig. X. Test of annealing temperature for primer *GlpA*3 and *GlpA*4 (1=48°C, 2=50°C, 3= 52 °C, 4= 53°C, 5=55°C, 6= 57°C, M= 100 bp DNA marker, + = positive control (GFP), - = RNase-free H_2O).

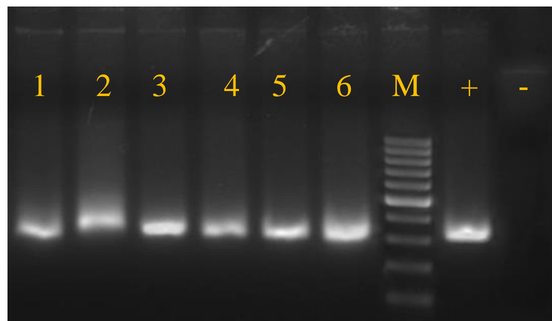

Fig. XI. Test of annealing temperature for primer *GlpB7* and *GlpB8* (1=52°C, 2=54°C, 3= 56 °C, 4= 58°C, 5=60C, 6= 62°C, M= 100 bp DNA marker, + = positive control (GFP), - = RNase-free H_2O).

The optimal annealing temperature for the GlpA primer set was considered to be 55°C and 56°C for the GlpB primer set.

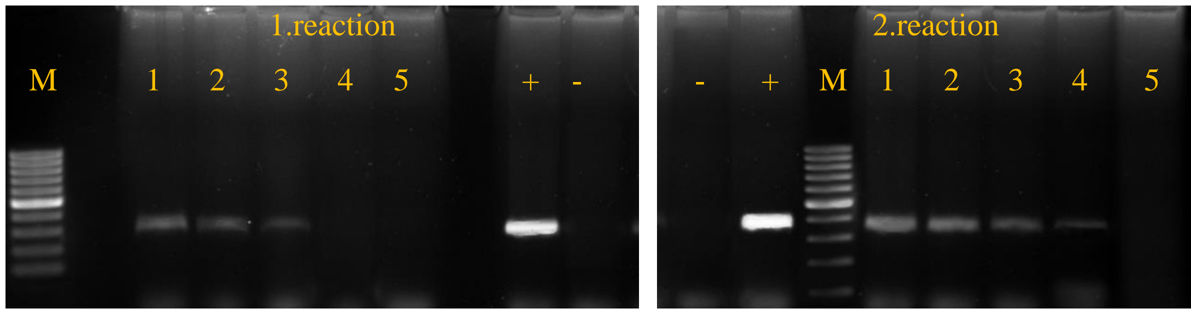

Fig. XII. Nested PCR of dilution series of gDNA of dormant form of GFP strain using *Glp*A-B primers. (M= 100bp DNA marker, 1= no dilution, 2= dilution 1:10, 3= dilution 1:100, 4= dilution 1:1000, 5= dilution 1:2000, + = positive control (GFP), - = RNase-free H_2O).

Results are showing that the first reaction of PCR using specific primers may reveal detection of *Borrelia* in maximum dilution ratio 1:100 (from previous original concentration gDNA of 2 ng). The second reaction is showing significant bands also when gDNA diluted to 1:1000.

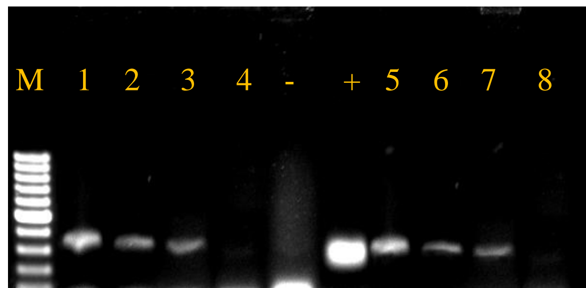

Fig XIII. Nested PCR of dilution series of gDNA of motile form of GFP strain using *Glp*A-B primers. (M= 100bp DNA marker, 1= no dilution, 2= dilution 1:10, 3= dilution 1:100, 4= dilution 1:1000, 5= no dilution, 6= dilution 1:10, 7= dilution 1:100, 8= dilution 1:1000, + = positive control (GFP), - = RNase-free H_2O).

GlpA-B primers in Nested-PCR reaction may reveal gDNA of *Borrelia* (spirochaetal form), even the bands are not as significant.

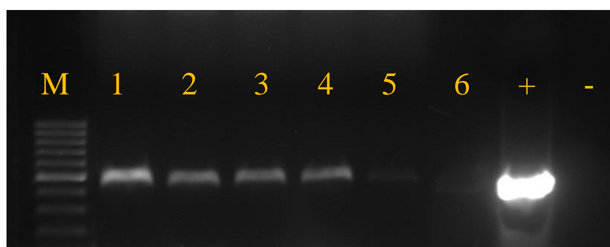

Fig. XIV. Dilution series of nested-PCR using *flagellin* set of primers for detection of motile form of *Borrelia* (M= 100bp DNA marker, 1= no dilution, 2= dilution 1:10, 3= dilution 1:100, 4= dilution 1:1000, 5= dilution 1:2000, 6= dilution 1:3000, + = positive control (GFP), - = RNase-free H_2O)

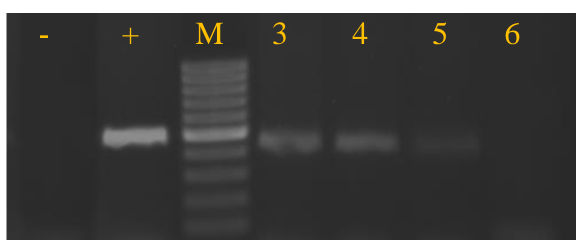

Fig. XV. Dilution series of nested-PCR using flagellin set of primers for detection of dormant form of *Borrelia* (M= 100bp DNA marker, 1= no dilution, 2= dilution 1:10, 3= dilution 1:100, 3= no dilution, 4= 1:10, 5= 1:100, 6= 1:1000 , + = positive control (GFP), - = RNase-free H_2O)

Results are showing both *flagellin* and *glp* primers are able to detect dormant and motile forms of borreliae spirochetes. Nevertheless, *glp* primers appear to be more sensitive to detection of dormant morhotype of borreliae cells in low concentrations than *flagellin*.

5.3. Antibiotics used against GFP spirochetes *in vitro*

After 2, 7, 14 and 21 days of emerging 96% RBs in cultures, samples transferred to fresh MKP medium showed different re-occurrence of spirochaetal cells. Filtered samples through a 0.45 µm filter did not indicate the presence of spirochetes. Therefore, the original antibiotic-treated cultures were considered 98% of the atypical forms with the minimum number of motile spirochetes.

According to darkfield and fluorescence microscopy, over 96% of motile spirochetes of the strain GFP transformed into RBs up to 72 hours from the Milli-Q water, amoxicillin and doxycycline treatment. Nevertheless, elongated cells of *Borrelia* did not completely

disappeared from the samples. These cells were non-motile. After 72 hours from the initial contact with anti-spirochete treatment, RBs occurred mostly single. As time progressed from 1-2 weeks, number and size of aggregates of these globular morphotypes increased. Simultaneously, the number of RBs decreased. *Borrelia* cultures from 96% atypical forms were able to reconvert in fresh MKP medium into mobile spirocheatal cells even after 3 weeks of water treatment (Tab XVIII., Tab. XIX), while there was no evidence of progressive growth of spirochetes. Dormant forms treated with anti-spirochete medium for less time were able to reconvert faster to spirochaetal forms. The scheme below describes samples which were exposed to fresh MKP medium 2, 7, 14 and 21 days after 96% RBs emerged in the original cultivation sample (Tab. XVII).

Tab. XVII. Type of treatment used for GFP culture: Milli-Q water.

Age of GFP-RBs	2 days	7 days	14 days	21 days
Reconversion time (days)	10	22	27	31

Tab. XVIII. Type of treatment used for GFP culture: Amoxicillin with concentrations 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml.

Concentration of ATB	Age of GFP-RBs	2 days	7 days	14 days	21 days
	Reconversion time (days)				
25 μg/ml		4	10	13	15
50 μg/ml		5	16	20	23
100 μg/ml		7	17	25	32
200 μg/ml		9	21	27	41

Tab. XIX. Type of treatment for GFP culture: Doxycycline with concentrations 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml.

Concentration of ATB	Age of GFP-RBs	2 days	7 days	14 days	21 days
	Reconversion time (days)				
25 μg/ml		2	6	10	12
50 μg/ml		4	12	16	14
100 μg/ml		8	13	21	36
200 μg/ml		11	16	23	48

The original GFP cultivation tubes treated with doxycycline showed the presence of active spirochetes even 37 days after ATB exposion (25/50 µg/ml). The concentration of motile spirochetes reached density of 4.6 x 10 4 cells / ml during 21. day from 96% RBs formation. Motile spirochetes were found in these cultures in ratio 1: 8 to the atypical form of *Borrelia*. This observation suggests that spirochetes were able to reconvert into the active form even without fresh MKP medium treatment. Therefore, observation of RBs cultures transferred from original tubes (25, 50 µg/ml of ATB concentration) to fresh MKP medium on day 2 revealed the reverse process occurring much faster than for ATB treatment of concentrations 100, 200 µg/ml.

This study *in vitro* has been applied for incoculation of mice (with doxycycline and Milli-Q water treated RBs) as a control experiment *in vitro* and the same results have been revealed. Consequently, the next chapter describes *in vivo* experiment of RBs inoculation to laboratory models.

5.4. Infection of laboratory model with dormant form of GFP strain

Mice inoculated with dormant morhotype of GFP strain did not develop clinical symptoms. Positive control suffered from loss of hair during the 5-6 week after inoculation with motile spirochetes.

5.4.1. Cultivation of spirochetes from tissues

Motile spirochetes were not isolated from any tissue of mice even after 3 months of cultivation in MKP medium. The lack of observation of spirochetes in the culture medium could be caused by progressively increasing contamination.

5.4.2. Rapid immunochromatographic test

Blood serum for 1 mouse of each experimental group was tested for the detection of antibodies of *Borrelia burgdorferi* s. l.: 1-1, 2-1, 3-1, 4-1, 5-1, 6 (positive control), 7 (negative control) (Tab XX.). All results were negative, including positive control.

Tab XX. Concentration of anti-spirochete treatment (doxycycline, Milli-Q water, which was inoculated to mice model when reached 96% RBs forms and inoculation material used for mouse chosen for positive and negative control.

		Mice group	Number of mice
Anti-spirochaetal treatment	Doxycycline 20 µg/ml	1	2
	Doxycycline 50 µg/ml	2	4
	Doxycycline 100 µg/ml	3	4
	Doxycycline 200 µg/ml	4	4
	Milli-Q water	5	4
Positive control	Well-grown GFP culture	6	1
Negative control	MKP-medium	7	1

Nested-PCR

Detection of *Borrelia burgdorferi* s. l. in blood and tissues of mice exposed to dormant and active morhotypes (positive control) of the spirochetes was performed with nested-PCR method modified in previous study, using *GlpA*7- *GlpB*2 , and *GlpB*1-*GlpA*8. The efficiency of these primers was compared to commonly used set of primers aimed to detect *Borrelia burgdorferi s.l.* in infected tissues, *flagellin* out/in. Results are shown in the scheme below (Tab. XXI)

Group of mice	Total number of mice	*Glp*A-B +/-			*Flagellin* +/-		
		blood	bladder	joint	blood	bladder	joint
		1,2/3,4	1,2/3,4	1,2/3,4	1,2/3,4	1,2/3,4	1,2/3,4
1	2	-+	--	-+	++	-+	-+
2	4	-+/-+	--/--	+-/+-	++/++	++/++	-+/-+
3	4	-+/-+	-+/-+	++/++	++/++	++/++	++/++
4	4	-+/-+	--/--	++/++	++/++	++/++	--/--
5	4	-+/-+	--/--	--/--	++/++	-+/-+	-+/-+

6	1	+	+	+	+	+	+
7	1	-	-	-	-	-	-

+ positive result

-negative result

Mice inoculated with GFP strain with over 90% RBs, achieved by previous exposure of a culture to 25 µg / ml concentration of doxycycline, showed the following results with *glpA-B* primers: In the first mouse, the presence of *Borrelia* was not detected in investigated tissues and blood. Tissues of the same mouse were screened again, using *flagellin* primers, and blood was detected for *Borrelia* as positive. In the second mouse, blood and joint were detected positive with *Glp* primers, while *flagellin* revealed borreliae DNA in all investigated organs.

The second group of mice tissues revealed following results. Blood and bladder of two mice have been negative for *Borrelia,* while PCR of their joints confirmed presence of *Borrelia* with *Glp* primers. The same mice tissues tested for flagellin revealed blood and bladder as positive. The other two mice tested with glp primers were positive in blood and for all investigated organs.

The third test group was positive for blood, bladder and joint using *flagellin* and for joint, using *Glp* primer. The Glp primers also confirmed a negative result for blood and bladder for two mice and a positive result for the other two mice.

In the fourth experimental group, two mice tested with *Glp* primers were negative for blood and urinary bladder but positive for bone. The other half of the experimental group was positive for blood and bone. *Flagellin* revealed blood and bladder positivity of all mice of the given group.

The fifth group revealed positive results of blood of all mice with *flagellin*, whereas *Glp* primers detected *Borrelia* only in blood of 2/4 mice. Half of the mice were positive for *Borrelia* in bladder and joint with *flagellin* and negative for both organs by *Glp* primers.

Positive control revealed the presence of *Borrelia* in all investigated organs including blood using both primer sets. The negative control showed no signs of *Borrelia*.

Individual primer sets showed different results in specific tissues. The results suggest that inoculated dormant borelia were able to induce organism infection and proliferation of

typical form of *Borrelia*-spirochetes in at least the blood of all experimental individuals. These results were revealed by *flagellin*. At the same time, *glp* primer revealed the presence of dormant type of *Borrelia* in the blood and various tissues (see Tab. XXI).

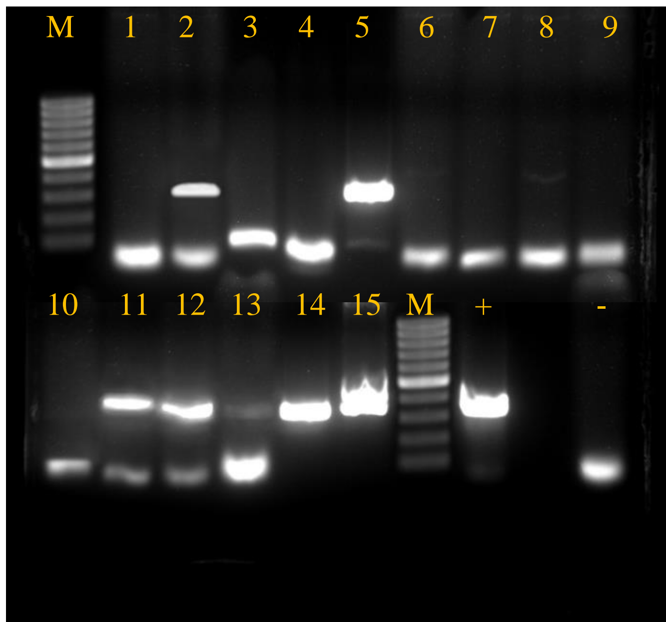

Fig. XVI. Nested-PCR of tissues of laboratory model using *glp* primers ((M= 100bp DNA marker, 1= 1st group of mice- bladder, 2= 1st group of mice- joint , 3= 2nd group of mice- bladder, 4= 2nd group of mice joint, 5= 3rd group of mice- bladder, 6= 3rd group of mice- joint, 7= 4th group of mice- bladder, 8= 4th group of mice- joint, 9= 5th group of mice- bladder, 10= 5th group of mice- joint, 11= blood serum of first mice group, 12= blood serum of second mice group, 13= blood serum of third mice group, 14= blood serum of fourth mice group, 15= blood serum of fifth mice group, + = positive control (gDNA of positive control mice), - = RNase-free H_2O).

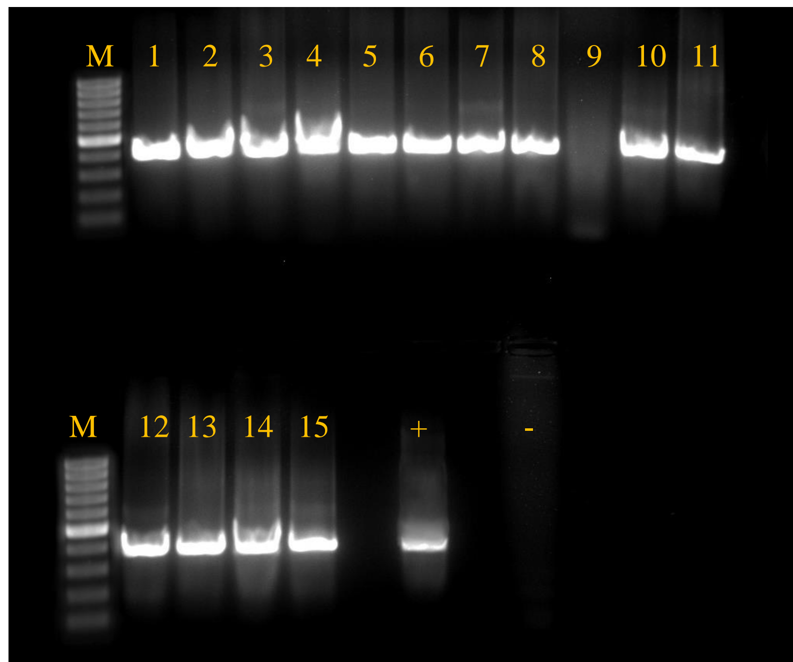

Fig. XVII. Nested-PCR of tissues of laboratory model using *flagellin* primers ((M= 100bp DNA marker, 1= 1st group of mice- bladder, 2= 1st group of mice- joint , 3= 2nd group of mice- bladder, 4= 2nd group of mice joint, 5= 3rd group of mice- bladder, 6= 3rd group of mice- joint, 7= 4th group of mice- bladder, 8= 4th group of mice- joint, 9= 5th group of mice- bladder, 10= 5th group of mice- joint, 11= blood serum of first mice group, 12= blood serum of second mice group, 13= blood serum of third mice group, 14= blood serum of fourth mice group, 15= blood serum of fifth mice group, + = positive control (gDNA of positive control mice), - = RNase-free H_2O).

In the experimental model of water-induced RBs, spirochetes proliferated in both tissues and blood, but atypical forms did not occur in tissues.

Spirochaetal form borreliae was present in 100% of experimental models inoculated with 200 µg / ml doxycycline-induced RBS for blood and bladder. *Flagellin* did not reveal the presence of *Borrelia* in joints. The *glp* primer set detected joints of mice as positive for *Borrelia*. It can be assumed that joints of these mice contained predominantly a dormant form of *Borrelia*.

5.4. Real time recordings of transformation of *Borrelia* spirochetes from active to dormant forms and back to vital form

For the long-term observation of spirochetes and their possible metamorphoses (within one hour), a specific method has been proposed, but not yet very successful. It was possible to observe spirochaetal cells when converting to an atypical form (see Appendix 15). The video was recorded after 20 minutes of exposure of the rabbit serum to spirochetes. The reverse process could not be recorded, while the necessary volume of fresh BSK-II medium aimed to change the morhotype of *Borrelia* back to motile spirochte moved the cells from their fixed position.

6. DISCUSSION

It has previously been reported that *B. burgdorferi* spirochaetes may occur in various morphotypes in response to non-optimal environmental conditions (Barbour & Hayes 1986; Brorson & Brorson 1997, Burgdorfer & Hayes 1989; Kersten et al. 1995). Atypical forms of Borrelia such as round bodies have been observed in vitro in human CSF, while it is uncertain whether these objects possessed exactly the same morphological and fuctional features (Brorson & Brorson, 1998a). It has been shown in previous study, that inoculation material containing predominantly round body form of *Borrelia* may induce infection of laboratory model (Gruntar et al. 2001). If such morphotypes may form in human organism, they may serve as individuals representing a strategy of possible survival of *Borrelia* cells during stress conditions in human tissues. Therefore, it is discussed that atypical forms of *Borrelia* spirochetes may be responsible for chronic Lyme borreliosis infections (Kersten et al.1995). In spite of studies revealing possibility of borrelia spirochetes to recultivate from hosts tissues after months of antibiotic treatment, there is an intensive discussion, questioning the existence of persistent form of *Borrelia* in human host. In addition to capability of Borrelia to morphological changes, an escape strategy was observed in laboratory model. Penetration of endothelial cell monolayers of umbilical vein with spirochetes of *B. burgdorferi* was shown by an electron microscopy (Comstock & Thomas 1989).

This study monitored closer relationship between the specific stress conditions and the formation of different types of spirochetes *in vitro* in order to apply particular stress conditions and atypical morphotypes of *Borrelia in vivo*. The laboratory model was examined for the presence of borreliae using a specific PCR procedure and a method for monitoring alive spirochetes during morhotypic changes was studied, resulting in collection of images showing transformation of borreliae cell from spiral to round body.

The non-optimal pH used in cultivation series of *Borrelia* was inspired by the pH values found in the human organism of localities where *Borrelia* spirochetes have already been observed. These tissues include skin, heart, joints and more (Brorson & Brorson 1998a). All *Borrelia* strains used in this study were able to change their shape at lower pH than optimal for their growth. Subsequently, they were able to reconvert into a typical spirochaetal form after treatment with fresh cultivation media. Mild proliferation of spirochetes also occurred during reconversion phase. Result indicate the probability that spirochetes may survive in the

extreme pH conditions which spirochetes may theoretically be exposed to in human body. Further experiments in this study aiming to determine the parameters of the formation of different spirochete morphotypes have also shown that atypical forms of borreliae may persist for weeks in this specific morphology. Furthemore, cultivation tubes of dormant forms appeared to change to motile spirochaetal forms when exposed to better growth conditions.

The conditions for using primers (*glpA, glpB*) in this study were optimized and utilized in the detection of *glp* protein expressed by dormant forms of borreliae inoculated *in vivo*, using a laboratory model. Primers detected dormant type of *Borrelia* in both tissue and blood of the experimental model. In addition, a primer set (flagellin out/in used to routinely detect borreliae in culture or tissue has been used. Both sets of primers were able to detect *Borrelia* in various tissues of mice. It can be assumed that the tissues where *Borrelia* DNA has been detected by both sets of primers have been infected with both atypical form of spirochetes, namely RBs and spiral cells.

Tissues tested for detection of *Borrelia* were collected 40 days post inoculation of the material. In future studies, it would be advisable to collect material (such as ear tissue, blood) from mice exposed to dormant forms of *Borrelia* throughout the experiment and to test these samples for the presence of different forms of *Borrelia* cells. Moreover, during this experiment, it would be appropriate to monitor at least one mouse of each test group for possible symptoms of the disease, such as hair loss.

In order to see if one spirochete can change its morphotype to an atypical shape, and subsequently back to the spirochetal form, the spirochetes were monitored and recorded, exposed to various conditions inducing individual morphotype changes.

During live recording of transformation of spirochetes into various morphotypes, the reverse process could not be recorded, whereas the necessary volume of medium aimed to create optimal conditions for spirochetes to induce their transformation moved the cells from their fixed position and thus away from the field of observation.

Therefore, it is necessary to modify the methodology in further studies, including modifications of microscopic slides, another ratio of medium needed to change the spirochetes to the motile form, etc. With the second suggestion, the possible modification of the medium is aiming to faster changes of the morhotypes of *Borrelia.* Another possibility is to create conditions for the formation of morhotypes of spirochetes with different concentrations of anti-spirochete treatment with intention to use a lower volume of BSK II medium when the goal is to record the reverse process of spirochetes.

Although the reverse process of spirochetes was not captured during the monitoring, a spirochete changing its shape from spiral to round body was recorded.

During this study, various morphotypes of *Borrelia* were observed under different *in vitro* stress conditions. Under subsequently improved conditions, typical spirochetal cell forms appeared in the cultures. We cannot determine from these experiments with certainty whether the atypical morphotypes of *Borrelia* would be able to escape the immune mechanism of the living organism and change shape and function during the improvement of the conditions, which could result in manifestations like the aforementioned chronic borreliosis. In any case, further experiments in which borreliae cells were exposed to antibiotics used in human Lyme disease treatment and subsequently converted to atypical forms demonstrate infection of the laboratory model. *Borrelia* cells were detected in blood, bladder and joint. *Borrelia* spirochetes were exposed to doses of antibiotics that are higher than those recommended in LB. Nevertheless, they were capable of inducing infection. Collected tissues from laboratory models were unable to cultivate *Borrelia* even after three months of culture. The error could have occurred according to the selection of the medium chosen for the cultivation and gradually increasing contamination in the culture tubes. However, PCR detection of mice organs has demonstrated the presence of borreliae DNA.

The results of this study support previous studies and suggest that atypical forms of *Borrelia* are not only different in shape but also in function in compare to spirochaetal cells, and that their presence may result in infection of the organism.

7. CONCLUSIONS

In this study, the relationship between morhotypes of *Borrelia burgdorferi* s. l. and related stress conditions (pH changes, changes of temperature of cultivation, and exposure to antibiotics) in different types of cultivation media was recognized. In general, lowering the optimal pH of cultivation media induced production of blebs, biofilms and round bodies, while reducing the optimal temperature caused only the production of blebs and biofilms. Subsequent observation of the reconversion of the atypical morhotypes to motile spirochetes during pH changes revealed the most effective medium for the reverse process of individual strains of *Borrelia* is BSK II medium. In case of temperature, the most successful medium for proliferation of spirochetes and reverse process was detected as the MKP-F medium. *Borrelia burgdorferi* s. s. treated with doxycycline induced production of round body form of cells. Simultaneously, the ATB treatment showed the presence of active spirochetes even 37 days after ATB exposure, indicating that spirochetes were able to reconvert into the active spirochaetal form even without fresh cultivation medium treatment.

Furthermore, the optimal PCR conditions for new sets of primers targeting for dormantn types of spirochetes were designed.

The newly designed conditions of above mentioned primers were used for detection of the infectious potential of atypical morhotypes of *Borrelia burgdorferi* s. s. in tissues of laboratory model and the infection with dormant types of *Borrelia* spirochetes was confirmed. Changes in morhotypes under specific stress conditions were observed and recorded, including spirochaetal and round body forms of *Borrelia burgdorferi* s. s. cells.

8. LIST OF ABBREVIATIONS

ACA	Acrodermatitis Chronica
ATB	antibiotic
BIOCEV	Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University
BODIPY	boron-dipyrromethene
BSK-H	Barbour-Stoenner-Kelly medium
BSK-II	Barbour-Stoenner-Kelly medium II
BYS	Baggio-YOshinari syndrome
CB43	*Borrelia afzelii* strain
CSF	cerebrospinal fluid
DIC	differential interference contrast microscopy
EM	erythema migrans
gDNA	genomic DNA
GFP	green fluorescent protein-strain of *Borrelia burgdorferi* sensu stricto
LB	Lyme borreliosis
LD	Lyme disease
LPS	lipopolysaccharide
MKP	Modified Kelly-Pettenkofer
MKP-F	Modified Kelly-Pettenkofer (MKP) medium containing 10% fetal calf serum
NaCL	sodium chloride
glpA	anaerobic glycerol-3-phosphate dehydrogenase, subunit A
glpB	anaerobic glycerol-3-phosphate dehydrogenase, subunit B
OspC	the outer surface protein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PTLDS	post-treatment Lyme disease syndrome
RB/s	round body/ies- morphotype of *Borrelia burgdorferi* sensu lato
SCW53	*Borrelia burgdorferi* sensu stricto strain

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10. APPENDICES

Appendix 1. List of the currently valid species of the genus *Borrelia* with the specific vectors and hosts including their life cycle and geographical distribution.

***Borrelia* species**	**Vector (Ixodes tick)**	**Host (Reservoir)**	**Geographic distribution**	**Reference**
B. afzelii	*I. persulcatus, I. ricinus*	rodents	Europe, Asia	Canica et al. 1993
B. americana	*I. minor, I. pacificus*	birds	United States	Rudenko et al. 2011
B. andersonii	*I. dentatus*	cottontail rabbit	United States	Marconi et al. 1995
B. bavariensis	*I. ricinus*	rodents	Europe	Margos et al. 2009
B. bissettii	*I. ricinus, I. scapularis*	rodents	Europe, United States	Postic et al. 1998
B. burgdorferi. s. s.	*I. pacificus, I. ricinus, I. scapularis*	birds, lizards, big mammals, rodents	Europe, United States	Baranton et al. 1992
B. californiensis	*I. pacificus, I. spinipalpis*	kangaroo rat, mule deer	United States	Postic et al. 2007
B. carolinensis	*I. minor*	birds, rodents	United States	Rudenko et al. 2009a
B. finlandensis	*I. ricinus*	unknown	Europe	Casjens et al. 2011
B. garinii	*I. ricinus, I. persulcatus*	birds, lizards, rodents	Europe, Asia	Baranton et al. 1992
B. chilensis	*I. stilesi*	long-tailed rice rats	Chile, South America	Ivanova et al. 2014
B. japonica	*I. ovatus*	rodents	Japan	Kawabata et

				al. 1993
B. kurtenbachii	*I. scapularis*	rodents	Europe, United States	Margos et al. 2010
B. lanei	*I. pacificus, I. spinipalpis*	rabbits	United States	Margos et al. 2017
B. lusitaniae	*I. ricinus*	Lizards, rodents	Europe, North Africa	Le Fleche et al. 1997
B. mayonii	*I. scapularis*	unknown	midwestern United States	Pritt et al. 2016
B. sinica	*I. ovatus*	rodents	China	Masuzawa et al. 2001
B. tanukii	*I. tanuki*	unknown- possibly dogs, cats	Japan	Fukunaga et al. 1996
B. turdi	*I. turdus*	birds	Japan	Fukunaga et al. 1996
B. spielmanii	*I. ricinus*	rodents	Europe	Richter et al. 2006
B. valaisiana	*I. granulatus, I. ricinus*	birds, lizards	Europe, Asia	Wang et al. 1997
B. yangtze	*I. granulatus, Haemaphysalis longicornis*	rodents	China	Chu et al. 2008

Infectious potential for humans

Uncertain infectious potential for humans

Non-infectious genospecies for humans

Appendix 2: Scheme of growth rate of strain SCW53 strain at pH= 7.0 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK-II	100000	16666	116000	350000	500000
MKP-F	100000	5882	60000	78000	170000
MKP	100000	8400	10500	17000	40000

Appendix 3: Scheme of growth rate of strain SCW53 at pH= 7.1 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK-II	100000	117000	165000	250000	345000
MKP-F	100000	45000	95000	128000	145000
MKP	100000	7250	60000	115000	130000

Appendix 4: Scheme of growth rate of strain SCW53 at pH= 7.3 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK-II	100000	130000	1900000	4000000	5000000
MKP-F	100000	12195	97600	609000	800000
MKP	100000	100000	13000	140000	150650

Appendix 5: Scheme of growth rate of strain GFP at pH= 7.0 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK II	100000	16900	117200	400000	600000
MKP-F	100000	7000	67000	95000	180000
MKP	100000	9450	14520	31000	62300

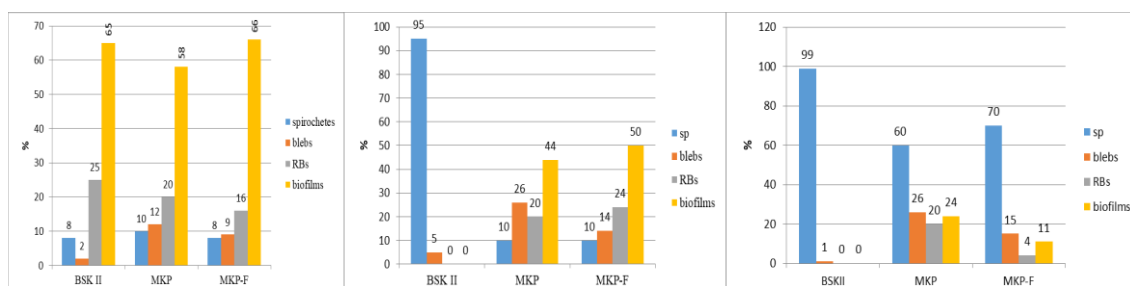
Appendix 6: Scheme of growth rate of strain GFP at pH= 7.1 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK II	100000	117500	172000	273000	374000
MKP-F	100000	52000	102000	136500	164000
MKP	100000	63000	84000	124600	148560

Appendix 7: Scheme of growth rate of strain GFP at pH= 7.3 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK II	100000	136000	2200000	4120000	8000000
MKP-F	100000	35000	130000	700620	1850000
MKP	100000	67000	150000	142500	1500000

Appendix 8. Ratio of morhotypes of GFP induced by pH changes 7.0, 7.1, 7.3 after 5 days of low-pH exposure.



Appendix 9. Graph of growth rate of strain CB43 at pH= 7.0 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK II	100000	16502	90000	150000	190000
MKP-F	100000	4800	50260	72123	120000
MKP	100000	10200	150000	32000	3700

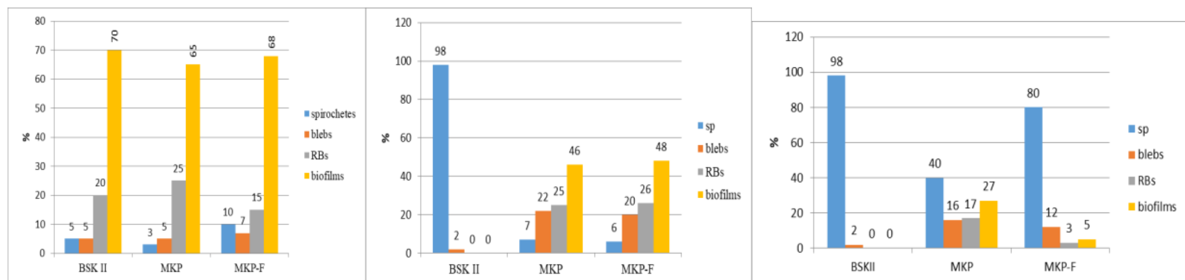
Appendix 10. Graph of growth rate of strain CB43 at pH= 7.1 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK II	100000	19000	123000	165000	194000
MKP-F	100000	6320	67000	81000	124600
MKP	100000	10560	22460	31000	45200

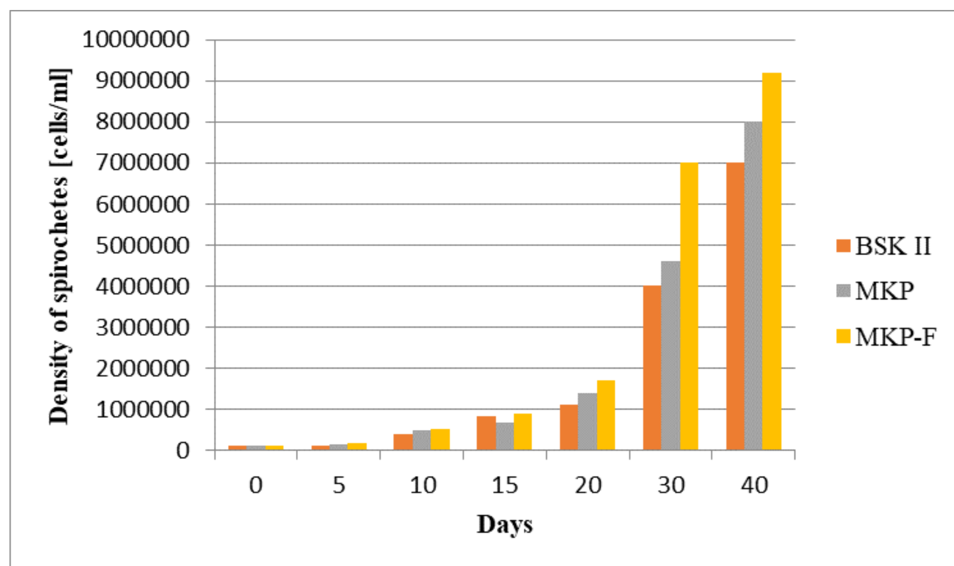
Appendix 11. Graph of growth rate of strain CB43 at pH= 7.3 cultivated in BSK II, MKP-F and MKP medium.

Medium	Initiation phase [cells/ml]	After 5 days [cells/ml]	5 days after fresh-medium treatment [cells/ml]	10 days after fresh-medium treatment [cells/ml]	20 days after fresh-medium treatment [cells/ml]
BSK II	100000	28000	133000	180500	212000
MKP-F	100000	11050	85400	96000	138600
MKP	100000	100000	10562	20000	36400

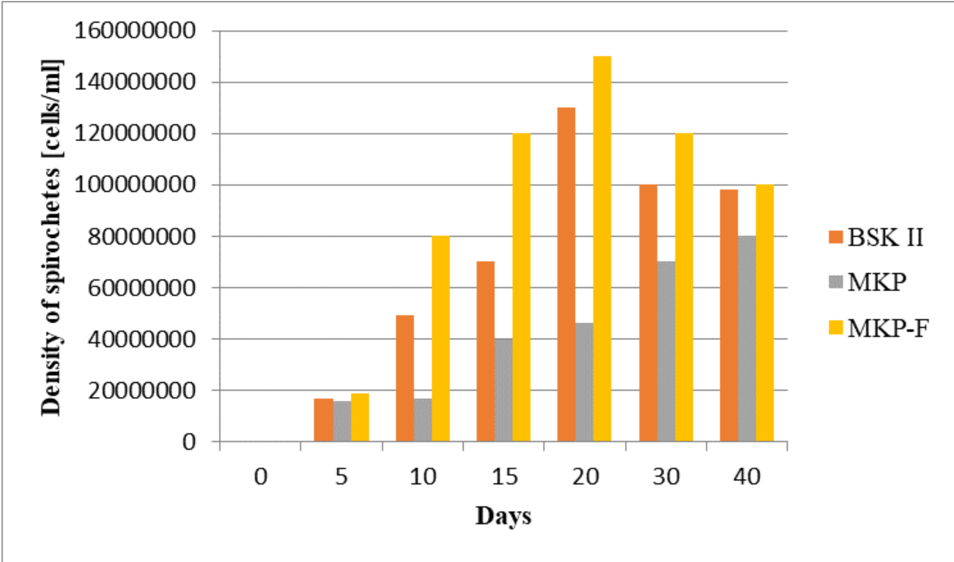
Appendix 12. Ratio of morhotypes of CB43 induced by pH changes 7.0, 7.1, 7.3 after 5 days of low-pH exposure.



Appendix 13. Graph of *Borrelia burgdorferi* s.l. cultivated at 21°C in BSK II, MKP, MKP-F.



Appendix 14. Graph of Borrelia burgdorferi s. l. cultivated at 33.5 °C in BSK II, MKP, MKP-F.



160000000
140000000
120000000
100000000
80000000
60000000
40000000
20000000
0
Density of spirochetes [cells/ml]
0
5
10
15
20
30
40
Days
BSK II
MKP
MKP-F

Apendix. 15. Change of spirochaetal cell of GFP strain from spirochete to round body shape, including bulbs emerging at the membrane. Size of recording field: 142,41 x 106,37 µm.