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Establishing a tick-mouse model for the relapsing fever pathogen *Borrelia duttonii*

Bachelor Thesis

Laboratory of Molecular Ecology of Vectors and Pathogens
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Annotation:

The aim of this work was to establish a tick-mouse model for the relapsing fever spirochete *Borrelia duttonii*. This model, the first for *B. duttonii* using the soft tick vector *Ornithodoros moubata* and mice was successful in showing the acquisition and transmission of the spirochete by its vector. Thus, this model helps to provide an avenue for studying the biology of the spirochete within the tick vector and the mammalian host.

Affirmation:

I hereby declare that my submitted bachelor thesis was written independently by myself without any external assistance. All additional information and sources are fully listed in the reference section as well as all paraphrased quotes are credited in this paper.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form resulting from deletion of indicated parts to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

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CONTENTS

1 INTRODUCTION

1.1. Borrelia

1.1.1. Characteristics

Borrelia are parasitic bacteria, belonging to the phylum of the phylogenetically- distinct spirochetes. They are made usually long and helically coiled and can have multiple flagella, depending on the spirochete species. The bacteria appear wavy with a length of about 10 µm and a diameter between 0.2 – 0.5 µm.

Furthermore, *Borrelia* possess high motility, due to their axial filaments located in their periplasmic space, the region between the protoplasmic cylinder complex and the outer membrane of the cell.[1,2] Above all, the bacteria can survive complete enzootic cycles, which involve acquisition and transmission of the pathogen by arthropod vectors like ticks and the mammalian hosts.

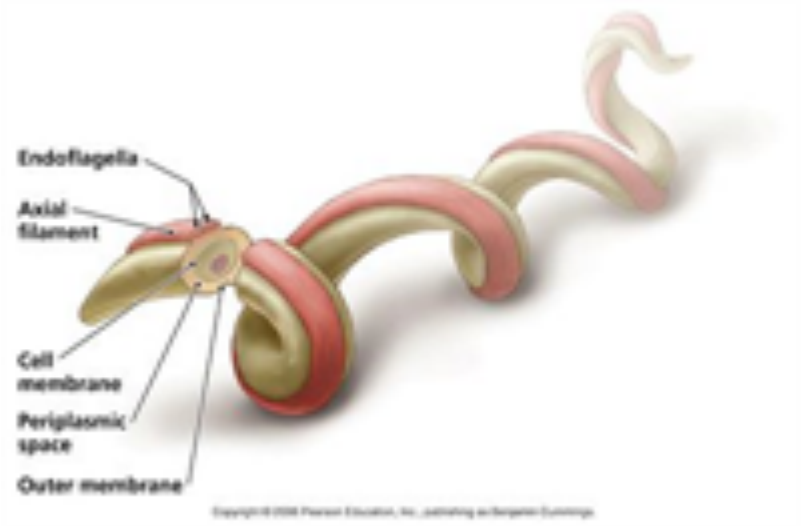

***Figure 1**: Scheme of the cell structure of a spirochete (Pearson Education Inc., 2006)*

At least 34 different non-pathogenic or pathogenic *Borrelia* species are known worldwide, differing in their geographical distribution and their transmission vector.[4] *Borrelia* act as agents of 2 major diseases: Lyme Borreliosis (LB)[5] and relapsing fever (RF)[6].

1.2. Relapsing Fever *Borrelia*

Relapsing fever (RF) is a neglected disease, which was first identified in Africa but has evolved to spread through other parts of the world. Both Tick-Borne Relapsing Fever (TBRF) and Louse-Borne Relapsing Fever (LBRF), transmitted either by ticks or via the human body louse respectively, have been recognized as major diseases arising from RF *Borrelia* infection.[6-8]

However, TBRF also needs to be sub-categorized according to its arthropod vectors. If transmitted by argasid (soft) ticks, the sickness is called Soft Tick-Borne Relapsing Fever (STBRF) and named as Hard Tick-Borne Relapsing Fever (HTBRF), when transmitted by ixodid (hard) ticks.[31,33]

In each of those cases, the spirochetes are transmitted through an enzootic cycle from mammals (reservoir hosts) to other mammals or even humans (end hosts) by ticks or lice.

LBRF has an epidemic potential, especially in regions with pour hygiene standards, whereas TBRF is called to be endemic. That can be explained from the fact, that the only host (reservoir as well as end-host) for LBRF are humans, thereby possibly causing an epidemic outcast. [16] However, due to improvement in standards of living within the last decades resulting in massive reduction of body lice, the majority of the world was able to overcome the epidemics of LBRF. [6,15,17]

Relapsing fever, in general, is characterized in an influenza-like pattern, showing recurrent and acute episodes of fever, as well as non-specific symptoms like abdominal complaints, headache, shaking chills or myalgia. The reason for these recurrent fever episodes is the immunological response of the host to the surface proteins of the pathogen.

Figure 2: *Graphical representation of a relapsing fever trend with febrile and afebrile episodes.*[47]

The disease pattern containing both symptomatic febrile and asymptomatic afebrile periods, can be explained from the changes in these proteins, causing the immune response of the host. Due to the fact, that the pathogenic organism tries to avoid eradication by its host, the organism modifies the outer surfaces of these proteins, causing new febrile episodes.[6,8,12]

During symptomatic episodes, RF *Borrelia* are detectable in the blood under a dark-field microscope and the estimated average blood concentrations in animals and humans within this periods are 10^5 to 10^6 *Borrelia* organisms per milliliter blood whereas during asymptomatic intervals, the spirochetes are microscopically undetectable.

Experimental data from researches suggests, that in these afebrile episodes the pathogens are sequestered in the internal organs of the host, such as the liver or central nervous system.[12]

The symptoms, in general, do not show a significant difference from an infection with malaria or other febrile diseases, but the disease can be lethal, if untreated. Hence there is a quite unspecific display for recognizing a *Borrelia* infection; it is barely distinguishable if someone is actually infected with RF or malaria. [18]

Relapsing fever was classified into two different sub-categories, before molecular tools were even available; the “old world” and the “new world” taxonomic clades according to geographical, as well as phylogenetic differentiations.

2.1.1. Old World Relapsing Fever

The term “old world” relapsing fever, results from a classification in a geographical manner comprising mostly Africa, but also European and Asian regions, where these already well-known species have occurred. Most important *Borrelia spp*. belonging to the “old world” clade, are *Borrelia recurrentis*, *Borrelia duttonii*, *Borrelia crocidurae*, *Borrelia persica*, respectively.

One of the first RF species described is *B. recurrentis,* which is the only known bacterial pathogen causing LBRF and is transmitted by the human clothing louse (*Pediculus Humanus Humanus*). [19]

B. duttonii, the sub-saharan Old World Relpsing Fever agent, is transmitted via the soft-bodied tick *Ornithodorous moubata moubata*.[20,21] The reservoir hosts for this species are suggested to be small rodents, though, not scientifically proven yet. [22]

Another important species belonging to the “old world” family is known as *B. crucidurae*, mostly found in West Africa, especially in Senegal and thereby being the primary cause of RF in this region.[23] The vector serving for infection with this species is the *Ornithodorous erraticus sonrai* and the reservoir hosts are usually rodents and insectivores.

A crucial *Borrelia* member of the “old world” which is not only located in Africa, but also located in Spain, Portgual and Marocco, called *B. hispanica*. The most commonly known vector of *B. hispanica* is the *Ornithodorous erratic erraticus* and the reservoir hosts for this spirochete species are mostly pigs. [24,25]

However, the majority of the clinical infections with RF *Borrelia* in Eurasia is attributed to the *B. persica* species, which is transmitted by the vector named *Ornithodorous tholozani*.

Nevertheless, besides the “old world” family, RF-*Borrelia* were also found to be present in North and South America, constituting the “new world” clade.[46]

Figure 3: *Relapsing fever around the world and its geographical distribution.* [46]

2.1.2. New World Relapsing Fever

Borrelia spp. contributing to the "new world", as already been said, are found in North and South America. Although this may be true, it is important to memorize the fact, that the separation of these species into "new" and "old" world classifications are not possible only according to their geographical findings, but also due to their phylogeny and genetics.[47] The major contributors belonging to the "new world" which are mentioned here are *Borrelia hermsii*, *Borrelia turicatae*, *Borrelia parkeri*, *Borrelia coriaceae* and *Borrelia miyamotoi*. [26]

The "new world" RF pathogen *B. hermsii* and its vector *O. hermsi* are the most studied RF species in the regions of North and South America. The typical reservoir hosts for *B. hermsii* are most commonly small rodents like squirrels and chipmunks, but can also be deer mice or woodrats.[17,26]

In the southern regions of the USA (Florida, Texas and Kansas), the *Borrelia* species *B. turicatae* has been isolated from its primarily likeable vector *O. turicata*, which is also a commonly known vector for the African Swine Fever Virus.[17,27]

B. parkeri, the *Borrelia* spp. which is preferably located in the western United States and transmitted by the *O. parkeri*, is also a RF causing agent, where researches suggested a possible pathogenicity to horses.[28]

B. coriaceae (first isolated in California, 1985), named after its tick vector *O. coriaceus*, was found in Northern California and suggested to be a plausible cause for epizootic bovine abortion (EBA).[29,30]

B. miyamotoi, one of the most known and studied RF *Borrelia* in the U.S., is

the only known RF causing agent to be transmitted and carried over several *Ixodes* tick species all over the globe. *B. miyamotoi* is the only representative *Borrelia* spp. belonging to the Hard-Tick-Borne Relapsing Fever (HTBRF) clade and is commonly habited in its reservoir hosts like birds or small rodents.[31]

***Table 1**: Summary and comparison of "old world" and "new world" Borrelia spp. according to their vectors, geographical distributions over the globe, their reservoir hosts and their causing diseases.*[40]

Species	Vector	Geographical distribution	Hosts (reservoir)	Disease
New world				
B. hermsii	*O. hermsi*	North America	Rodents, Birds	STBRF
B. turicatae	*O. turicata*	North America	Cattle, Snakes, Prairie dogs, Gopher Tortoise, Pigs, Squirrels	STBRF
B. parkeri	*O. parkeri*	North America	Unknown	STBRF (possibly)
B. coriaceae	*O. coriaceus*	North America	Bovine, Columbian black-tailed deer	EBA (possibly)
B. miyamotoi	*Ixodes* spp.	Eurasia, North America	Birds, Rodents	HTBRF
Old world				
B. recurrentis	*P. humanus humanus*	Ethiopia, Sudan (pot. worldwide)	Humans	LBRF
B. duttonii	*O. moubata moubata*	Sub-saharan Africa, Tanzania	Rodents	STBRF
B. crocidurae	*O. erraticus sonrai*	West and North Africa	Rodents	STBRF
B. hispanica	*O. erraticus erraticus*	Africa, Spain, Portugal, Marocco	Pigs, Rodents	STBRF
B. persica	*O. tholozani*	Eurasia	Rodents	STBRF

1.2.3. Soft ticks vs. /hard ticks

Ixodidae and *Argasidae* (hard and soft ticks) are not only distinguishable by their causative diseases and transmission ability of different *Borrelia* species, but also have clearly defined differences in their life-cycle as well as their behavior.
When mentioning argasid ticks, the *Ornithodoroinae* genus is mainly taken into focus, especially the *Ornithodoros moubata*, which was used during this work.
However, *Ixodidae* and *Argasidae* vary also in morphological aspects and their number of molting stages. Argasids undergo 2-7 nymphal stages prior to molting into the adult stage (reproduction phase) depending on the argasid tick species, whereas Ixodids are only capable of molting into one nymphal stage and then directly into the adult stage.[48,49]

Morphologically, even though both tick species have six-legged larvae, soft ticks are easily recognizable and thereby distinguishable due to their lacking scutum and their leathery cuticle. One of the major recognizable morphological differences is the capitulum of Argasids. It is in contrast to Ixodids, located in a ventral, subterminal position, hidden from a dorsal view and only visible by looking at the tick ventrally (shown in Figure 4).[48-50]

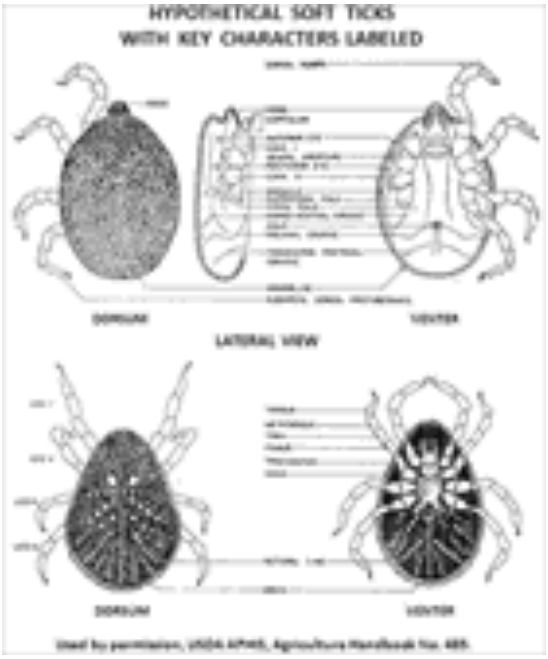

Figure 4: *Morphology of a female and male argasid tick.*[50]

Despite the morphological differences, the tick species vary in numerous other aspects such as feeding behavior, transmission ability and starvation (Table 2).

***Table 2**: Diffrences between ixodid and soft tick species.*[41-44]

	Hard tick (*Ixodidae*)	**Soft tick (*Argasidae*)**
Feeding	4-5 days	30mins – few hours
Anatomy	Capitulum visible, shield	Capitulum not visible, no shield
Transmission (Borrelia)	54 – 72 h	min. 1 minute – few hours
Molting stages	Larvae, Nymph, Adult (Feeding – One per stage)	Larvae, Nymph (2-7stages), Adult (can feed numerous times)
Hosts	1, 2 or 3	Multiple (up to 10)
Starvation	several months	Years (up to 11)

1.2.3.1. Argasid life cycle

. *Argasidae* can undergo a multiple-host life cycle, whereas *Ixodidae* can undergo a one, two or three-host life cycle. A significant difference between the tick species is that the life cycle of Ixodids are season and time-dependent whereas the argasid tick life cycle is not.[51,52]

Argasids are able to undergo numerous nymphal stages and are even able to have various blood meals as adults, thereby undergoing a multiple-host life cycle, as pictured in Figure 5.[49-51]

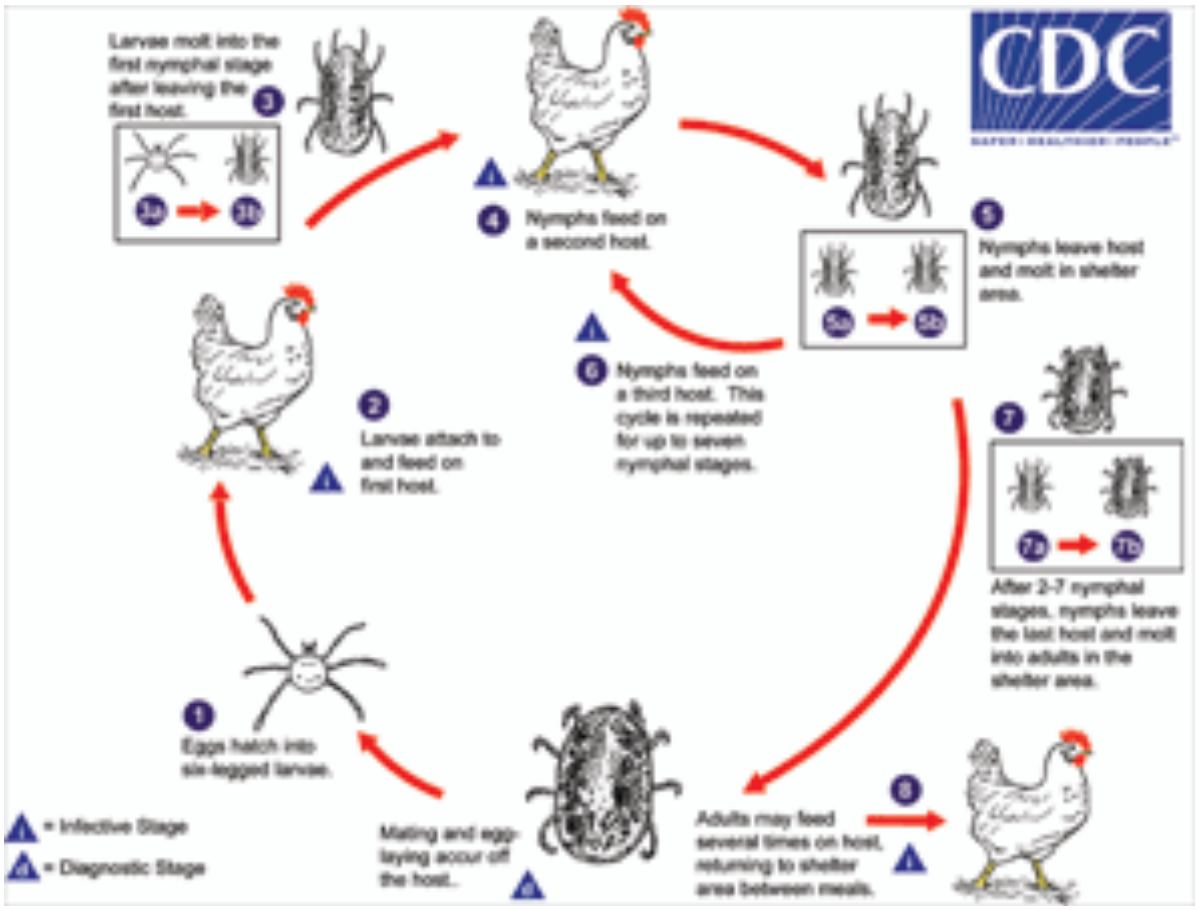

Figure 5: *Graphical representation of a multiple-host life cycle of argasid ticks.*[51]

Figure 5 shows the hatch of the six-legged larvae from eggs that have been laid from a female tick in the first step of the cycle (1). Then, after attaching and feeding on the first host (2), the tick leaves the host and molts into an eight-legged 1st stage nymph. (3, 3a, 3b). Those nymphs then feed on a second host (4), molt in a sheltered area (5, 5a, 5b) to a higher stage and feed on a third host as 2nd stage nymphs (6). This cycle can be repeated up to seven nymphal stages.

However, depending on the argasid tick species, after reaching the last nymphal stage and leaving the last host the nymphs molt in a sheltered area to adults (7, 7a, 7b).

Those adults are, as previously mentioned, capable of having continuous blood meals on different hosts and returning to shelter between each blood meal where the females lay eggs after every blood meal (8).[51,52]

1.2.3.2. *Ornithodoros moubata*

O. moubata is a soft tick belonging to the *Ornithodorinae*, a genus of *Argasidae* family of ticks found in Africa. It is a vector of different pathogenic organisms that can cause several severe diseases to animals but also humans. The major pathogen transmitted by it is the African Swine Fever Virus (ASFV). The virus is persistent in the southern half of Africa either in wildlife reservoirs, warthogs or soft ticks (*O. moubata*) or as an enzootic disease between domestic pigs, though it does not give symptoms or any effect, neither on warthogs nor the ticks and only causes an actual disease in domestic pigs.[20,27,32]

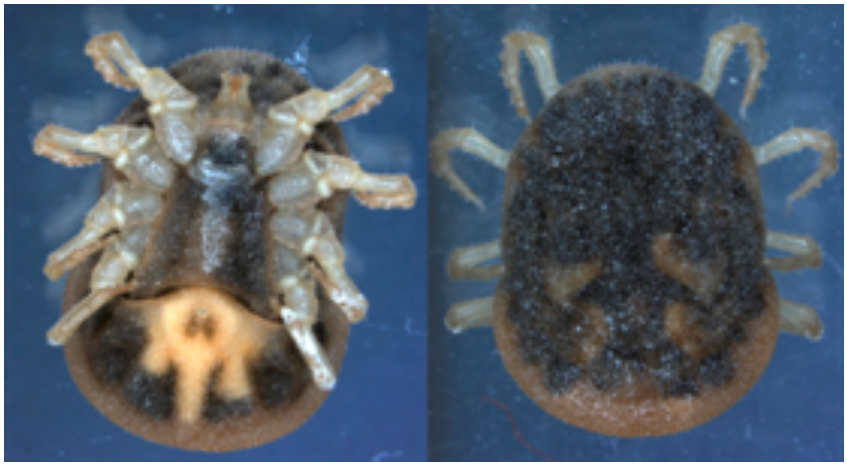

Figure 6: *Ornithodoros moubata in a ventral (left) and dorsal view (right). (Image: CSIC)* [46]

This argasid tick is a vector of *B. duttonii*, and was used for this project to gain further knowledge about. *B. duttonii*..

2 AIMS OF THE WORK

For creating a tick-mouse model and thereby confirming the successful infection between mice/ticks and *Borrelia duttonii*, the following objectives were attempted:

1. Confirming the identity of the *B. duttonii* strain *1120K3* and *Ly* using *flab* and *glpQ* primers, then sequencing and analyzing them

2. Infecting healthy BALB/c & C3H/HeN mice by needle inoculation of the *B. duttonii* isolates and verifying their infection via blood analysis and antibody detection (Western Blot).

3. Feeding of healthy larval & 1st stage *O. moubata* nymphs on infected C3H/HeN & BALB/c mice (*1120K3*) and after molting to the next stage stage confirming acquisition by crushing them and performing PCR on tick DNA using *flaB* and *glpQ* primers.

4. Allowing the infected ticks to feed on naïve mice to see if transmission is possible of *B. duttonii*.

3 MATERIALS & METHODS

3.1. Bacterial Strains

For establishing the animal model of the spirochete *B. duttonii*, the strains *1120K3* and *Ly* were used (provided by Sven Bergström, University of Umea, Sweden). For the cloning and transformation step, *E. coli* with the competent cells DH5α (Thermo Fisher®) was used.

3.2. Bacterial Growth Conditions

Both of the *Borrelia* species *1120K3* as well as *Ly* have been taken from glycerol stocks and then cultivated in BSK II and MPK II media in an incubator at 34°C until reaching the mid-log phase (1 x 10^7 *Borrelia*/ml).. The *E. coli* cultures were grown in LB media using an orbital shaker at 37°C.

3.3. Genomic DNA Isolation

Borrelial gDNA samples were prepared from *Borrelia* cultures using the Promega® kit 'Isolation of Genomic DNA from Gram Positive and Gram Negative Bacteria' and its adaptions from the manufacturer manual.

First, 10 ml of the cultivated *Borrelia* (taken from the incubator) was transferred into a 15 ml centrifugation tube and then centrifuged at 9000 rpm for 10 minutes to pellet the cells. The supernatant was then poured off and residues were removed using a pipette. Subsequently, 600 µl of Nuclei Lysis Solution were added, re-suspended and transferred into a 1.5 ml Eppendorf ® micro tube.

To lyse the cells, the mixture was incubated at 80°C for 5 minutes and rapidly cooled for 2 minutes using an ice bucket. Then, 3 µl of RNase Solution was added to the cell lysate, shaken by inverting the tubes 2-5 times and incubated at 37°C for 15 minutes. After the solution has cooled to room temperature, 200 µl of Protein Precipitation Solution were added to the cell lysate, followed by vigorous shaking using a Vortex shaker and cooling on ice for 5 minutes.

Next, the sample was centrifuged at 13000 rpm for 5 minutes at 4-8°C and the supernatant, containing the DNA, transferred to a clean 1.5 ml micro centrifuge tube containing 600 µl isopropanol (room temperature). The mixture was gently shaken by inversion of the tube until visible DNA thread-like strands formed and then centrifuged again (13000rpm, 4-8°C, 10 minutes).

After centrifugation, the supernatant was poured off, drained on clean adsorbent paper, suspended with 600 µl ethanol (room temperature) and centrifuged again (13000rpm, 4-8°C, 10 minutes). The ethanol was discarded carefully and small residues were removed using clean adsorbent paper.

In the last step, the pellet was air-dried for 10-15 minutes, 100 µl of DNA Rehydration Solution was added and finally stored at 2-8°C.

3.4. DNA Isolation from tick samples

In order to perform analytical methods to receive data about infected ticks for this project (PCR and Gel electrophoresis, Chapter 3.5 & 3.6), DNA needed to be isolated from ticks and was done using the QIAGEN Supplementary Protocol and the DNeasy® Blood & Tissue Kit for detection of *Borrelia* DNA.

First, the tick was placed into a 2 ml homogenizing tube with 180 µl of ATL Buffer including a small steel grinding ball. Then, using the TissueLyser II (QIAGEN), the tube was placed into an adapter set (2 x 24), which was previously cooled in the freezer. Subsequently, the adapter set is shaken with high speed at 20-30 Hz for 2 minutes, disrupting and homogenizing the tick completely. The tube was then rapidly centrifuged to collect all tissue pieces of the tick at the bottom. After addition of 20 µl Proteinase K and thorough vortexing, the tube was incubated at 56°C until the tissue was completely lysed. After vortexing again, 200 µl AL Buffer were added, mixed and incubated at 70°C for 10 minutes. Then, 230 µl of 96-100% ethanol were added and thoroughly mixed. The mixture was then pipetted into a DNeasy Mini Spin column placed into a 2 ml collection tube and centrifuged at 8000 rpm for 1 minute.
The column was then placed into a new collection tube (2 ml) and after addition of 500 µl AW1 Buffer centrifuged again using the same conditions (8000 rpm, 1 min.) and the flow-through was discarded. Afterwards, the column was again placed into a new collection tube (2 ml) with 500 µl AW2 Buffer and centrifuged (14000 rpm, 3

min.) to dry the membrane. After discarding the flow-through again, the DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 35 µl (for small ticks) or 80 µl (for large ticks) AE Buffer was pipetted directly on the DNeasy membrane.

The mixture was incubated at room temperature for 1 minute and centrifuged (8000 rpm, 1 min.). Then, another 30 µl (small ticks) or 50 µl (large ticks) of AE Buffer was added directly onto the DNeasy membrane, incubated at room temperature for 1 minute and centrifuged again (8000 rpm, 1 min.) to elute the purified g DNA.

3.5. Polymerase Chain Reaction (PCR)

The PCR method was used to amplify the *glpQ* and *flaB* genes located in the *Borrelia* strains *1120K3* and *Ly* using *glpQ* and *flaB* primers. Furthermore, PCRs were performed using the same primer sets when analyzing a *Borrelia* infection in them. In Table 3, the primers involved in those amplification reactions are listed.

Table 3: *Corresponding primers (forward and reverse), their melting temperatures and sequences.*

Primer	T_m [°C]	Sequence
flaB forward BOR1	49.4°C	5' – TAA TAC GTC AGC CAT AAA TGC – 3'
flaB reverse BOR2	48.0°C	5' – GCT CTT TGA TCA GTT ATC ATT C – 3'
glpQ fw B. dutt	55.0°C	5' – CCA TTA ATT ATA GCT CAC AGA GGT GC – 3'
glpQ rev B. dutt	54.2°C	5' – TCT GTA AAT AGG CCA TCT ACT TTT GC – 3'

In order to amplify the *flaB* and *glpQ* genes of *1120K3* and *Ly*, the 'One Taq Hot Start DNA polymerase' with corresponding buffers from New England BioLabs® was used. However, the only possible method for amplification was the use of this polymerase with fail-safe buffers; premix A,D & E of the 'Fail Safe PCR Kit' from Epicenter® resulting in successful amplification, transformation and cloning.

In the following tables, the PCR program settings as well as the master mixes are listed.

***Table 4**: Master Mix ingredients (fail safe) and their corresponding volumes per reaction.*

Master Mix – Fail safe	**Amount/reaction**
Primer F	1 µl
Primer R	1 µl
Premix E/A/D (fail safe buffer)	12.5 µl
One Taq Hot Start Polymerase	0.2 µl
dNTP's	0.4 µl
MiliQ H_2O	8.9 µl
DNA	1 µl

***Table 5:** Settings for PCR using 'One Taq Hot Start Polymerase'.*

PCR settings – Fail safe	
Initial denaturation:	95°C for 2 min.
30 cycles:	95°C for 45 sec.
	50°C for 45 sec.
	68°C for 90 sec.
Final extension:	68°C for 10min.
Hold:	16°C

3.5.1. Agarose Gel Electrophoresis

The PCR samples were visualized using gel electrophoresis. Together with 1kb+ DNA Ladder (standard) from Invitrogen®, the samples were loaded on a 5% agarose gel with 10xSYBR, a fluorescent dye. The gel was placed into the electrophoresis apparatus together with a buffer (1xTAE). After applying voltage of 80-120V for 1-1.5 hours and thereby separating the samples according to size, the DNA was visualized and examined using UV light.

3.6. Cloning and Transformation in *E. coli*

After successful PCR, the products of the PCR were inserted into the Invitrogen's pCR®2.1-TOPO® vector and transformed into *E. coli.* The kit used for transformation and cloning was the Invitrogen's pCR®2.1-TOPO® kit.
This was performed by preparing the following reaction mixture:

Table 6: *Reagent mixture for cloning into E. coli using the 2.1-TOPO vector.*

pCR®2.1-TOPO® vector	
DNA insert (from PCR)	2.0 µl
Salt solution	0.5 µl
2.1-TOPO vector	0.5 µl
MiliQ H_2O	2.0 µl

After setting up the reaction mixture, according to Table 6, the sample was incubated for 5 minutes, 1.5 µl of this mixture was added into a 75 µl *E. coli* cell tube with competent cells (DH5α, Thermo Fisher®). Then, after gently flicking the tube, the reaction was kept on ice for 30 minutes, heat-shocked at 42°C in a water bath for 30 seconds, and immediately put on ice for 2 minutes again. When the mixture was cooled, 200 µl of S.O.C.-Outgrowth media was added and the tube was horizontally shaken for1.5 hours. Meanwhile, pre-warmed agar plates containing Carbenicillin (50µg/ml) were prepared by placing a 40 µl X-Gal layer on the plates with a sterilized loop.

Finally, 75 µl and 150 µl of the transformed *E. coli* cells were plated on the prepared plates, incubated for 5 minutes at room temperature and kept overnight in the incubator at 37°C.

3.7. Plasmid DNA Isolation

To perform the isolation of plasmid DNA from the samples, the agar plates from section 3.5 were used, where bacterial colonies of *E. coli* have been grown on. White colonies were picked from the plates by using pipette tips and incubated in 6 ml of LB media at 37°C.

The actual plasmid DNA isolation process was performed using 'NucleoSpin® Plasmid DNA Purification kit', as well as the 'QIAprep Spin Miniprep kit', according to the protocols provided by the manufacturers.

3.8. Restriction Digestion

To confirm the presence of an insert within the cloning vector, the isolated plasmid DNA was tested using restriction digestion EcoRI from New England Biolabs®.

***Table 7**: Restriction digestions reagents and their corresponding volumes in the mix.*

Restriction digestion using EcoRI	
EcoRI buffer	2.0 µl
EcoRI enzyme	0.5 µl
MiliQ H_2O	14.5 µl
DNA	3 µl

The reagents were mixed according to Table 7 and incubated at 37°C for 30 minutes. Then, the mixture was placed into an agarose gel and examined for an insert using gel electrophoresis (as described in section 3.4.1).

3.9. Bioinformatic Analysis of *B. duttoni 1120K3 & Ly*

In order to confirm the identity of the strains *Ly* & *1120K3* from *B. duttonii*, two different plasmids containing the glycerophosphoryl diester phosphodiesterase (*glpQ*) gene, as well as two different plasmids each containing the flagellin (*flaB*) gene of *Ly* & *1120K3* were sent for sequencing and then compared to homologous nucleotide sequences using the BLAST method to identify them.

The identification of the strains was done by comparing the sample sequences of the genes with genes from the NCBI databank. The accession numbers of the used nucleotide sequences are listed in Table 8.

***Table 8**: B. duttonii strains, their genes and their corresponding accession number from the NCBI databank.*

Strain	Gene	Gene Accession number
B. duttonii Ly	*glpQ*	ACH93196
B. duttonii Ly	*flaB*	DQ346833
B. duttonii 1120K3	*glpQ*	GU357577
B. duttonii 1120K3	*flaB*	GU357617

3.10. Mice, intradermal injection of *B. duttonii and blood sample collection*

In total, 7 eight week-old female C3H/HeN mice (5 inoculated with *1120K3*, 2 with *Ly*) and 7 eight week-old female BALB/c mice (3 inoculated with *1120K3*, 4 with *Ly*), which were kept in individually ventilated cages at the animal facility in the Institute of Parasitology, BC CAS, were each injected with 1 x 10^6 *B. duttonii* per mouse in a 250 µl volume (150 µl inter-peritonealy and 100 µl subcutaneously). The infection of mice was investigated daily by performing tail-snips. Therefore, the tail of each mouse was cut and a few microliters of blood were collected using a pipette. The collected blood samples were placed onto a glass slide and -the amount spirochetes for every 10 fields of view in the dark-field microscope was determined.

To obtain mouse sera, blood of the infected mice was collected into a 1.5 ml Eppendorf® Tube via retro-orbital bleeding, after anesthesia.[34]

After an incubation time of 15 minutes at room temperature, the collected blood was centrifuged for 7 minutes at 9000 rpm and the serum (upper phase) was extracted and transferred into a new tube and stored at -20°C. The produced sera from the blood samples were then used for detection of antibodies in serological analysis (Immunoblotting, Chapter 3.12).

3.11. Tick colonies and transmission studies

The uninfected *O. moubata* tick colonies used in this work were kept at 27°C and 90% relative humidity. In order to initiate acquisition, 10 uninfected larval and 10 uninfected 1st stage nymphal *O. moubata* ticks were put on each of the 14 previously infected C3H/HeN and BALB/c mice on 3rd day post-inoculation , when the spirochete peaked.

After the molting process, which took about 3-4 weeks, 12 of the 1st stage nymphs, previously fed as larvae, from one C3H/HeN and one BALB/c mouse (6 ticks each) and 12 of the 2nd stage nymphs, previously fed as 1st stage nymphs, from one C3H/HeN and one BALB/c mouse (6 ticks each) were crushed and the tickDNA was then isolated (Chapter 3.4) in order to subsequently determine the presence of *Borrelia* in them.

When the presence of spirochetes in the crushed ticks was confirmed by PCR and evaluation with a dark-field microscope, the infected 1st stage nymphal ticks from the same colony were put on 5 naive C3H/HeN mice and the infected 2nd stage nymphal ticks from the same colony on 4 naïve C3H/HeN mice (10 ticks/mouse).

3.12. SDS – PAGE

The already prepared *Borrelia* lysates were heated up to 95°C for 10 minutes and then 12 µl of these lysates were loaded into a SDS-PAGE (12% separating gel, 5% stacking gel and a PageRuler™ Plus Prestained Protein Ladder, ThermoFisher®). The electrophoresis was performed at 120 V for approximately 90 minutes. For SDS-PAGE analysis, the gel was then stained using Coomassie® Blue R-250 (ThermoFisher®) staining solution, according to manufacturer's protocol.

***Table 9**: Preparation of one Polyacrylamide Gel for SDS-PAGE or Western Blot analysis.*

	12% Separating Gel	5% Stacking Gel
30% Acrylamide	2 ml	0.17 ml
4x Separation Buffer	1.25 ml	-
4x Stacking Buffer	-	0.25 ml
dH_2O	1.7 ml	0.57 ml
10% APS	50 µl	10 µl
TEMED	2 µl	1 µl

- Buffer ingredients:

  4x Seperation Buffer:
  1.5 M Tris
  0.4 % SDS
  pH 8.8

  4x Stacking Buffer:
  0.5 M Tris
  0.4 % SDS
  pH 6.8

3.13. Western Blot (Immunoblotting)

For serological analysis, the separated proteins from the electrophoresis in the SDS-PAGE were electro-blotted onto a Nitrocellulose membrane using Trans-Blot® Semi Dry Electrophoretic Transfer Cell (Bio-Rad). The blotting process was performed according to the Western Blot standard protocol, 25V for 1 hour. After successful transferring, the membrane was placed into a blocking buffer solution (5% non-fat dry milk in 200 ml 1x TBS + 0.05 % Tween 20) for 1.5 hours at room temperature. The Nitrocellulose membrane was cut according to the different lysates used, to separate them from each other. Each of those cut pieces was initially handled and placed into different small plastic bags, filled with 1ml of blocking solution mixed with 5 µl of primary antibody solution (mouse sera, prepared in Chapter 3.10) and kept at 4°C overnight.

The next day, the membrane was washed with 1x TBS + 0.05 % Tween 20 three times for 15 minutes and then incubated with 50ml 5x blocking solution containing 5 µl of 1:10000 diluted , secondary anti-mouse antibody solution (Sigma-Aldrich®) for 1 hour.

Subsequently, the membrane was washed three times with 1x TBS + 0.05 % Tween 20 for 15 minutes each and stained with a substrate solution, SYPRO® Ruby protein blot stain (Bio-Rad), following the manufacturers instructions.
Finally, the membrane was visualized with a UV-Transilluminator, photographed and the protein bands were examined using Image Lab® software.

4.1. Confirmation of the *B. duttonii* strains *1120K3* & *Ly*

For identification, the *glpQ* and *flaB* genes of *1120K3* and *Ly* were amplified by PCR (Chapter 3.5) and then visualized and examined using Agarose Gel Electrophoresis (Chapter 3.5.1).

The gel electrophoresis picture represented in Figure 7 was performed after successful cloning and transformation of the previously amplified and visualized *1120K3* & *Ly* DNA (*glpQ* and *flaB*) into *E. Coli* using the Invitrogen's pCR®2.1-TOPO® vector (Chapter 3.6) and subsequent restriction digestion using the restriction enzyme EcoRI from New England Biolabs® (Chapter 3.8).

In total, 4 samples from *Ly glpQ*, 4 samples from *1120K3 glpQ*, 2 samples from *Ly flaB* and 1 sample from *1120K3 flaB* was used in this electrophoresis and then sent for sequencing.

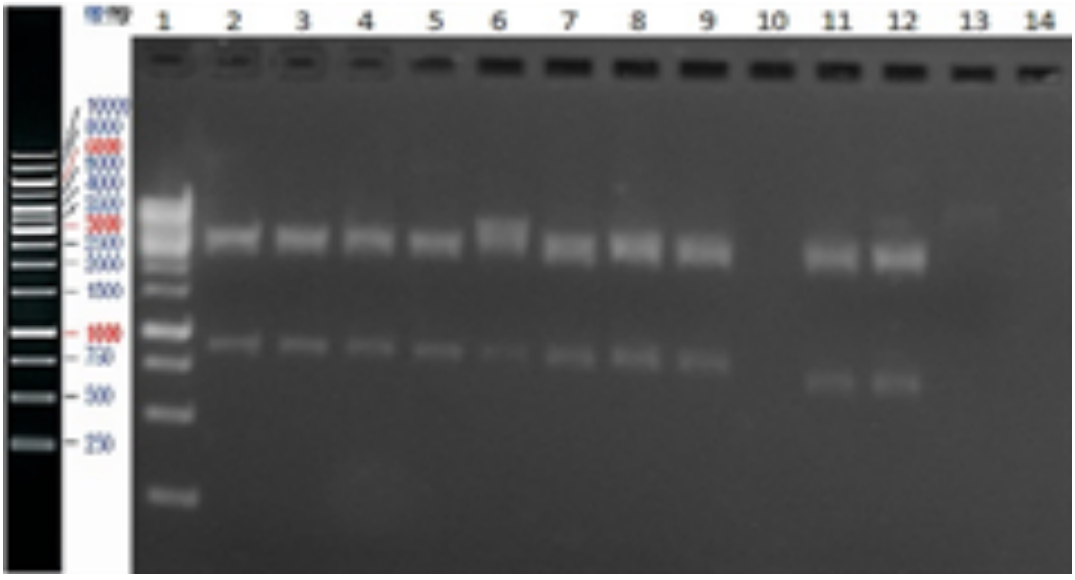

Figure 7: *Agarose gel electrophoresis after restriction digestion of plasmid DNA. (Lane 1: GeneRuler™ 1kb DNA Ladder, Lane 2-5: Ly glpQ 1-4, Lane 6-9: 1120K3 glpQ 1-4, Lane 10. No result, Lane 11-12: Ly FlaB 1-2, Lane 13: negative glpQ, Lane 14: negative FlaB)*

All samples which showed bands of the expected size for the *glpQ* gene (Lane 2-9) gave a DNA fragment at ~800 bp length, and the plasmid vector at ~3 kbp. The samples containing the *flaB* gene (Lane 11-12) gave bands at ~600 bp length and also

the plasmid vector at ~3 kbp. All of the samples with positive results were subsequently sent for sequencing and evaluated.

For the electrophoresis shown in Figure 8, 7 samples from restriction digestion of *1120K3 flaB* were taken, to get as many samples as possible that can be sent for sequencing.

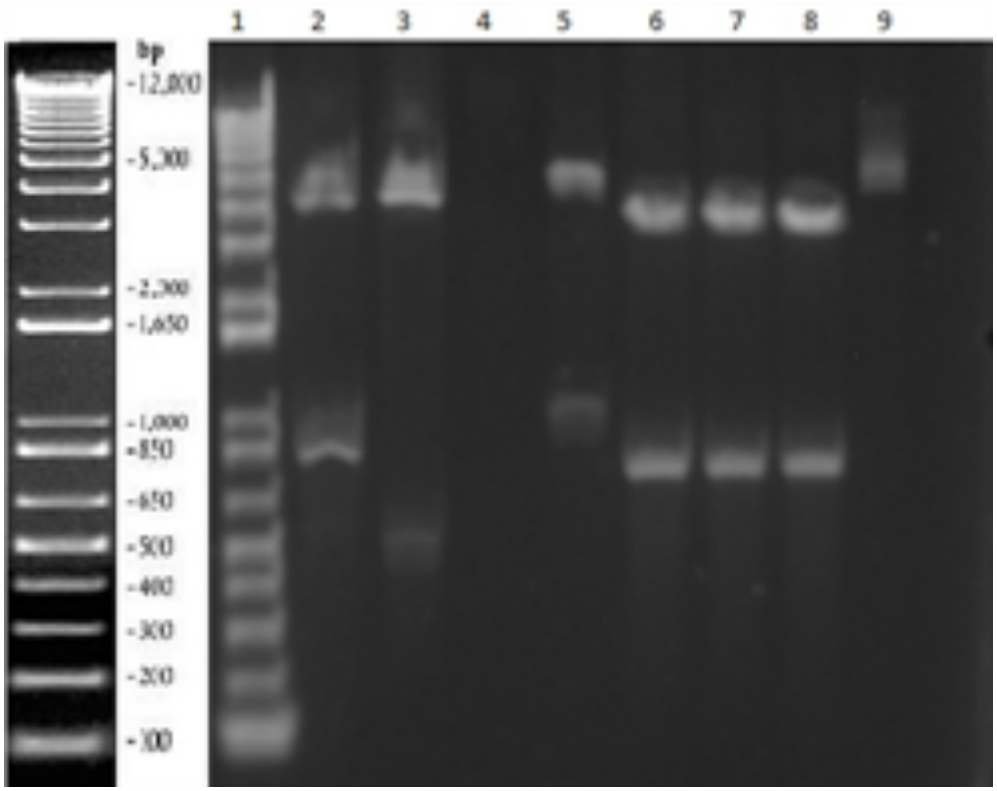

Figure 8: *Agarose gel electrophoresis after restriction digestion of plasmid DNA (FlaB). (Lane 1: GeneRuler™ 1kb plus DNA Ladder, Lane 2: 1120K3 FlaB sample, Lane 3: no result, Lane 4: no result, Lane 5: no result, Lane 6-8: 1120K3 Flab samples, Lane 9: negative FlaB)*

As pictured in Figure 8, it can be seen that only 4 of the 7 samples (Lane 2 and 6-8) gave reasonable results with DNA fragments (*flaB*) at ~700 bp length and the plasmid vector at ~3 kbp. Thus, those samples were sent for sequencing and all others were not used any further for subsequent analysis.

The obtained nucleotide sequences of the *glpQ* and *flaB* genes in the strains *1120K3* and *Ly* showed high identity scores when comparing the sequences of those genes in the databank (NCBI), represented in Chapter 3.9. In order to receive meaningful data, only hits with e-values below a threshold of 10^{-40} and sequence query above 68% were taken into account. The sample sequences compared with the values of the databank (DB) resulted in more than 100% identity.

All of the samples that were sent for sequencing were bioinformatically analyzed, aligned and used for BLAST. Still, for simplification, only one of those samples that were sent for sequencing was used here in Table 10 to confirm the identity of the gene (*glpQ* or *flaB*) embedded in the corresponding strain (*1120K3* or *Ly*).

4.2. PCR detection of *B. duttonii* in *O. moubata ticks*

After the first successful infection of the BALB/c and C3H/HeN mice *with B. duttonii 1120K3* and *Ly* in this work (Chapter 3.10, 4.3), larval, as well as 1st stage nymphal ticks were put on mice infected with both *1120K3* as well as *Ly* and then subsequently molted to a higher stage (Chapter 3.11). Then, the tick DNA was isolated (Chapter 3.4) and PCR as well as agarose gel electrophoresis was performed (Chapter 3.5, 3.5.1) in order to determine a successful spirochete infection of the ticks with *1120K3*, since *Ly* showed up as non-infective (by looking blood and tick DNA-PCR).

In Figure 9 and 10, the results of the electrophoresis of infected 1st and 2nd stage nymphal ticks infected with *B. duttonii 1120K3* are shown, where isolated DNA was amplified by PCR using *flaB* (Figure 9) and *glpQ* (Figure 10) primers and then visualized.

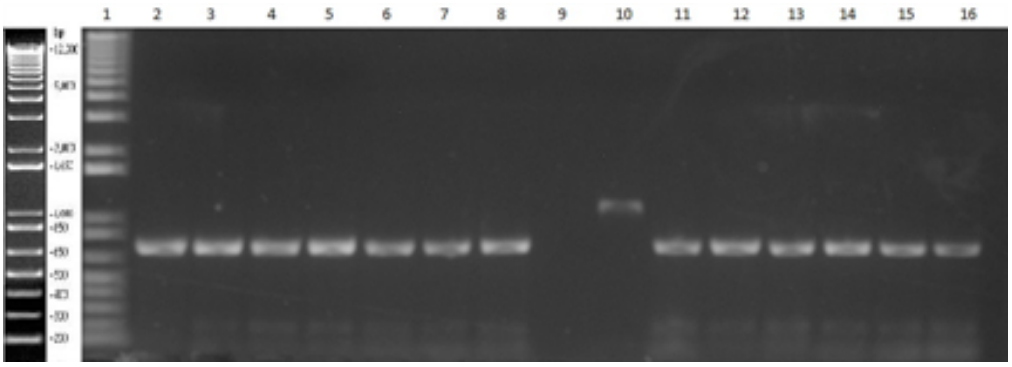

***Figure 9**: Agarose gel electrophorsis of DNA from 1st (first 6) and 2nd (last 6) stage nymphal ticks infected with 1120K3 using FlaB primers. (Lane 1: positive 1120K3 FlaB, Lane 2-8: 1st stage nymphs with 1120K3 FlaB, Lane 9: empty, Lane 10: negative FlaB, Lane 11-16: 2nd stage nymphs with 1120K3 FlaB)*

When evaluating Figure 9, there are *Borrelia* DNA fragments (*flaB*) at ~700 bp length in all lanes (*flaB*), suggesting that all of the tick DNA samples (1st stage as well and the 2nd stage nymphs have fed as 1st stage nymphs on infected C3H/HeN (inoculated with 1 x 10^6 *Borrelia*) and got successfully infected by those mice.

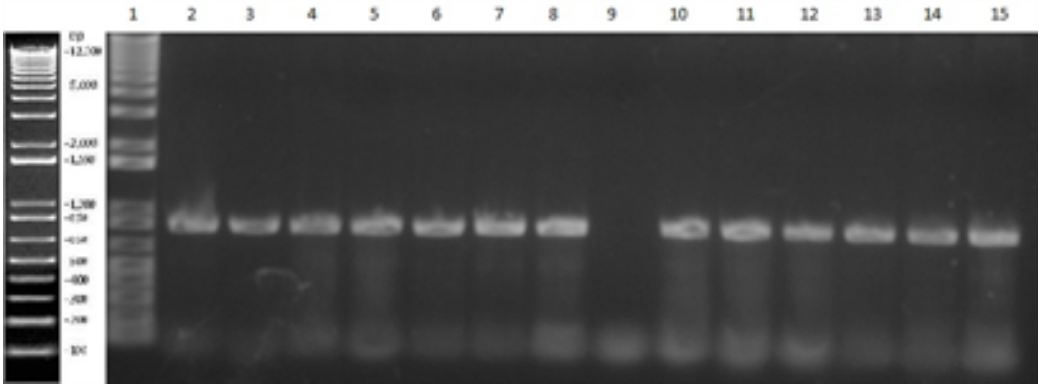

Figure 10: *Agarose gel electrophoresis of DNA from 1st (first 6) and 2nd (last 6) stage nymphal ticks Infected with 1120K3 using glpQ primers. (Lane 1: positive 1120K3 glpQ, Lane 2-8: 1st stage nymphs with 1120K3 glpQ, Lane 9: negative glpQ, Lane 10-15: 2nd stage nymphs with 1120K3 glpQ)*

In Figure 10, all of the tick DNA samples show *Borrelia* DNA fragments (*glpQ*) at ~800 bp length.

That gave clear results of an *1120K3* infection of the 1st stage and 2nd stage nymphs. The infected 1st stage and 2nd stage nymphal ticks used for this electrophoresis previously fed on infected BALB/c mice (also inoculated with 1 x 10^6 *Borrelia* organisms) as larvae and 1st stage nymphs and got successfully infected by those mice.

4.3. Antibody detection and infection kinetics of *B. duttonii* in mice

The first step in the tick-animal model was the inoculation of BALB/c and C3H/HeN mice with 1 x 10^6 *Borrelia*/mouse (Chapter 3.10). After the first try of inoculation with *1120K3* and *Ly* in C3H/HeN mice , we decided to continue the experiment only further using the *1120K3* strain of *B. duttonii* due to the fact that *Ly* showed to be non-infective when performing blood analysis of the inoculated mice. Nevertheless, in the subsequent two immunoassays involving new C3H/HeN and BALB/c mice, 6 mice (4 BALB/c and 2 C3H/HeN) received intradermal injection with *Ly* and 8 mice (3 BALB/c and 5 C3H/HeN) got inoculated with *B. duttonii 1120K3*.

The Western Blot image obtained from the first infection of the bacteria in the mice, showed clear infection of mice with *1120K3*. However, the mice which got inoculated with *Ly* showed to be poorly or even non-infective to them.

Due to this observation, we concluded that only the usage of the *1120K3* strain

of *B. duttonii* for further experiments and for the establishment of the tick-animal model would lead to promising results.

Nonetheless, after this experiment showed a clear infection of C3H/HeN mice with *1120K3*, larval ticks were put on those, as well as on the mice which got inoculated with *Ly*. After performing PCR detection of *Borrelia* in those ticks, the results were negative and did not show an infection of the ticks. That being observed, new C3H/HeN and BALB/c mice were inoculated with *Ly* and *1120K3* to re-start the experiment. The infection of the mice was then investigated by performing daily tail-snips, pipetting some microliters of blood onto a glass slide and observing the amount of spirochetes using a dark-field microscope (Chapter 3.10).

Figure 11 shows the spirochete infection of C3H/HeN and BALB/c mice infected with 1 x 10^6 *1120K3* (Mouse 1-3 BALB/c, Mouse 10-14 C3H/HeN) and *Ly* (Mouse 4-7 BALB/c, Mouse 8-9 C3H/HeN) post-inoculation. The numbers in the table correspond to the amount of *Borrelia* per 10 fields of view in the dark-field microscope. The *Borrelia* infection analysis of the mice was carried out over a span of 21 days in order to obtain the peak of spirochetemia, which was needed to be observed for the subsequent tick-feeding. However, for infection analysis, the data of the first 9 days was used in order to obtain better results.

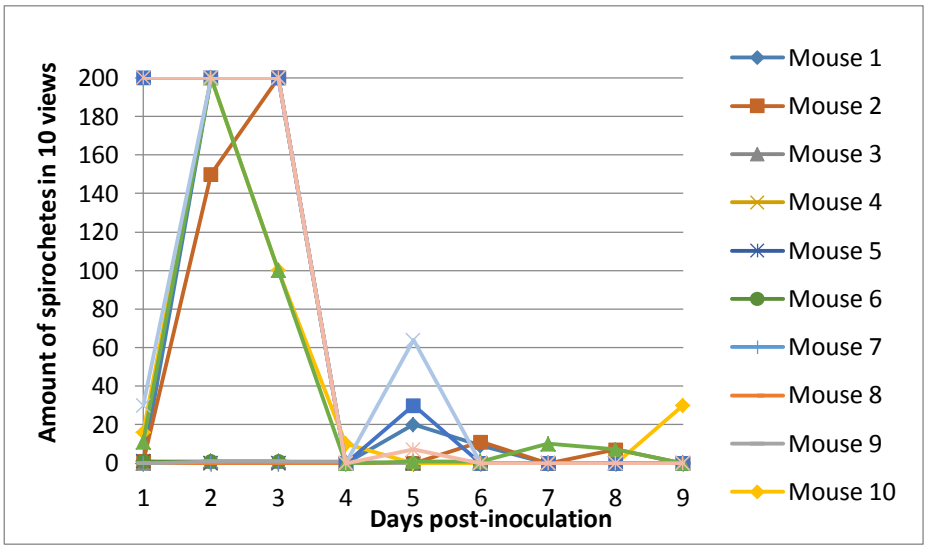

***Figure 11**: Infection kinetics of C3H/HeN and BALB/c mice post-inoculation with 1120K3 and Ly.*

When evaluating Figure 11, it is easily recognizable that the mice that were inoculated with *Ly* (Mouse 4-9) showed no *Borrelia* in their blood and thus were not considered to be infected. After observing this data, the mice which were inoculated with *Ly* were

not used for further experiments in the tick-animal model.

The mice which were inoculated with *1120K3* showed a high amount of *Borrelia* in their blood and showed their peak of spirochetemia on days 2 and 3 with approximately 200 *Borrelia* in 10 views, obtained from the spirochete determination method in Chapter 3.10.
Thus, the larval and 1st stage *O. moubata* nymphs were put on them on this day.
The subsequent measurements after day 7 were done to evaluate the re-occurrence of spirochetes in blood during the febrile episodes, but gave no promising results.

However, even though there were no visible *Borrelia* during the counting's in the blood of the mice, the Western Blot of those mice gave positive results and showed possible infection followed by clearance of *B. duttonii Ly*. Still, due to the previous negative results that came from either immunoassays or the blood investigation of the mice which showed no spirochete infection, those infected mice were not further used for the tick-animal model.

The results of the Western Blot, in which the mice got inoculated with *1120K3*, gave clearly infected BALB/c and C3H mice with *B. duttonii 1120K3* and also gave promising results in the previous blood analysis.

Subsequently, after the confirmation of spirochete acquisition, the transmission cycle was continued by putting uninfected ticks (larvae and 1st stage nymphs) on C3H/HeN and BALB/c mice infected with *1120K3*. After molting for 3-4 weeks, determining their infection and the successful confirmation of spirochetemia by PCR (Chapter 4.2), they were put on naïve C3H/HeN mice.

In order to close the infection cycle of *1120K3*, the newly molted 2nd stage nymphs (previous 1st stage nymphs) fed on 4 naive C3H/HeN mice (10 ticks each) and the 1st stage nymphs (molted from larvae) were put on 5 naïve C3H/HeN mice (10 ticks each) to initiate infection of healthy mice. Those mice were then serologically analyzed by performing daily tail-snips and Western Blots.
In Figure 12, the blood analysis of C3H/HeN mice post-transmission is shown; where 1st and 2nd stage *O. moubata* nymphs fed on them. The analysis was carried out over a span of 5 days, in order to obtain the spirochetemia of the mice with high numbers of spirochetes in their blood.

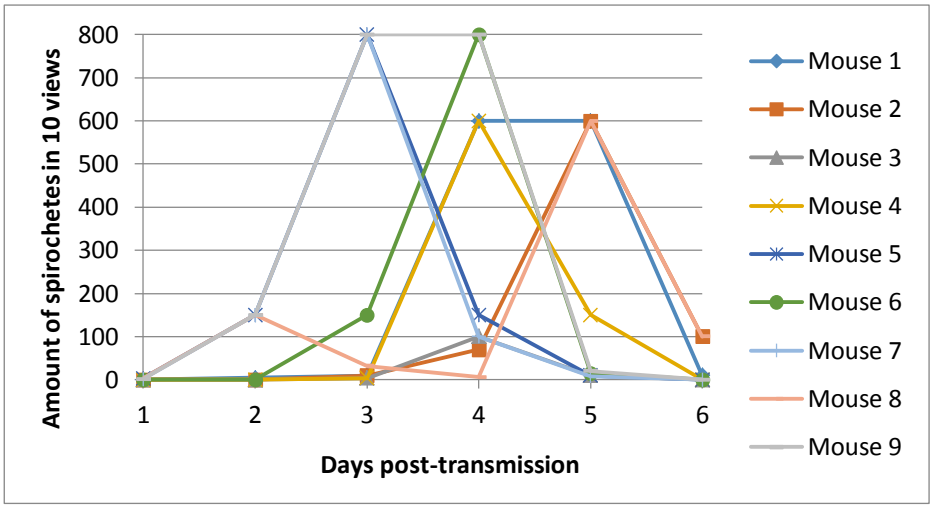

***Figure 12**: Infection kinetics of C3H/HeN mice post-transmission from infected 1st and 2nd stage O. moubata nymphs.*

All of the mice from Figure 12 showed a peak of spirochetemia, even if they were not on the same day and not always the same amounts of *Borrelia*. The highest amount of spirochetes counted was 800 *Borrelia* in 10 views, using the determination method from Chapter 3.10. The infected C3H/HeN mice were subsequently bled in a retro-orbital fashion to collect their blood, which was needed for confirming their infection via Western Blot.

After performing the Western Blot, all C3H/HeN mice, where 1st stage nymphs as well as 2nd stage nymphs had fed, finally confirmed the infection with *1120K3*, meaning that the ticks were able to successfully transmit the *Borrelia.*

Figure 13 shows an example of a Western Blot picture, where all of the C3H/HeN received an infection of *B. duttonii 1120K3* by 2nd stage *O. moubata nymphs.*

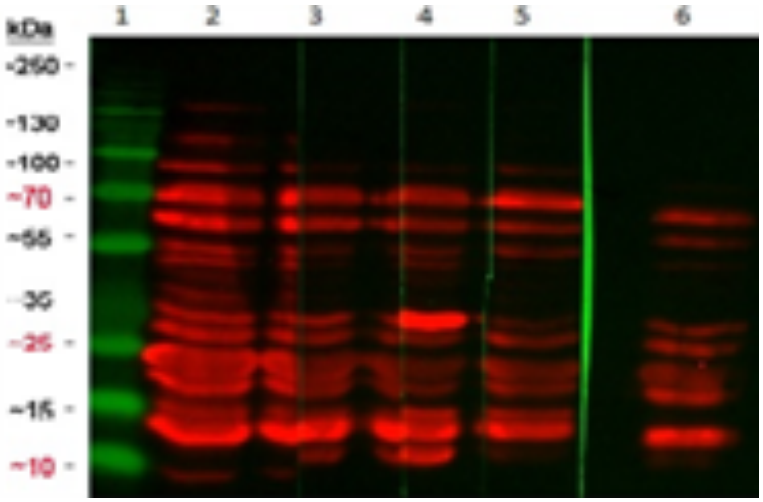

***Figure 13:** Western Blot of C3H/HeN mice infected with 1120K3 by 2nd stage nymphs. (Lane 1: PageRuler^{TM} Plus Prestained Protein Ladder, Lane 2: positive 1120K3, Lane 3-6: C3H/HeN mice infected with 1120K3)*

5 DISCUSSION

Borrelia duttonii, is next to other relapsing fever agents belonging to the *Borrelia* genus, not only a very dangerous, but also a very infective bacterial organism. Since relapsing fever has become a very much neglected tropical disease worldwide and is barely distinguishable from other diseases like malaria for instance, the sickness has become even more threatening since there are no vaccines yet available.[16,19] Therefore, animal models plays a crucial role in understanding the biology of pathogens like spirochetes or viruses that are transmitted via tick bites.
Until today, several vector-borne diseases that result from parasitic organisms such as *Plasmodium* or *Borrelia*, or even viruses such as Phleboviruses or Flaviviruses, were investigated by developing different animal models in different kinds of animals such as guinea pigs, monkeys and mice in order to understand the severity of the pathogens to the host immune system. [35–37]

Nevertheless, there have only been very few actual vector-host transmission studies, such as tick-animal models carried out when taking a closer look on relapsing fever *Borreliae.* Most of the animal models that have been done involving transmission of spirochetes via ticks, are represented in Lyme disease studies.[5] This can be explained by the fact, that Lyme disease as well as TBEV are the most commonly known tick-borne diseases and therefore also the sicknesses gaining most of the attention within our society (in Europe, Asia and the US). Still, there have been some tick-animal models with *O. moubata*, using Flaviviruses such as WNV or the ASFV. [20,32,38]

The only known tick-animal model that has been done with *B. duttonii* and *O. moubata* was done using gerbils and gave successful results in transmission.[33] . This studies main objective was to look at the possibility of transovarial transmission. For the first time we have developed a tick-mouse model using C3H/HeN mice infected with *B. duttonii* with *O. moubata* as a vector, which will help lead to a further understanding of the biology in host-pathogen interaction of African relapsing fever *Borrelia* and their ability to infect mice, as well as further knowledge about the competence of *O. moubata* as a vector of vector-borne diseases.

Regarding our work, we were able to scientifically prove, after receiving the provided passages of the *B. duttonii* strains *1120K3* and *Ly* (from Sven Bergström, University of Umea, Sweden) and re-cultivating them in our laboratory, the identity of this *Borrelia* species and the strains we worked withby designing specific *glpQ* and *flaB* primers to amplify specific genes in the genomes of *1120K3* and *Ly*. Moreover, we confirmed, that an intradermal injection of *B. duttonii* organisms (*1120K3*) into healthy C3H/HeN as well as BALB/c led to successful infection of the mice and thereby verifying *1120K3* as infectious, when inoculated. The infection of those C3H/HeN and BALB/c mice was controlled by performing daily tail-snips and investigating blood samples under a dark-field microscope.

After first inoculation of *Ly* into C3H/HeN mice, we identified the strain as poorly infective according to the obtained Western Blot results (poor antibody expression). Even when new mice got inoculated with 1 x 10^6 *Borrelia duttonii Ly* again (re-trying the experiment), we were not able to identify any spirochetes in the blood samples of the mice infected with *Ly* when performing microscopic analysis of the blood. Still, after final retro-orbital bleeding of those mice, which came up negative in blood analysis, the immunoassay showed next to no infection of the mice with *B. duttonii Ly*.

However, the experiment was continued by putting uninfected larval and 1st stage nymphal *O. moubata* ticks on the C3H/HeN and BALB/c mice that were infected with *1120K3* (3 days post-inoculation) and confirmed us successful transmission of the spirochetes into the tick vectors after molting into a higher stage (via PCR).

After the infection of the ticks was verified, the molted and infected ticks were put on naive C3H/HeN mice and it was confirmed the successful transmission of *1120K3* into the vertebrate hosts (via Western Blots). That resulted into a closed tick-animal infection cycle, stating that *B. duttonii 1120K3* is infective in mice and can be transmitted by the *O. moubata* from infected to healthy C3H/HeN mice.
Nonetheless, it is important not to forget, that the *B. duttonii* strain *Ly* still can have infective potential (due to infection via inoculation) when using tick-mice models, even though it did not give promising results in this work.

However, as already mentioned, this has been the first time that a tick-mouse model for *B. duttonii 1120K3* was established and successfully performed.

We are confident, that this work will help in taking a bigger step forward in relapsing fever research for Old World RF *Borrelia* species. It is important to gain further understanding how *Borrelia* can avoid the ticks and hosts immune system, how the bacteria can even survive and how it can be beaten or preserved (by vaccines, for instance).

Establishing and working on more animal models with different kinds of rodents and bacterial strains would generate a lot brighter insight as well as a wider spectrum of biological knowledge about parasitic organisms that are able to do severe damage, or even kill animals and humans. Therefore, it is important to investigate all of the unknown as accurately as possible and to not keep sicknesses such as relapsing fever a forgotten disease, but to rather find diagnostic methods for detecting the disease as well as possible vaccines for in order to prevent this disease.

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