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Understanding the interactions between *Borrelia duttonii* and the tick *Ornithodoros moubata* as well as the mammalian host at the *in vitro* and *in vivo* level

Bachelor Thesis

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Annotation: The aim of this Bachelor thesis was to study the interactions between the spirochete *B. duttonii*, which is a causative agent for relapsing fever in Africa, and its vector, the soft tick *O. moubata*. Several experiments were conducted including passaging, membrane feeding and keeping infected ticks at different temperatures. Microscopy methods were used to visualize the presence of *B. duttonii* in mammalian blood cells and in the salivary glands of the *O. moubata* tick.

Affirmation: I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. 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 shortened form resulting from deletion of the sections materials and methods, results, discussion and conclusion 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. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Abstract

Relapsing fever is a disease that has been long neglected when it comes to the research of its causative agents, the RF Borrelia spirochetes.

The goal of this thesis was to study the interactions between the spirochete *B. duttonii*, which is a causative agent for relapsing fever in Africa, and its vector, the soft tick *O. moubata*. In the course of this thesis several different experiments using various methods were conducted.

By passaging a *B. duttonii* culture, the effects of prolonged in-vitro cultivation were studied. The protein expression between the passages was compared to the wildtype by SDS-PAGE.

To design a tick feeding model that does not involve the use of animals, a working model for infectious in-vitro membrane feeding of *O. moubata* was validated. The feeding and moulting behavior of the ticks was studied and the presence of *B. duttonii* inside the ticks post feeding was analyzed.

O. moubata ticks infected with *B. duttonii* were kept at two different temperatures and the difference in tick infections was studied by seeing if the Borrelia within the ticks were infectious.

We confirmed the presence of *B. duttonii* in the salivary glands of the *O. moubata* tick by conducting an immunofluorescence assay followed by visualization with a fluorescence microscope.

Lastly, scanning electron microscopy was used to visualize the interactions between Borrelia spirochetes and red blood cells inside mammalian blood.

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Content

1 Introduction

1.1 Relapsing fever Borrelia

Relapsing fever Borrelia (RF Borrelia) are motile bacteria characterized by their helical and cork-screw like shape [1]. They belong to the spirochete phylum and are responsible for causing the disease relapsing fever. Alongside Lyme disease Borrelia, they form the Borrelia genus [2].

Different Borrelia species vary in morphology with a length of 10-30µm and a helical width of 0.2-0.5µm. Looking at the structure of a spirochete, a protoplasmic cylinder is surrounded by an outer membrane with periplasmic space in between, where up to 30 flagellates reside [2,3]. The protoplasmic cylinder further consists of an outer peptidoglycan layer, an inner cell membrane and cytoplasm [3].

1.1.1 Species and Geographical distribution of RF Borrelia

RF Borrelia species are classified as being either tick-borne (TBRF) or louse-borne (LBRF), determined by its vector. As of today, 23 different species of RF Borrelia are confirmed, although new species are still found regularly, for example in Japan [4] or in Australia [5]. Only 1 species, namely *B. recurrentis*, belongs to the LBRF group, it is transmitted by the clothing louse *Pediculus humanus.* All other species of RF Borrelia have soft ticks as their respective vectors with the exception of *B. miyamotoi* which is transmitted by hard ticks of the Ixodes species [6,7].

RF Borrelia are well distributed around the world, they can be found in the United States, Mexico and Central America, around the Mediterrean Sea, the Middle East, Central Asia, parts of Japan and in Africa. There is a strong regional correlation between the distinct species of RF Borrelia and their respective vectors, so a species can naturally be found in the area where its vector resides. NoTable examples of RF Borrelia species include *B. hermsii* and *B. turicatae* in North America, *B. venezuelensis* in Central and South America or *B. baltazardii* and *B. persica* in Asia and the Middle East. In Africa there is a exceptionally wide variety of RF Borrelia species, including for example *B. crocidurae* in North and West Africa, *B. hispanica* in North Africa and additionally South Europe, *B. recurrentis* in East Africa, *B. graingeri* in East Africa and *B. duttonii* in Central and East Africa [1].

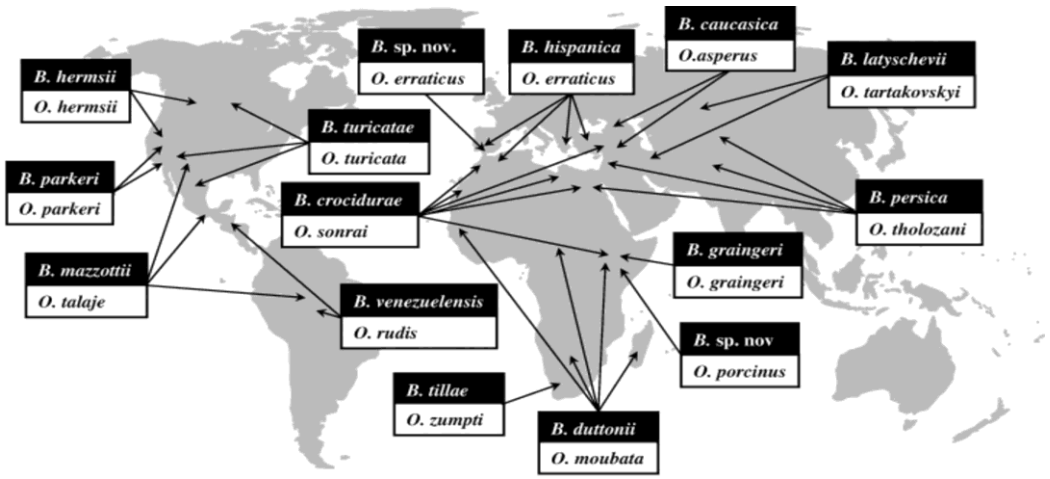

Figure 1: Distribution of tick-borne relapsing fever agents and their specific vectors of the genus Ornithodoros, adapted from [8]

1.1.2 Tick-Borne Relapsing fever

Tick-Borne Relapsing fever (TBRF) is an illness that is characterized by recurring episodes of fever and other symptoms like headache, myalgia and tachycardia, interrupted by afebrile episodes, generally called relapses [9]. During the febrile periods of the illness the amount of Borrelia in the blood is very high, up to 10^7 spirochetes per mL of blood; during the periods of well-being this number is significantly lower, being undetectable under dark-field microscopy [9, 10].

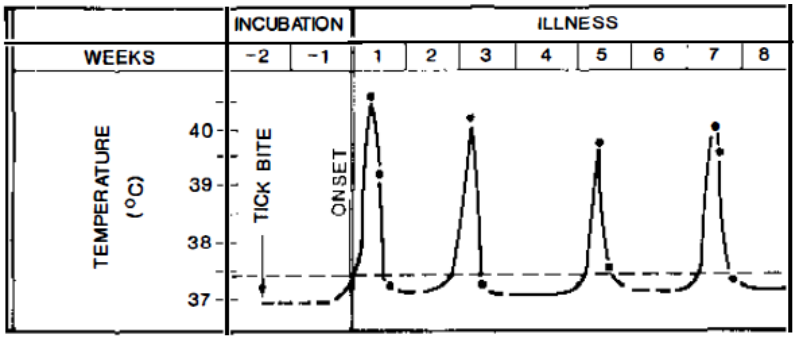

Figure 2: Body temperature over 8 weeks of a patient with relapsing fever infected with *B. hermsii*, adapted from [10]

The incubation period lies between 4 and 18 days with a mean of 7 days [9]. Patients usually experience between 3 and 7 relapses, the interval between 2 relapses can vary from 4 to 28 days. The first febrile episode has shown to be the most severe both in terms of fever height and fever duration.

Studies on laboratory rats and mice also showed that the number of spirochetes is approximately 10-fold lower in subsequent relapses compared to the initial one [10]. The mortality rate lies between 2 and 5%, although it depends on the specific Borrelia species [11].

The diagnosis of RF is difficult as many of its symptoms show high similarity to those of malaria which is an illness especially prevalent in countries where *B. duttonii* is the main RF agent like Togo or Senegal [12]. There are several methods to diagnose Tick-Borne Relapsing fever and identify RF Borrelia spirochetes in a suspected infected patient. Direct microscopic methods including dark field microscopy or the usage of stains such as Wright–Giemsa or acridine orange are useful for symptomatic untreated patients at the beginning of their infection [11, 13]. PCR-based detection provides more specific diagnosis methods like real-time PCR using Borrelia flagellin DNA (flaB) or Multispacer typing (MST) of intergenic spacers [11]. Serological detection can be used in the case of an ongoing infection, a widely used method here is ELISA to screen for antibodies against the glpQ-protein which is expressed in RF Borrelia. However, this assay is only applicable in later stages of the disease when an immune response for antibody production has already happened [11,14].
Relapsing fever in patients is commonly treated by the administration antibiotics such as tetracycline, erythromycin of doxycycline, preferably starting at the first febrile period of the disease [15].

1.1.3 Natural Reservoirs of RF Borrelia

Natural reservoirs of RF Borrelia spirochetes include mammals like rodents, birds like chickens, but also bats and reptiles [1]. A study conducted in a rural area in Senegal showed that roughly 18% of rodents in this region were infected with *B. crocidurae* [16]. Despite that, the strong association between RF Borrelia led some authors to suggest that the soft tick vectors are their original natural reservoir, this is supported by the fact that transovarial transmission has been shown for *B. crocidurae* in their vector *O. erraticus* for instance [17,18]. However, RF Borrelia of the *B. duttonii/B. recurrentis* complex have so far only been associated with humans as a reservoir, although *B. duttonii* DNA has also been detected in domestic pigs and chicken in Tanzania [17].

1.2 Soft ticks (the *Argasidae* family)

Soft ticks of the *Argasidae* family are the vector of most Borrelia species, as mentioned in paragraph 1.1.1. These ectoparasitic and bloodsucking animals belong to the *Arachnida* class in its subclass *Acari*. The *Argasidae* family includes five genera with an estimated 193 species, although only 2 genera include a larger amount of species, namely the *Ornithodoros* and the *Argas* genus [19, 20].

Soft ticks mostly live in animal burrows and human dwelling and are nocturnal, thus attaching to their vector at night and then hiding in small holes and rifts after their blood meal. [21]

The main morphological difference to ticks of the *Ixodidae* family is the lack of a dorsal scrotum in both nymphal and adult stages. They possess a leathery cuticle and a centrally located dorsal plate coved in tiny mamillae. The capitulum (structure bearing the mouth parts) lies subterminally and hidden from a dorsal view. The eyes, if present in a species, are located on the ventral face of the body [22]

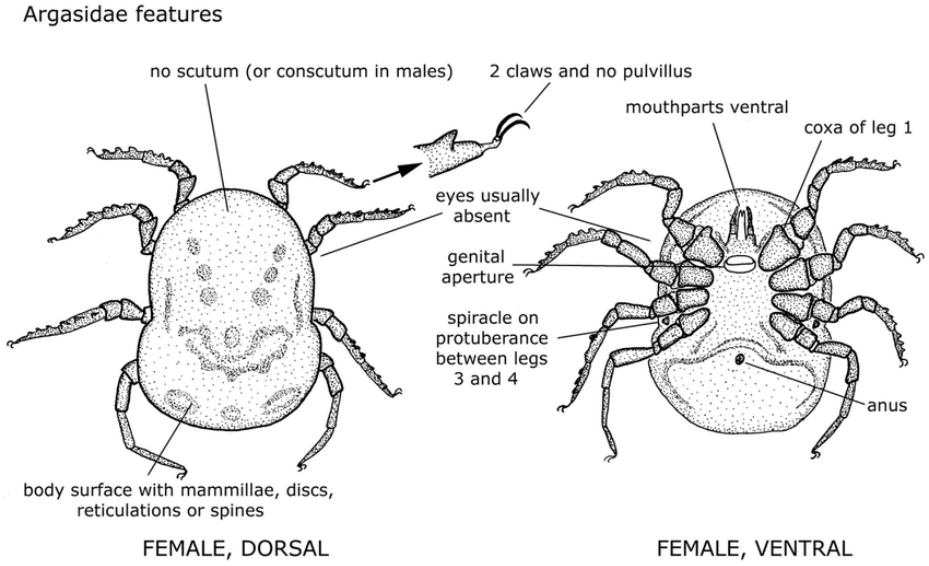

Figure 3: Morphological features of soft ticks (Family *Argasidae*) on the example of an adult female of the *Ornithodoros* genus, adapted from [23]

A primary characteristic of soft ticks is the especially short duration of their blood meal, ranging from 15-60mins for nymphal and adult stages. Argasid ticks could do this to reduce exposure to disadvantageous external conditions that might occur when the host leaves the tick's microhabitat. The moulting process happens only a few weeks after the blood meal which conveniently makes soft ticks very suitable for scientific research as analysis times are short compared to hard ticks [24].

1.2.1 Ornithodoros moubata

O. moubata, otherwise known as the African hut tampan is a tick belonging to the *Argasidae* family. It occurs in central and eastern Africa in countries like Kenya, Tanzania, Uganda, Congo and many more [25]. Besides being the main vector of the spirochete *B. duttonii*, which is the main focus of this thesis, it is also responsible for transmitting the African swine fever virus in pigs and the West Nile virus [26, 27]. Its main hosts while transmitting these diseases are pigs, humans, rodents and birds [28].

1.2.1.1 Life cycle of a soft tick on the example of *O. moubata*

In contrast to hard (*Ixodes*) ticks, which life histories and population dynamics have been extensively studied, it is difficult to model the life cycle of a soft-bodied argasid tick. *O. moubata* ticks are opportunistic feeders, meaning that they hide in burrows and feed on the first possible host in their reach. Unlike their hard tick counterparts that strictly undergo just three life stages, the *O. moubata* can go through up to six nymphal stages before moulting into an adult [28]. After hatching from an egg, a tick larvae moults into the first nymphal stage without a need for a meal and from there on subsequently moults into the next respective nymphal stage and finally to the adult stage upon the completion of a blood meal [28, 29]. An adult female tick can lay up to 500 eggs in one clutch after feeding on a large host and it can lay up to three clutches in her lifetime [28]. The life cycle of an argasid tick is depicted in Figure 4.

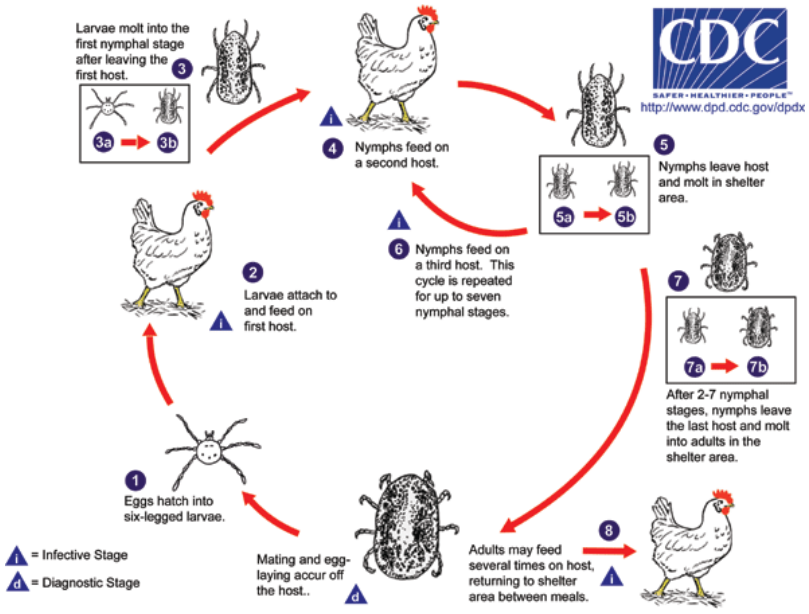

Figure 4: Life cycle of a soft (*argasid*) tick, larvae feeding is not necessary in *O. moubata*, adapted from [29]

1.3 Microscopy

1.3.1 Electron microscopy

Electron microscopy (EM) is a commonly used way to determine the interactions between pathogens and cells or tissues. It works by the use of a beam of accelerated electrons to visualize its ultrastructure. EM achieves sub-nanomenter spatial resolution better than 0.05 nm which makes it more than 4000 times better than light microscopy in that aspect [30]. Because SEM has been used for the work in this thesis, it will be discussed in greater detail.

1.3.1.1 Scanning electron microscopy (SEM)

First, accelerated electrons with high energies between 500 eV and 30 keV are produced in an electron gun. The electron beam is focused onto the sample and scans the specimen point for point to map an image of its surface. The electrons interact with the atoms of the sample, resulting in a loss of energy of a primary electron. The interaction volume is hereby the 3-dimensional space in which primary electrons interact with the sample. While travelling through the interaction volume, the electrons scatter and change direction while losing. Two types of scattering can be observed: Elastic scattering, where the energy loss is minimal but a change in direction occurs or inelastic scattering, where significant and defined amounts of energy are lost, but the direction remains virtually unchanged. Inelastic scattering results in the emission of secondary electrons, Auger electrons, and x-rays amongst other things and elastic scattering results in the emission of backscattered electrons. These emitted radiations are then detected and amplified to produce an image of the mapped surface [31]. Depending on the used operation mode, either the secondary electrons or the backscattered electrons are captured to produce an image, x-ray detector is optionally used for unfixed, hydrated biological samples [32]. Figure 5 shows a typical SEM instrumental setup.

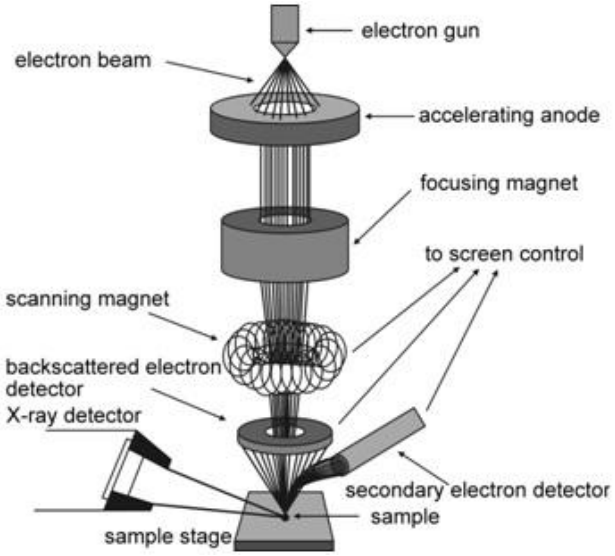

Figure 5: Setup of a scanning electron microscope, adapted from [33]

1.3.2 Fluorescence microscopy

Fluorescence microscopy is another widely used method in life sciences to study the properties of organic substances since a lot of molecules in living organisms can either adopt fluorophores or possess autofluorescencing properties and also because it offers a high degree of specificity [34].

The principle can be summarized as followed: Light of a specific wavelength, mostly of the blue, green or ultraviolet region of the visible spectrum is produced in a lamp source, passes through a selective excitation filter, gets reflected by a dichromatic mirror (beamsplitter) and passes through the objective to illuminate the specimen [34]. After an indicator, mostly a fluorophore within the sample, has absorbed the light energy, it emits light with lower energy and longer wavelengths than the excitation light, called fluorescence. The wavelength difference between the excitation light and emission light is described by the Stokes shift [35]. The emitted light is now transmitted through the dichroic mirror, passes through an emission filter that blocks out unwanted light and meets the eye to create bright image of the specimen against a dark background [35, 36]. Figure 6 (left picture) shows the schematic setup of a fluorescence microscope.

1.3.2.1 Confocal Fluorescence Microscopy

Confocal microscopy is an imaging technique that offers much higher resolutions than conventional wide field microscopy and is therefore commonly used in tissue engineering [37]. It works by focusing the excitation light of a given wavelength on a specific point in the sample and collecting the emitted fluorescence through a pinhole, therefore ensuring that only emitted light from the excited focal spot is generating the image [38]. This allows for spatial resolutions of up to ~0.8 µm in the axial direction and up to ~0.3 µm in the lateral direction. By choosing the right set of fluorescent dyes and illumination/detection settings, a multichannel registered image can be created that allows a 3D visualization of several subcellular structures [37]. Figure 6 shows the general setup on a confocal microscope in comparison to a regular widefield fluorescence microscope, the important difference here is the pinhole aperture allowing only in focus light to pass through.

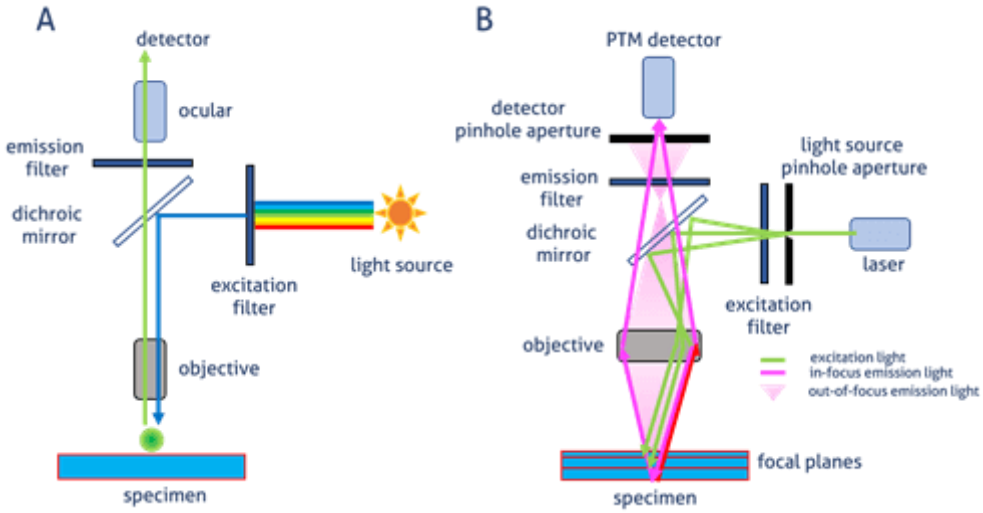

Figure 6: Schematic setups of a widefield fluorescence microscope (left) and a confocal microscope (right), adapted from [39]

2 Aims of the work

- Monitoring the effects of prolonged in-vitro cultivation on *B. duttonii* via passaging
- Establish a working model for infectious in-vitro membrane feeding of *O. moubata* ticks
- Monitoring the effects of different temperatures on *B. duttonii* infected *O. moubata*
- Visualization of *B. duttonii* in the salivary glands of the *O. moubata* tick via immunofluorescence
- Visualization of *B. duttonii* interaction with blood cells in mammalian blood via scanning electron microscopy

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