Pathobiology of African relapsing fever *Borrelia*

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Paper II

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Paper VI

Phylogeny of old world relapsing fever Borrelia
Relapsing fever (RF) is a disease caused by tick- or louse-transmitted bacteria of the genus *Borrelia*. It occurs worldwide but is most common in Africa where it is one of the most prevalent bacterial diseases. The main manifestation is a recurring fever which coincides with massive numbers of bacteria in the blood. Severity ranges from asymptomatic to fatal.

RF is usually considered a transient disease. In contrast, *B. duttonii* causes a persistent, residual brain infection in C57BL/6 mice which remains long time after the bacteria are cleared from the blood. The host gene expression pattern is indistinguishable from that of uninfected animals, indicating that persistent bacteria are not recognized by the immune system nor do they cause noticeable tissue damage. This is probably due to the quite low number of bacteria residing in the brain. The silent infection can be reactivated by immunosuppression allowing bacteria to re-enter the blood. To investigate if the residual infection is in a quiescent state or if the bacteria are actively dividing, mice with residual brain infection were treated with the cell-wall disrupting antibiotic ceftriaxone, which is only active against dividing bacteria. Since all mice were cured by ceftriaxone we conclude that the bacteria are actively growing in the brain rather than being in a latent, dormant state. The brain is used as an immunoprivileged site to escape host immune defence and probably as a reservoir for bacteria.

RF is a common cause of pregnancy complications, miscarriage and neonatal death in sub-Saharan Africa. We established a murine model of gestational relapsing fever to study the pathological development of these complications. *B. duttonii* infection during pregnancy results in intrauterine growth retardation as well as placental damage and inflammation. Spirochetes cross the maternal-foetal barrier, resulting in congenital infection. Further, pregnancy has a protective effect, resulting in milder disease during pregnancy.

A clinic-based study to investigate the presence of RF in Togo was performed. Blood from patients with fever were examined for RF by microscopy, GlpQ ELISA and PCR. About 10% of the patients were positive by PCR and 13% had antibodies to GlpQ. Many RF patients originally had a misdiagnosis of malaria, which resulted in ineffective treatment. The inability of microscopic analysis to detect spirochetes demonstrates the need for tests with greater sensitivity. To provide simple, fast, cheap and sensitive diagnostics using equipment available in small health centres, a method based on enrichment of bacteria by centrifugation and detection by Giemsa staining was developed which detects <10 spirochetes/ml.

To study the phylogeny of RF, IGS and *glpQ* were sequenced and neighbor joining trees were constructed. *B. persica* and *B. hispanica* were distant from the other species whereas *B. crocidurae* appeared to be a heterogeneous species. *B. duttonii* is polyphyletic in relation to *B. recurrentis* suggesting that the two species may in fact be the same or have a polyphyletic origin.
PAPERS IN THIS THESIS

This thesis is based on the following articles and manuscripts which will be referred to by their roman numbers I-VI.


PAPERS NOT INCLUDED


INTRODUCTION

1. The history of relapsing fever

The year is 1868 and Berlin is suffering from an epidemic of recurring fever. The 25-year-old physician Dr. Otto Obermeier at the Berlin Charité Hospital is examining blood from fever patients in the laboratory. His hypothesis is that the disease is caused by a fungus, but instead he sees “threads of different lengths in constant moving among blood cells” in the microscope. Borrelia is discovered (Burgdorfer 2001).

Obermeier published his findings in 1873 (Obermeier 1873) which soon were repeated and confirmed by others. Obermeier continued his work to find the agents of infectious diseases. He died five years after his discovery of relapsing fever (RF) after having injected himself with blood from a dying patient.

The French microbiologists Sergent and Foley showed that the human body louse *Pediculus humanus humanus* is the vector of relapsing fever spirochetes (Sergent and Foley 1910). Several scientists, among others Robert Koch, failed to establish the bacterium in various laboratory animals. Finally monkeys were found to be sensitive to the spirochete and could thereby fulfil Koch’s postulates.

The bacterium was first named *Protomyctum recurrentis* by Lebert in 1874. The year after it was re-named to *Spirochaeta obermeieri* and for a while it was also called *Treponema recurrentis* until it became known by its, hopefully, final name *Borrelia recurrentis* in honour of Amatée Borrel, not for contributing to the relapsing fever field, but rather for his eminent position at the Pasteur Institute and French Academy of Science.

The famous explorer Dr. David Livingstone who was supposedly lost in the jungle of Africa and found by the American reporter Stanley with the
legendary phrase, “Dr. Livingstone, I presume?” was in 1857 the first to describe tickborne relapsing fever which he referred to as “human tick disease”. He got the idea from the natives who suspected the recurring illness to be caused by tick bites. Later, in 1904-1905 several scientists, Cook in Uganda, Ross and Milne in the same area, and Dutton and Todd in the Congo, independently found Dr. Livingstone’s tick fever to be caused by spirochetes. They assumed it to be the same as *Spirochaeta obermeieri*, transmitted by the tick *Ornithodoros moubata* (Ross and Milne 1904; Dutton and Todd 1905). The symptoms of the disease were described as well as the fact that it could be transmitted from infected ticks to their newly hatched larvae (Dutton and Todd 1905). Dutton and Todd who described the disease as having “low mortality except under circumstances of great fatigue” both contracted the disease. Dutton became accidentally infected during an autopsy and died. *B. duttonii* was named in his honour (Figure 1).

![FIGURE 1. The British physician Joseph Everett Dutton (1877-1905). Dutton was one of the first to describe tickborne relapsing fever. He died from the disease after accidentally cutting himself during an autopsy. *Borrelia duttonii* was named in his memory.](image)

The finding of RF spirochetes in an African tick was of course followed by several investigations worldwide and several new *Borrelia* species in several tick vectors were discovered. The Lyme disease agent *B. burgdorferi* was not discovered until 1981, when it was found in the midgut of *Ixodes dammini* ticks during a search for spotted fever *Rickettsia* (Burgdorfer, Barbour et al. 1982).

It is impossible to know what was RF and what was not before Otto Obermeier’s discovery in 1868 although the “ardent fever” described by Hippocrates, the father of medicine in ancient Greece, is thought to have been RF (Felsenfeld 1971). The first clinical description of what is thought to have
been RF was presented in Dublin 1739. The term “relapsing fever” was first used in the 1843-1948 epidemic in Edinburgh (Southern and Sanford 1969).

During the Swedish-Russian 1788 war, the Swedish navy conquered the Russian 74-cannon battleship Vladimir and its 783 man crew at a battle in the Finnish bay near the island Hogland. The ship was brought to Helsinki in victory, but several of the Russian prisoners onboard were sick. The louseborne RF spread rapidly in the Swedish Fortress Sveaborg and among Helsinki civilians. People were constantly dying. Five hundred sick soldiers were shipped to Karlskrona, the main Swedish naval base which also got heavily plagued by RF (Felsenfeld 1971; Huldén 2006).

2. General characteristics

Relapsing fever is caused by spirochetes belonging to the genus Borrelia (Figure 2). The bacteria have a wave-like appearance with a length of about 10µm. The length is very variable, especially during in vitro cultivation where it differs with growth rate but also between single bacteria. The diameter is between 0.2-0.5µm and is fairly constant within a strain (Barbour and Hayes 1986). Some species can be easily recognized just by studying them in the microscope, e.g. the “chubby” B. persica (authors’ observation).

2.1 Motility

The spirochetes are very motile due to their flagellae situated in the periplasmic space between the cytoplasmic membrane and the outer membrane (Figure 2). The periplasmic flagellae are anchored in the cytoplasmic membrane on both ends of the bacterium by a hook basal body. By rotating the flagella clockwise or counter-clockwise, directional movement is achieved. The number of flagella varies between species (Charon, Greenberg et al. 1992).

The structure and function are very similar to that of other extracellular bacterial flagellae. It also functions as a sort of backbone giving the cells their wave-like shape. Mutants lacking flagellae are straight and non-motile (Sadziene, Thomas et al. 1991).
2.2 Genomic organisation

*Borrelia* spirochetes are odd in that their genome is divided among several genetic elements with all or part of their genome on linear replicons. *B. burgdorferi* has 22 replicons, one linear chromosome of about 1 Mb and several circular and linear plasmids of 10-200 kb, a size that would place the larger genetic elements as mini-chromosomes rather than plasmids. Several essential genes are situated on plasmids (Barbour 1993). The plasmids are named according to their size and whether they are linear (lp) or circular (cp). Some species such as *B. anserina*, *B. parkeri*, *B. coriacae* and *B. duttonii* contain exclusively linear DNA elements (Marconi, Samuels et al. 1993; Schwan, Raffel et al. 2005). The ends of linear plasmids are AT-rich inverted repeats, creating small hairpins. When such a plasmid is denatured it forms a single-stranded circular molecule (Barbour and Garon 1987). The origin of linear replicons in *Borrelia* is unknown. Similar hairpin telomere sequences occur in some viruses, e.g. the iridovirus causing the porcine disease African swine fever (Barbour 1993). Interestingly, this virus is transmitted by the tick *O. moubata*, which also is the vector for *B. duttonii*. Perhaps spirochetes have acquired linear DNA from an iridovirus in the tick at some time point during
evolution? The tick is probably a suitable place for genetic exchange between spirochetes but nothing is known about this.

Some plasmids in *B. burgdorferi*, e.g. the cp32-plasmids are prophages. They can be induced to enter the lytic cycle *in vitro* if cultures are treated with 1-methyl-3-nitro-3-nitrosoguanidine (MNNG) or Ciprofloxacin (Neubert, Schaller et al. 1993; Eggers and Samuels 1999; Eggers, Casjens et al. 2000). Ciprofloxacin is a gyrase inhibitor and MNNG is a DNA alkylating agent. The conditions that activate phages in nature are unknown so far. Whether plasmids of RF *Borrelia* are functional prophages is uncertain. We have treated *B. duttonii* and *B. recurrentis* cultures with MNNG, Ciprofloxacin and Mitomycin C without being able to recover phage particles (unpublished).

Lyme *Borrelia* loses virulence in about 10-15 passages of *in vitro* growth due to loss of plasmids (Schwan, Burgdorfer et al. 1988). Interestingly, despite prolonged, repeated passages (>70) RF *Borrelia* seems to always keep its pathogenicity (author’s observation). However, it has been reported that the RF agent of avian spirochaetosis, *B. anserina* loses virulence during repeated culturing in a manner similar to Lyme *Borrelia* (Levine, Dykstra et al. 1990).

### 2.3 Cell envelope and membrane proteins

The *Borrelia* cell envelope consists of an outer membrane and an inner membrane separated by a periplasmic space where the flagellae are located. The inner membrane is connected to a peptidoglycan layer. The membrane is fluid consisting of the glycolipid β-monomoglycosyldiacylglycerol (MGalDAG) and phosphatidylglycerol, phosphatidylcholine, and cholesterol (Östberg, Berg et al. 2007). Compared to other bacteria, *Borrelia* has few membrane spanning proteins, but many lipoproteins (Walker, Borenstein et al. 1991; Radolf, Bourell et al. 1994; Fraser, Casjens et al. 1997). The most abundant protein in the RF outer membrane is the variable major protein Vmp. It is a lipoprotein which can be expressed as different variants due to antigenic variation. The biology of antigenic variation will be discussed further in the host-pathogen interactions section.

Bacteria need to transport molecules over the membranes, especially *Borrelia* with its limited biosynthetic capacity. This is performed by the few membrane spanning proteins forming more or less substrate specific pores, so-called porins. Several porins such as P66, Oms28, P13 and A01 have been
identified in *B. burgdorferi* (Skare, Champion et al. 1996; Skare, Mirzabekov et al. 1997; Östberg, Pinne et al. 2002). By analysing an outer membrane fraction extracted with octyl-glucopyranoside from *B. duttonii*, a so-called B-fraction (Magnarelli, Anderson et al. 1989), in a lipid bilayer assay for pore activity, a novel pore-forming protein was discovered (Figure 3). The pore is very small, only 80pS (most porins are a few nS). A related gene encoding a 50pS pore has been found in *B. burgdorferi*. Ongoing work aims to identify substrate specificity and to clone and knock out the gene (unpublished).

**FIGURE 3. The first *B. duttonii* porin.** In a black lipid bilayer assay electrodes measure electric conductance over a lipid membrane. As channels are formed by porins in the lipid bilayer, conductance increases. Each insertion is detected as a step up in conductance. The height of each step reflects the size of the inserted pores, in this case 80pS. By adding various compounds to the KCl buffer, substrate specificity can be tested.
2.4 In vitro culture

Culturing *Borrelia* is both science and art (Barbour 1984). The medium formula was developed and thereafter improved by Barbour, Stoenner and Kelly, hence the name BSK-medium (Barbour 1984). The major components are cell culture medium (CMRL 1066), salts, buffering agents, bovine serum albumin (BSA), N-acetylglucosamine and rabbit serum. For some reason, the quality of the BSA is crucial. Different batches of BSA from the same vendor may differ dramatically. To ensure optimal culturing conditions, several BSA batches must be tested. It is not known what differs between the good and the bad batches. An experiment analysing glycolipid composition of several good, bad and intermediate BSA batches by thin layer chromatography gave no conclusive result (unpublished).

If 1/5 volume of 7% gelatin is added, the bacteria reach the same or an even slightly higher concentration as in "pure" BSK. By diluting expensive BSK with inexpensive gelatin, medium costs are decreased. Gelatin makes the media more viscous, facilitating bacterial motility and thereby avoiding aggregation of bacteria. Borreliae are microaerophilic and are best cultured in fully filled cell culture flasks with tightened caps. The optimal temperature to grow Lyme disease spirochetes is 30-37°C and the most common culture temperature is 35°C (Barbour 1984). Since *Borrelia* has a biphasic life style, alternating between ticks (cold, alkaline) and vertebrates (~37°C, pH 7.4) culture conditions have a tremendous impact on which proteins the bacteria express. A pH shift from 7 to 8 results in numerous alterations in the outer membrane protein profile of *B. burgdorferi*, e.g. decorin-binding adhesins (Carroll, Garon et al. 1999; Carroll, Cordova et al. 2000). By shifting temperature from 37° to 23° C, *B. hermsii* converts from bloodstream associated Vmp8 to the tick phase Vmp33, which is structurally similar to OspC of *B. burgdorferi*, but functionally corresponds to OspA (Schwan and Hinnebusch 1998). We can conclude that culture conditions matter. To best model human infection, culture should be performed at 37°C. *B. duttonii*, *B. recurrentis*, *B. crocidurae* and *B. hermsii* grow at room temperature and up to 39°C. It is interesting that 40°C inhibits growth even though they apparently survive even 42°C in fever patients (unpublished).
3. Strains and phylogeny

The spirochetes represent a separate phylum in the kingdom *Eubacteria*. They are divided into 13 genera of which *Borrelia* is one. Among the other genera bacteria such as *Treponema* are found with e.g. the agent of syphilis (*T. pallidum*) and the genus *Leptospira* causing leptospirosis (*L. interrogans*). Spirochetes are also found in diverse natural environments, e.g. the giant *Cristispira* which with its length of up to 180µm is almost visible to the naked eye. It lives exclusively within a certain digestive organ in some bivalve molluscs. Other genera of spirochetes are symbionts living in the guts of termites (Margulis, Nault et al. 1991).

The genus *Borrelia* is divided into two groups, both pathogenic to humans: the Lyme disease (LD) and the relapsing fever *Borrelia*. A summary of the most important species is presented in table 1. Classification, identification and phylogeny of *Borrelia* are not simple tasks. Before the dawn of modern molecular biology, systematics and identification of species were performed by criteria such as morphology or the host animals in which the isolates were infective. These methods of course resulted in the description of several “new” species, and it is often impossible today to elucidate to which species older scientific reports refer.

Gordon E. Davis, one of the leading *Borrelia* scientists in the mid-20th century, developed a model assuming that each tick species carried its own specific spirochete that could not be transmitted by other species of ticks, or in the case of *B. recurrentis*, lice (Davis 1952). By using this model, bacterial species was determined by the tick from which it was isolated. The Davis model has been confirmed with modern methods to fit quite well for American RF (called new world species) but to be less useful for African, European and Asian (old world) species. The strict tick-*Borrelia* vector specificity model has been widely used to determine species of bacterial isolates. Sometimes the presence of a certain vector in the area has been enough for species identification, even in modern days, e.g. in Davis, Vincent et al. (Davis, Vincent et al. 2002). Nature is rarely as organized as we want it to be. With modern methods, *Borrelia* phylogeny will have to be partly re-written. However, phylogeny based on DNA sequences is not an exact science either. The results are very dependent on which gene is used, what part of the
sequence is analysed and which statistical tools are applied. All of these non-biological factors have a massive impact on the outcome of phylogenetic trees. This is very important to keep in mind when validating phylogenetic data.

*Borrelia* phylogeny on the species level is usually performed by sequencing the 16S rRNA or flagellin genes (Ras, Lascola et al. 1996). To discriminate among strains within a species or closely related species, the non-coding intergenic spacer between 16S and 23S rRNA genes (IGS) (Bunikis, Tsao et al. 2004) or the glycerophosphodiester phosphodiesterase (glyP) gene is generally used. The IGS is highly variable since it is non-coding and may also be of variable length. The *glyP* gene is used since it is RF specific and therefore not cross-reactive to other bacteria such as *Treponema* or Lyme *Borrelia*.

![FIGURE 4. RF of the world.](image)

*FIGURE 4. RF of the world.* The geographic distribution of RF is local and scattered due to the ecology of vectors and hosts. Presence of RF in general, but especially in South America and Southern/ Eastern Asia, is largely unknown. Therefore a more detailed map would be too speculative. Only species causing human disease having a fairly known distribution has been included. Although restricted to Ethiopia and Sudan, *B. recurrentis* may establish foci wherever there are lice.
3.1 New world relapsing fever

The "new world" is defined as South and North America. The traditional division of RF into old world and new world species was performed before molecular tools were accessible. It assumes American strains to be alike whereas strains in the old world; Africa, Europe and Asia constitute another group. This separation works fine for the old, well-known RF strains, but as we will see, this geographic classification has its limitations, especially when it comes to species discovered more recently.

*B. hermsii* is the most common cause of RF in North America (Figure 4). The bacterium is transmitted by the tick *O. hermsi*, and is present in coniferous forests of western USA and southwestern Canada. Humans cases are usually associated with sleeping outdoors, in caves, or huts where rodents live (Schwan, Policastro et al. 2003). The species is divided into two phylogenetic sub-groups. Most work on antigenic variation has been performed on *B. hermsii* (Barbour, Dai et al. 2006; Stoenner, Dodd et al. 1982).

*B. turicatae* has been isolated in Texas, Kansas and Florida from the tick vector *O. turicata* and from dogs, but the numbers of findings are too scarce to predict a geographic distribution (Schwan, Raffel et al. 2005). No human cases of *B. turicatae* have been verified although it is likely that the species is pathogenic to man, e.g. in Davis, Vincent et al. 2002. *B. turicatae* is infectious in mice and used to study neuroborreliosis (Cadavid, Pennington et al. 1997).

*B. parkeri* is closely related to *B. turicatae* but its pathogenicity to man is even more uncertain. The species has only been isolated in California from its tick vector *O. parkeri*. One case of equine abortion caused by a spirochete similar to *B. parkeri/B. turicatae* points to medical, or at least veterinary, importance (Walker, Read et al. 2002). A rare feature of *B. parkeri* is the absence of circular plasmids (Schwan, Raffel et al. 2005).

*B. coriaceae* has been isolated a few times from the tick *O. coriaceus* in California. Although not definitively proven, it has been suggested to be a cause of bovine abortion (Lane, Burgdorfer et al. 1985). Also *B. coriaceae* contains only linear plasmids (LeFebvre and Perng 1989).
**B. miyamotoi** was first isolated in Japan from the hard tick *Ixodes persulcatus* (Fukunaga, Takahashi et al. 1995). Later, similar strains were also found in the USA (Scoles, Papero et al. 2001) and Sweden (Fraenkel, Garpmo et al. 2002). *B. miyamotoi* has been found also in *I. ricinus* and *I. scapularis*, ticks that frequently feed on humans, although ability of *B. miyamotoi* to infect humans has never been demonstrated.

**B. lonestari** was discovered in the Ixodid Lone star tick, *Amblyomma americanum*, collected in the USA. The bacterium is infectious to white-tailed deer but not to mice, dogs or cows (Moyer, Varela et al. 2006). It is thought to cause the Lyme disease-like southern tick-associated rash in man (Moore, Varela et al. 2003; Varela, Luttrell et al. 2004).

**B. theileri** causes the disease tick spirochaetosis in cattle, sheep and horses. The disease occurs in Africa and is presented as mild fever, loss of appetite, weakness and anaemia (Felsenfeld 1971). Very few studies have been performed on *B. theileri* despite the fact that it was discovered back in 1902 (Felsenfeld 1971). Recently it has been shown to be most closely related to *B. lonestari* (Rich, Armstrong et al. 2001).

**B. anserina** is an odd member of the RF Borrelia group since it is transmitted not by a single *Ornithodoros* species, but by several *Argas* species. Further, the hosts are various birds and *B. anserina* causes avian spirochaetosis, a diarrhoeal disease with little in common with ordinary RF. *B. anserina* occurs worldwide (Felsenfeld 1971). Also this species lacks circular plasmids (Schwan, Raffel et al. 2005).

Other new world *Borrelia* species are suggested to be present in South American ticks such as *O. rudis* (B. venezolensis) and *O. talaje* (B. dugesii, *B. mazzotti*) although these have not been studied and characterized recently (Felsenfeld 1971). In 2004 a novel species was isolated from *O.m. porcinus* ticks in Tanzania. This strain was distantly related to the new world RF, even more distant than odd strains such as *B. lonestari* and *B. miyamotoi* when comparing *flaB* sequences, but monophyletic to *B. anserina* when looking at IGS (Kisinza, McCall et al. 2003; Mitani, Talbert et al. 2004). Since the two phylogenetic
analyses differed so greatly, it is impossible to know the true identity of the isolate. From this two things can be concluded: firstly, never trust single sequence phylogeny 100%, secondly, the division into new world and old world RF is obsolete and will have to be revised. Further, it is obvious that both terms “new world” and “relapsing fever” have their limitations since not all species cause relapsing fever and not all of them are restricted to or are even present in the new world. The separation into old world and new world works fine for those *Borrelia* species causing human RF but is more problematic when avian and cattle borreliosis are included, grouping with new world RF. Although e.g. *B. hermsii* and the old world species *B. crocidurae* cause very similar infections and have similar ecology, *B. hermsii* is closer related to odd species such as *B. anserina* and *B. theileri*, not infectious in man.

### 3.2 Old world relapsing fever

*B. recurrentis* was the first RF species described and has a unique position among borreliae since it is transmitted by lice and not ticks. *B. recurrentis* infections used to occur worldwide in massive epidemics with the latest during the two World Wars. Today the only endemic area is the highlands of Ethiopia with sporadic cases in Sudan (Figure 4) where it is associated with natural disasters, famine and refugee camps (Bryceson, Parry et al. 1970; Felsenfeld 1971). However, *B. recurrentis* can be established wherever there are lice and has therefore high potential to cause epidemics worldwide (Cutler 2006). RF is often divided into tickborne versus louseborne species, thereby grouping all RF except *B. recurrentis* together (Southern and Sanford 1969; Cook and Zumla 2002). Therefore, it is interesting that *B. recurrentis* is closely related to *B. duttonii* as shown in paper VI and Figure 5. Other researchers have suggested that *B. recurrentis* and *B. duttonii* are actually the same species based on phylogenetic studies (Cutler, Scott et al. 2005). Early experiments have shown that *B. recurrentis* can be transmitted by *O. moubata* and that *B. duttonii* can be adapted to lice. Some go even further, proposing an ecological model in which the *B. duttonii*/*recurrentis* spirochete uses ticks as a reservoir and lice for epidemic spread (Heisch and Garnham 1948; Felsenfeld 1971).
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*B. duttonii* is present in sub-Saharan Africa, with most cases occurring in the eastern part of the area, and is transmitted by the tick *O. moubata moubata*. The tick lives in the walls and floor of huts where people live and feeds on the inhabitants at night. It is regarded as anthropophilic, preferring humans rather than animals as host (Sonenshine 1997). As mentioned above, this species is closely related if not identical to *B. recurrentis* (Figure 5).

**FIGURE 5.** Neighbor-joining phylogenetic trees of old world RF *Borrelia* based on IGS and *glpQ*. Note that Tanzanian *B. duttonii* (La, Ku, Wi) group together with *B. recurrentis* in both trees, excluding *B. duttonii* from the Congo (8151, 1120K3, 1079). Also note the placement of *B. crocidurae* CR2 compared to the other two *B. crocidurae* strains.

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Although *B. recurrentis* and *B. duttonii* have only been found in their vectors and man, several animals have been suggested to be reservoirs. *B. duttonii* is probably the species causing most of the *Borrelia* cases in the world.

**B. crocidurae** is transmitted by *O. erraticus* sonrai. The reservoir is several types of small mammals in the Sahel region and West and North Africa. Earlier it was considered a “subgroup” of *Borrelia* species such as *B. merionesi*, *B. dipodilli*, *B. microti*, etc. Today this division into species is questionable. *B. crocidurae* is probably a quite heterogeneous species and, as our data indicates, perhaps not even monophyletic (Paper VI) and (Figure 5). *B. crocidurae* causes less severe disease than do *B. duttonii* and *B. recurrentis*. In an impressive epidemiological study performed in Senegal, Mauritania and Mali, *B. crocidurae* infection occurred at an incidence of 11 per 100 person-years were found. This is the highest incidence described for any bacterial disease in Africa (Vial, Diatta et al. 2006).

**B. hispanica** is found in the Mediterranean region and is transmitted by *O. erraticus erraticus*. It is outside the monophyletic group of *B. duttonii*, *B. recurrentis* and *B. crocidurae* but related to them (Figure 5). The disease caused by *B. hispanica* is reported to be one of the less severe RF’s (Cadavid and Barbour 1998).

**B. persica** is found in Asia, but the distribution is uncertain. Most cases are reported in Israel. It is transmitted by *O. tholozani*. *B. persica* seems to be quite distant phylogenetically to the other old world RF species since *B. duttonii*, *B. recurrentis*, *B. crocidurae* and *B. hispanica* form a monophyletic group excluding *B. persica* (Figure 5), (Ras, Lascola et al. 1996).

**Novel species** have been found in several ticks despite the supposed rigid tick-vector relations according to Davis model (Davis 1952). In Spain a non-culturable RF spirochete related to but outside the monophyletic group of *B. duttonii*, *B. crocidurae* and *B. hispanica* was isolated from both RF patients and the *B. hispanica* vector *O. erraticus erraticus* (Anda, Sánchez-Yebra et al. 1996). In Turkey, a fast-growing spirochete named *B. turcica*, was isolated from *Hyalomma aegyptium* ticks infesting tortoises. This species groups together with
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RF but outside the monophyletic group consisting of all other known species (Guner, Watanabe et al. 2004). The status of species sometimes described in the literature such as *B. caucasia*, *B. latyschevii* in Asia and the “*B. crocidurae* complex” described above, *B. graingeri* and *B. tillae* in Africa is questionable.

**TABLE 1.** Some of the characterised RF *Borrelia* strains, their vectors, host range and clinical presentation.

<table>
<thead>
<tr>
<th>Species</th>
<th>Vector</th>
<th>Geographical distribution</th>
<th>Host range</th>
<th>Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>New World</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. hermsii</em></td>
<td><em>O. hermsi</em></td>
<td>North America</td>
<td>Mammals,</td>
<td>RF</td>
</tr>
<tr>
<td><em>B. turicatae</em></td>
<td><em>O. turicata</em></td>
<td>North America</td>
<td>Mammals</td>
<td>RF</td>
</tr>
<tr>
<td><em>B. parkeri</em></td>
<td><em>O. parkeri</em></td>
<td>North America</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><em>B. coriacae</em></td>
<td><em>O. coriacous</em></td>
<td>North America</td>
<td>Bovine</td>
<td>Bovine abortion?</td>
</tr>
<tr>
<td><em>B. miyamotoi</em></td>
<td><em>Ixodes spp.</em></td>
<td>Europe, Asia, North America</td>
<td>Mouse,</td>
<td>?</td>
</tr>
<tr>
<td><em>B. lonestari</em></td>
<td><em>Amblyomma americanum</em></td>
<td>USA</td>
<td>Deer, ?</td>
<td>STARI: Southern</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tick Associated</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Rash Illness</td>
</tr>
<tr>
<td><em>B. theileri</em></td>
<td><em>N. theileri</em></td>
<td>Africa, Ruminants, Horses</td>
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<td>Tick spirochaetosis</td>
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<td><em>B. anserina</em></td>
<td><em>Argas spp.</em></td>
<td>Worldwide</td>
<td>Birds</td>
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</tr>
<tr>
<td>Old World</td>
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<tr>
<td><em>B. recurrentis</em></td>
<td><em>P. humanus</em></td>
<td>Ethiopia, Sudan, Potentially worldwide</td>
<td>Man</td>
<td>RF</td>
</tr>
<tr>
<td><em>B. duttoni</em></td>
<td><em>O. moubata moubata</em></td>
<td>Sub-Saharan Africa</td>
<td>Man</td>
<td>RF</td>
</tr>
<tr>
<td><em>B. crocidurae</em></td>
<td><em>O. erraticus erraticus</em></td>
<td>West and North Africa</td>
<td>Mammals</td>
<td>RF</td>
</tr>
<tr>
<td><em>B. hispanica</em></td>
<td><em>O. erraticus erraticus</em></td>
<td>Mediterranean region</td>
<td>Mammals</td>
<td>RF</td>
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<tr>
<td><em>B. persica</em></td>
<td><em>O. thelezi</em></td>
<td>Asia</td>
<td>Mammals</td>
<td>RF</td>
</tr>
</tbody>
</table>

**4. Vectors**

All *Borrelia* have a biphasic lifecycle, alternating between vertebrate hosts and their tick or louse vector. Every now and then other vectors are reported, usually for Lyme borreliosis, such as mites, mosquitoes, blackflies and other blood-sucking insects. Of the reports on “new vectors” I have seen, none was worth the paper it was printed on or worth referring here. Usually *Borrelia* DNA is detected by a questionable PCR implicating importance as vector. However, to have vector competence the infectious agent must be 1: taken
up, 2: survive in the vector, and 3: passed over to a new host. If any of these steps fail, the arthropod can not function as vector. The three prerequisites are reminiscent of the Koch’s postulate principles. Serious attempts to break old, scientific dogmata is important and impressive, but must be supported by extraordinarily convincing data.

Due to the low infectious dose (one, single bacterium) the risk of laboratory infection is great when working with syringes, cover glasses or when sectioning. In the case of accidents it is wise to always undergo prophylactic treatment. Never underestimate the risk. There are cases where laboratory personnel have become infected by clotted blood kept for six days at room temperature (Felsenfeld 1971). Despite five such accidents, mostly during animal injections but also including pouring a late log-phase *B. recurrentis* culture in my eye the first week in the lab, I have never had RF. Thanks to doxycycline.

4.1. Ticks

The suborder *Ixodida* consists of three families, *Ixodidae* (hard ticks) to which the Lyme disease transmitting species belong, *Argasidae* (soft ticks) and the single-species intermediate family *Nuttalliella*. *Argasidae* comprises 5 genera of which the RF transmitting *Ornithodoros* is the largest with its about 100 species (Figure 6). These ticks normally inhabit sheltered environments such as rodent burrows, bird nests, caves or man-made shelters in close proximity to their hosts. In contrast to *Ixodes* ticks, the Lyme borreliosis vector, which attach to their host for days, *Ornithodoros* ticks feed very quickly, completing their bloodmeal in about 30 minutes (Table 2). The bite is painless and usually occurs at night. Thus, RF patients may often be unaware that they have been bitten by a tick (Sonenshine 1997).

When ingested into the tick gut, RF spirochetes rapidly invade several organs such as the coelomic cavity, the central ganglion, salivary glands, and the urine secreting coxal gland. They are instantly transmitted to the new host by the saliva and/or by coxal fluid contaminating the wound from the bite. In contrast, Lyme disease *Borrelia* inhabits the midgut of *Ixodes* ticks and becomes activated to penetrate the midgut wall and enter the salivary glands when the tick takes its bloodmeal. This process takes about 48 hours, consequently no bacteria are transmitted in the first two days (Ribeiro, Mather et al. 1987).
Some RF spirochetes also invade gonads. They penetrate the follicular layer of the eggs to become intracellular and reside there throughout embryogenesis, resulting in transovarial transmission of spirochetes. The borreliae multiply and invade coxal fluid and salivary glands making the larvae infectious from its first bloodmeal. The process can continue for several transovarial passages without vertebrate hosts (Felsenfeld 1971). Transovarial transmission is very rare for Lyme disease spirochetes and if *B. burgdorferi* enter tick eggs they usually disrupt the embryogenesis (Sonenshine 1997).

**Ornithodoros moubata**

*O. moubata* (Figure 6) is a complex of several species. Walton hypothesized that *O. moubata moubata* was domesticated from *O. moubata porcinus* in the Neolithic age when man started to live in semi-permanent huts in the Kenya highlands and brought ticks home on warthog prey (Walton 1962). The “true”
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O. moubata moubata prefer humans as a host even if animals are accessible nearby (Walton 1957). This is probably the reason why B. duttonii never have been found in any animals despite being infectious to e.g. rodents in the lab. The optimal climate condition for O. moubata is a temperature of 22°C and a relative humidity of 84%. To obtain this microclimate in a mud hut a constantly burning fire in the centre is required. Related species like O. m. porcinus have different host range and are attracted to light, gathering at the entrance of burrows, in contrast to O. m. moubata which is negatively phototactic. O. m. moubata has been found in natural habitats, but Walton claimed these were morphologically different and also had a different behaviour than the domestic form (Walton 1962). The B. crocidurae vector O. erraticus sonrai could, despite being morphologically and behaviourally identical to one another, be divided into four sub-groups based on the DNA sequences corresponding to 16S and 18S rRNA.

| TABLE 2. Comparison of Argasid and Ixodid biology (Sonenshine, 1997). |
|--------------------|------------------------------------------------------------------|
| Feature            | Argasidae                                                        | Ixodidae                                                        |
| Habitat            | Sheltered. Burrows, nests, caves, cracks and crevices, man-made shelters | Open. Grass and bushes                                          |
| Host seeking       | Lives in close proximity of the host                             | Waiting for a host to pass by                                   |
| Host range         | Narrow                                                          | Wide                                                            |
| Life span          | Long                                                            | Short                                                           |
| Size of blood meal | 2-6x body weight                                                 | 100x body weight                                                 |
| Number of blood meals | Many                                                            | Few                                                            |
| Feeding time       | 30 minutes                                                      | Several days                                                    |
| Mode of feeding    | Secretion of excess water by coxal fluid                       | Regurgitation                                                   |
| Nymphal instars    | Many (2-8)                                                      | One                                                            |
| Time required for Borrelia transmission | Instant                                                        | >48 hours                                                      |
| Number of eggs     | Few (200-300)                                                    | Many, up to 23,000                                              |
| Female can lay eggs several times |                                                   | Female dies after oviposition                                 |
| Transovarial transmission of Borrelia | Yes, in many species                                             | No (extremely rare)                                            |
All sub-groups harboured *B. crocidurae* but to significantly differing extents, suggesting differences in vector competence of the four groups (Vial, Durand et al. 2006). It is possible that ticks of the *O. moubata* complex may also differ in vector competence which would explain some oddities in *B. duttonii* and *O. moubata* ecology.

4.2. Lice
The human body louse *Pediculus humanus humanus* (Figure 6) is the only non-tick *Borrelia* vector. It transmits *B. recurrentis*, sometimes causing massive epidemics. The louse is strictly human specific and lives on the body and in clothing. A population of lice can increase by 11% per day, giving a hint to how rapidly an outbreak may spread in, for instance, a refugee camp (Bryceson, Parry et al. 1970; Raoult and Roux 1999). Compared to the elaborate relationship between *Ornithodoros* and tickborne RF, it is a mystery how the awkward louseborne RF could cause global epidemics. The louse only feeds on human blood and it needs to feed everyday, otherwise it dies (Raoult and Roux 1999). A bloodmeal is about 1µl, consequently the spirochtemia of the patient must be relatively high for the louse to become infected. In the louse, *B. recurrentis* crosses the gut epithelium to invade the coelomic cavity where it multiplies. *Borrelia* does not invade ovaries or salivary glands of the louse and is therefore not transovarially transmitted or transmitted by the louse bite itself, nor is it transmitted through faeces. Instead, the route of transmission is through infected lice being crushed and the coelomic fluid containing bacteria entering the bite wound by scratching (Southern and Sanford 1969; Felsenfeld 1971). Hence, the louse can only transmit disease once, and the lifespan of a louse, about 3 weeks, is anyway too short to constitute an important reservoir of bacteria over time. To maintain a *B. recurrentis* population it takes lice and a constant availability of RF patients with high spirochetemia. Lice can acquire and transmit several other RF species, although with variable success. The species other than *B. duttonii* which seems to adapt best to lice is *B. duttonii* (Heisch and Garnham 1948; Felsenfeld 1971).
5. Epidemiology

The epidemiology of RF is still quite poorly understood. For a vector-borne disease to be successful it needs competent vectors, sensitive hosts and a reservoir. This might seem trivial but when taking factors such as vector and host competence, persistence, seasonal dynamics, herd immunity, reservoir hosts, their ecology, etc. into account, the issue gets complicated. Complicated and extremely interesting.

5.1 Endemic relapsing fever

The majority of the approximately 20 known RF *Borrelia* species are endemic in wild animal populations, such as small mammals, which serve as reservoirs for the disease. Human infection is frequent in endemic areas where people live in close proximity to burrows of host animals. Vial and co-workers found a relapsing fever incidence of 11 per 100 person-years in West Africa (Vial, Diatta et al. 2006). This is the highest incidence ever described for any bacterial disease in Africa. Travellers who have spent time in such areas are also at risk (Rebaudet and Parola 2006). Otherwise the disease is sporadic, occurring after visiting an infested cave, abandoned hut or sleeping outdoors in a tick-infested area (Schwan, Policastro et al. 2003). An exception is *B. duttonii* which has never been detected in any wild animals, only in humans and its tick vector *Ornithodoros moubata*. Nevertheless, the species is infectious in several laboratory animals such as mice. Hence, the bacteria are not human specific (Larsson, Andersson et al. 2006). It is probably the anthropophilic behaviour of *O. moubata*, living in human settlements and strongly preferring humans as a host, that has resulted in the apparent human specificity (Sonenshine 1997). *B. duttonii* is endemic in sub-Saharan Africa and is a common cause of disease in many areas. This is probably the species causing the majority of RF cases in the world.

5.2 Epidemic relapsing fever

Instead of ticks, *B. recurrentis* uses the human body louse, *Pediculus humanus humanus*, as its vector. Today, *B. recurrentis* infection occurs as sporadic epidemics in Sudan and Ethiopia, usually in connection with refugee camps,
natural disasters and famine, but the disease used to be common worldwide with the latest massive epidemics during the two World Wars (Felsenfeld 1971). However, it may re-establish anywhere in louse-infested human populations (Raoult and Roux 1999; Cutler 2006). Louseborne RF is generally described as more severe than tickborne RF. Perhaps the more severe illness caused by \textit{B. recurrentis} compared to \textit{B. duttonii} can be explained by its appearance in a population which has not encountered the disease before and is thereby lacking the partial immunity of a population living in an endemic area. Moreover, the adverse conditions in which it occurs, famine, war and disasters, may be another explanation (Southern and Sanford 1969). The observation that \textit{B. duttonii} infection is more severe in visitors than in local inhabitants of an endemic area supports this theory (Felsenfeld 1971; Goubau 1984).

6. Human infection

One of the major difficulties in RF diagnostics is the variable disease presentation. The outcome may range in severity from asymptomatic to fatal and is generally most severe in young children (Southern and Sanford 1969). Patients are often unaware that they have been bitten by a tick. The infectious dose is low: a single bacterium is sufficient to establish infection in mice (author’s observation).

6.1 Acute phase

Usually, the incubation time between tick bite and the first symptoms is about 7 days (Southern and Sanford 1969) with sudden onset of fever, typically between 38.7°-40° (-41°) C, and chills (Figure 7). The first fever period lasts on average 4-7 days (Bryceson, Parry et al. 1970; Felsenfeld 1971). Common complaints are malaise and general ache, often myalgia and headache. Nausea occurs frequently, sometimes with vomiting, sometimes with diarrhoea. Skin rashes, sometimes petechial or even hemorrhagic are common. Several old world RF species form aggregates of erythrocytes and bacteria, disrupting the microcirculation by causing microthrombosis in arterioles anywhere in the body (Figure 10) (Shamaei-Tousi, Martin et al. 1999). This phenomenon will be more thoroughly described in section 10.4. Frequently, more or less severe
bleeding is observed. Hemorrhagic phenomena such as nose bleed, haemoptysis, bloody diarrhoea, haematuria, sub-arachnoid, cerebral and retinal haemorrhages are also observed. Hepatomegaly and liver tenderness are common and often accompanied by jaundice. In some cases the spleen might be enlarged with microabscesses and even ruptured. Respiratory symptoms with cough as well as myocarditis are common.

Various neurological symptoms may occur during RF, but again, symptoms are variable. *B. duttonii* and *B. turicatae* have been reported to be the species most notoriously causing neurological symptoms of which meningitis and facial palsy are the most common. In several fatal RF cases with neurological involvement, the most common sequelae were oedema and subarachnoid and parenchymal brain haemorrhages (Judge, Samuel et al. 1974; Salih, Mustafa et al. 1977; Ahmed, Abdel et al. 1980). In tickborne RF patients, a meningeal and brain parenchymal perivascular mononuclear infiltrate with monocytes and lymphocytes is common. Also ocular complications may occur (Cadavid and Barbour 1998). Haematological changes are variable, depending on the infecting strain and changing with the phases of the disease. Thrombocytopenia is often striking, as well as low haemoglobin and erythrocyte count.

**FIGURE 7. Typical RF spirochetemia and fever pattern.** The first spirochetemia is highest, followed by relapses of lower magnitude with longer and longer time spans between them. The image is generated from *B. duttonii* infection in a mouse (black line). Typical human fever pattern (grey) is added for clarity.
A progressive decline of the general condition, sometimes with extreme weakness and weight loss, can occur if successive relapses arise without sufficient treatment.

### 6.2 Jarisch-Herxheimer reaction

The Jarisch-Herxheimer reaction (JHR) may occur after treatment of spirochaetal diseases. The exact mechanism is not known, but it is thought to be an endotoxic shock caused by sudden lipoprotein release from lysed bacteria. Generally, the clinical and pathophysiological signs of this reaction are similar to those of a classic endotoxin reaction. Typical symptoms are increased fever, rigors, headache, malaise, hypotension, sweating and increased respiration resulting in alkalosis, with an onset about one hour after treatment. The crisis lasts for 1-2 hours. JHR is an “all or nothing” reaction and occurs in 54% of louseborne, and 82% of tickborne RF patients. It usually resolves by itself within 12-24 hours, but a cardiovascular collapse with a fatality rate of about 5% may also occur (Bryceson, Parry et al. 1970; Bryceson, Cooper et al. 1972). Several studies have suggested that the choice of antibiotic affects the JHR severity, but results have been inconclusive. Most promising is anti-TNF-α treatment prior to antibiotics which has been shown to reduce the severity of JHR (Pound and May 2005).

### 6.3 Residual infection

It is beneficial for *Borrelia* to persist in the host as long as possible, in order to enhance the possibility of transmission by a vector from one host to another. After entering the blood system the bacteria can be transported within a few minutes to a vascular bed in any part of the body. In small vessels such as capillaries and sinusoids, where blood flow is slow, there is a chance for the spirochetes to be arrested, extravasate and invade neighbouring tissues. Although suspected but not proven in humans, animal experiments have shown RF spirochetes to be able to persist silently in the brain for an extended time. During this period the bacteria usually are undetectable in the blood, making diagnostics difficult or even impossible (Cadavid and Barbour 1998; Larsson, Andersson et al. 2006). Cutler has observed low numbers of *B. duttonii* in blood from apparently healthy villagers in Tanzania, further
indicating the importance of silent, residual or asymptomatic infection (Cutler 2006). The possibility of residual bacteria behind the blood-brain barrier should be taken into account when planning the treatment regime. It cannot be excluded that other organs/tissues also harbour persistent, residual *Borrelia* although neither testis (Larsson, Andersson et al. 2006), nor eye (unpublished) harboured late residual infection in our experiments.

### 6.4 Relapsing fever in pregnancy

About one fourth of neonatal deaths in the developing world are caused by infectious disease (WHO/UNICEF/UNU. 2001). Pregnancy complications such as low birth weight, preterm delivery, spontaneous abortion and perinatal death are commonly caused by *B. duttonii* in sub-Saharan Africa (Goubau 1983; Brasseur 1985; Melkert and Stel 1991; Dupont 1997; Jongen 1997; van Holten, Tiems et al. 1997). In the Congo, 6.4% of pregnant women seeking healthcare at a maternity ward were diagnosed with RF (Dupont 1997).

Many observers claim/suspect intrauterine infection, but only in rare cases can infection at or after birth be excluded (Fuchs and Oyama 1969). Animal experiments have shown that the bacteria do infect the foetus *in utero*. In mice infected with *B. duttonii*, 73% of foetuses were infected before birth (Larsson, Andersson et al. 2006). Intrauterine growth retardation probably caused by placental damage and inflammation, impaired foetal circulation and decreased maternal haemoglobin were found to be the cause of pregnancy complications besides the transplacental transmission of bacteria (Larsson, Andersson et al. 2006). Some claim that RF infection is more severe in pregnant women, as is common in many other diseases (Goubau and Munyangeyo 1983; Melkert 1988). However, no convincing data has been published which show this to be the case. This statement is frequently encountered in reviews, usually referring to other reviews. In contrast, Fuchs and Oyama report a case of congenital RF where the mother had a low grade fever, thought to be an uncomplicated upper respiratory tract infection, three weeks before labour. The child died from RF 1½ days after birth (Fuchs and Oyama 1969). In mouse experiments, pregnant mice had lower levels of bacteria in the blood and less severe symptoms than non-pregnant (Larsson, Andersson et al. 2006). If this is also the case in human infection, diagnosis may be more difficult in pregnant women than in other patients.
7. Diagnostics

Since RF has a very variable presentation diagnosis is often hard to perform based on symptoms alone. Moreover, in developing countries mixed infections, sometimes with several causative agents, is common (Nordstrand, Bunikis et al. 2007). This must be kept in mind when examining patients. Due to its sudden onset, and since the first fever attack is the most dangerous, lengthy diagnostic procedures such as *in vitro* culturing and animal inoculation are of only academic interest.

7.1 Laboratory diagnosis

Serology is of little use since it takes at least one week to mount an antibody response, and the disease has progressed significantly by then. Further, the majority of the antibodies are directed towards the variable Vmp protein. A third disadvantage of serology in endemic areas is remaining antibodies from earlier cleared infections, resulting in false positive results. Moreover, several RF proteins are cross-reactive to *Treponema* and Lyme *Borrelia*. The RF-specific protein GlpQ has been used in epidemiologic studies (Schwan, Schrumpf et al. 1996; Porcella, Raffel et al. 2000; Nordstrand, Bunikis et al. 2007) and would be the protein of choice if serological methods were to be used.

The standard method for detection of *Borrelia* spirochetes is Giemsa-staining of thin blood smears. However, the protocols used for malaria diagnostics in which water is used to lyse erythrocytes, usually also lyse spirochetes and are therefore useless for RF diagnostics. Phase contrast or dark field microscopy directly on 10-fold diluted blood is useful, although sensitivity is not great. Due to the spirochetes’ thin and transparent morphology, ordinary light microscopy is of little use.

During the major fever peaks spirochetes are numerous and easily detected but between peaks the bacteria are scarce (Figure 7). Acridine orange–coated quantitative buffy coat tubes, centrifugation, and fluorescence microscopy have been successfully used (van Dam, van Gool et al. 1999; Cobey, Goldbarg et al. 2001). However, this technique requires expensive equipment generally not available in RF endemic areas.
I have developed a centrifugation-based enrichment method to provide inexpensive and simple, but powerful RF diagnostics (Figure 8). About 10 ml of blood is drawn into a tube with anticoagulant (EDTA, heparin, sodium citrate etc.) and transferred to a 15 ml plastic centrifuge tube. The sample is then centrifuged 5 minutes at 500 x g to pellet erythrocytes but not bacteria, and the plasma phase is carefully transferred into a new centrifuge tube avoiding erythrocyte contamination. Thereafter the plasma is centrifuged 10 minutes at 5000 x g or faster to pellet spirochetes. If the centrifuge cannot rotate as fast as 5000 x g the time can be extended to maximize recovery. After centrifugation the plasma is aspirated, leaving only the nearly invisible pellet and a few microliters of plasma at the bottom of the tube which is resuspended and smeared onto a clean microscope glass to air-dry. The sample is then fixed by heating a few times over a flame followed by a 30 second dip in methanol. Staining is performed by standard Giemsa staining. May-Grünwald- and Wright Giemsa stain bacteria well. Gram staining does not stain Borrelia. Bacteria are then detected by microscopy at 1000x magnification. The method has been established by using blood spiked with cultured RF spirochetes. From 10 ml blood spiked with 100 B. duttonii (10/ml) about 10% were recovered on the glass, demonstrating the sensitivity of the
method. Note that stained bacteria sometimes may lose their evenly wave-like appearance.

PCR is a useful diagnostic method since it is rapid and sensitive although it may not be accessible or may be too expensive to use in many clinical laboratories. RF specific primers directed to DNA sequence corresponding to 16S rRNA have been designed by Ras et. al (5'-ATGCTAGAAAC TGCATGA-3' and 5'-TCGTCTGAGTCCCCCATCT-3') in a protocol consisting of denaturation at 93°C for 1 min, annealing at 46°C and extension at 72°C 1 min for 35 cycles and finally an extension step consisting of 7 min at 72°C, resulting in a 523-bp product (Ras, Lascola et al. 1996). Halperin et. al used the glpQ primers 5'-CAGAACATACCTTAGAAGCTCA AGC-3' and 5'-GTGATTTGATTTCTGCTAATGTG-3' in a PCR protocol using five initiation cycles (40 s at 95°C, 50 s at 45°C, 1 min at 72°C), and thereafter 25 cycles of 40 s at 95°C, 50 s at 51°C, 1 min at 72°C followed by a 5 min final extension at 72°C resulting in a 212-bp amplicon (Halperin, Orr et al. 2006).

Nested PCR can be used to increase sensitivity. The enrichment method described earlier can also be used to concentrate samples prior to DNA extraction for PCR.

7.2 Differential diagnosis

RF is often misdiagnosed as malaria, especially in malaria endemic areas (Nordstrand, Bunikis et al. 2007). Other possible differential diagnoses include typhoid fever, yellow fever, leptospirosis, viral hepatitis, meningococcal meningitis, African tick-bite fever, typhus, trench fever or virtually any disease causing fever. The relapsing pattern may be similar to malaria (mainly the relapsing species Plasmodium vivax and P. ovale) and typhoid fever (Cook and Zumla 2002). Giemsa stained specimens might be confused with Onchocerca volvulus or trypanosomes. Thus, the multitude of illnesses with similar presentations and the possibility of mixed infections can complicate diagnosis.

8. Prevention

Louseborne RF is best prevented by good hygiene, thereby avoiding louse infestation. During epidemics isolation and delousing of RF patients and their personal belongings is important (Raoult and Roux 1999). Ornithodoros ticks
are always close to their hosts, and tickborne RF is best prevented by avoiding sleeping in close vicinity to e.g. rodents’ burrows. In the long run decimation of the rodent population might be effective, but in the shorter perspective it can lead to ticks instead seeking humans for feeding. Tick bites can be avoided by using insect repellent containing N,N-diethyl-m-toluamide and permethrine-treated mosquito bed nets, also effective against malaria. A bed elevated above the ground is preferable to sleeping on the floor to restrict contact with questing ticks. Human settlements can be sprayed with insecticides, e.g. permethrine, to eradicate tick infestation. Ticks may survive or repopulate the hut from external sources. Therefore, treatment must be repeated e.g. once a year (Sonenshine 1997). Best, yet not affordable to everyone, is housing made of concrete instead of clay or straw, providing an uninhabitable environment for ticks.

9. Treatment

RF *Borrelia* is so sensitive to antibiotics that a single dose often is recommended (Perine, Krause et al. 1974; Cook and Zumla 2002). This is often sufficient and may be justified during epidemics when drug availability is low. However, these recommendations were written when RF was considered a disease solely of the blood. Today more and more evidence points to silent and residual infections of sites difficult to reach by antibiotics, such as the brain (Cadavid and Barbour 1998; Larsson, Andersson et al. 2006). Antibiotic treatment for a few days may eliminate circulating *Borrelia*. However, the brain and possibly other sites might function as reservoirs from which the bacteria may re-enter the blood after insufficient treatment. Several observations show that RF might relapse after one or two days’ treatment. This persistence has been reported to be more common in tickborne than louseborne RF (Cadavid and Barbour 1998). Since RF shows close resemblance to Lyme disease in its ability to cause persistent infection of sites which might be hard to reach by antibiotics, we suggest therapy similar to that given to Lyme disease patients. For Lyme disease treatment, oral amoxicillin or doxycycline is usually sufficient if facial nerve palsy is the only neurological sign. In more severe cases, intravenous injections with ceftriaxone or cefotaxime is recommended, or as a third alternative, penicillin G (Table 3), (Wormser, Nadelman et al. 2006).
INTRODUCTION

Oral administration should be avoided in patients with nausea. Louseborne RF epidemics often occur as mixed infections with epidemic typhus (*Rickettsia prowazekii*) and trench fever (*Bartonella quintana*) (Perine, Wolde-Gabriel et al. 1975). When proper diagnosis is difficult RF may also be mistaken for malaria. Under such circumstances, doxycycline is a suitable antibiotic since it is effective both against *Rickettsia, Bartonella* and *Plasmodium*.

Table 3. Recommended treatment (Wormser, Nadelman et al. 2000)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Adult dose</th>
<th>Pediatric dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxycycline</td>
<td>100 mg PO bid x 14-21days ≥8 yrs 1-2 mg/kg PO</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>500 mg PO tid x 14-21days 25-50 mg/kg/day PO divided bid</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2 g IV once x 14-28 days 75-100 mg/kg once/day IV</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>2 g IV/day q8h x 14-28 days 150-200 mg/kg/day IV in 3-4 doses</td>
<td></td>
</tr>
<tr>
<td>Penicillin G</td>
<td>18-24 million units IV/day q4h 200,000-400,000 units/kg/day IV q4h</td>
<td></td>
</tr>
</tbody>
</table>

1 Not for children younger than 8 years or pregnant or breastfeeding women. Causes photosensitivity.
2 Also efficient against malaria and louse-borne typhus (Perine, Krause et al. 1974).
3 For more serious neurological symptoms.
4 Possible alternative to ceftriaxone or cefotaxime for patients with neurological symptoms. Dose should be reduced for patients with impaired renal function.
10. Host-pathogen interactions
Within the tick, RF spirochetes reside in virtually all organs, where they express a certain tick-specific Vmp, in B. hermsii called Vmp33. Interestingly, the gene is structurally related to Lyme disease (LD) *ospC* but functionally related to *ospA*! It has its own promoter in contrast to the *vmp* genes expressed in the host undergoing antigenic variation (Carter, Bergström et al. 1994; Schwan and Hinnebusch 1998). During feeding, spirochetes are transmitted by the tick coxal fluid and/or saliva (Sonenshine 1997). In the mammalian host *vmp33* is silenced in favour of the *vmp* at the expression site for *vmp*’s undergoing antigenic variation (Schwan and Hinnebusch 1998).

The tick saliva is a cocktail of several bioactive substances helping the tick obtain its blood meal. The saliva contains vasodilators, anti-platelet factors, anticoagulants, anti-inflammatory substances and proteins inhibiting complement (Valenzuela 2004). It is likely that some of these compounds may be helpful for the bacteria entering the host.

10.1 The first contact
The first line of defence encountered by the spirochetes is the innate immune system. The response is quick and does not require previous contact with the bacteria. *Borrelia* are readily ingested and killed by polymorphonuclear cells and monocytes/macrophages without complement or antibody opsonisation. They are probably recognised and engulfed by lectin-mediated phagocytosis (Cinco, Cini et al. 2001). Toll-like receptors recognise conserved pathogen structures, so called pathogen-associated molecular patterns (PAMPs). Recognition by Toll-like receptors, especially TLR-2, plays an important role in activating the immune system, e.g. mounting an antigen-specific IgM-response (Bolz, Sundsbak et al. 2006; Alugupalli, Akira et al. 2007).

10.2 Complement resistance
Another part of the innate immune system is complement. The complement system is divided into three pathways, the classical pathway reacting to antibody-antigen complexes, the lectin pathway activated by sugar moieties on microbial surfaces, and the alternative pathway initiated by C3 hydrolysis, and
C3b binding on the surface of a pathogen. C3b binding leads to opsonization and thereby greater internalization by phagocytic cells. Complement activation also leads to the formation of a membrane attack complex in the bacterial surface, lysing the pathogen. To protect self cells the complement system needs to be tightly regulated. The major fluid phase regulator of the classical pathway is C4b-binding protein (C4BP), and factor H (fH) for the alternative pathway. C4BP regulates complement by acting as a cofactor for factor I in cleaving C4b, and fH by cleaving C3b. To evade complement attack, B. recurrentis, B. duttonii and several other species, bind complement regulators, keeping their biochemical properties intact. They are therefore insensitive to complement in sera whereas others such as B. anserina and B. coriaceae do not bind fH or C4bp (McDowell, Tran et al. 2003; Meri, Cutler et al. 2006). B. duttonii, B. recurrentis and B. crocidurae are resistant to complement from humans, mouse, rat, guinea pig, goat and hen. The host specificity of the first two species can not be explained by differences in complement resistance (unpublished).

The LD agent B. garinii is sensitive to human complement in vitro. Still it is infectious in humans. Experiments using fH-deficient mice have demonstrated that complement evasion by fH binding is not essential for B. burgdorferi to cause infection (Woodman, Cooley et al. 2007). Several studies have shown that RF is also completely cleared by the immune system without complement (Newman and Johnson 1981; Connolly and Benach 2001). Clearly, there is more to borrelial complement evasion than just binding of fH and C4BP.

10.3 Antibodies vs. Antigenic variation
RF spirochetes multiply in the blood with a generation time of about 6 hours. Consequently, when infecting a mouse with a single bacterium, it takes about 5 days to reach $1 \times 10^6$ bacteria/ml (Barbour 1989). The average incubation time in humans is approximately 7 days (Southern and Sanford 1969).

In mice, an antibody response directed to the antigenic Vmp is mounted about 7 days post-infection which eliminates all bacteria expressing this protein. The first peak is cleared by complement-independent bacteriocidal IgM antibodies killing bacteria by direct damage of their outer membrane (Connolly and Benach 2001; Connolly and Benach 2005). By a process called
antigenic variation, in which a small population, perhaps only one or a few bacteria, shift their Vmp to a novel variant not recognized by the antibodies the spirochetes evade the immune response (Figure 9). This new serotype repopulates the blood until a new antibody response against the second Vmp is mounted and again only the few bacteria that have undergone antigenic variation survive. Antigenic variation is the mechanism behind the relapses in RF.

![Figure 9. Antigenic variation](image)

**FIGURE 9. Antigenic variation.** RF increases its persistence in blood by shifting the surface protein Vmp. When antibodies are mounted against the initial serotype (red) all bacteria expressing it are killed by Vmp-specific antibodies. Only those that have shifted to another serotype (yellow) survive and multiply to cause the first relapse. This battle continues until the host dies or the bacteria are eradicated from the blood. Antigenic variation is the mechanism causing the recurring fever which gave the disease its name. Remember that relapses rarely consist of one, single serotype.

and can go on for several cycles until the bacteria eventually are eradicated, or the host is killed (Stoenner, Dodd et al. 1982).

Antigenic variation occurs also in other microbes such as *Neisseria*, *Plasmodium* and Trypanosomes causing sleeping sickness. The antigenic gene locus has structural similarities to RF *vmp*’s (Burman, Bergström et al. 1990). In *B. hermsii*, the single, active *vmp* gene is located at an expression site near the
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telomere of a 28 kb linear plasmid (Kitten and Barbour 1990). A copy of the expressed gene and several (59 or even more?) other silent \( \text{vmp} \) genes are present on linear plasmids. Antigenic variation occurs when a silent gene is duplicated and replaces the active \( \text{vmp} \) in the expression site. The frequency of this shift has been estimated to occur at a rate of approximately \( 10^{-4} - 10^{-3} \) per cell per generation (Stoenner, Dodd et al. 1982; Dai, Restrepo et al. 2006).

It could be hypothesized that a randomized serotype switch in several individual bacteria would result in several populations of bacteria displaying the whole repertoire of Vmp’s simultaneously. Instead, the serotype switch is semi-predictable, resulting in relapses of somewhat mixed serotypes but usually just a few, up to 7 (Stoenner, Dodd et al. 1982). If, for instance, the first serotype is 1, the probability is highest that the first relapse is 7. Serotype 17 and 24 are less likely, but relapses consisting of other serotypes are very rare following serotype 1. This results in a semi-predictable order of serotypes, decreasing the numbers of different serotypes developing in the blood simultaneously. Another consequence is that some serotypes are common in early infection whereas others are more likely later (Barbour, Dai et al. 2006). The antigenic variation itself is not regulated or affected by antibodies (Stoenner, Dodd et al. 1982), nor has the coding sequence of the \( \text{vmp} \) genes anything to do with the pattern of serotypes (Barbour, Dai et al. 2006). The mechanism behind semi-predictable antigenic variation is due to properties of the flanking regions upstream homology sequence (UHS) consisting of 61 nucleotides near the start codon and the downstream homology sequence (DHS), a 214 nucleotide region downstream of the \( \text{vmp} \) gene. If a silent \( \text{vmp} \) has high UHS sequence identity and a short distance to the DHS of the active \( \text{vmp} \), recombination is likely. Opposite, if the UHS similarity is low and DHS is far away recombination is unlikely (Barbour, Dai et al. 2006; Dai, Restrepo et al. 2006).

**Other functions of Vmp’s?**

The fact that some certain serotypes usually occur early in the infection whereas other occur late, has raised the question of whether there may be more to Vmp function than antigenic variation. Vidal and co-workers demonstrated different purified Vmp’s from three \( B. \text{recurrentis} \) isolates to differ in the ability to induce TNF-\( \alpha \) in the monocyte-like cell line MonoMac-
6 (Vidal, Scrugg et al. 1998). In later experiments they showed the lipid part of the Vmp to be the TNF-α inducer (Scrugg, Kwiatkowski et al. 2000). By using several *B. duttonii* serotypes from a clonal origin, differences in TNF-α induction of MonoMac-6 cells could be confirmed; different isogenic serotypes do vary in TNF-α induction. However, the serotypes did not differ in the ability to infect either wt or SCID (Severe Combined ImmunoDeficiency) mice and spirochetemia levels were equivalent (data not shown). Although differences are seen *in vitro*, the serotypes are equivalent *in vivo*. This indicates that the difference between serotypes in infection may be neglectable or an *in vitro* phenomenon.

In experiments where SCID mice were infected with the two different *B. turicatae* serotypes Bt1 (previously VmpA) and Bt2 (previously VmpB) the infections were similar in onset, spirochetemia and eye and heart involvement. However, the Bt2 serotype caused more severe arthritis as measured by tibiotarsal joint swelling whereas Bt1 were better at penetrating umbilical endothelial cells and infecting brain, although Bt2 were also found in brain at the latest time point, 31 days p.i. (Cadavid, Thomas et al. 1994). Bt1 was shown to be the most common serotype in brain, whereas Bt2 was the predominant in skin (Cadavid, Pachner et al. 2001). In other experiments, Bt2 was found to cause eightfold higher spirochetemia than Bt1, which corresponded to the more severe joint swelling (Pennington, Allred et al. 1997). Several studies verifying the neurotropism of Bt1 and the more severe manifestations and arthritis of Bt2 have been published (Sethi, Sondey et al. 2006; Gelderblom, Londono et al. 2007; Gelderblom, Schmidt et al. 2007). Further, Bt1 penetrated monolayers of human umbilical vein endothelial cells (HUVEC) more efficiently than Bt2 (Cadavid, Thomas et al. 1994). It is definitely proven by now that Bt1 and Bt2 behave differently both *in vitro* and *in vivo*. However, they are two serotypes of the same strain and RF in immunocompetent animals and man undergo antigenic variation. Since the serotypes occur in a semi-random order with some serotypes being likely early in infection whereas others occur more frequently later, it is tempting to speculate about further properties of different Vmp’s. A late serotype could for instance be better at persistence and brain invasion although there are no experimental evidence of this. On the contrary, several different serotypes can reside in brain, including the skin- and blood-associated Bt2 (Cadavid,
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Thomas et al. 1994; Larsson, Andersson et al. 2006). Individual serotypes are difficult to study in wt animals since it is in their nature to constantly change and relapses generally are a mix of several serotypes. This issue is extremely interesting and the importance of Vmp functions beside antigenic variation in RF Borrelia infection biology remains to be proven.

10.4 Erythrocyte rosetting

Some old world RF species such as B. crocidurae, B. duttonii and B. hispanica aggregate erythrocytes (Mooser 1958). This phenomenon, later named “rosetting” after a similar phenomenon in P. falciparum malaria, has been proposed to be a mechanism of immune evasion by avoiding contact with immune cells such as e.g. T cells and phagocytic cells (Figure 10). B. crocidurae render higher spirochetemia and a delayed antibody response compared to the non-rosetting B. hermsii which has been proposed to be an effect of the rosetting (Burman, Shamaei-Tousi et al. 1998). However, the use of erythrocytes to hide from immune cells seems unlikely, and the delayed immune response may be due to other factors rather than the rosetting per se since B. crocidurae and B. hermsii are two separate species. Immune evasion by rosetting could be tested by a non-rosetting mutant or by expressing the “rosetting gene”, if such a gene exists, in e.g. B. hermsii. The rosetting of spirochetes and erythrocytes is reversible and the spirochetes can easily swim back and forth between the blood cells. Therefore, the interaction should rather be called “interaction” than “binding”. Various proteins, lipids and carbohydrates have been considered as receptors and adhesins, but our attempts to identify the rosetting adhesin and the erythrocyte receptor have all so far been unsuccessful. When following spirochetemia in animal blood daily,
rosetting mainly occurs when bacteria are numerous and growing, whereas the spirochetes do not interact with erythrocytes when the numbers are low (author's observation). The bacteria are numerous during peaks, sometimes even outnumbering the erythrocytes. It is obvious that massive amounts of nutrients are consumed by the growing bacterial population and that metabolites may be depleted in the serum. It is possible that the bacteria are grazing erythrocytes for metabolites rather than adhering to them. Pettersson and co-workers have studied the purine metabolism of *Borrelia* and found that RF can utilise purines, e.g. hypoxanthine, for synthesis of DNA and RNA, nucleotides that are unavailable to Lyme *Borrelia*. Hypoxanthine is the most abundant purine in human plasma and is produced by erythrocytes. Pettersson et al. speculate that the ability to utilise hypoxanthine may be crucial for the spirochetes to reach high densities in blood. They further speculate that rosetting spirochetes may be grazing hypoxanthine from the surface of erythrocytes (Pettersson, Schrumpf et al. 2007). This is extremely interesting.
and the theory is tempting but needs to be tested experimentally. Further, it
does not explain why some RF borreliae are rosetting whereas others are not.

10.5 Invasion
RF spirochetes invade tissues such as brain and kidney as early as 2 days p.i.
(Nordstrand, Shamaei-Tousi et al. 2001). At this time point, the number of
bacteria is still too low to be detected in blood by direct microscopy.

Penetration of tight barriers
RF *Borrelia* invade organs with tight endothelial barriers such as the blood-
brain barrier, blood-testis barrier and they pass the blood-placenta barrier
(Nordstrand, Shamaei-Tousi et al. 2001; Shamaei-Tousi, Collin et al. 2001;
Larsson, Andersson et al. 2006; Larsson, Andersson et al. 2006). The bacteria
seem to reside in the extracellular matrix of tissues. By performing a double
immunofluorescence staining of murine brain sections for *B. duttonii* and the
tight junction protein ZO-1 the mode of endothelial passage could be studied.
We found that the bacteria could penetrate cerebral vessel walls both in the
tight junctions and by transversing cell cytoplasm (unpublished). We can
conclude that RF spirochetes are both efficient and fast at invading various
tissues.

Hostile takeover of host proteases
Two important factors making *Borrelia* invasive is its pointed shape and
efficient motility, drilling and twirling its way through the tissue. A subtler
factor is the binding of plasminogen to the *B. burgdorferi* cell surface (Coleman,
Sellati et al. 1995; Coleman, Gebbia et al. 1997; Coleman, Roemer et al. 1999).
The bound plasminogen is transformed into proteolytically active plasmin by
the hosts own urokinase-type plasminogen activator (uPA). When associated
to bacteria the plasmin is stabilised and protected from inhibitors. Plasmin is a
broad spectrum serine protease, degrading fibrin-rich extracellular matrix. Its
normal function is in tissue remodelling and cell migration. By binding this
protease the bacteria increase tissue invasion. Binding of plasmin(ogen) and
uPA has been demonstrated in several *Borrelia* species, both Lyme and RF.
This interaction was most prominent on the tips of the spirochetes indicating
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its importance for penetration (Klempner, Noring et al. 1996). The dissemination of *B. crocidurae* is delayed in plasminogen knock-out mice (*plg-/*) showing that plasminogen binding is important but not essential for tissue penetration (Nordstrand, Shamaei-Tousi et al. 2001). The novel RF species isolated in Spain described by Anda et al. (Anda, Sánchez-Yebra et al. 1996) was used to study the importance of plasminogen binding in an animal experiment using *plg-/-* mice. They found fewer bacteria in the brain and heart of *plg-/-* mice compared to wild type mice 25-30 days p.i. Furthermore, the plasmin-deficient mice had less inflammation of the leptomeninges (Gebbia, Monco et al. 1999).

Host matrix metalloproteinases (MMP’s) has been suggested to be used by *Borrelia* to improve its invasive capacity. Most reports on the subject show differential expression of several MMP’s *in vivo* or *in vitro* rather than spirochetes really using the MMP’s for penetrance. It is demonstrated that pro-MMP-9 is activated by both LD and RF spirochetes binding plasmin *in vitro* (Gebbia, Coleman et al. 2001). Convincing evidence of spirochetes using MMP’s for penetrance remains to be demonstrated.

10.6 Persistence
Vector-borne pathogens are dependent on constant availability of both vector and susceptible hosts/carriers. It is of greatest importance for the bacteria to maximise exposure in the blood to increase the chances of being transmitted by a feeding vector. The antigenic variation in RF is an obvious mechanism to increase persistence in the blood. Less studied is the persistence after the last relapse. The bacteria invade virtually all tissues, including the brain where they can remain for long period time (Sergent 1945; Cadavid and Barbour 1998; Cadavid, Sondey et al. 2006; Larsson, Andersson et al. 2006). Before cryopreservation was invented, RF *Borrelia* were often maintained in laboratories as residual brain infection in rodents (Felsenfeld 1971). When injecting infected brain tissue into naïve animals, the bacteria start to grow in their blood. This simple method called mouse infectivity test (MIT), has been used by us to assess persistent infection since it is more accurate and sensitive than e.g. PCR. Interestingly, animals injected with brain harbouring residual *B. duttonii* infection have frequently developed spirochtemia even later than would be expected if the brain contained only one, single spirochete (about 5
days). In some cases the first bacteria were seen later than a week after infection (unpublished observation). Looking into why residual bacteria are so slow-growing after MIT would be extremely interesting.

Bacteria isolated from brain are sensitive to the serum from this animal (Steiner and Steinfeld 1925). This indicates that the bacteria in the brain are of a serotype to which the immune system has made antibodies and memory B-cells, likely during the blood phase of the disease. As described in paper II B. duttonii in brain are killed by ceftriaxone, an antibiotic killing only actively dividing bacteria (Larsson, Andersson, et al. 2006). It seems as the bacteria are restrained in the brain by antibodies or in other words, eradicated from tissues where antibodies can reach them. This seemingly inadvertent persistence may be epidemically important since spirochetes may leak from the brain out into the blood to enable further transmission. The mechanisms of this recolonisation have not yet been studied. The bacteria might shift their antigen in the brain, seeding the blood with new serotypes. Since immune suppression with corticosteroids induce relapses, the immune system seems to be important in the process (Larsson, Andersson et al. 2006).

11. Animal models for relapsing fever

Animal experimentation is a controversial issue. Even though experiments are designed to reduce animal use and minimise their suffering, use of animals in research always is a violation of their natural behaviour. In all animal experiments the importance of the knowledge gained from the experiment must be weighed against the suffering of the animals. If questions can be answered by in vitro experiments, e.g. with cell lines, this is always preferred. In vitro experiments are not only preferable in an ethical respect. They are also cheaper, less labor intensive and more predictable than in vivo experiments.

In this thesis most experiments aim at describing complex interactions between the mouse as an organism and the bacteria. Placental damage and residual brain infection cannot be modelled effectively in vitro. Hence, use of animals cannot be avoided to answer such questions.

The purpose of animal models is to mimic human disease as closely as possible. When performing animal experiments we want to be able to draw conclusions valid also for human RF. If this can not be done, the animal
model is of very limited importance and the experiments may not be defendable either ethically or scientifically. To perform accurate and stringent animal experiments it is crucial to understand the differences between animals and man. “In vivo veritas” is not a universal truth. What is true in animals is not automatically true in man.

11.1 Mice as laboratory animals
The mouse, *Mus musculus* is the most frequently used laboratory animal. It is easily maintained, simple to breed and, most importantly, well characterised. There are several inbred strains, outbred strains, mutants and knock-outs. Its genome is sequenced and an arsenal of various antibodies, arrays and protocols are available.

11.2 Human vs. murine relapsing fever
Mice are the reservoir of several RF *Borrelia* species in nature. The fact that RF is a natural pathogen of mice is a great strength of our model. As for humans, mice develop RF with high spirochetemia and relapsing disease. Typical human symptoms such as hepato- and splenomegaly, haemorrhage and thrombocytopenia are seen also in mice. When infected with RF, the mice tend to be less active and have more ruffled fur than uninfected controls during peak spirochetemia. Between peaks and when disease is mild they look and behave like uninfected mice. On the other hand, the infection may also be lethal. To summarize, human and murine RF seem to be very similar.

SCID mice have often been used in RF experiments. These mice lack B- and T-cells and can consequently not produce antibodies. Use of SCID mice can be meaningful and defendable in many cases. However, when studying an organism capable of antigenic variation and escape from antibody response as the major virulence factor, the conclusions that can be drawn from such experiments are often rather limited.

11.3 Human vs. murine immune defence
The human and murine immune systems are very similar although there are numerous differences that may or may not be important depending on what is studied. Mice have a larger fraction of lymphocytes in their blood while
humans have more neutrophils. Another difference is the pathways in which macrophages are induced to produce NO. Some differences are also seen in antibody subclasses. Interestingly, C57BL, SJL and NOD mice do not express IgG2a but instead expressed IgG2c not present in other mouse strains. There are also differences between mouse strains! In humans, both Th1 and Th2 cells can express IL-10 whereas it is only expressed by Th2 cells in mice. Several other differences have been found as reviewed by Mestas and Hughes. Still, the similarities between human and mouse are more pronounced than the differences (Mestas and Hughes 2004).

11.4 Human vs. murine pregnancy
The murine gestation period is 19-21 days compared to 9 months in humans. The time span of infection as well as gestational phase has to be taken into account when comparing the two systems. Both mice and humans get an increased blood volume and lower haemoglobin concentration when pregnant. Humans usually have one foetus whereas mice have a litter size of 10-12, all with their own placenta. Both human and murine placentas are hemochorial and the vascular bed is built up not by endothelial cells, but by trophoblasts merging together to form the impermeable blood-placenta barrier. In all major aspects, human and murine placentas are very similar. There are differences, mainly in, development, but that is of minor importance when studying its physiological and barrier functions (Adamson, Lu et al. 2002).

11.5 Other animals in relapsing fever research
Almost all animal experiments on RF have been performed in mice. Other animals are used mainly if a certain technique requires so, e.g. in vivo microscopy of Borrelia-erythrocyte rosetting in testicular blood vessels of rats (Shamaei-Tousi, Collin et al. 2001). Rats are also used if a larger amount of serum or cerebrospinal fluid is needed. Primates have been used to study B. recurrentis infection (Judge, La Croix et al. 1974 I-III). Guinea pigs, rabbits etc. are seldom used nowadays but were common earlier (Felsenfeld 1971).
12. The African enigma

The ancestors of all humanity evolved in Africa. All of us non-Africans originate from a small group of perhaps fewer than 50 persons leaving Africa about 100,000 years ago to populate the rest of the planet. Africa has seen great civilizations throughout the history such as the Nile civilizations, Aksum in Ethiopia, and the Mali- and Zimbabwe empires rise and fall. Africa has the world’s greatest reserves of chromium and platinum-group metals. Africa has potassium, oil, uranium and diamonds. The sea is full of fish. The climate and soil are in many areas optimal for agriculture, producing cacao, wine, coffee, peanuts and other crops. More than half of all gold mined throughout history originates from Africa. Still, the poorest people in the world live there and continue to die from starvation and infectious disease (Reader 1998). It must be questioned how the incredible wealth of Africa is distributed.

It is a common misunderstanding that infectious diseases are more frequent in Africa than e.g. Europe and America because of the warmer climate. This may be true in some cases, e.g. in tickborne RF, sleeping sickness etc. with vectors restricted to a certain habitat. What often is forgotten is that typical diseases of developing countries were widespread also in cold Europe not long ago. Louseborne RF, leprosy, tuberculosis, syphilis, cholera and other diseases nowadays are sorted under “tropical medicine” were very common and responsible for numerous deaths even in cold Sweden (Högberg 1983). Also tropical disease number one: malaria, has been common in Sweden, even in the northern parts (Linné 1735). The mosquito vector is still here, but the parasite is eradicated. Climate has very little to do with the burden of infectious disease. The secret is in economy and the healthcare, education and infrastructure it brings.
Throughout my work, my own curiosity has been the guiding star. I have asked myself, what about relapsing fever do I really want to know and what kind of knowledge is needed?

All along I have been thinking a lot about the residual brain infection. Why do the bacteria remain in the brain? What are they doing there? Are they latent? How is the host affected?

Pregnancy complications have been well documented, but surprisingly little was known about the underlying mechanisms. This felt very interesting and important to get to the bottom of.

Relapsing fever is a prevalent disease in West Africa but has never been reported from the southern parts. We wanted to test our hypothesis that RF may be frequent also in areas where it is unknown. We also suspected that it is misdiagnosed as malaria. This resulted in two months of field work at hospitals in Togo which changed my view of life.

The stunning finding that RF causes about 10% of the fever cases in Togo made it clear that good diagnostic tools are urgently needed. I have thought a lot about how to increase the sensitivity of diagnostics in a way that can be routinely used in rural, African health centres.

Some experiments were performed more spontaneously or just because I got the opportunity. Others should perhaps not have been done at all, but generally my own interest and curiosity has led the way.

Writing the introduction has been a challenging task. What should I write about and what can be excluded? Who will read the introduction besides,
AIMS OF THIS THESIS

hopefully, my supervisor, the opponent, and Jenny and Betty who more or less was forced to help me with proof reading? My intention has been to write the introduction for new graduate- and undergraduate students in the thrilling field of relapsing fever. I have tried to include the information I would have wanted when I started my work. I can just extend my congratulations for a thrilling time ahead of you. There are SO many exciting RF experiments left to do!

Specific aims

- **Elucidate the pathobiology of residual brain relapsing fever infection.**

- **Identify the mechanisms by which relapsing fever causes pregnancy complications.**

- **Examine if relapsing fever Borrelia causes illness in Togo, West Africa and if so, describe the epidemiology, diagnostics and treatment regimens.**

- **Develop a simple, fast, inexpensive and sensitive method for relapsing fever diagnostics.**
RESULTS AND DISCUSSION

PAPER I

Persistent brain infection and disease reactivation in relapsing fever borreliosis

Before cryopreservation at -80°C came into use, Borrelia strains were maintained in laboratory animals. Strains were kept for months or even years in animals as residual brain infections. When bacteria were needed for an experiment, the animal was killed and the brain injected into another animal which developed spirochetemia in a few days (Sergent 1945; Felsenfeld 1971; Cadavid and Barbour 1998). However, the pathobiology and medical importance of residual brain infection has been poorly studied. Therefore we developed an animal model based on immunocompetent C57BL/6J mice to study the life of several RF Borrelia species in brain and how the host harbouring the bacteria is affected.

B. turicatae, the species most widely studied in brain-RF interactions, usually in SCID mice, caused only a transient disease with spirochetes seen for only a few days; and several animals were not infected at all. B. hermsii infection resulted in high spirochetemia with few and low-grade relapses. B. crocidurae and especially B. duttonii did not cause as high spirochetemia as B. hermsii initially, but had higher bacterial titers in the relapses and stayed positive for a longer period of time. This is advantageous for the borreliae
since the more bacteria there are and the longer they inhabit the blood, the larger the chance to be taken up by a feeding tick and be further transmitted.

**B. duttonii reside in mouse brain in moderate numbers**

By efficient penetration into host organs, the borreliae access the brain where they may persist further for a long period of time. However, the difference between various species was prominent. *B. helmsii* do not persist in the brain, and *B. crocidurae* is eradicated by day 50 p.i. *B. duttonii* on the other hand, can reside in mouse brain for long time, at least 270 days at moderate numbers, about 1000 bacteria, as measured by real-time PCR day 80 p.i. This amount may seem low, but is approximately the same as the number of *B. burgdorferi* in an infected tick (Wang, Liveris et al. 2003). Consequently, it is enough to constitute a source of bacteria but they are probably too few and too scattered to evoke the immune response.

*B. duttonii* have humans solely as host whereas the other species in this study mostly infect small mammals such as rodents. Although these animals may reach an age of a few years in captivity, the life span in nature is probably only a few months. A residual brain infection may be less beneficial in short-lived hosts. In long-lived hosts such as humans, residual brain infection may constitute a reservoir. Bacteria may escape from the brain, leaking out in the blood at low concentrations to enable tick transmission. *O. moubata* reside in people’s homes and feeds frequently. Consequently, brain as reservoir, in combination with the long life span of *O. moubata* and its transovarial transmission of bacteria is a plausible explanation for how *B. duttonii* maintains its population. The observation of small amounts of *B. duttonii* spirochetes in the blood of apparently healthy people in Tanzania may support this model (Cutler 2006).

**Several different serotypes can reside in brain**

Several similar experiments on *B. turicatae* in immunodeficient mice have demonstrated the bacteria to have one blood-associated and one brain-associated serotype (Cadavid, Pachner et al. 2001). We used *B. duttonii* in immunocompetent mice and found several different serotypes residing in the
brain, in some cases also several serotypes simultaneously. An infected brain is probably inhabited by all or most serotypes that have been in the blood. The spirochetes are arrested there by the hostile immune defence outside the blood-brain barrier. Serotypes obtained from residual brain infection did not differ from one another or from the original 1120K3 serotype in infectivity, spirochetemia or \textit{in vitro} growth rate (data not shown).

\textbf{The infection can be “reactivated” by immunosuppression}

By suppressing the immune system with methylprednisolone the bacteria residing in brain can be reactivated to re-enter the blood, causing a spirochetemia as high as the primary peak. As stated in paper II the bacteria are active but arrested behind the blood-brain barrier. Consequently, the term “reactivation” is inappropriate since it indicates that the bacteria are in a non-active, latent state. Immunosuppression is probably a more suitable description of the phenomenon. In that case, the “reactivated” bacteria leak out into the blood and since the animal is immune suppressed, they reach higher densities than in an animal with normal immune system. This model is supported by the observation of \textit{B. duttonii} in the blood on day 80 p.i. in 1/20 non-suppressed animals, but not detectable by microscopy, only by MIT. Perhaps the methylprednisolone is not reactivating infection but rather letting the bacteria enter the blood and grow to detectable titers by compromising host immune response.

\textit{B. duttonii} \textbf{seems to be unnoticed by the host immune defence}

Murine brains with residual \textit{B. duttonii} infection were analysed with a cDNA microarray and compared to a pool of uninfected controls. Out of about 15,000 sequences, only a false positive (coding for variable region of a monoclonal antibody to a human transcription factor) was found. Not even when looking into the non-significant hits were any differences of interest found. We concluded that the residual brain infection probably is sub-clinical and does not evoke the brain immune defence. In so-called “latent syphilis” it has been suggested that it takes a critical bacterial mass to induce
immunological reaction to the spirochetes, and the *T. pallidum* are therefore unnoticed since they reside in too low numbers (Lafond and Lukehart 2006). The approximately 1000 *B. duttonii* inhabiting the murine brain may be too few to be noticed. There may be some sort of equilibrium between bacterial numbers and immune response.

In an experiment with *B. turicatae* in B-cell deficient mice, brain microgliosis without detectable injury but with high CXCL 13 was observed (Gelderblom, Londono et al. 2007). CXCL 13 is a B-lymphocyte chemoattractant chemokine and it is tempting to speculate that an upregulated B-cell chemoattractant in a B-cell deficient mouse would have more to do with the mouse phenotype than the bacteria. There are small amounts of B-cells in normal brain but whether these cells have any immunological function is not known (Anthony, Crawford et al. 2003). In experiments with early (day 0-14) *B. crocidurae* infection in brain, no increase in brain B-cells were seen (Andersson, Nordstrand et al. 2007). However, my colleague Andersson observed something that appeared to be B-cell germinal centres in the brain of one of my *B. duttonii* infected mice day 80 p.i. However, only one animal was accessible and we can therefore not draw any conclusions from this single observation (Marie Andersson, personal communication). The role of cerebral B-cells, germinal centres and antibody production within the brain needs to be studied further.

We showed *B. duttonii* to causes a long-term persistent infection in brain with small amounts of bacteria not evoking any immune reaction. The results are important for our understanding of the pathobiology and ecology of *B. duttonii*, including epidemiological and medical applications.
PAPER II

Residual brain infection in murine relapsing fever borreliosis can be successfully treated with ceftriaxone

In previous experiments (paper I) we described the residual silent brain infection of *B. duttonii*. The arising question was; in what kind of state are the residual bacteria? Are they active in the brain or are they in a resting, dormant state? The idea of a latent state was tempting considering the long time it takes for a residual infection to start growing in the blood after MIT. Perhaps resting bacteria needed time to be revitalised from a quiescent state to start growing again?

To investigate whether residual *B. duttonii* is latent or not, we treated mice with ceftriaxone, a bacteriocidal β-lactam antibiotic inhibiting bacterial cell wall synthesis. Our hypothesis was that actively growing cell-wall producing bacteria are killed by ceftriaxone, while latent, dormant, non-growing bacteria survive.

Ceftriaxone is the drug of choice for treating meningitis and Lyme neuroborreliosis (Table 3), (Wormser, Nadelman et al. 2000). It is one of the best drugs for treating cerebral infection and has good CNS penetrance. It has also been demonstrated that ceftriaxone kills actively growing RF spirochetes in mice (Kazragis, Dever et al. 1996). In this study, the dose, longevity and route of administration were adapted to mimic typical human neuroborreliosis treatment (100mg/kg, 14 days, i.v. respectively).

Of the 10 control mice receiving saline, 4 (40%) were positive whereas none of the 20 treated mice had bacteria in the brain. This was demonstrated by the Mouse Infectivity Test (MIT), in which brain tissue of the experimental animal is homogenised and injected into naïve mice, thereafter screened daily for spirochetemia.

We show that ceftriaxone clears residual RF infection which is valuable knowledge when it comes to treatment, but the experiment also tells us something about the pathobiology of RF. If the bacteria were in a latent state...
they would be unaffected by the cell wall inhibitor ceftriaxone. Instead all bacteria are killed, showing that they are not latent, but actively growing in the brain.

To further test the hypothesis of non-growing bacteria surviving ceftriaxone treatment, growth was inhibited in vitro by the bacteriostatic antibiotic chloramphenicol. The majority of these bacteria survived and many of them were still motile although protein synthesis was stopped (or at least lowered). A 24h pre-treatment with 40µg/ml chloramphenicol rescued bacteria from 12µg/ml ceftriaxone which is more than enough to completely kill actively dividing B. duttonii. This supports our prediction that non-growing bacteria survive ceftriaxone treatment. However, we cannot predict whether the residual bacteria are growing more slowly, just that they are somewhat active in cell wall synthesis and not dormant.

In previous experiments (Paper I), low numbers of bacteria were found in the blood late in the infection, sporadically also at day 80 p.i. At this time point, B. duttonii could also be reactivated by cortisone injections to re-enter the blood. This indicates that the bacteria are captured in the brain by the immune defence, probably due to the antibodies in the serum. This is further supported by the early observation that bacteria derived from brain are sensitive to serum from the same animal as they were isolated from (Steiner and Steinfeld 1925). We conclude that the bacteria use the brain as an immune privileged site from where they can leak out into the blood to facilitate further transmission.

It is possible that B. duttonii can enter a latent state after day 80 p.i., but this seems unlikely, especially since the frequency of brain infection in mice is decreasing with time (Paper I).

To test if the results from our mouse model are applicable also in humans in endemic areas would take several brain biopsies from healthy persons, something which would be neither technically nor ethically possible. We may get a hint from Cutler, who found low amounts of B. duttonii in apparently healthy villagers in rural Tanzania (Cutler 2006). Perhaps man, the only B. duttonii host found in nature, is an important reservoir in whose brain the bacteria can remain in low numbers. They may not cause disease, but slowly divide to re-enter the blood and infect feeding ticks.
We show that the residual *B. duttonii* infection is active rather than latent. It is probably trapped in the immunoprivileged brain by the external immune response. Our results support use of ceftriaxone in RF *Borrelia* treatment. Substances with poor CNS penetrance, single-dose treatments and bacteriostatic antibiotics should be avoided.
PAPER III

Pregnancy complications and transplacental transmission of relapsing-fever borreliosis

Complications during, and after pregnancy are very common in developing countries. About ¼ of these complications are caused by infectious diseases (WHO/UNICEF/UNU. 2001). At a Congolese hospital, 6.4% of the pregnant women seeking healthcare were diagnosed as having RF. Spontaneous abortion, stillbirth and neonatal RF were common findings (Dupont 1997). Several cases of maternal and neonatal RF have been reported and this is obviously a great problem in many regions (Brasseur 1985; Barclay 1990; Jongen 1997; van Holten, Tiems et al. 1997).

Surprisingly, no one had looked into the pathobiology of pregnancy complications caused by RF so this felt like an urgent task. We developed a mouse model to study how B. duttonii infection interferes with pregnancy.

B. duttonii causes intrauterine growth retardation in pregnant mice

There is an inverse linear relationship between spirochetal burden during pregnancy and foetal weight. The decreased birth weight may be caused by several factors. We found decreased haemoglobin levels in infected animals which may contribute to lower oxygenation of the foetus, especially since pregnancy itself cause a certain amount of anaemia. We also found placental tissue damage and inflammation which obstruct blood flow and result in lowered exchange of oxygen and metabolites. Large amounts of spirochetes reside in the placental labyrinth and it is likely that they adhere to erythrocytes, even further obstructing placental circulation.

The bacteria invades the foetus in utero

Several infectious diseases may be detrimental to pregnancy but very few cause congenital infections. It is well known that RF causes complications of pregnancy and there are several reports of neonatal RF but the reports of intrauterine infection of the foetus are scarce. In many cases the neonate may
RESULTS AND DISCUSSION

have been infected at or after birth, e.g. when cutting the umbilical cord. In some cases even transfer by tick bite is likely. There are cases, such as Fuchs and Oyama reporting human, and Walker et al. reporting equine transplacental transmission, both in new world RF (Fuchs and Oyama 1969; Walker, Read et al. 2002). However, a handful of cases do not indicate that this is a major problem. In our mouse experiments, we show that 73% of the foetuses from infected mothers are infected by *B. duttonii* before birth. We thereby conclude that congenital transfer is an important factor of RF in pregnancy.

**Disease manifestations are milder during pregnancy**

Generally pregnancy is considered an immune-suppressed state in which the immune system is “down regulated” to protect the non-self foetus. This would then result in infectious diseases being more severe during pregnancy as seen in for instance malaria (Goldenberg and Thompson 2003), listeriosis (Abram, Schluter et al. 2002), and also reported to be the case in human RF (Goubau and Munyangeyo 1983). To our surprise, the pregnant mice were not very affected by the infection. They had significantly lower spirochetemia than non-pregnant controls. We tried several different mouse strains more closely mimic human disease. All strains tested gave similar results. There may be a difference between human and murine RF in this aspect but if so, that needs to be proven. When scrutinising the Goubau and Munyangeyo paper reporting RF as being more severe during pregnancy, it is obvious that no convincing evidence that this would be the case is presented (Goubau and Munyangeyo 1983). Perhaps also women are more prone to seek health care when pregnant than they would be otherwise?

The view of pregnancy as an “immunosuppressive state” has been outdated for years. Instead immunology of pregnancy has been refined to instead suggest an altered Th1/Th2 balance during pregnancy, still protecting mother and foetus but avoiding harmful reactions.

The Th1 immune defence is dominated by e.g. the cytokines TNF-α and IFN-γ and a cell-mediated defence with phagocytic cells effective against intracellular pathogens such as malaria and *Listeria*. A strong Th1 dominance, resulting in phagocytic cells attacking foreign cells is detrimental to pregnancy. Therefore, the immune system is shifted towards a Th2 profile during
pregnancy. With a dominant Th2 profile the immune system is more prone to use e.g. antibodies, thereby better at fighting extracellular pathogens such as *Borrelia*. We hypothesize that the shift towards a Th2 cytokine pattern can explain the milder maternal RF since the Th2 profile would be better at fighting a *Borrelia* infection.

Milder symptoms might seem to be something positive, but may result in neglecting to seek healthcare as reported by Fuchs and Oyama when a pregnant women mistakes RF for an upper respiratory tract infection and gives birth to a spirochetemic baby which later dies (Fuchs and Oyama 1969).

This work shed light on the previously unknown pathobiological factors causing pregnancy complications during RF infection. It is important for our understanding of the disease and for diagnostics and treatment.
Tickborne relapsing fever diagnosis obscured by malaria, Togo

In 2002, RF was unheard of in Togo.

According to the epidemiological database Gideon, 2000 and 2800 cases were reported in 1981 from neighbouring countries Nigeria and Burkina Faso respectively (GIDEON 1999). In other countries in the same region, nothing was known about RF occurrence. Our hypothesis was that RF may exist in Togo, perhaps misdiagnosed as malaria. We thought it might occur mainly in the dry woodland and savannah in the north.

**FIGURE 11: Mouba village in the Savanes region of Northern Togo.** The big pots containing food attract rodents which together with dogs, chickens and other domestic animals constitute hosts for RF transmitting ticks. The open houses made of mud and straw provide shelter for rodents and ticks.
First finding of RF in Togo

We performed a clinic-based study during 2002-2004 by collecting epidemiological data and blood samples from patients with fever. No spirochetes were found by microscopy of Giemsa-stained blood smears. However, about 13% of the samples contained antibodies against the RF specific protein GlpQ, and about 10% were positive in a PCR for 16S. When sequencing the 16S gene of positive samples 19/21 (90%) were found to be \textit{B. crocidurae}. More surprising was the two sequences identifying the causative agent as \textit{B. duttonii}, the East African RF. Both \textit{B. duttonii} cases were from the northern savannah area. However, taking the high similarity of \textit{B. duttonii} and \textit{B. crocidurae} 16S into account (only one nucleotide difference), the limited amount of published sequences and the confusion of what some of the old RF strains maintained in laboratories really are, caution has to be taken in interpretation of this single nucleotide. Several attempts were made to sequence IGS and \textit{glpQ} of these samples but all the DNA was of too low quality to render any PCR products. Indeed, \textit{B. duttonii} seems to occur in Togo, but better documentation would be desirable.

Surprisingly, RF was slightly more common in the southern, tropical parts of Togo than in the dryer northern parts which we hypothesised would be preferred by the tick vector. This may be explained by all patients being children in the north whereas patients from all age groups were included in the south. The results demonstrate the paucity of epidemiological knowledge about African RF. We probably found \textit{B. duttonii} in West Africa for the first time and we found about 10% of the fever cases to be caused by RF. This is remarkable if keeping in mind that the disease was totally unheard of in the area until present study was performed. Ten percent of the fever patients were infected and the disease agent was unknown!

A questionnaire documenting living conditions, ethnicity, occupation and demographic data was collected for each patient in the study. RF was most frequent among cow herders belonging to the Peuhl (Fulani) ethnic group. They are traditionally nomads of the Sahel grasslands moving with their cattle. When spending many nights in the outdoors, the risk of being bitten by \textit{O. erraticus} ticks is increased. RF was also more common among people living in mud houses than those living in houses built of cement (Figure 11).
RF is misdiagnosed and inefficiently treated

Despite the impressive skills of the laboratory technicians, none of the patients infected with RF were correctly diagnosed in the clinic and only 1/18 received treatment efficient against RF. As suspected, most fever patients had malaria, but about 4.5% of these were coinfectected with RF. From another perspective, 33% of the RF patients also had malaria. Malaria diagnostics is not always easy, either. Therefore it is usually performed by using thick blood smears. In this technique, the sample is treated with water to lyse erythrocytes, making the malaria parasites more concentrated and easier to see. Unfortunately, spirochetes are also lysed by the water treatment.

We can conclude that RF is obscured by malaria and they often occur together. It is obvious that improved diagnostic tools are needed. The most striking result of this study is how little is known about RF epidemiology and distribution. If the illness is this prevalent in Togo, how common is it in other locations?
PAPER V

Diagnostics and treatment of relapsing fever borreliosis

In paper IV we emphasize the need for improved techniques for RF diagnostics.

Paper V is intended as a short, easy-to-read, summary of what the clinician encountering a suspected RF patient needs to know. The introductory part is included in the thesis introduction in a revised form. Therefore only the novel RF diagnostic method will be presented and discussed here.

RF can be anything from deadly to asymptomatic, and disease symptoms also vary between these two extremes. The similarity to malaria is striking, but RF can be confused with virtually any fever disease. Diagnosis is usually performed by Giemsa staining of thin blood smears, using the same dye as for malaria diagnostics. This method is fast, cheap and simple. However, it is performed at 1000x magnification on a very thin blood film. Thereby only a tiny volume of blood is screened, resulting in low sensitivity (paper IV) and (Trape, Duplantier et al. 1991; van Dam, van Gool et al. 1999; Kisinza, McCall et al. 2003). To increase sensitivity in malaria diagnostics, a method called thick smear is often used which makes it possible to analyse a larger volume of blood in each microscopic field. Water or other erythrocyte-disrupting solutions is often used to lyse red blood cells, simplifying detection of malaria parasites. Unfortunately, this procedure also lyses spirochetes, making it useless for RF diagnostics.

PCR is an excellent method for RF diagnostics. Briefly, DNA is extracted from, for example, 100µl blood, analysed by PCR using *Borrelia*-specific primers, separated on an agarose gel, or, if using real-time PCR, directly on the screen. The method is fairly quick and sensitive if the instrument and all reagents, including specific primers, are accessible. The major drawback with PCR is the cost of the machine and all the reagents needed. Very few, if any, hospitals and health centres in the endemic areas where RF is present have the possibility to use PCR in their diagnostics.
ELISA is a valuable scientific tool to screen sera for antibodies against pathogens. An ELISA utilising the RF-specific protein GlpQ identifies patients who have antibodies to it (Schwan, Schrumpf et al. 1996; Porcella, Raffel et al. 2000; Nordstrand, Bunikis et al. 2007). RF is an acute disease and in most fatal cases people die during the first attack. Otherwise the first spirochetemia lasts for a few days and thereafter it takes another couple of days until the fever drops as a result from the host antibody response. It is not until this time point that an ELISA can detect RF, making ELISA a useless diagnostic tool in the most critical phase of the infection. Further, ELISA may give false positive results as a result of antibodies from past infections. This can lead to misdiagnosis of other diseases. ELISA is expensive, labour intensive and takes some practice to perform. It is valuable for less acute diseases and for epidemiological studies, but not for RF diagnostics.

The ideal diagnostic method should be fast and simple to perform. It should be cheap and possible to use in small rural health centres, preferably with existing equipment. It should also be sensitive to detect as low bacterial titers as possible. Several strategies were considered until we came up with the method described in paper V.

About 10ml blood is drawn into anticoagulant (e.g. heparin, sodium citrate, EDTA etc.) and transferred to a centrifuge tube. The blood is centrifuged 5 minutes at 500xg to pellet erythrocytes but keeping the spirochetes in the plasma supernatant. The supernatant is transferred to a new tube, preferably with v-shaped bottom, and centrifuged 5 minutes at 5000xg to collect the bacteria. The supernatant is thereafter aspirated, keeping the bacteria in the bottom of the test tube. This small bacterial pellet is dissolved in the few remaining µl of plasma, transferred to a glass slide, and an ordinary Giemsa staining is performed.

By this method, the bacteria are concentrated which increases the sensitivity drastically. In 10ml blood spiked with 100 spirochetes, about 10 could be counted at the glass slide. This implies that 90% of the bacteria were lost during the processing but on the other hand, that we have sensitivity even higher than PCR, considering the sample size for DNA extraction and the volume of eluted DNA usually used.

During the Togo study we realized there is an urgent need for improved diagnostic tools. We considered a PCR kit with all reagents and primers
prepared as a “PCR-bead”, GlpQ ELISA or Western detection. Another approach would be to perform a gradient centrifugation of blood together with coloured beads of same density as spirochetes. These beads would then be a marker for the purified spirochete band. All these approaches would probably all be functional, but none of them would be widely used since they are too technically advanced, too expensive, and require materials not available where good RF diagnostics is really needed. Despite its almost childish simplicity, or perhaps just because of that, I regard this to be my most important contribution and what I am most proud of in this thesis. I hope my method will come to be used and improve RF diagnostics in all those areas where it is so urgently needed.
PAPER VI

Phylogeny of old world relapsing fever
Borrelia

Clinically, RF is usually divided into tickborne vs. louseborne RF due to the differences in epidemiology and disease manifestations (Southern and Sanford 1969; Felsenfeld 1971; Cook and Zumla 2002). Using this division, e.g. B. duttonii and B. hermsii are grouped together as opposed to B. recurrentis. A biological division was developed by Davis who classified RF Borrelia according to their vectors (Davis 1952). The idea that each tick species transmits one, single Borrelia species seems neat and orderly and fits quite well in the new world. However, nature is rarely neat and orderly, and there are several exceptions to the Davis model, especially in the old world.

In paper VI we sequenced the glpQ gene and IGS, aligned them and made neighbor-joining phylogenetic trees. This has been done earlier with these and additional sequences by others, and our results are well in line with theirs. (Fukunaga, Okada et al. 1996; Ras, Lascola et al. 1996; Cutler, Moss et al. 1997; Cutler, Akintunde et al. 1999; Cutler, Scott et al. 2005; Scott 2005). Our study started mainly as an internal validation of our old world RF strain collection. Naturally, strains isolated in a small geographic area tend to be quite homogenous. Usually a few isolates of species A from a certain area is compared by a few species B from another area and conclusions are made. The novelty in our approach is that we have used strains obtained from various sources and it is due to this that we have gained interesting results. We have three B. crocidurae strains in our strain collection named Achema, Tenatao and CR2A. The first two were isolated from Mauretania and Senegal respectively and provided by the Pasteur institute. The origin of CR2A is unknown but the strain comes from the collection at Rocky mountain laboratories, NIH. The Achema and Tenatao strains differed by five nucleotides in each of glpQ and IGS which seems reasonable for two different strains of the same species. Interestingly, the CR2 strain did not group together with the other B. crocidurae isolates. Instead, it grouped with B. duttonii and B. recurrentis in both the IGS and glpQ tree. Either this strain was
inaccurately identified as *B. crocidurae* when first isolated or otherwise the species is very heterogeneous, perhaps even polyphyletic.

As mentioned above, RF *Borrelia* are often divided into tickborne and louseborne varieties. Therefore it is interesting that the tickborne *B. duttonii* and the louseborne *B. recurrentis* are closely related. In our results, the *B. duttonii* strains from Tanzania are monophyletic with *B. recurrentis*, excluding the three *B. duttonii* isolates originating from the Congo. This supports the theories that louseborne RF has evolved from *B. duttonii* (Heisch and Garnham 1948; Felsenfeld 1971). It could also support the somewhat wilder theory that *B. recurrentis* and *B. duttonii* is the same species, being able to be transmitted by both ticks and lice (Cutler, Scott et al. 2005). More isolates from other geographic areas have to be included to come to a final conclusion on RF phylogeny. This work may raise more questions than answers, but that is also an important part of the scientific process, perhaps the most important.
CONCLUSIONS

I. *B. duttonii* causes a residual, persistent infection of the brain.

The bacteria reside in low numbers and can be reactivated by immunosuppression.

The residual infection does not evoke noticeable host immune response.

II. Residual *B. duttonii* brain infection is actively growing rather than in a dormant state since it is cleared by the cell wall disrupting antibiotic ceftriaxone.

III. *B. duttonii* causes pregnancy complications.

The mechanisms are decreased maternal haemoglobin and placental inflammation and damage, resulting in impaired foetal circulation and intrauterine growth retardation.

The bacteria invade the foetus *in utero*.

Disease manifestation is milder during pregnancy.
IV. Relapsing fever borreliosis was found for the first time in Togo, West Africa.

The diagnosis is obscured by malaria.

About 10-13% of fever patients were found to be infected with relapsing fever.

Improved, more sensitive diagnostic procedures are needed.

V. A simple, cheap and sensitive method for relapsing fever diagnostics was developed.

VI. Both IGS and \( glpQ \) are useful in phylogenetic studies of old world relapsing fever and give similar results.

\( B. duttonii \) has a polyphyletic relation to \( B. recurrentis \).
SAMMANFATTNING PÅ SVENSKA

Swedish summary

*Borrelia* är bakterier som orsakar två sjukdomar, Lyme borrelios som är den vi i Sverige kallar ”*Borrelia*” samt återfallsfeber, vilken är den som denna avhandling avhandlar.


Sjukdomen sprids med fästingar eller löss och finns i nästan alla tropiska- och subtropiska områden men är absolut vanligast i Afrika där fästingarna lever i människors bostäder.

I **artikel I** undersöktes *B. duttonii*, en återfallsfeberborrelia som finns söder om Sahara. M.h.a. djurförsök visar vi att bakterierna kan ligga kvar i hjärnan lång tid (åtminstone 270 dagar) efter att bakterierna försvunnit från blodet och djuret blivit ”friskt”. Vi visar att det finns ungefär 2000 bakterier/gram hjärna. Experimenten tyder på att bakterierna är helt dolda och inte hittas av immunförsvar. Om däremot immunförsvar nedsätts kan bakterierna växa i blodet igen.


I artikel V utvecklas därför en enkel och billig metod för att snabbt kunna hitta även små mängder *Borrelia* i blodet. Diagnostikmetoden är framtagen för att kunna användas på laboratorier med minimal tillgång på utrustning. Artikeln innehåller också en sammanfattning över diagnostik och behandling som kan användas av klinisk personal.

sig på olika grenar utan flyter in i varandra. Tidigare har man antagit att det är olika arter eftersom de sprids på så olika sätt. Kanske är det en och samma art som kan spridas med både löss och fåstingar?
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