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Pathogenesis of Relapsing Fever

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Abstract

Relapsing fever (RF) is caused by several species of *Borrelia*; all, except two species, are transmitted to humans by soft (argasid) ticks. The species *B. recurrentis* is transmitted from one human to another by the body louse, while *B. miyamotoi* is vectored by hard-bodied ixodid tick species. RF *Borrelia* have several pathogenic features that facilitate invasion and dissemination in the infected host. In this article we discuss the dynamics of vector acquisition and subsequent transmission of RF *Borrelia* to their vertebrate hosts. We also review taxonomic challenges for RF *Borrelia* as new species have been isolated throughout the globe. Moreover, aspects of pathogenesis including symptomology, neurotropism, erythrocyte and platelet adhesion are discussed. We expound on RF *Borrelia* evasion strategies for innate and adaptive immunity, focusing on the most fundamental pathogenetic attributes, multiphasic antigenic variation. Lastly, we review new and emerging species of RF *Borrelia* and discuss future directions for this global disease.

Introduction

Relapsing fever (RF), or what we believe to be RF, was historically known by different names including “Febris recurrentis”, “recurrent fever”, “famine fever”, “spirillum fever”, “spirochetal fever”, “saddleback fever”, “vagabond fever”, “fowl nest fever”, “Karlskrona fever”, “gharib ghez”, “Giesinger’s bilious typhoid”, and “kimputu.” RF was recognized as a disease by physicians in ancient Greece at the time of Hippocrates (Felsenfeld, 1971). Later, between 1485 and 1551, five epidemics of “sweating sickness” that swept through England probably included outbreaks of RF. One of the best recorded descriptions of RF came from the physician John Rutty, who kept a detailed diary during his time in Dublin, where he described the weather and illnesses in the area during the mid-1700’s (Rutty, 1770). Interestingly, the fatality rate was very low and most of the affected people did recover after two or three relapses.

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RF symptoms also were described in detail by field medics during the 1788 Swedish-Russian war. The Swedish navy conquered the Russian 74-cannon battleship Vladimir and its 783 men crew at a battle in the Finnish bay near the Hogland Island. Following the victory, the ship was brought to Helsinki, but several of the Russian prisoners aboard were sick. The louse-borne RF spread rapidly in the Swedish Fortress Sveaborg and among Helsinki civilians. Five hundred sick soldiers were shipped to Karlskrona, the main Swedish naval base, which then also became heavily plagued by RF (Felsenfeld, 1971; Huldén, 2006). From the documentation, it is possible that many soldiers were afflicted by concomitant infection with *Rickettsia prowazekii* and possibly other microorganisms. Difficulty in clinically distinguishing RF from other vector-borne infections continues to this day. For example, RF and malaria are often mistaken in areas where both exist. Also, dengue and Colorado Tick Fever, present with similar symptoms to RF such as headache, constitutional symptoms, and “saddleback fever”-like (biphasic fever that may suggest a relapse).

The definite cause of RF was not established until 1868 when the German scientist Otto Obermeier identified a spirochete as the etiologic agent of an epidemic fever outbreak in Berlin. The organism causing this epidemic disease was first named *Spirocheta obermeieri* but later renamed *Borrelia recurrentis*. The largest outbreaks occurred during World War I over large areas in Europe, Asia and Africa. The epidemics among the soldiers and civilians were probably due to poor hygienic conditions and the forced migration of refugees (Felsenfeld, 1971).

RF is either tick-borne (TBRF) or louse-borne (LBRF) (Felsenfeld, 1971). LBRF is caused by *B. recurrentis*, and TBRF is caused by several different *Borrelia* species. Many of the RF outbreaks described by Ruty and others in Northern Europe were likely louse-borne. These outbreaks occurred when there were optimal conditions for the body louse - the weather was turning cooler and people were adding more layers of clothing, the preferred niche for the human body louse. Also, during wartime, people are more vulnerable to attack by lice because of reduced hygiene, including the lack of clean clothes and bed linen.

Since the initial descriptions of LBRF, there have been millions of human cases in large and small epidemics (Bryceson et al., 1970). However, endemic transmission of the agent continues to persist in the Horn of Africa and adjacent areas (Borgnolo et al., 1993; Mitiku and Mengistu, 2002; Sundnes and Haimanot, 1993). As expected for a disease with this degree of public health importance, there have been numerous clinical, epidemiologic, and entomologic studies of LBRF, its agent, and vector. Many of these reports date to the first half of the last century and provide a wealth of information about the clinical aspects of LBRF.

The reservoir of *B. recurrentis* is probably restricted to humans, and reports of experimental animal infections with *B. recurrentis* have been scarce (Barbour and Hayes, 1986). For example, *B. recurrentis* may cause a spirochetemia in weanling mice, newborn rabbits, and grivet monkeys (Judge et al., 1974a, b, c). More recently, Larsson and coworkers successfully developed a LBRF model in immunodeficient mice (Larsson et al., 2009).

The findings demonstrated that, in addition to humoral immunity, innate immune responses could limit the pathogens in the mammalian host.

TBRF is a vector-borne zoonosis found in all regions of the world, apart from Oceania and Antarctica. TBRF accounts for thousands of cases of RF globally each year. Most of the studies on the pathogenesis and immunity of RF have been carried out on TBRF species, whose natural reservoirs are rodents and other mammals. However, the natural reservoir of the RF species *B. duttonii* has not yet been defined. For the TBRF *Borrelia* species, mice and rats serve as suitable experimental animal models.

This review by necessity emphasizes studies of TBRF, especially those of two Nearctic species, *B. hermsii* and *B. turicatae*, and two Afro-Tropical/Paleartic species, *B. duttonii* and *B. crocidurae*. This review also covers important aspects of LBRF infection and its causative agent *B. recurrentis*, which is descended from the tick-borne species *B. duttonii* or a close relative (Lescot et al., 2008). Thus, the findings of experimental studies of tick-borne species might be applicable to *B. recurrentis* and LBRF. Furthermore, this review is an updated version of the publication by Barbour and Guo (2010). We have included a more comprehensive section on vector biology and an update on new and emerging hard tick-transmitted RF species, *Borrelia miyamotoi*.

Transmission-vector and reservoirs

Harry Hoogstraal, a medical acarologist and parasitologist, suggested that the *Borrelia* lineage began as symbionts of ticks rather than as parasites of vertebrates (Hoogstraal, 1979). This is an interesting hypothesis but, as Barbour and Hayes stated, “As it is metaphorically true for the chicken and the egg, it may not be known whether *Borrelia* were originally parasites of arthropods or of vertebrates” (Barbour and Hayes, 1986). Thus, spirochetes are found as endosymbionts in several invertebrates, e.g., termites and mollusks. From that viewpoint, infection of vertebrates is the main role for transmission of the spirochetes to another arthropod host, in this case soft-bodied (argasid) ticks of the genus *Ornithodoros* (Cooley and Kohls, 1944; Felsenfeld, 1971; Hoogstraal, 1979). On the other hand, one piece of evidence that favors an arthropod origin for the genus *Borrelia* is the finding of a high frequency of transovarial transmission observed for TBRF species, with between 35 - 100% of offspring, in most but not all species (Burgdorfer and Varma, 1967; Davis, 1943, 1952). When the *Borrelia* spirochete enters the ovary, it invades the developing oocyte-yolk complex before the impermeable shell is formed around the egg (Aeschlimann, 1958).

TBRF spirochetes, are transmitted either by argasid or ixodid ticks and their life cycles in each vector differ. In argasids, TBRF spirochetes enter the tick when feeding on an infected vertebrate host. While it is not completely clear if replication occurs in the vector, the spirochetes persistently colonize the tick midgut and salivary glands of argasids in the genus *Ornithodoros*, (Boyle et al., 2014; Krishnavajhala et al., 2017; Policastro et al., 2013; Schwan and Hinnebusch, 1998). Since *Ornithodoros* ticks are rapid feeders, completing the blood meal within 60 minutes, the population of TBRF spirochetes that colonized the salivary glands enters the host’s skin and blood within seconds of attachment (Boyle et al.,

2014; Burgdorfer, 1951). This was demonstrated when *Ornithodoros turicata* ticks engorged on the ears of mice, and after feeding the bite site was removed and placed into culture medium (Boyle et al., 2014). All the animals became infected, and spirochetes also were cultivated from ear biopsies. These results indicated that, while some spirochetes enter the bloodstream, others that remain at the bite site after feeding. While most species of TBRF are transmitted through the saliva of *Ornithodoros* ticks, transmission of *O. moubata* may occur through contamination of the bite site with infected coxal fluid (Varma, 1956).

Recent work with the ixodid tick vector of TBRF spirochetes suggests a slightly different life cycle compared to argasid ticks. Studies with *Ixodes scapularis*, the vector of *B. miyamotoi*, indicated that the bacteria persistently colonize the midgut and salivary glands of the tick (Breuner et al., 2017). Upon feeding infected ticks, spirochete DNA was detected in 10% of mice after 24 hours. This indicates that spirochetes in the salivary gland are the first to enter the vertebrate host. Interestingly, by the time ticks had completed feeding, several days later, 73% of the animals were infected. This suggests that unlike argasid-transmitted TBRF spirochetes, the population of *B. miyamotoi* that colonizes the midgut migrates to the salivary glands then plays a role in vertebrate infection as has been well established for the causative agent of Lyme disease.

The transmission dynamics of LBRF spirochetes are unique compared to the tick-transmitted species. After ingestion by the louse, LBRF spirochetes pass from the midgut to the hemolymph where they multiply and persist for the ~3-week lifespan of the louse. Subsequent transmission of LBRF spirochetes to humans does not occur through the bite or the saliva of the human body louse (*Pediculus humanus humanus*). LBRF spirochetes are transmitted mechanically and transovarial transmission of *B. recurrentis* does not occur (Bryceson et al., 1970; Felsenfeld, 1965). Only when the louse is crushed does contaminated hemolymph or feces enter the human (Houhamdi and Raoult, 2005). This occurs through contamination of the conjunctivae, abrasions in the mucous membranes of the mouth, or the louse bite site.

The vertebrate reservoir hosts of the TBRF *Borrelia* spp. are mostly different species of rodents (Nicolle and Anderson, 1927) but may also include animals like pigs, goats, sheep, rabbits, bats, opossums, armadillos, foxes, cats, dogs, and birds (Felsenfeld, 1971; Hoogstraal, 1985; Pavlovsky, 1963; Thomas et al., 2002). These animals usually become infected during the bite of an *Ornithodoros* or related soft-bodied tick. Accidental peroral transmission through consumption of livers and brains of infected animals also has been reported (reviewed in Barbour and Hayes, 1986). Transplacental transmission occurs also in mammals and may cause congenital infection with subsequent placental damage and stillbirths (Larsson et al., 2006a). However, the peroral and transplacental routes of RF transmission are rare, and their importance for transmission in nature is probably insufficient for the maintenance of the pathogen. Likewise, for humans, RF is nearly always acquired through contact with a tick or louse carrying the spirochetes. Rarely the disease is acquired through (i) accidental inoculation of infected blood; (ii) contact of abraded or lacerated skin, mucous membranes, or conjunctiva with infected patient or animal blood (Herms and Wheeler, 1935); or (iii) transplacental or perinatal transmission from mother to fetus (Fuchs and Oyama, 1969; Yagupsky and Moses, 1985). Additionally, pregnancy complications

connected to RF infection have been reported, especially *B. duttonii* infections in the sub-Saharan region of Africa (Brasseur, 1985; Dupont et al., 1997; Goubau and Munyangeyo, 1983; Jongen et al., 1997; Melkert and Stel, 1991; van Holten et al., 1997). Importantly, transmission does not occur by aerosols, fomites, saliva, urine, feces, or sexual contact.

***Borrelia*-arthropod interactions: Life inside the tick!**

In the early and mid-twentieth century, there were many studies on the colonization and movement of *Borrelia* spirochetes in ticks and lice, reviewed in (Barbour and Hayes, 1986; Burgdorfer and Varma, 1967; Felsenfeld, 1965; Felsenfeld, 1971). An example was the comprehensive investigation by Willy Burgdorfer assessing *B. duttonii* colonization in its tick vector, *O. moubata* (Burgdorfer, 1951). In that study, he showed that a high density of spirochetes was found around ganglia and in the salivary glands of the tick (Burgdorfer, 1959). Interestingly, Burgdorfer also showed that the transmission of RF *Borrelia* occurred not only via a tick bite, but also through contamination from infected coxal fluid (Burgdorfer, 1951, 1959).

As efforts have focused on generating spirochete isolates from the field, we are gaining refined insights into vector competence of TBRF spirochetes. For example, multilocus sequencing of ~50 *B. hermsii* isolates distinguished two genomic groupings, GGI and GGII. A series of tick acquisition studies was performed to determine whether co-infection of GGI and GGII impacted vector colonization and subsequent transmission frequencies from *O. hermsi* (Policastro et al., 2013). Policastro and colleagues first infected cohorts of ticks with either GGI or GGII spirochetes. After molting, they fed ticks again to allow them to become infected with the opposite genomic group. Finally, in the third tick feeding, individual ticks were assessed to determine the impact of superinfection in transmission. Interestingly, ticks that were first infected with GGII then GGI transmitted only GGII spirochetes. However, prolonging the interval between the second and third feeding resulted in co-transmission of GGI and GGII spirochetes (Policastro et al., 2013). Thus, transmission of different populations of *B. hermsii* depends upon the order of tick acquisition and the interval between feedings.

Additional studies with *B. turicatae* further demonstrate that specific populations of TBRF spirochetes colonize disparate environments within the tick. *B. turicatae* producing GFP established a persistent infection for at least 18 months in the tick midgut and salivary glands (Krishnavajhala et al., 2017). While the population colonizing the salivary glands is the one responsible for transmission to the vertebrate host (Boyle et al., 2014; Schwan and Hinnebusch, 1998), the role of the midgut population remains unclear. Given the rapidity of *Ornithodoros* feeding and TBRF spirochete transmission, there is an insufficient time for the midgut population to be transmitted through the saliva. There are at least two potential roles for the midgut population. First, it may be involved with spirochete replication in the midgut after infected ticks feed. Alternatively, the midgut population may have a role in transovarial transmission to developing eggs.

Advances in high-throughput methodologies have begun to provide insight into the selective pressures and molecular mechanisms of vector colonization by TBRF spirochetes. Mans and

colleagues seminal work produced the first salivary gland transcriptomes of *Ornithodoros* ticks, reporting the identification of immune regulators that may aid in tick feeding and TBRF spirochete transmission (Mans et al., 2008a; Mans et al., 2008b). Recent studies on the proteome of fed and unfed *O. erraticus* midguts indicated the production of proteins involved with antioxidant defenses (Oleaga et al., 2015). These findings were expanded further with the salivary gland transcriptome of *O. turicata* (Bourret et al., 2019). The expression of 57 genes involved in oxidant metabolism or antioxidant defenses were identified. Of these, the genes encoding glutathione peroxidase, thioredoxin peroxidase, manganese superoxide dismutase, copper-zinc superoxide dismutase, and catalase were further evaluated in fed and unfed midguts and salivary glands. These findings indicated that the midguts and salivary glands of *O. turicata* were highly nitrosative and oxidative, respectively. Interestingly, the work also determined that *B. turicatae* was hyper-resistant to reactive oxygen species compared to Lyme disease (LD)-causing spirochetes (Bourret et al., 2019). Collectively, omics studies have begun to provide insight into TBRF adaptation within the tick vector; however, additional work is needed to understand the mechanisms utilized by the pathogens in the tick-mammalian infectious cycle.

As molecular tools have improved and become available for genetically manipulating TBRF spirochetes, the events of vector colonization are becoming clearer. The most significant advance on the molecular interactions between the arthropod vector and *Borrelia* was performed with *O. hermsi* and *B. hermsii*. Schwan and Hinnebusch began to unravel the intricacies of vector colonization and transmission with the identification of the variable tick protein (Vtp) (previously denoted pIc, VmpC or Vsp33) (Schwan and Hinnebusch, 1998). They demonstrated that Vtp was preferentially produced by spirochetes in the salivary glands of unfed *O. hermsi* ticks and that its production could be induced *in vitro* by lowering the incubation temperature to 23° C. Interestingly, they reported that when spirochetes producing Vtp enter a new mouse host from the tick, they reverted back to the mammal-associated variant they last manifested in the mouse host (Schwan and Hinnebusch, 1998). Raffel and co-workers genetically inactivated the *vtp* locus and it became clear that the protein is involved with preadapting *B. hermsii* for mammalian entry (Raffel et al., 2014). Mutants lacking Vtp were able to colonize the salivary glands of *O. hermsi* ticks but failed to establish an infection in mice after tick feeding (Raffel et al., 2014). While the function of Vtp is unclear, the protein is homologous to the Vsp proteins of RF *Borrelia* spp. (see below) and OspC of Lyme borreliosis species (Carter et al., 1994; Marconi et al., 1993). Interestingly, the finding that the Vtp proteins from different *B. hermsii* strains are highly polymorphic suggests positive selection involving antibody responses or for the need to survive in different biological niches like rodents, canines, etc. While Vtp is essential in the establishment of early mammalian infection, less is known regarding the molecular mechanism of persistent vector colonization.

Transcriptional studies have been implemented to begin to understand how the TBRF spirochetes establish an infection in the tick vector. Work in *B. turicatae* suggested that the linear megaplasmid is primarily involved in vector colonization and/or initiating mammalian infection (Wilder et al., 2016). *B. turicatae* was grown under ambient temperature mimicking the tick and 67% of the genes localized on the linear megaplasmid were upregulated in these conditions compared to spirochetes isolated from infected murine blood. The expression

of a cluster of genes on the 3' end of the plasmid was investigated further in the tick because they were predicted to code for surface proteins. While the results demonstrated up-regulation in infected ticks compared to infected blood, the proteins are of unknown function. With advances in genetic systems for TBRF spirochetes, the identification of genes essential in vector colonization is now possible (Battisti et al., 2008; Lopez et al., 2013b).

RF *Borrelia* speciation: Old and New World borreliosis

Currently, there is no systematic way to classify RF *Borrelia*; nomenclature has been subject to change, and differences exist between the speciation of Old and New World pathogens (Figure 1). A comprehensive description of classified RF *Borrelia* species is found in the *Borrelia* chapter of Bergey's Manual of Systematics of Archaea and Bacteria as well as in the recent review by Talagrand-Reboul (Barbour and Schwan, 2018; Talagrand-Reboul et al., 2018). Well characterized Old World species include *B. duttonii* and *B. crocidurae*, which were named after Dr. Joseph Everett Dutton and the vertebrate host in the genus *Crocidura*, respectively. The Old World species, *B. recurrentis* (LBRF), was speciated based on its biology in the human host.

The speciation of New World RF spirochetes has been somewhat more systematic and primarily based on the species tick vector. For example, *B. hermsii*, *B. parkeri*, and *B. turicatae* were originally speciated according to their argasid vectors, *O. hermsi*, *O. parkeri*, and *O. turicata*. This method of speciation was based on the observed vector specificity of TBRF spirochetes. However, discrepancies do exist with other New World species. For example, *B. mazzottii*, which was detected in *O. talaje* from northern Mexico and likely distributed in the southern United States and other regions of Latin America, was named after Dr. Luis Mazzottii (Davis, 1956; Lopez et al., 2016).

Historically, confusion has surrounded the speciation of RF *Borrelia* and their tick vectors in Central and South America. For example, it was not clear whether *O. talaje*, *O. rudis*, or *O. venezuelensis* were independent species, and this led to the uncertainty in the speciation of their respective RF spirochetes (Davis, 1955). As the ambiguity regarding the tick vectors was clarified, it became apparent that *Borrelia neotropicalis* was synonymous with *B. venezuelensis* (Davis, 1955). Within the last 15 years, significant advances have been made toward identifying and speciating *Ornithodoros* ticks from Central and South America. Venzal and coworkers provided detailed morphological characteristics of larval *Ornithodoros* ticks and demonstrated the importance in characterizing this developmental stage for speciation (Venzal et al., 2008). Recently, Munoz-Leal and colleagues identified *O. rudis* in Brazil and this resulted in the first isolation of a TBRF spirochete from the continent (Munoz-Leal et al., 2018). Given the historic association of *B. venezuelensis* with *O. rudis*, this novel *Borrelia* isolate was designated *B. venezuelensis*. Interestingly, the molecular characterization of this spirochete indicated that it was most closely related to the North American species, *B. turicatae*.

Speciating TBRF spirochetes in Eurasia has posed similar challenges. This is primarily because a few loci are sequenced for speciation, while genetic, geographical, ecological, and biological factors are not considered when designating a new species of TBRF spirochete.

The need for a refined systematic classification of TBRF spirochetes from Eurasia and the Middle East was demonstrated recently with the first isolation of *B. caucasica* from Ukraine (Filatov et al., 2020). Filatov and co-workers performed a phylogenetic analysis of *B. caucasica* comparing several single loci. The results indicated that, depending on the locus sequenced (16S rRNA, flagellin, and DNA gyrase), this species could have been designated as *B. hispanica* or *B. persica*. However, the spirochete was speciated as *B. caucasica* given the tick vector from which it was isolated. Moreover, while the intragenic spacer sequence (IGS) was not evaluated in that study, there is accumulating evidence that sequencing this locus may provide the best resolution for speciation of TBRF spirochetes (Bunikis et al., 2004; Cutler et al., 2010; Nieto and Teglás, 2014; Schwan et al., 2007).

Clinical presentation- the crisis phenomenon and the Jarisch-Herxheimer reaction

The clinical hallmark of RF is periods of very high fever spaced by periods of relative well-being, i.e., the recurrent fever episodes. The incubation period is between 5-8 days for LBRF, but varies much more with TBRF (Southern and Sanford, 1969). The reported mortality rates for untreated RF differ (Barbour et al., 2006), with the largest variability seen in LBRF. The high mortality was probably caused by factors associated with wars, famine, and situations within refugee camps, i.e., conditions of malnutrition and insufficient health care. Patients with RF usually complain of headache, backache, muscle pain, arthralgia, and abdominal pain. Sometimes, the clinical examination reveals jaundice and tenderness over the liver and spleen. A gradual decline of general fitness, with extreme weakness and weight loss can occur if successive relapses occur without adequate treatment (Goodman et al., 1969). If death occurs during RF, it is most common during a crisis episode or within hours after the start of antibiotic therapy. The crisis phenomenon results from the rapid disintegration of spirochetes within the blood stream because of the increase of neutralizing antibodies. Antibiotic treatment of spirochetal infections provides a similar effect, which was termed the Jarisch-Herxheimer reaction (J-HR) after its first description by the dermatologists Adolf Jarisch and Karl Herxheimer. They both observed this reaction during antimicrobial treatment with mercury in patients with secondary syphilis. Interestingly, Balfour (1911) observed that chickens experimentally infected with *B. anserina* also produced an “artificial crisis” after treatment with Salvarsan (arsenic compound 606). Treatment of both LBRF and TBRF with antibiotics was shown to commonly result in the J-HR (Bryceson, 1976; Bryceson et al., 1972; Warrell et al., 1983; Webster et al., 2002). Several studies have tried to resolve if the type of antimicrobial used is important for the generation and severity of J-HR. It is logical to envision that a bactericidal antibiotic is more prone to induce a J-HR response as large amount of cell fragments are released from the bacteria. However, there is still no conclusive result differentiating the J-HR response of a bacteriostatic from a bactericidal antibiotic (Butler, 2017). With the appearance of either neutralizing antibodies or the administration of antibiotics, spirochetes in the blood are lysed, releasing proinflammatory bacterial cell fragments into the circulation. Within a couple of hours after initiation of antibiotic treatment, the patient often presents with acute symptoms, including headache, rigors, malaise, hypotension, and sweating. Subsequently, symptoms might even worsen with an increase of temperature by 1-2° C, tachycardia,

and a rise in blood pressure. This period of worsening symptoms occurs over the next couple of hours, followed by diaphoresis, exhaustion, a decline in temperature, hypotension, and leukopenia. This apparent shock-like condition, with dilated vasculature and increased oxygen demands with fever (Warrell et al., 1983), may be exacerbated by myocardial dysfunction if the spirochetes have invaded the heart.

Given the similarity of J-HR to septic shock, early clinical observations predicted endotoxins to be the cause of the J-HR phenomenon (Bryceson et al., 1972). However, *Borrelia* spirochetes have an outer membrane architecture different from other Gram-negative bacteria and lacking endotoxin, i.e., LPS is not present (Ben-Menachem et al., 2003; Takayama et al., 1987). Instead, spirochetes contains ample amounts of glycolipids (Ben-Menachem et al., 2003; Hossain et al., 2001; Schroder et al., 2008) as well as a large amount of surface located lipoproteins. Another hypothesis about the crisis was that it was attributable to antigen-antibody complexes (Galloway et al., 1977), but changes in complement levels were not detected in patients with LBRF (Warrell et al., 1983). A more likely explanation for J-HR and the crisis is the action of liberated spirochetal lipoproteins on the toll-like 2 receptors (TLR2) of macrophages and other cells (Scragg et al., 2000; Vidal et al., 1998). In patients with RF, the levels of tumor necrosis factor (TNF- α), interleukin (IL)-6, and IL-8 rose several fold over pre-treatment levels during the J-HR in patients treated for LBRF (Negussie et al., 1992). Importantly, in a clinical trial, treatment with anti-TNF antibodies before the penicillin therapy reduced the severity of J-HR (Fekade et al., 1996). In mice experimentally infected with *B. turicatae* or *B. hermsii*, serum concentrations of the anti-inflammatory cytokine IL-10 reach high levels (Crowder et al., 2016; Londono et al., 2008). In patients with LBRF, IL-10 was highly elevated, however, treatments with recombinant IL-10 neither prevented J-HR nor inhibited the elevation of TNF, IL-6 or IL-8 (Cooper et al., 2000).

Mammalian infection: RF *Borrelia* pathogenesis

The different periods of time spent feeding by soft and hard ticks (minutes versus days, respectively) clearly separates the biology of transmission of RF *Borrelia* and LD *Borrelia*. Under these very restricted time periods of transmission, a single spirochete may be enough to initiate an RF infection in experimental animals (Beunders and Van Thiel, 1932; Schuhardt and Wilkerson, 1951; Stoenner et al., 1982). The bacteria multiply in the blood at an estimated rate of one cell division every ~6 hours until they number between 10^6 to 10^7 , sometimes as many as 10^8 , per milliliter of blood at their peak (Figure 2) (Cadavid et al., 1994; Crowder et al., 2016; Schuhardt and Wilkerson, 1951; Stoenner et al., 1982). Cultivable RF species achieve similar generation times in broth medium, such as BSK II (Barbour, 1986). The interval between relapses in experimental infections is usually 2 to 7 days. Untreated LBRF tends to have fewer relapses than does TBRF (Bodman and Stewart, 1948; Borgnolo et al., 1993; Brown et al., 1988; Bryceson et al., 1970; Garnham et al., 1947; Salih et al., 1977; Sundnes and Haimanot, 1993). The reason for this discrepancy in the number of consecutive relapses is not known, but the finding that *B. recurrentis* have a reduced genome compared to *B. duttonii*, suggests that the former could have lost genetic traits related to prolonged relapsing (Elbir et al., 2014; Lescot et al., 2008).

The incubation period between exposure and onset of fever or other signs of illness is from 3 to 12 days. During relapses, the number of spirochetes in the blood increases again, but usually not to the same density as during the first episode of spirochetemia. The lower concentration of cells in a relapse does not appear to be due to attenuated virulence of the relapse serotypes. Russell described the relapse populations as being as virulent as the “attack” serotype in naïve animals (Russell, 1936). Moreover, Crowder *et al.* observed that the relapse population of *B. hermsii* increased more slowly than the serotype that initiated the infection in mice; however, when each serotype was used to infect naïve mice, their growth rates were indistinguishable (Crowder *et al.*, 2016). The existence of this growth retardation was corroborated by the finding that changes in host factors, involving the innate immune responses, could influence the growth rate during infection (Crowder *et al.*, 2016). Further evidence for this was the observation that, during co-infection of experimental animals with a RF *Borrelia* species and an African trypanosome species, the population growth of the parasite was slower than during single infections (Felsenfeld and Wolf, 1973).

The growth rates and maximum cell densities of *Borrelia* species vary by species and environmental conditions. Most notably, TBRF species and *B. recurrentis* differ by a factor of 1,000 or more in the cell densities they achieve in the blood of immunocompetent as well as immunodeficient mice (Barbour and Bundoc, 2001; Crowder *et al.*, 2016; Sadziene *et al.*, 1993). However, during *in vitro* cultivation in the same type of medium, representative TBRF spirochetes and *B. recurrentis* have similar growth rates and reach stationary cell densities as high as $\sim 10^8$ per milliliter (Barbour, 1988; Cadavid *et al.*, 1994; Crowder *et al.*, 2016; Pennington *et al.*, 1997). Also, expression profiles analyzed by genome-wide arrays revealed little difference between cells of *B. hermsii* harvested from BSK II broth medium or in the blood of mice, when the temperature and pH of the broth approximated that of blood, and cell densities at the time of collection were comparable (Zhong and Barbour, 2004). Given these observations, genetic differences in metabolic pathways or other inherent capabilities plausibly account for observed differences between TBRF and LBRF species in peak burdens in the blood. Four pathways that differ between the two groups of species are in the acquisition of glycerol-3-phosphate (Bacon *et al.*, 2004; Schwan *et al.*, 2003; Schwan *et al.*, 1996), pyrimidine biosynthesis (Zhong *et al.*, 2006), purine salvage (Barbour *et al.*, 2005; Lescot *et al.*, 2008; Pettersson *et al.*, 2007), and glutamate-arginine-proline biosynthesis (Lescot *et al.*, 2008).

Greater persistence in the blood presumably increases the fitness of RF *Borrelia* for transmission from an infected vertebrate to a naïve set of tick vectors (Barbour and Restrepo, 2000). However, RF spirochetes also leave the vascular compartment through the endothelium to invade the central nervous system, the eye, the liver, testes, the placenta, and other organs and tissues (Cadavid and Barbour, 1998; Cadavid *et al.*, 2001; Nordstrand *et al.*, 2001; Shamaei-Tousi *et al.*, 2001). The borreliae survive in these extravascular sites when they have been eliminated from the blood (Cadavid *et al.*, 1993; Cadavid *et al.*, 2006; Kazragis *et al.*, 1996; Larsson *et al.*, 2008). Persistence in extravascular niches may also serve to enhance transmissibility, because invasion of the blood from these sites can recur under conditions of immune suppression (Larsson *et al.*, 2006b). Also, Larsson and coworkers suggest certain immune privileged sites can serve as a location for silent

infections that provide a reservoir for reactivation of infection as a result of external signals (Larsson et al., 2006b; Larsson et al., 2008; Lundqvist et al., 2010).

In animal models, tissue invasion appears to be facilitated by recruitment of plasminogen to the borrelial cell surface. Once converted to enzymatically active plasmin, local degradation of extracellular matrix barriers in conjunction with efficient spirochetal motility enable spread to distant organs. Consequently, in plasminogen-deficient mice, *B. crocidurae* invasion of brain and kidney was delayed (Nordstrand et al., 2001). Another mechanism of tissue invasion occurs indirectly because of erythrocyte rosette formation and microemboli-mediated hemorrhage (Figure 3). In experimental rats, pressure-induced capillary rupture leads to leakage of aggregates of spirochetes and erythrocytes into adjacent tissue (Shamaei-Tousi et al., 2001).

When spirochetes are found in tissues, they generally are found in the interstitial spaces between cells, except when they have been engulfed by phagocytes. There is no compelling evidence that RF borreliae are facultative, let alone obligatory, intracellular pathogens. The pathologic findings and subsequent identification of RF spirochetes are mainly from autopsies of fatal human cases and from histologic examinations of experimentally-infected animals (Bryceson et al., 1970; Cadavid and Barbour, 1998; Southern and Sanford, 1969). Extracellular spirochetes were demonstrated in tissues, often in perivascular locations (Cadavid et al., 2001; Duray, 1987; Pennington et al., 1997). The liver and spleen were often enlarged and showed inflammation, with microabscesses in the spleen (Anderson and Zimmerman, 1955; Judge et al., 1974b; Thomas et al., 2002). The microabscesses, areas of necrosis, and petechiae may be the consequence, in part, of microemboli of spirochetes and blood cells, and transendothelial migration of neutrophils (Shamaei-Tousi et al., 2000; Shamaei-Tousi et al., 1999).

Intracranial hemorrhage has been observed in both LBRF and TBRF (Anderson and Zimmerman, 1955; Babes, 1916; Dewar and Walmsley, 1945; Judge et al., 1974d; Thomas et al., 2002). When spirochetes in the brain were noted, they were more common around vessels and adjacent to leptomeninges (Anderson and Zimmerman, 1955; Cadavid et al., 2001; Garcia-Monco et al., 1997). Although cardiac abnormalities are not usually associated with RF, myocarditis with the presence of numerous spirochetes has been demonstrated in experimental infections of both TBRF and LBRF (Breitschwerdt et al., 1996; Cadavid et al., 1994; Judge et al., 1974d; Londono et al., 2005; Wengrower et al., 1984). In experimental *B. turicatae* infection, the severity of illness correlated with the numbers of spirochetes in the blood (Pennington et al., 1997).

In the blood of infected patients, leukocyte counts are usually in the normal range or only slightly elevated (Parsons, 1947). Leukopenia and platelet counts below 50,000/mm³ may occur during the crisis. Hemorrhagic complications, such as epistaxis, are common in LBRF (Dennis et al., 1976; Perine et al., 1971). The erythrocyte sedimentation rate often is moderately elevated. There may be laboratory evidence of hepatitis, such as elevated serum concentrations of aminotransferases and prolonged prothrombin and partial thromboplastin times (Barbour, 2011). In the cerebrospinal fluid there may be mildly elevated numbers of mononuclear cells and protein levels, but glucose levels are typically normal.

Neurotropism

Both RF and LD *Borrelia* affect the neurological system, resulting in various neurological and pathognomonic symptoms. LBRF often presents a more severe course, but TBRF, at least for some species of *Borrelia*, such as *B. duttonii* and *B. turicatae*, results in a higher frequency of neurologic complications, including symptoms like facial palsy, weakness, radiculopathy, and, occasionally, stupor or coma (Cadavid and Barbour, 1998). Also, neuropsychiatric symptoms with psychotic events, including confusion, anxiety and hallucination has been described (Aubin et al., 1947). Neurotropism has also been observed experimentally in mammals (Cadavid and Barbour, 1998; Cadavid et al., 1993; Dubois and Pearson, 1939; Kazragis et al., 1996; Larsson et al., 2006b; Shamaei-Tousi et al., 1999; Velu et al., 1930). RF spirochetes are seen in the brain of infected mice as early as the second day after inoculation (Andersson et al., 2007). *B. crocidurae* cells remain actively growing in the brain and persist there in numbers of ~1000 per mouse brain (Larsson et al., 2006b; Larsson et al., 2008). A similar phenomenon may also occur in the tick vector, as *B. duttonii* spirochetes accumulate around the central ganglion cells of *O. moubata* (Burgdorfer, 1951).

In both human cases and experimental infections of rodents, neurotropism is more common following infection by some species than by others (Cadavid and Barbour, 1998). For example, the greater incidence of neurologic involvement in cases of RF due to *B. duttonii* in Africa and *B. turicatae* in North America has long been noted (reviewed by Cadavid and Barbour, 1998). Also, *B. duttonii* appears to cause persistent brain infection that could be reactivated by immune suppression as well as with concomitant infection with malaria (Lundqvist et al., 2010). These findings also present the importance of humans as reservoir for certain RF *Borrelia* species and a relevant biological signal for reactivation of silent RF borreliosis. A newly described species of RF *Borrelia* in Spain appears to be particularly neurotropic (Garcia-Monco et al., 1997). This isolate was later taxonomically defined as *B. hispanica* (Toledo et al., 2010).

Neurotropism also varies between variants of a given strain. Cadavid et al. demonstrated in an experimental animal model that some variants of a *B. turicatae* strain were present in higher numbers in the brain compared to other variants of the same strain (Cadavid et al., 2001; Cadavid et al., 1997; Cadavid et al., 1994). However, neurotropism is not necessarily associated with greater virulence as is evident from the finding of residual brain infection up to several months for RF *Borrelia* such as *B. duttonii* (Larsson et al. 2006). A neuroinvasive variant of *B. turicatae* had a lower fatality rate among experimentally infected mice than an isogenic variant with a lower potential for invasion of the brain (Cadavid et al., 1994; Pennington et al., 1997). Moreover, the noticeable neurological disease symptoms in certain cases of RF is aggravated by the massive influx of immune cells, including T cells that are important for neuropathogenesis (Liu et al., 2010).

Not only do neurological complications impact treatment, they also can lead to a misdiagnosis of Lyme disease; consequently, caution should be exercised when diagnosing LD in historically nonendemic regions. For example, in Mexico, where *B. turicatae* is distributed (Cooley and Kohls, 1944; Donaldson et al., 2016), serological responses in patients presenting with neurological symptoms (facial palsy) were screened for LD (Gordillo-Perez et al., 2017; Gordillo-Perez et al., 2018). These tests used whole

B. burgdorferi protein lysates or commercially available kits. Unfortunately, given the serological cross reactivity with patients infected with RF and LD, assays should have targeted antigens specific for TBRF species, like glycerophosphodiester phosphodiesterase (GlpQ) or the *Borrelia* immunogenic protein A (BipA) (Lopez et al., 2010; Schwan et al., 1996). Moreover, there is increasing evidence that serological responses generated during *B. turicatae* infections cross-react with commercially available LD assays (Gettings et al., 2019). Interestingly, when TBRF-specific antigens are utilized in Mexico, serologic studies point to ongoing circulation of TBRF spirochetes (Vazquez-Guerrero et al., 2019).

Adhesins

Adherence to host cells is an important bacterial virulence mechanism. This enables microbes to gain a foothold on surfaces and resist mechanical removal. Binding of RF *Borrelia* to cellular and soluble blood components, such as integrins and glycoaminoglycans, has been described (Alugupalli et al., 2001; Magoun et al., 2000). However, in the absence of knock-out mutants, the functional roles of these associations for infectivity and pathogenesis still remain to be confirmed. The function as well as mechanism for these host ligands in mediating adherence of spirochetes to host tissues and cells are likely, and would be consistent with, similar, more established pathogenetic mechanisms of other pathogens, including LD *Borrelia* (see Radolf and Samuels, 2021).

Some species of RF spirochetes have been noted to form aggregates or rosettes with platelets or erythrocytes in the blood of infected animals (Alugupalli et al., 2003b; Burman et al., 1998; Mooser, 1958). Figure 2 shows a rosette of spirochetes and erythrocytes. Rosettes are best viewed when spirochete density in the blood is at its highest. For *B. crocidurae*, aggregation of spirochetes and erythrocytes into rosettes appears to delay immune clearance; antibodies appear and rise in the blood more slowly than is observed in experimental infection with *Borrelia* spp. that do not aggregate erythrocytes (Burman et al., 1998). Erythrocyte and spirochete rosettes also activate endothelial cells and cause microvascular thrombi and emboli that lead in turn to pathologic changes in tissues (Shamaei-Tousi et al., 2000; Shamaei-Tousi et al., 2001; Shamaei-Tousi et al., 1999). Erythrocyte aggregation has also been noted in experimental infections with *B. duttonii* (Takahashi et al., 2000).

B. hermsii attachment to platelets occurs via the integrin $\alpha_{IIb}\beta_3$ (Alugupalli et al., 2001; Alugupalli et al., 2003b), and *B. crocidurae* binds to erythrocytes via the neolactoglycan lacto-N-neotetraose (Guo et al., 2009). The bacterial ligands for these interactions have not yet been identified for RF species. One possible spirochete ligand is P66, or Oms66, an outer membrane protein porin of both LD and RF *Borrelia* species (Bunikis et al., 1998; Bunikis et al., 1995; Shang et al., 1998; Skare et al., 1997). P66 of *B. burgdorferi* has been reported to be an adhesin for the β_3 -integrin and as such important for dissemination in the infected host (Coburn et al., 1999; Coburn and Cugini, 2003). Two other possible adhesins are products of open reading frames BH0512 and BH0553 of *B. hermsii*. Both proteins are characterized by multiple repeating domains. Indirect evidence for the adhesin function of these proteins was the finding that antisera to these proteins diminished attachment of *B. hermsii* to erythrocytes (Guyard et al., 2005). One of the surface lipoproteins of *B. turicatae*,

Vsp1, binds to glycosaminoglycans to a greater degree than other Vsp proteins of the same species or of *B. hermsii* (Magoun et al., 2000; Zuckert et al., 2004).

Serum, complement, and phagocyte resistance

Early investigators noted that RF *Borrelia* were susceptible to non-immune sera from some species of animals but resistant to the sera from other species. For example, *B. duttonii* survived in fresh sera from mice, guinea pigs, rabbits, pigeons, chickens, and horses but not in fresh sera from cow, goat, pig, or sheep (Cuboni, 1929). If the latter sera were first heat-inactivated, viability of the spirochetes was retained, an indication that the alternative complement pathway was involved. This is similar to the differential serum resistance demonstrated by LD *Borrelia* species (see Radolf and Samuels, 2021). Factor H is a major complement regulator in the serum that prevents C3b formation by accelerating decay of the C3 convertase and by facilitating the irreversible inactivation of C3b. Bacterial acquisition of factor H could enable complement evasion by inactivating C3b or preventing its deposition and, thus, phagocytosis. Some RF *Borrelia* species, including *B. hermsii*, but neither *B. anserina* nor *B. coriaceae*, display factor H binding activity (McDowell et al., 2003; Meri et al., 2006). This activity was attributed to plasmid-encoded proteins of ~20 kDa called FhbA proteins (Hovis et al., 2004). *B. hermsii* also binds to factor H-like protein 1, an alternative splice variant of factor H (Hovis et al., 2006; Lopez et al., 2008). FhbA elicits IgM and IgG responses during experimental infection of mice (Colombo and Alugupalli, 2008). The BhCRASP-1 protein of *B. hermsii* is a 20-kDa outer membrane lipoprotein that is orthologous to the CRASP protein BBA68 of *B. burgdorferi*. BhCRASP-1 appears to bind factor H as well as plasminogen (Rossmann et al., 2007). Finally, *B. duttonii* and *B. recurrentis* are reported to associate with C4b-binding protein in the serum (Meri et al., 2006). Several subsequent studies have identified additional complement factors, including FhbA, HcpA and CihC, which affect different regulators of complement activation. These factors make RF *Borrelia* resistant to killing by complement and provide other important virulence properties (Brenner et al., 2013; Fine et al., 2014; Grosskinsky et al., 2009; Grosskinsky et al., 2010; Schott et al., 2010; Woodman et al., 2009)

Antigenic variation

Vsp and Vlp proteins—The steady increase of spirochetes in the blood, followed by a rapid and deep drop in their numbers, and then their reappearance in the blood (Figure 2) are the direct result of the bacterium's unique capacity for antigenic variation. Investigators as early as 1918 noted that each new crop of spirochetes differed antigenically from the population it succeeded and the population to succeed it (Jancso, 1918; Meleney, 1928; Russell, 1936). The individual variants of a given strain are called serotypes. By definition, there is little or no antigenic cross-reactivity between serotypes with polyclonal antisera obtained either from infected patients or immunized animals (Coffey and Eveland, 1967; Stoenner et al., 1982). A serotype that had disappeared from an RF *Borrelia* population under the force of immunity could reappear again if even a single of the surviving population was transmitted to a naïve animal (Barbour, 1987); thus the hereditary information conferring serotype identity is not lost from a cell. This feature of RF indicated that antigenic variation was not simply an accelerated evolutionary process with selection for a succession of mutants, as typified by influenza A virus.

B. hermsii, a North American species that is most commonly encountered in the mountainous areas of the West (see Radolf and Samuels, 2021), has been the subject of the most extensive studies of antigenic variation (Barbour, 2002). Several serotypes of the HS1 strain of this species were derived from a single cell injected into a mouse (Barbour and Stoenner, 1985; Restrepo et al., 1992; Stoenner et al., 1982). Stoenner raised specific antisera in mice to each of 24 *B. hermsii* serotypes and used these reagents to study the relapses that occurred during experimental infections (Stoenner et al., 1982). With this battery of antisera in immunofluorescence assays of blood smears from infected animals, he identified the serotypes of ~80-90% of the variants that appeared during relapses in hundreds of mice. Examination of the bulk of *B. hermsii* chromosomal sequences and plasmids revealed additional *vsp* and *vlp* genes for 59 different serotypes (Dai et al., 2006).

Serotype identity is determined by expression of one of a repertoire of polymorphic lipoproteins (Barbour et al., 1982) (see Radolf and Samuels, 2021). Similar to other bacterial lipoproteins, the processed N-terminus is acylated and anchors the protein in the outer membrane (Carter et al., 1994; Scragg et al., 2000). The known set of about 40 proteins, for which expression is documented, is divided about equally between two different families: variable large proteins (Vlp) of about 36 kDa and variable small proteins (Vsp) of about 20 kDa (Barbour et al., 1983; Burman et al., 1990; Carter et al., 1994; Restrepo et al., 1992). These are encoded by *vlp* and *vsp* genes, respectively. Full-length Vsp and Vlp proteins may have near-identical N-terminal sequences, but after the conserved signal peptide has been cleaved off, the processed proteins are highly divergent. Vsp and Vlp proteins are orthologs of the OspC proteins and VlsE proteins of LD-causing *Borrelia* species, respectively (Carter et al., 1994; Marconi et al., 1993; Zhang et al., 1997). Another ortholog of Vsp is the Vtp protein that is expressed in ticks (see above).

Figure 4 represents the structure of a Vsp protein dimer. The Vsp and Vlp proteins are not obviously homologous at the primary sequence level, but they resemble each other at the conformational level, being predominantly α -helical in secondary structure (Burman et al., 1990; Zuckert et al., 2004) and having similar folds (Lawson et al., 2006). Vsp proteins exist as dimers (Zuckert et al., 2001; Zuckert et al., 2004) and in this configuration resemble the deduced structure of a single Vlp protein (Eicken et al., 2002). The C-terminus is not anchored but is close to N-terminus near the cell's surface for both Vsp and Vlp proteins. The loops between the four α -helices of each monomer are exposed to the environment as ligands or epitopes. The first and fourth alpha helical chains are conserved in sequence, while the second and third chains and loops between the chains comprise four variable regions of the molecule. The Vlp family of proteins is further divided into four sub-families with less than 70% sequence identity among them: α , β , γ , and δ (Burman et al., 1990; Dai et al., 2006; Hinnebusch et al., 1998; Restrepo et al., 1992). Different strains of *B. hermsii* from distant geographic origins in western North America have the same four sub-families (Hinnebusch et al., 1998).

The considerable diversity among the sets of *vsp* and *vlp* genes contained in each *Borrelia* cell is the consequence of multiple rounds of intragenic recombination among the alleles within each cell (Rich et al., 2001b). This process was likely preceded by horizontal transfer of alleles between strains, for otherwise there would not be a fund of heterogeneous

sequences upon which recombinational processes could operate. Horizontal transfer could be accomplished by the bacteriophage observed in association with *B. hermsii* (Barbour and Hayes, 1986), which may be homologous to the 32-kb circular plasmid prophages of *B. burgdorferi* (Eggers and Samuels, 1999; Stevenson et al., 2000; Zhang and Marconi, 2005). If an ancestor of RF species which possessed a single proto-*ospC/vsp* gene became merodiploid through acquisition of a second ortholog, then with subsequent rounds of recombination and gene duplications, the number of alleles might increase from two to several within a single lineage.

Genetic mechanisms for antigenic variation—Recognizing the cumulative studies of the biology, biochemistry, and immunology of RF, a modeler of the genetic mechanism of serotype-switching should account for the following features of the phenomenon, reviewed in (Barbour, 2002, 2003) (1) The variation is reversible; a serotype once cleared by an animal can appear again in another animal to whom the pathogen, even a single cell, is passed. (2) It is multiphasic, that is, involving three or more different antigens. (3) Serotype identity is conferred by a set of proteins, only one of which is expressed at a time. (4) Serotype-specific epitopes of the protein are located in different parts of the protein, not just in one region. (5) When there is an antigenic switch in a cell during infection, all or nearly all of the protein changes, not just a limited region of the protein, as in VlsE variation in *B. burgdorferi*. (6) Antigen switches are effectively random and spontaneous. In other words, they are not an effect of a change in the environment, such as inoculation into a mouse from culture medium or the appearance of neutralizing antibody in the blood (Stoener et al., 1982).

Reversibility is achieved by archiving the antigen genes at locations where they are silent and, thus, presumably not under direct selection by immune systems and other host factors (Kitten and Barbour, 1990; Meier et al., 1985; Plasterk et al., 1985). For an antigen gene to be expressed, it must be copied and moved to a site where it can be transcribed (Barbour et al., 1991a; Burman et al., 1990; Sohaskey et al., 1999). This site may be altered by subsequent DNA rearrangements, leading to loss of the DNA that was located there, but a copy of that gene remains in the archive for future activation. The archived genes and the expression site for the duplicated gene are located on linear plasmids of 28 to 32 kb in *B. hermsii* (Ferdows et al., 1996; Kitten and Barbour, 1990; Plasterk et al., 1985) and on plasmids of other sizes in other RF species (Lescot et al., 2008; Pennington et al., 1999; Tabuchi et al., 2002). There are multiple copies of each type of plasmid in each cell with the genomes distributed evenly within the approximately 20 μm long spirochetal cell (Kitten and Barbour, 1992). The number of copies varies due to growth and milieu, but the mean was calculated both *in vivo* and *in vitro* to be within the range of 16 to 22 genomes per cell (Kitten and Barbour, 1992).

Sequencing of the linear plasmids that bear *vsp* and *vlp* genes in *B. hermsii* further defined the organization and arrangement of the expression site and the several archival sites (Burman et al., 1990; Dai et al., 2006; Restrepo et al., 1992). There appears to be a complete or nearly complete *vsp* or *vlp* gene for each Vsp or Vlp protein that a spirochete is capable of expressing. The report by Dai et al. provides full physical maps of large regions of the plasmids (Dai et al., 2006). In brief, the archived *vsp* and *vlp* genes are, with

a few exceptions, found in clusters, usually in the same orientation, i.e., head-to-tail, but head-to-head at some locations. Several of the silent genes are full-length, that is, with a start codon and the signal peptide. Most of these complete genes at silent sites also have consensus ribosomal binding sequences and transcriptional start sites, but not discernible promoters. Other archived genes are lacking some sequence at the 5' end. Some *vsp*- and *vlp*-like sequences are likely pseudogenes; they are missing 3' ends and/or have indels with frameshifts. The expression site for *vsp* and *vlp* genes is located near the telomere of a 28-kb plasmid (Kitten and Barbour, 1990); it has a σ^{70} -type promoter (Barbour et al., 1991a) and an upstream T-rich region that appears to enhance transcription (Barbour et al., 1991b; Sohaskey et al., 1999). Similar arrangements of the repertoires of *vsp* and *vlp* genes have been found on linear plasmids of various sizes in the RF species *B. duttonii* and *B. recurrentis* (Lescot et al., 2008; Tabuchi et al., 2002; Vidal et al., 2002).

In *B. hermsii*, two mechanisms can account for all the observed features of antigenic variation (Figure 5). The first and most common mechanism is a non-reciprocal recombination between a linear plasmid with an array of silent, archived *vsp* and *vlp* genes and another linear plasmid with a transcribed *vsp* or *vlp* gene (Kitten and Barbour, 1990; Meier et al., 1985; Plasterk et al., 1985). In *B. hermsii*, as well as in *B. turicatae*, what is effectively a gene conversion results in the replacement of the expressed gene for a variable major protein at a site downstream from a promoter at an expression site (Barbour et al., 1991a; Cadavid et al., 1997; Pennington et al., 1999; Pennington et al., 1999). An archived *vsp* gene can replace an expressed *vlp* gene or vice versa. The boundaries for the recombination are regions of sequence identity between silent and expression sites around and flanking the extreme 5' and 3' ends of the expressed and silent *vsp* or *vlp* genes (Barbour et al., 2000; Dai et al., 2006). In some cases, recombination at the expression site occurs within a *vsp* or *vlp* gene (Dai et al., 2006; Kitten et al., 1993). However, if the resultant chimeric protein retained one or more specific epitopes of the original protein, it presumably would still be recognized by circulating antibodies elicited during the preceding spirochetemia (Barstad et al., 1985). In *B. hermsii*, the longest length of converted sequence that has been observed is about 2 kb (Kitten and Barbour, 1990; Restrepo et al., 1992). In *B. turicatae*, a more extensive gene conversion involving 10 or more kb downstream of the promoter on the expression-site plasmid occurs (Pennington et al., 1999). The mechanism of intermolecular recombination between plasmids first demonstrated in Nearctic species, like *B. hermsii*, also seems to occur in Afro-Tropical and Palearctic species. Rearrangements incorporating long lengths of plasmids have also been noted in *B. duttonii* (Takahashi et al., 2000).

The second mechanism for expressing a different variable major protein is an intramolecular DNA rearrangement of the plasmid with the expression site (Figure 5). This mechanism has only been noted when there is another variable major protein gene just downstream of an expressed *vlp* gene (Restrepo et al., 1994). In such instances, the expressed gene and the non-transcribed downstream gene are in the same orientation. A deletion between short direct repeats at the 5' ends of each of the tandemly arrayed genes effectively excises the sitting *vlp* gene, and the downstream gene effectively takes the place of the deleted gene next to the promoter. After this deletion, the newly expressed gene is susceptible to small gene conversions that provide further diversity of variable membrane proteins (Restrepo and

Barbour, 1994), but the significance of this phenomenon for further immune avoidance is not known.

The activation of the *vtp* gene when *B. hermsii* is in the tick occurs by a mechanism that does not require recombination or transposition. This is a third mechanism: modification of transcripts by expression site switching (Figure 5). The *vtp* gene is located at another locus on a different plasmid than the expression site. At that location, *vtp* has its own promoter (Barbour et al., 2000; Carter et al., 1994). There is only one copy of *vtp* per genome. The mechanism of differential transcription between the *vtp* locus and the expression site locus is unknown.

Programming of antigenic variation—The process of switching starts in one of the 16 to 22 genome equivalents of the spirochetes, which means that during a certain period the cell is expressing more than one Vmp. This hybrid spirochete would be considered recessive until it becomes homozygous for the new serotype gene because of the antibody response generated against the prior Vmp (Crowder et al., 2017). Moreover, the overall serotype switch rate for *B. hermsii* is an estimated 10^{-4} to 10^{-3} per cell per generation. That is, by the time a given serotype reaches a population size of 1,000 to 10,000 cells in an animal, at least one cell of a different serotype has likely appeared in the population (Barbour and Stoenner, 1985; Stoenner et al., 1982). However, the rates for individual switches, e.g., from serotype 3 to serotype 7 or from serotype 17 to serotype 19, vary over about a thousand-fold range (Barbour et al., 2006; Barbour and Stoenner, 1985). The rates in each direction are not equivalent. For instance, switches from serotype 7 to serotype 19 are much less frequent than a switch from serotype 19 to serotype 7. These differences in switch rates among all possible antigenic variation events could account for the rough ordering of the appearance serotypes during individual infections that has been observed (Coffey and Eveland, 1967; Frank and Barbour, 2006; Stoenner et al., 1982)

The organization of the *vsp* and *vlp* genes on the plasmids provided clues to the possible mechanisms for the observed differences in frequencies of serotypes during the first relapse (Dai et al., 2006). The more frequent *B. hermsii* serotypes had Vsp or Vlp proteins that were encoded by genes with certain characteristics in their silent or archival locations (Barbour et al., 2006). One important characteristic was the extent of identity between the 5' end of archived *vsp* or *vlp* sequence and the 5' end of the expression site (Figure 6). This region of identity of approximately 50 nucleotides is called the UHS region and is the usual site for the upstream crossover during recombination (Dai et al., 2006). The second major variable was the spatial relationship of a given archived gene to an extragenic repetitive sequence of ~200 nucleotides called the DHS element, which directly follows some but not all archived genes and, when present, is at various distances downstream of a silent allele. The DHS contains the downstream crossover site for the recombination (Dai et al., 2006).

The serotypes that were most frequently observed during the first relapse of infection in laboratory mice were specified by *vsp* or *vlp* genes which, in their archived locations, had the following properties: (1) a UHS sequence that was identical or near-identical to the UHS at the expression site, and (2) a DHS element 50-200 nucleotides downstream. Archived *vsp* or *vlp* alleles that lacked both properties were rarely expressed during the first or

early relapses. Silent genes with either property were intermediate in frequency (Barbour et al., 2006). Presumably, possession of both (1) and (2) fostered recombination between the archived sequence and the expression site.

Immunity

Immunity in RF has been the subject of study since the late nineteenth century, as reviewed in (Barbour, 1987; Barbour and Restrepo, 2000). The relapse phenomena observed in experimental animals and in patients with neurosyphilis undergoing fever therapy with RF spirochetes provided evidence for early immunologists, such as Paul Ehrlich, of the fine specificity of the adaptive immune response. The role of immunity in bringing about clearance of spirochetes from the blood was demonstrated by findings that the initial spirochetemia of mice or rats can be prolonged by gamma irradiation (Barbour et al., 1983; Newman and Johnson, 1984), treatment with cyclophosphamide (Newman and Johnson, 1984; Stoenner et al., 1982), the severe combined immunodeficiency (SCID) phenotype (Cadavid et al., 1994), or splenectomy (Meleney, 1928).

Figure 2 shows the appearance of anti-*Borrelia* antibodies in response to infection and the proliferation of spirochetes. This process was thoroughly described in a study by Crowder and coworkers, which infected <10 cells of *B. hermsii* in a group of mice and obtained a spirochetal peak of $\sim 10^8$ followed by a clearance of spirochetes that coincides with the rise of variant-specific agglutinating antibodies (Crowder et al., 2016). This first immune response does not prevent the growth of a second wave of spirochetes. As Figure 2 suggests, adaptive immunity to RF species is largely, if not exclusively, variant-specific. If there is an immune response that provides protection against several antigenic variants of a strain or species of RF *Borrelia*, it has not to our knowledge been reported. This may be one reason why there has not been an effective vaccine against RF, although the agent has been known for over 120 years. Aristowsky and Wainstein immunized humans with one variant of a strain; this provided protection against homologous challenge, but not against heterologous variants (Aristowsky and Wainstein, 1929). The variant-specificity of immunity may also explain the paucity of useful serologic assays for confirmation of the diagnosis of RF or for seroepidemiologic surveys. The most promising subunit serologic assays for RF are based on the GIpQ and BipA proteins, which are found in RF *Borrelia* spp. but not in LD *Borrelia* spp. (Lopez et al., 2010; Lopez et al., 2013a; Porcella et al., 2000; Schwan et al., 1996).

In 1896, Gabritchewsky showed the importance of humoral immunity for clearance of spirochetes during RF when he demonstrated the lytic action of immune serum on *Borrelia* (Gabritchewsky, 1896). Novy and Knapp reported in 1906 that they passively protected animals against infection with immune serum (Novy and Knapp, 1906). Calabi subsequently found that serum taken at the time of clearance reduced the viable count of spirochetes in suspension by up to one million-fold (Calabi, 1959). The lesser importance of cellular immunity for resolution of infection was indicated by the findings that T cell deficient nude mice cleared spirochetemia as effectively as wild-type animals and that infection was cleared following reconstitution of lethally irradiated mice with B cells alone, but not T cells (Barbour and Bundoc, 2001; Newman and Johnson, 1984).

As the foregoing experiments would suggest, IgM antibodies from infected animals are alone sufficient to limit or eliminate infection in the blood (Alugupalli et al., 2003a; Arimitsu and Akama, 1973; Barbour and Bundoc, 2001; Connolly and Benach, 2001; Newman and Johnson, 1981, 1984). Within 24 to 48 hours after the spirochetes reach a peak of 10^6 to 10^7 organisms per milliliter of blood, they are undetectable by light microscopy in the blood of an immunocompetent animal. When antibodies are administered intravenously, the spirochetes are cleared from the blood within one hour (Barbour and Bundoc, 2001; Cadavid et al., 1993). As earlier empirical studies had suggested, the neutralizing antibody response is serotype-specific: the animal remains susceptible to other serotypes of the same strain or of different strains (Barbour, 1987; Barbour and Restrepo, 2000). Up to 10 different relapses have been recorded during experimental infections (Felsenfeld, 1965). Whether recovery is due to the development of antibodies to all or most serotypes during the infection's course or to the eventual rise of antibodies directed against antigens expressed by all serotypes is unknown. In endemic areas, newcomers or visitors appear to be at greater risk of symptomatic TBRF than long-term residents, an indication that long-lasting immunity to the local strain(s) may occur among humans.

Specific antibodies, even F_{ab} monomers, recognizing outer membrane proteins of *B. hermsii* and other RF species can kill the spirochetes in the absence of complement or phagocytes (Connolly and Benach, 2001; Connolly et al., 2004; Sadziene et al., 1994). The bactericidal action lies in the variable region (LaRocca et al., 2008), as it does for bactericidal antibodies directed to a surface protein of *B. burgdorferi* (Sadziene et al., 1994). Incubation of the antibody with *Borrelia* cells leads to bacterial lysis following outer membrane disruption and excessive bleb formation (LaRocca et al., 2008).

B cell memory commonly is dependent on stimulation by helper T cells. The fact that *Borrelia* clearance is not dependent on T cell function indicated that a different B cell type was responsible for generating bactericidal antibodies. B1b lymphocytes were shown to confer long-lasting immunity independent of a T cell response via rapid generation of *Borrelia*-specific IgM (Alugupalli et al., 2003a; Alugupalli et al., 2004). Induction of this response occurs by way of toll-like receptor stimulation and B cell antigen receptor activation (Alugupalli et al., 2007).

During infection, both experimental animals and human patients develop antibody responses against several different spirochetal components, including the periplasmic flagella and heat shock proteins. However, only antibodies directed against variant-specific proteins appear to be effective in controlling infection (Barbour and Bundoc, 2001; Cadavid et al., 1993; Stoenner et al., 1982). Moreover, a host response that eradicates the spirochetes of the predominant serotype from the blood is often not successful in clearing spirochetes from the brain, cerebrospinal fluid, or eye (Barbour, 1987; Cadavid and Barbour, 1998; Larsson et al., 2006b). Unfortunately, given the mechanisms of antigenic variation, targeting the Vmps in a vaccine is not practical.

An alternative approach to generating a vaccine against RF spirochetes would be to neutralize them during early mammalian infection, as they enter the host during tick feeding. Since it takes several generations for antigenic variation to commence, this approach

targets more conserved surface proteins that RF spirochetes produce while still colonizing the salivary glands. The efficacy of this approach was demonstrated initially in the *B. hermsii* model (Krajacich et al., 2015). Groups of mice were immunized with one of two antigenically distinct *vtp* variants and were subsequently challenged by tick bite (Krajacich et al., 2015). The animals were protected against homologous variant challenge, but not against heterologous challenge. Regardless, this study demonstrated that preventing the establishment of early mammalian infection is a promising approach toward generating a vaccine against RF spirochetes.

Other RF *Borrelia* species: Emerging diseases

Borrelia miyamotoi is a RF spirochete that, despite its discovery in 1995 (Fukunaga et al., 1995), has only recently been shown to be pathogenic to humans (Platonov et al., 2011). Unlike other RF *Borrelia*, *B. miyamotoi* is not transmitted by soft ticks or lice, which mostly reside in the Southern hemisphere, but instead by hard ticks of various *Ixodes* species that also serve as vectors for *B. burgdorferi* sensu lato (Wagemakers et al., 2015). The main *Ixodes* vectors for both *Borrelia* species live in the temperate climate zones of the Northern hemisphere: *Ixodes pacificus* and *Ixodes scapularis* (North-America), *Ixodes ricinus* (Europe and Asia) and *Ixodes pacificus* (Russia and Asia). In contrast to *B. burgdorferi* sensu lato, which is not transmitted from the female adult tick to her offspring (Steere et al., 2016), *B. miyamotoi* is efficiently transmitted transovarially (Han et al., 2019). Infection rates of nymphs, the *Ixodes* life stage that is most notorious to bite humans, differ for *B. miyamotoi* and *B. burgdorferi* sensu lato, with for instance average infection rates of *I. ricinus* nymphs with *B. burgdorferi* sensu lato of approximately 12% (Strnad et al., 2017) compared to approximately 2% for *B. miyamotoi* in Europe (Cutler et al., 2019; Wagemakers et al., 2015).

Similar to other *Borrelia* species, *B. miyamotoi* has a complex genome with one linear chromosome and a variable number of linear and circular plasmids (Hue et al., 2013; Kingry et al., 2017a; Kingry et al., 2017b; Kuleshov et al., 2018). Due to apparent genetic differences between isolates, the broader term *B. miyamotoi* sensu lato was proposed (Krause et al., 2015). As for other RF *Borrelia*, multiple plasmids encode different Vmps. Vsps and Vlps belonging to the α , β , γ , and δ sub-families, of which only one Vmp is present and transcribed at the expression site in the linear plasmid p41 in any moment in time in a given RF spirochete (Kuleshov et al., 2020). *B. miyamotoi*, similar to other RF *Borrelia* (Barstad et al., 1985; Plasterk et al., 1985) may be able to evade host immune responses by full Vmp gene switching (Wagemakers et al., 2016), in which the initial Vmp is replaced by a different archival full length Vmp gene that is subsequently expressed (Wagemakers et al., 2015). This could explain the occasional spirochetemia relapses in mice experiments (Wagemakers et al., 2016) and in humans (Platonov et al., 2011; Sarksyant et al., 2015a). This could also explain why SCID mice, lacking T and B cells, are able to develop continuous spirochetemia (Wagemakers et al., 2016). Phylogenetic analysis of the available (partial) genomes showed that there are genetic differences between clinical or tick isolates of *B. miyamotoi* sensu lato from Europe, Asia and North America, presumably related to differences in tick vectors in the respective geographical locations (Kuleshov et al., 2019). Importantly, *B. miyamotoi* sensu lato is resistant to killing by the human host

complement system (Wagemakers et al., 2014). This is, at least in part, mediated through a protein designated complement binding and inhibitory protein A (CbiA), also called Factor H binding protein, that interacts with the complement regulators Factor H, C3, C3b, C4b, C5, and C9 (Rottgerding et al., 2017). CbiA inhibits the activation of the classical pathway and assembly of the terminal complement complex (Rottgerding et al., 2017) and can bind human plasminogen (Nguyen et al., 2018).

Although human exposure to, and infection with, *B. miyamotoi* appears to occur regularly (Jahfari et al., 2016; Kadkhoda et al., 2017; Krause et al., 2014; Sarksyian et al., 2015b; Sato et al., 2018; Smith et al., 2019; Wroblewski et al., 2017), human *B. miyamotoi* disease (BMD) is relatively infrequent. Nonetheless, case reports and case series of human BMD have been described in Europe, Asia and North America, with a considerable amount of cases originating from Russia and the United States (Chowdri et al., 2013; Crowder et al., 2014; Hoornstra et al., 2018; Jiang et al., 2018; Jobe et al., 2016; Karan et al., 2018; Koetsveld et al., 2017b; Krause et al., 2013; Krause et al., 2016; Molloy et al., 2015; Platonov et al., 2011; Sarksyian et al., 2015a; Sarksyian et al., 2015b; Sato et al., 2014; Sudhindra et al., 2016; Yamano et al., 2017). Generally, the clinical presentation is rather indistinct, but is characterized by fever a few days to weeks after a tick bite, accompanied by non-specific influenza-like symptoms, including chills, fatigue, headache, myalgia and arthralgia (Platonov et al., 2011). Relapses of fever, well known for other RF *Borrelia* infections, appear to only occur occasionally (Sarksyian et al., 2015a). Upon examination of blood, abnormalities, such as mild lymphocytopenia or thrombocytopenia and elevated liver enzymes and/or creatinine, can be observed. Thus, based on the clinical presentation and routine blood studies, it is difficult to differentiate BMD from other tick-borne diseases, or even from other non-tick bite related infectious or inflammatory conditions. In immunocompromised individuals, mainly those on B cell depleting therapy (e.g., anti-CD20 monoclonal antibody rituximab), BMD can present as a meningoencephalitis (Boden et al., 2016; Gugliotta et al., 2013; Hovius et al., 2013). However, a *B. miyamotoi*-induced meningoencephalitis also was described in a seemingly immunocompetent individual in Sweden (Henningsson et al., 2019).

B. miyamotoi disease requires a high level of suspicion among physicians, yet there is little public awareness and diagnostic tools are still experimental and scarcely available. Nonetheless, there are several diagnostic options. Firstly, although most likely of little clinical utility, it is possible to isolate *B. miyamotoi* from blood of infected individuals (Wagemakers et al., 2014). Secondly, and of greater clinical relevance, DNA from *B. miyamotoi* *sensu lato* can be detected by molecular methods in blood (plasma or serum) (Platonov et al., 2011) or cerebrospinal fluid (CSF) (Hovius et al., 2013) and, albeit less sensitive, spirochetes can be directly visualized by dark-field microscopy (Telford et al., 2019). The above-mentioned diagnostic modalities can be used only in febrile episodes, during which spirochetemia occurs. Outside of the spirochetemic episodes, especially with longer lasting symptoms, serology should be considered. Most studies have performed serological tests based on detection of antibodies against the (GlpQ) antigen expressed by RF *Borrelia*, but not by LD *Borrelia* (Schwan et al., 1996). Of note, GlpQ is also expressed in other gram-negative bacteria, although with low levels of similarity, and does not discriminate *B. miyamotoi* from other RF *Borrelia*. However, due to differences in

the geographical spread of hard-bodied *Ixodes* ticks and soft ticks and their associated RF *Borrelia*, this is of little concern, except in specific areas such as California, USA (Krause et al., 2018). Another concern with GlpQ as a diagnostic marker is the large variation in the sensitivity to detect *B. miyamotoi* disease; depending on the population tested, the techniques used to determine seroreactivity results in whether or not patients were treated with antibiotics (Jahfari et al., 2017; Koetsveld et al., 2018a; Krause et al., 2013; Molloy et al., 2015). Therefore, additional antigens, including Vmps, have been investigated (Wagemakers et al., 2016). Interestingly, combining GlpQ with a selection of Vlps and Vsps, results in a high specificity (100% for IgM and 98.3% for IgG) and sensitivity (79% for IgM and 86.7% for IgG) (Koetsveld et al., 2018a). Preliminary and unpublished observations from follow-up studies, which included culture confirmed Lyme borreliosis patients as controls, indicate that Vmps suffer from cross-reactivity (personal communication A.E. Platonov]. Additionally, and vice versa, antibodies against Vmps from BMD patients cross-react with the so-called C6 ELISA (Koetsveld et al., 2020; Molloy et al., 2018), which is based on a conserved peptide within *B. burgdorferi* sensu lato and is a widely used serodiagnostic test for Lyme borreliosis. Interestingly, when using two-dimensional electrophoresis, several immunogenic *B. miyamotoi* proteins were identified, including a putative lipoprotein that specifically reacted with mouse anti-*B. miyamotoi* and not anti-*B. burgdorferi* antibodies (Harris et al., 2019). Another promising antigen is BipA, which not only can discriminate between LD *Borrelia* and RF *Borrelia* (Lopez et al., 2010), but also may differentiate between different RF *Borrelia* species (Lopez et al., 2013a). Future research will be needed to determine whether BipA can distinguish infection caused by *B. miyamotoi* and other RF *Borrelia*.

Based on seroprevalence studies, a considerable number of human *B. miyamotoi* infections can be asymptomatic. However, when *B. miyamotoi* disease is suspected or confirmed, antibiotic treatment should be initiated, although in reported cases from Europe and the United States, individuals recovered fully without antibiotic treatment (Hoornstra et al., 2018; Sudhindra et al., 2016). Additionally, *in vitro* analysis indicated that *B. miyamotoi* laboratory strains and Russian clinical isolates are susceptible to doxycycline, azithromycin, and ceftriaxone (Koetsveld et al., 2017a; Koetsveld et al., 2018b). In these studies, a (relative) resistance to amoxicillin was shown. Although, this has not been confirmed *in vivo* and human patients with *B. miyamotoi* disease have been successfully treated with amoxicillin (Molloy et al., 2015), caution might be warranted using this antibiotic until further studies have been performed. As with LD and RF caused by other *Borrelia*, when central nervous system involvement is suspected or proven, treatment with intravenous antibiotics, e.g., ceftriaxone, should be considered (Henningsson et al., 2019; Hovius et al., 2013).

Other RF *Borrelia* species

B. anserina and *B. coriaceae* are two species transmitted by soft ticks but not known to cause RF in humans. *B. anserina*, which is the type species of *Borrelia*, is the agent of avian or fowl spirochetosis. It is transmitted by *Argas persicus* and related species and is global in distribution in poultry flocks (DaMassa and Adler, 1979; Diab and Soliman, 1977; Zaher et al., 1977). The disease causes severe anemia, weight loss, and high mortality in

affected poultry flocks and is the most widespread poultry disease in the world, so is of great economic significance. Heavy spirochetemia of the first episode of illness may extend for 4-6 days in chickens and is prolonged for weeks in bursectomized fowls (Bandopadhyay and Vegad, 1983; DaMassa and Adler, 1979). There are antigenic differences between strains of *B. anserina* and evidence of strain-specific immunity (DaMassa and Adler, 1979; Pachanian and Smith, 1970; Uppal and Rao, 1966). However, *B. anserina* has not been known for displaying antigenic variation during infection. The mortality of the early infection is often high, and many experimental infections were terminated after the first spirochetemia (Bandopadhyay and Vegad, 1983). Nevertheless, the occurrence of immune evasion is suggested by Balfour's report on "Spirochetosis of Sudanese fowls" (Balfour, 1911). He wrote that "in chicks relapses are common, and the disease therefore approaches mammalian spirochetosis in type." In experimental infections of chicks, Balfour noted that 2-3 day periods of heavy spirochetemia culminated in a "crisis." This was followed by disappearance of spirochetes from the blood, then reappearance of spirochetes in the blood, sometimes in "large numbers" after an interval of ~4 days. *B. anserina* has a Vsp-like lipoprotein of 22 kDa (Sambri et al., 1999). Chicks that were passively immunized with antisera to this protein were protected against subsequent homologous challenge with $\sim 10^5$ spirochetes, but antisera to a Vsp-like protein from another strain of *B. anserina* did not provide protection against heterologous challenge.

B. coriaceae occurs in *O. coriaceus* soft ticks in California (Hendson and Lane, 2000; Lane and Manweiler, 1988). These ticks feed on deer and cattle, but the role of these large mammals in maintenance of the infection remains to be determined. *B. coriaceae* was identified by PCR in the blood of a mule deer and a Columbian black-tailed deer in California (Lane et al., 2005; Yabsley et al., 2005). *O. coriaceus* has been associated with epizootic bovine abortion in California and Nevada (Schmidtman et al., 1976). *B. coriaceae*'s etiological role in this disease has been suggested but not proven (Lane et al., 1985).

B. theileri is an RF-like *Borrelia* species that is transmitted by hard ticks of the metastriate genus *Rhipicephalus* rather than by argasid ticks. *B. theileri* produces a microscopically-detectable spirochetemia and weakness, loss of appetite, and anemia in cattle in Africa, Australia, and Europe (Brocklesby et al., 1963; Sharma et al., 2000; Smith et al., 1978). According to Theiler's original description of the infection, there may be two attacks of fever, suggesting the occurrence of antigenic variation (Theiler, 1902). Transovarial transmission of *B. theileri* in *Rhipicephalus (Boophilus) microplus* occurs (Smith et al., 1978). Another *Borrelia* sp. that clusters with *B. theileri* was identified in *Rhipicephalus (Boophilus) microplus* in Brazil (Yparraguirre et al., 2007).

In the last decade, other RF group species that are transmitted by hard rather than soft ticks have been identified. These all cluster with *B. theileri* in a monophyletic group distinct from species transmitted by soft ticks. One of the newly discovered species is *B. lonestari*, which was found in the U.S. in *Amblyomma americanum*, another metastriate tick (Barbour et al., 1996; Rich et al., 2001a). The distribution in the southern and eastern US of *A. americanum* corresponds with the distribution of an illness characterized by a rash resembling erythema migrans of Lyme borreliosis, which has been called southern tick-associated rash illness or

STARI. The clinical association between STARI and *B. lonestari* came from a positive PCR test from an erythema of a patient with an attached *A. americanum*, suggesting that this spirochete might be the causative agent of STARI (Campbell et al., 1995). Further clinical and serological studies have clearly separated the erythema skin lesions presented during infection with *B. burgdorferi* and *B. lonestari*, respectively (Philipp et al., 2006; Wormser et al., 2005). Interestingly, a subsequent study by the same research group showed that neither *B. lonestari* nor *B. burgdorferi* or any other spirochete was the causative agent of an erythema migrans like condition in a specific area in Missouri (Wormser et al., 2005). The etiology of the erythema migrans like disease found in that area is still unclear. Recent reports on cases of STARI infections in other areas in the US will aid in the understanding of the epidemiology of *B. lonestari* (Feder et al., 2011). Also, the successful culture isolation of *B. lonestari* will promote the understanding of this spirochete (Varela et al., 2004). To further study the infection biology of *B. lonestari* (Moyer et al., 2006) used a low-passage culture isolate to infect white-tailed deer (*Odocoileus virginianus*), a common host of *A. americanum* ticks, as well as dogs, mice, and cattle. Only deer developed spirochetemia, as evidenced by blood smear and PCR; the spirochetemia lasted for 6-9 days. Yabsley et al. subsequently found that raccoons were refractory to infection with *B. lonestari*. (Yabsley et al., 2008) The failure of *B. lonestari* to infect mice is attributable to the bactericidal activity of naive mouse serum for this species (Grigery et al., 2005).

Future Directions

Many other species within the family of *Borreliaceae* probably have reservoir host-vector cycles that remain vague and their role in human infection is unknown. Consequently, these organisms have escaped attention. Of importance for the development of therapeutic, diagnostic, and preventative measures is the ability to isolate spirochetes. While isolation in culture medium can be challenging for RF spirochetes, once bacterial isolates are obtained the available tool-box of multiple omics techniques will enable further exploration to the unique life of these microorganisms. Importantly, with the increasing number of tools for genetic manipulation of RF *Borrelia*, we will further unravel mechanisms of pathogenesis. Also, the accumulation of knowledge on RF *Borrelia* pathogenesis will aid in development of anti-virulence drugs that might alter the use of broad-spectrum antibiotics and, as such, decrease the burden on the pandemic of antibiotic resistance.

As additional species of *Borrelia* are identified, they may include organisms that, like *B. miyamotoi* and *B. lonestari*, share features with both the RF species and LD species or have other novel features. New species may not include features linking these two groups of spirochetes, but they could be useful for further defining features distinguishing the pathogenesis of RF and LD. Finally, especially for those RF *Borrelia* species with relatively short-lived and low levels of spirochetemia, e.g., *B. miyamotoi*, novel molecular and serological diagnostic antigens are warranted. Ideally those would be able to discriminate between spirochetes belonging to the *B. burgdorferi* sensu lato group and those that belong to RF *Borrelia* and even amongst different RF *Borrelia* species.

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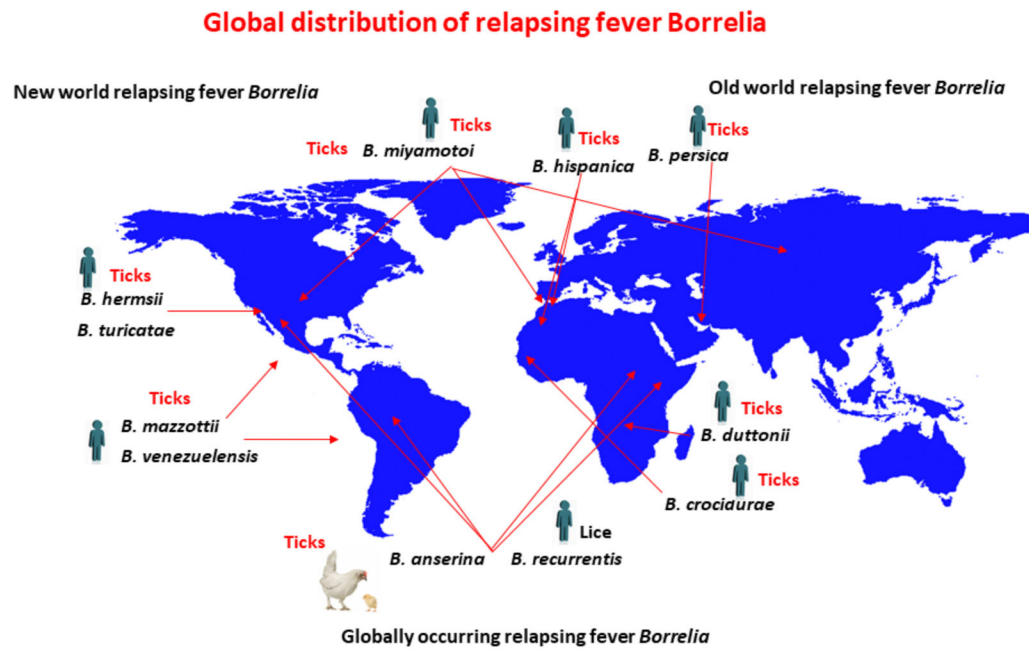


Figure 1.

Global distribution of RF *Borrelia*. Only certain representative RF *Borrelia* species are included in the figure. A more detailed description of currently defined species is found in (Barbour and Schwan, 2018; Talagrand-Reboul et al., 2018). Drawing was generated by Haitham Elbir. The global map was created using R software version 4.0.1

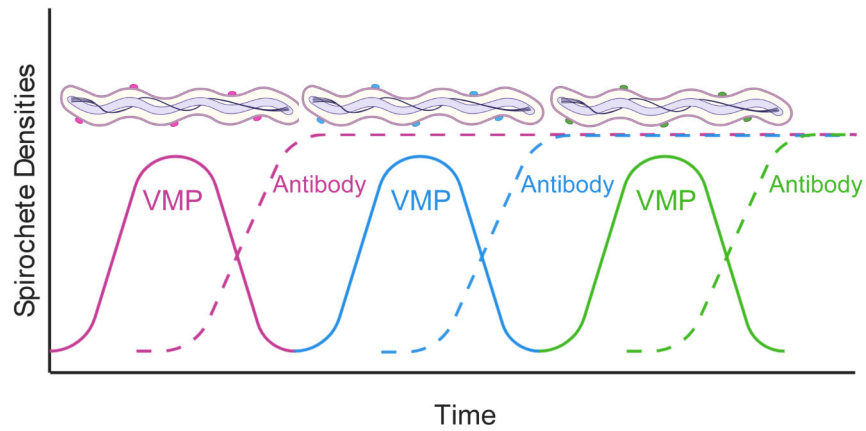


Figure 2. Antigenic variation and antibody response during RF borreliosis. Show are spirochete densities in the blood over time. As spirochetes replicate in the blood a predominant VMP variant emerges (pink line and spirochetes coated with pink proteins). The antibody response generated against this variant (pink dotted line) clears the population of spirochetes while a new population emerges (blue variant). Again, an antibody response is generated (blue dotted line) to clear the population, and yet another variant (green) emerges. The dynamics between antigenic variation and the host antibody response can continue for months. Drawing was generated using BioRender by Brittany A. Armstrong and Job Lopez.

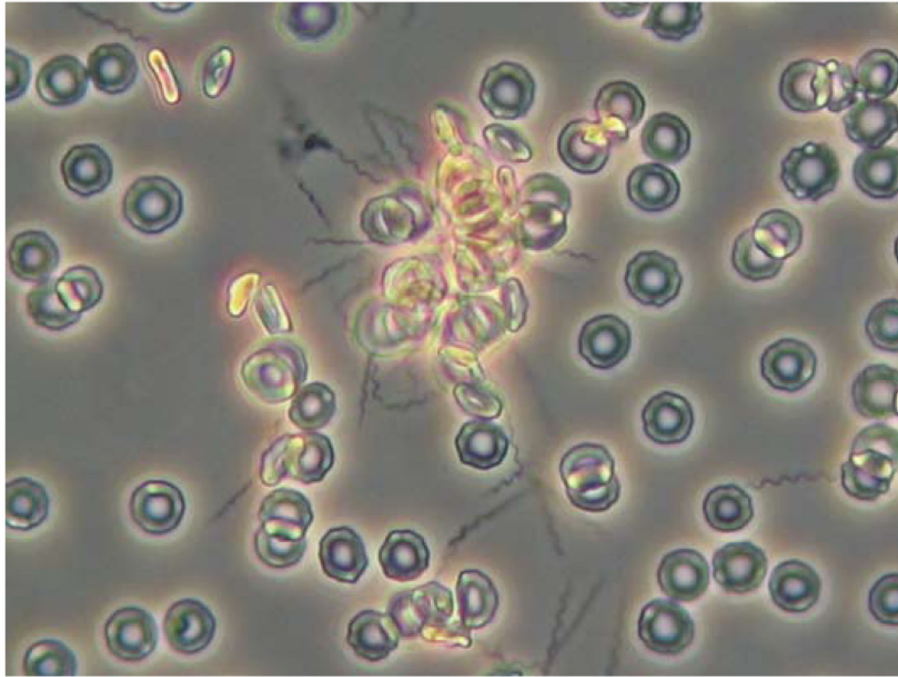


Figure 3. Phase contrast micrograph of rosette of spirochetes and erythrocytes formed during experimental RF. Blood was examined four days after inoculation of an adult C3H/HeN mouse with *Borrelia duttonii*. Magnification 400X. Photo courtesy of Marie Andersson, University of Umeå.

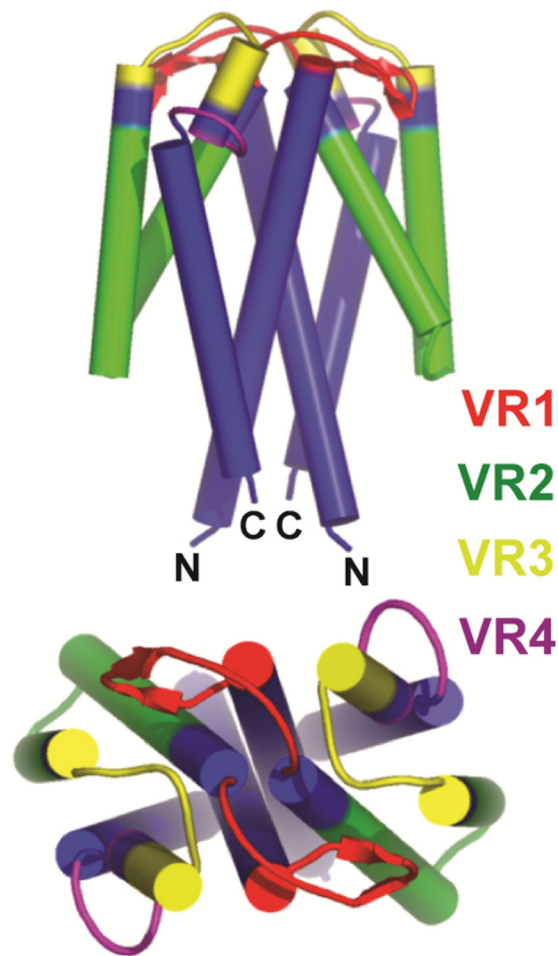


Figure 4.

Schematic representation of structure of a Vsp lipoprotein dimer of *B. turicatae*. The four major alpha-helical chains in each monomer are depicted by cylinders. The first and fourth alpha-helix (blue) in each Vsp are relatively conserved in sequence between different Vsp proteins of a given species (Dai et al., 2006; Restrepo et al., 1992). The second and third alpha-helical chain and the loops between all the chains are variable in sequence and are divided into variable regions (VR) 1 (red), 2 (green), 3 (yellow), and 4 (purple). The greatest variability is at the top or dome of the protein. Adapted from Lawson et al. (Lawson et al., 2006).

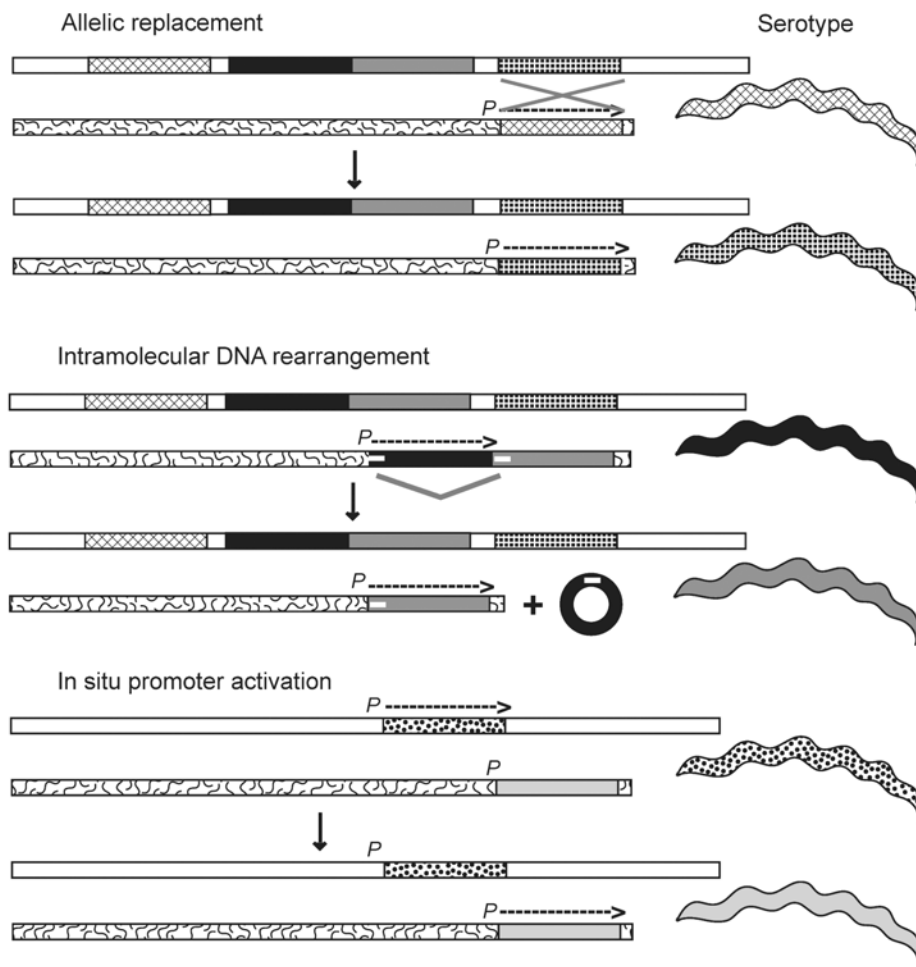


Figure 5.

Three mechanisms for serotype switches by *B. hermsii*: allelic replacement, intramolecular DNA rearrangement, and in situ promoter activation. The different mechanisms are discussed in the text. *P* indicates the promoter and the location of the expression site near the right telomere of a linear plasmid; an overhead arrow shows whether the gene is active and the direction of transcription. *vsp* and *vlp* genes at the expression site location or in archival locations on the same (squiggly line background) or another linear plasmid (white background) are schematically represented by different patterns and grayscale values. The serotype of the cell, similarly indicated by pattern or grayscale, corresponds to *vsp* or *vlp* gene, at the expression site. In the second mechanism a deletion by direct repeats (small white horizontal bars) occurs and results in a non-replicative circle. (Courtesy of Alan Barbour, UC Irvine).

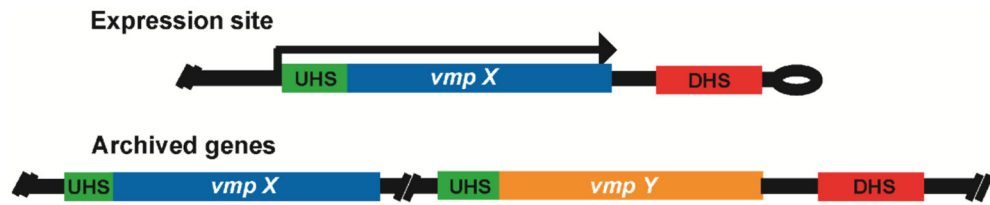


Figure 6. Organization of the expression plasmid (top) and one archival plasmid (bottom) in *B. hermsii*. Two examples of *vsp* or *vlp* genes are denoted by “X” and “Y” and different fill patterns. There is a single expression site on one plasmid, and silent variants of *vsp* and *vlp* genes are found on the same and other plasmids. The direction and extent of transcription of the duplicate gene at the expression site is indicated by the arrow. The UHS element at the expression site comprises 61 nt around the start codon of the variant gene (Barbour et al., 1991a; Dai et al., 2006; Kitten et al., 1993). Silent genes vary in the extent to which their UHS regions are identical to the expression site UHS; this is represented here by the relative length of the UHS block. There is a 214 nt noncoding DHS element downstream from the expression site and adjacent to the plasmid telomere and at various locations on the plasmids. The lengths of *vsp/vlp* gene examples and the UHS and DHS elements are not to scale. (Courtesy of Alan Barbour, UC Irvine).