

Cover Page



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Author: Burgel, Nathalie Daniëlle van

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Chapter 1

General introduction and outline

1.1 History of Lyme disease

The causative agent of Lyme disease, *Borrelia burgdorferi*, was first cultured from ticks and described in 1982 by Willy Burgdorfer et al.¹. By then many clinicians had already hypothesized a relation between Lyme disease and the tick and many of the clinical presentations had already been described.

Perhaps the earliest work describing Lyme disease is from the reverend John Walker describing a vector-borne illness much like Lyme disease in 1764 that crippled the community of Jura, a Scottish island². More evident reports are from the late 19th century when the physician Alfred Buchwald from Poland described a case of a patient suffering for 16 years from a degenerative skin disorder³. This disease is now called acrodermatitis chronica atrophicans (ACA). In 1896 Nikulin, from Moscow, reported the first case of ACA in a child⁴. In 1910 the Swedish dermatologist Arvid Afzelius described the first case of a ring like lesion which he called an erythema migrans (EM). He was the first person to associate this lesion with a recent bite from the European sheep tick (*Ixodes ricinus*)⁵. In 1911 the Swiss pathologist Jean Louis Burckhardt described the first case of what is now known as a borrelia lymphocytoma⁶.

Neurological syndromes associated with the EM were described as early as the year 1922 by the French physicians Charles Garin and Charles Bujadoux describing a farmer with a radiculitis after a tick bite and an EM⁷. The association with a tick bite and EM was not recognized until the association was proposed in 1930 by the Swedish dermatologist Sven Hellerström⁸. In 1941 the German neurologist Alfred Bannwarth described several cases of chronic lymphocytic meningitis and painful polyradiculoneuritis⁹. The tick-borne meningopolyneuritis became known as Garin-Bujadoux-Bannwarth syndrome.

The first reports of effective treatment of these clinical syndromes by the use of penicillin date from 1949^{10,11}. The effective treatment of the syndromes with penicillin prompted scientists to try to culture bacteria in order to fulfill Koch's postulates in which they continuously failed. For many years European doctors described the different syndromes in more detail but the actual causative agent was not isolated^{12,13}.

The first report of an EM in North America dates from 1970¹⁴. Soon after that, in 1975 in the towns Lyme and Old Lyme, a remarkable cluster of juvenile rheumatoid arthritis associated with an EM was identified. The disease was epidemiologically associated with tick bites and named Lyme disease¹⁵. The clinical manifestation of Lyme carditis was first described in 1980¹⁶. The first

placebo controlled trials for treatment with antibiotics were performed shortly after the discovery of the clusters¹⁷⁻¹⁹. It was in 1982 that the Lyme disease agent, *B. burgdorferi* sensu lato (sl) was isolated from *Ixodes scapularis* ticks²⁰. In that same year *B. burgdorferi* was isolated from the blood of several Lyme disease patients^{21,22}. In 1983 the first report of cultured spirochetes from Swiss *I. ricinus* ticks was published²³. The difference in clinical presentation between the North American and European patients was soon clear. Since then much research has been done on the pathogenesis, epidemiology, diagnostics and treatment of Lyme disease.

1.2 *B. burgdorferi* transmission and pathogenesis

Microbiology

B. burgdorferi is a spirally shaped gram negative, microaerophilic bacterium that belongs to the family of the Spirochaetaceae. The genus *Borrelia* consists of several species among which vector transmitted relapsing fever *Borrelia* and the tick transmitted *B. burgdorferi* sl. The *B. burgdorferi* sl complex consists of at least 17 genospecies, among which *B. burgdorferi* sensu stricto (ss), *B. afzelii*, *B. bavariensis*, *B. garinii* and *B. spielmanii* are known to cause disease in humans. Possibly *B. valaisiana*, *B. lusitaniae* and *B. bissettii* can also be pathogenic²⁴. Other genospecies in this group are *B. andersonii*, *B. japonica*, *B. turdi*, *B. tanukii*, and *B. lonestari*²⁵.

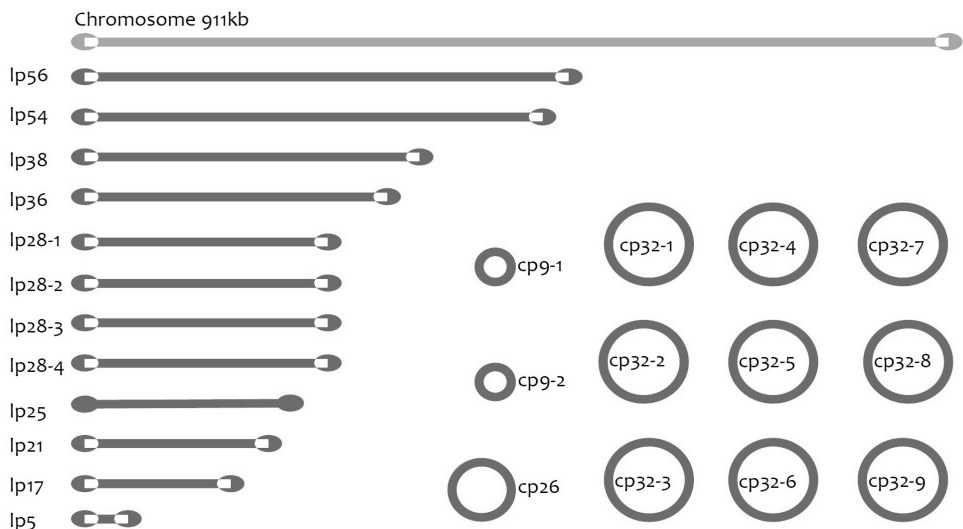


Figure 1: The genome of reference strain *B. burgdorferi* ss B31

The genome of *B. burgdorferi* sl has unique features. Primarily it has the largest number of plasmids known for any bacterium. An example of reference strain *B. burgdorferi* ss B31 is shown in figure 1. *B. burgdorferi* sl has a chromosome of about a million base pairs and is estimated to contain 853 genes that encode a basic set of proteins. The genome further consists of a large number of linear and circular plasmids with a combined size of more than 533,000 base pairs, containing approximately 926 genes²⁶⁻³⁰. The plasmids have approximately one copy number per chromosome³¹. The plasmids are unusual in that they contain many paralogous sequences and a large number of pseudogenes³⁰. Some of the plasmids contain essential outermembrane proteins necessary for infectivity and immune evasion in the vertebrate and arthropod host³². In addition, a number of the cp32 plasmids have features suggesting that they are prophages, which can facilitate exchange of genes³³.

The vector lifecycle

B. burgdorferi sl is transmitted to the mammalian host by infected ticks. Ticks belong to the phylum of the arthropoda and the class of arachnida. Ticks are classified as hard-bodied or soft-bodied ticks. Several hard body ticks can carry the different *Borrelia* species and they belong to the *Ixodes ricinus-persulcatus* complex, including *I. scapularis* (deer tick) in the Northeastern and Midwestern United States, *I. pacificus* in the Western United States, *I. ricinus* (sheep tick) in Europe, and *I. persulcatus* in Asia³⁴. In North America the only pathogenic species found is *B. burgdorferi* ss, while the biodiversity of pathogenic species in Eurasia is much larger³⁵.

Hard-bodied ticks have three distinct stages, for each stage only one blood meal is taken to molt into the next stage (figure 2). From the eggs larvae emerge, they usually obtain their blood meal from small rodents or birds. Typically a blood meal takes 4-7 days until the ticks drop off. After obtaining a blood meal they molt to the nymphal stage. Nymphs usually feed on medium to large sized vertebrate hosts and molt to the next and final stage - the adult. After feeding once more on a medium to large sized vertebrate host the adult female hard ticks lay one batch of thousands of eggs and then die. In central Europe nymphal ticks usually feed during spring and early summer, larval ticks later in spring and early autumn (figure 3). Activity in the summer is slightly lower and completely absent in winter, but this is very much depending on the local climate³⁵. Many hard bodied ticks can live for several months without feeding and the entire lifecycle can range from 2 to 6 years³⁶.

Transovarial transmission of *B. burgdorferi* s.l. does not occur, so the larva stage is assumed to be *B. burgdorferi* s.l. free³⁷. When the primary vertebrate host is infected with *B. burgdorferi* s.l. the larvae can become infected.

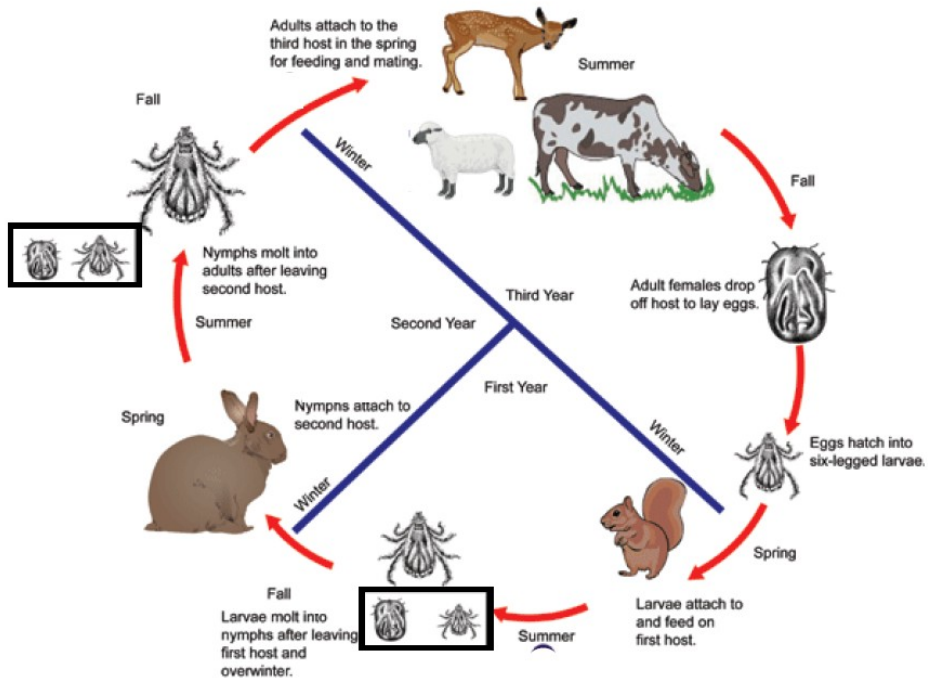


Figure 2: Tick lifecycle, adapted from www.cdc.gov

Pathogenesis

When the tick is feeding it acquires *B. burgdorferi* from the blood of the infected vertebrate host. Spirochetes are transferred from the vertebrate host to the tick and start to express high levels of outer surface protein A and B (OspA/B) which enable the spirochete to attach in the tick gut³⁸⁻⁴⁰. In between blood meals the spirochetes reside in the tick midgut. Over a period of months the tick molts into the next stadium. During the next feeding, the temperature of the tick rises and the pH in the midgut drops. This is a signal for the bacteria to migrate to the salivary glands. This is achieved by downregulating OspA/B and upregulate many proteins among which OspC⁴¹⁻⁴³. Tick saliva already contains substances that can suppress the host immune response⁴⁴. In the salivary gland OspC can bind to tick salivary protein (SALP) 15 which protects the spirochete against the host immune system⁴⁵⁻⁴⁸. During feeding the tick inoculates the spirochete in the blood of the host. After inoculation by the tick

the spirochetes give a local infection after which they disseminate. During infection spirochetes can be found throughout the infected host. However during prolonged infection the spirochetes can reside in preferred locations, for example joints or the central nervous system. It is generally presumed that *B. burgdorferi* are extracellular microorganisms, mainly located in the extracellular matrix.

On the membrane of the spirochete many proteins are expressed that are of importance for pathogenesis in the vertebrate host. Many proteins, of which some essential for effective transmission, are involved in binding host extracellular proteins like decorin (DbpA and DbpB), fibronectin (RevA and BBK32) and laminin (ErpX and BmpA-D) ⁴⁹⁻⁵³.

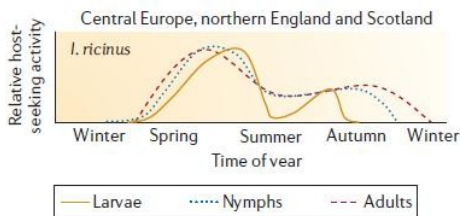


Figure 3: Seasonal activity of tick feeding in Europe. Adapted from Kurtenbach et al ³⁵

Immune evasion

Other proteins that are expressed at the surface of the spirochete are responsible for immune evasion later in infection. The best studied protein is the lipoprotein VlsE ^{54, 55}. The *vls* locus consists of 15 silent *vls* cassettes and the VlsE lipoprotein. VlsE consists of six variable and six invariable regions (IR) ⁵⁶. By applying unidirectional recombination events VlsE can display antigenic variation. This recombinational variation can potentially derive millions of antigenic variants and complicates detection by the immune system which facilitates persistent infection of the mammalian host ⁵⁷.

Borrelia can also bind to tick salivary proteins by OspC which can inactivate the mammalian host immune system like tick salivary protein (SALP) ¹⁵ ⁴⁶. Immediately after transmission many of the immunogenic outer surface proteins are downregulated, among which OspA/B (see figure 4) ⁵⁸.

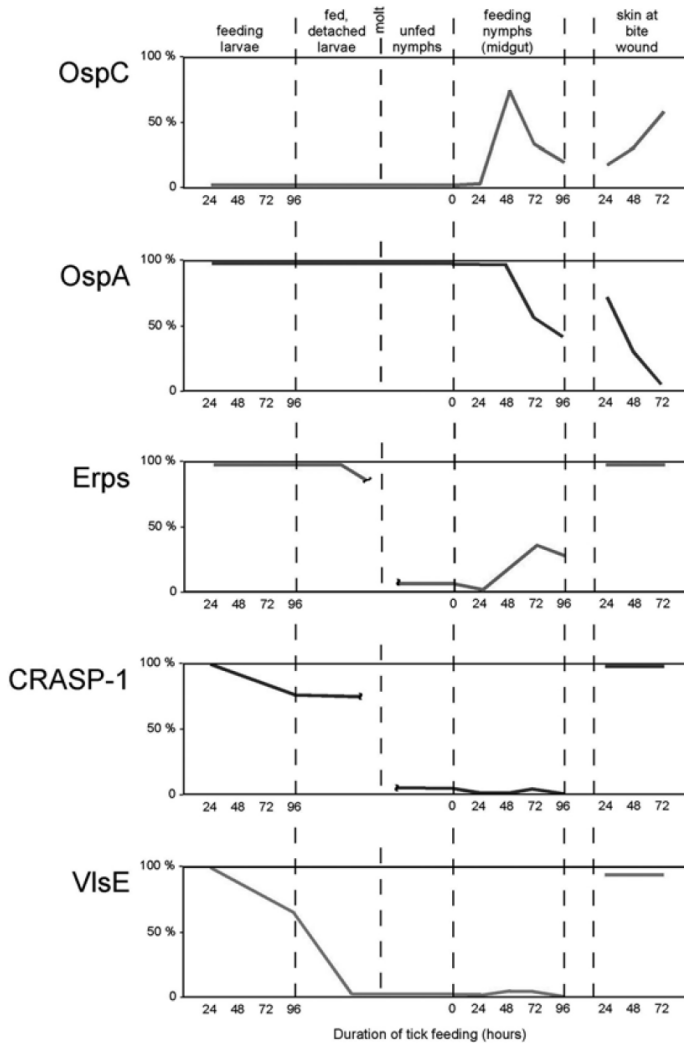


Figure 4: *B. burgdorferi* sl gene regulation at different time points in tick and host infection. Adapted from Stevenson et al.⁵⁸.

1.3 *B. burgdorferi* complement evasion

Complement is one of the first lines of defense against entering bacteria in the mammalian host. *B. burgdorferi* is primarily an extracellular pathogen⁵⁹. When a bacterium enters the mammalian host, three pathways can be activated; the alternative pathway (AP), the lectin pathway (LP) or the classical pathway (CP). The CP is activated by antibodies binding to the membrane of the pathogen. The LP is activated by binding of mannose binding lectin (MBL) to carbohydrates of the pathogen. The convertase for both these pathways

(C4bC2b) is composed of C4b and C2b. The alternative pathway is activated constantly and the convertase of the alternative pathway (C3bBb) is formed by C3b and factor Bb. Both C4bC2b and C3bBb cleave C3 to C3a and C3b. If activation progresses C3b is deposited on the nearest membrane. On the membrane of mammalian host cells deposition is prevented or C3b is inactivated by soluble regulators. If activation progresses C3b binds to the C3 convertases which generates the C5 convertase (C4bC2bC3b or C3bBbC3b respectively). By this convertase C5 is cleaved in C5a and C5b, the latter can activate the membrane attack complex (MAC) which consists of C6, C7, C8 and multiple C9 components. The assembly of the MAC leads to pore formation and cell lysis⁶⁰.

Borrelia can evade activation of the MAC. However, the extent to which spirochetes can resist complement differs markedly between the several *B. burgdorferi* ss species; *B. afzelii*, *B. burgdorferi* and *B. bavariensis* are relatively resistant to human serum, while *B. garinii* and *B. valaisiana* strains are usually sensitive. However in sera from other mammalian and avian species this resistance profile can be completely different⁶¹⁻⁶⁵.

Early this century, it was discovered that *borrelia* can inactivate human complement by binding two host derived fluid phase regulators of complement; factor H (CFH) and factor H-like protein 1 (FHL-1), also known as reconectin⁶⁶⁻⁶⁸. CFH and FHL-1, the main immune regulators of the alternative pathway of complement activation, are structurally related proteins composed of several protein domains termed short consensus repeats (SCRs)⁶⁰. CFH is a 150-kDa glycoprotein composed of 20 SCR domains. In contrast, FHL-1 is a 42-kDa glycoprotein corresponding to a product of an alternatively spliced transcript of the *CFH* gene and consists of seven SCRs and is present only in human serum in ten to 50 times lower concentrations than CFH⁶⁹. The seven N-terminally located SCRs of both complement regulators are identical with the exception of four additional amino acids at the C-terminus of FHL-1⁷⁰. CFH and FHL-1 in the human host are responsible for preventing binding of factor B to C3b, supporting the dissociation of the C3bBb complex and acting as a cofactor for factor I-mediated cleavage of C3b, the central component of all three complement activation pathways⁷⁰⁻⁷³ (see figure 5)

Serum resistant *borreliae* acquire CFH, FHL-1 and/or complement factor H-related proteins (CFHR) by direct interaction with outer surface proteins designated CRASPs (Complement Regulator-Acquiring Surface Proteins)⁷⁴. Previously, five different CRASPs have been described for *B. burgdorferi* ss and *B. afzelii*. No CRASP has been described for the also complement resistant *B.*

bavariensis. The CFH and FHL-1 binding CspA protein, also designated CRASP-1, is encoded by *cspA*, a gene located on the lp54 plasmid. Although the lp 54 plasmid of *B. burgdorferi* and *B. afzelii* carries multiple genes encoding a number of paralogous proteins, only the CspA is capable of binding human CFH and FHL-1⁷⁵⁻⁷⁷. CspA can also bind other proteins like plasminogen and different other extracellular matrix proteins⁷⁸. CspA is upregulated by spirochetes during the tick-mammalian transmission stage and downregulated during persistent infection^{79, 80}. Antibodies to CspA could be detected in sera from infected mice and from Lyme disease patients suggesting a potential prolonged expression of CspA in the mammalian host⁸¹⁻⁸³. Previously only CspA from complement resistant *B. burgdorferi* ss and *B. afzelii* has been described, but not a CspA from the human complement resistant species *B. bavariensis*.

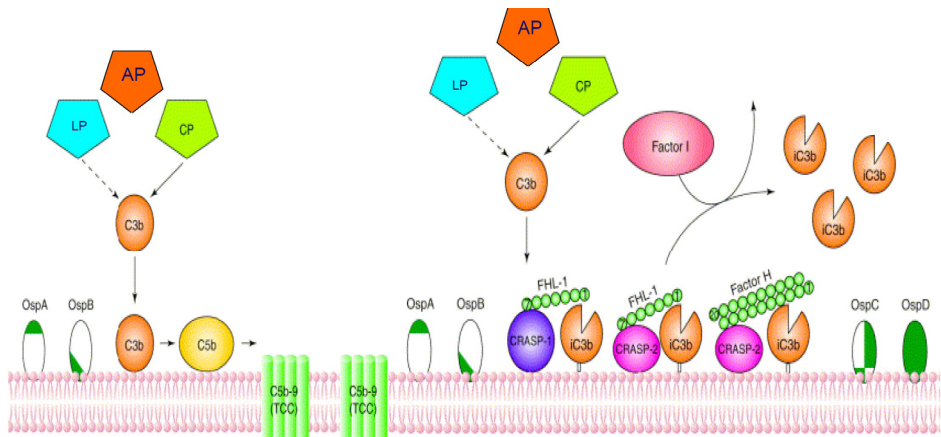


Figure 5: adapted from Kraiczy et al.⁶⁸. Complement evasion strategy of *B. burgdorferi* sl. Left: a normal situation of activation of complement with the eventual deposition of C3b on the membrane and assembly of the membrane attack complex (C5b-C9). Right: By expressing FHL-1 and factor H binding proteins on the membrane C3b is inactivated (iC3b) and the membrane attack complex is not formed. (LP: lectin pathway, AP: alternative pathway, CP: classical pathway)

Another protein that can bind human CFH and FHL-1, CspZ, also designated CRASP-2, is a distinct protein encoded by the *cspZ* gene located on plasmid lp28-3 and is expressed at higher levels during the mammalian infection than in bacteria residing in ticks or during laboratory cultivation⁷⁹. Anti-CspZ antibodies can be detected as early as two weeks post infection in mice infected by ticks⁸⁴. CspZ has been shown to bind other yet unknown proteins and therefore can have multiple functions^{63, 80, 84, 85}. However, the protein is not essential to cause effective transmission to, and infection of, the mammalian host⁸⁶. The CFH-binding CRASP proteins BbCRASP-3, -4, and -5 belong to the OspE-related proteins (Erp) paralogous family and their respective genes are located on different cp32 circular plasmids⁸⁷. Erp proteins are expressed in

tissues in the host during disseminated mammalian infection. Erp proteins have also been shown to be able to bind to CFHR proteins and plasminogen^{53, 88-97}.

In a mouse model where CFH deficient mice were infected with *B. burgdorferi* ss there were no major effects of the infectivity of *B. burgdorferi* ss injected in factor H deficient mice concluding that mice lacking factor H were as efficiently infected by *B. burgdorferi* as WT mice⁹⁸. Since in these mice, complement-mediated killing of spirochetes would not be inhibited by CFH binding, these findings suggested that complement did not play an important role in host defense against *Borrelia* infection. A problem with that model, however, is the fact that factor H deficient mice practically do not have C3, compensating for their factor H deficiency and can not kill invading spirochetes by complement activation⁹⁹.

Additionally, when entering the host *B. burgdorferi* can bind proteins from the tick saliva that can inactivate or modulate complement activation¹⁰⁰⁻¹⁰². Also expression of a CD59-like protein is described to modulate complement activation¹⁰³.

It is not clear whether the inactivation of complement by *B. burgdorferi* is essential for the infection of the mammalian host. It is also not known whether resistance to complement aids *B. burgdorferi* sl to cause a persistent infection and whether it causes a specific pattern of dissemination in the mammalian host.

1.4 Clinical presentation of Lyme borreliosis

The clinical presentation of Lyme Borreliosis (LB) can vary greatly between patients. The clinical presentation of patients that are infected in North America differs significantly from patients infected in Europe, most likely due to the different species that are prevalent and associated with specific clinical manifestations¹⁰⁴⁻¹⁰⁶. There are several ways of classification of LB. One commonly used classification is the classification in three stages, stage I is an early localized infection like an erythema migrans (EM). Stage II is an early disseminated disease like multiple EM lesions. Stage III is the late persistent disease¹⁰⁷. Other classifications primarily make a difference between early localized and later manifestations^{108, 109}.

For a diagnosis of LB to be considered the patient must have been exposed to the risk of a tick bite. This means having been to woody, grassy or bushy area in an endemic country^{108, 110}. The history of actual tick bites is important for the

clinical diagnosis. However, in clinical practice only a minority of patients remembers getting a tick bite ¹¹¹⁻¹¹⁴. There is usually a bimodal distribution of patients with LB mainly affecting children and elderly people, most likely due to risk behavior for attracting ticks (playing, gardening, hiking) ^{112, 114-117}.

Incidence of LB in Europe seems to be gradually increasing within the last decades; this might be due to increased awareness, as well as to an actual rise in infections ¹¹⁸⁻¹²⁰.

Erythema migrans

Erythema migrans (EM) is a typical sign of LB, usually presenting several days to weeks after a tick bite. The classical EM lesion is a round to oval expanding erythema that fades in the center, leaving only the annular border erythematous; the classical bulls eye (see figure 6). For some patients however, a homogeneous erythema may persist. The size of the ring, usually 5-10 cm, or the distance the lesion has migrated is positively related to the duration of the disease ¹²¹. Sometimes the EM may also remain stationary for a long time. Successful cultivation of spirochetes from skin biopsies have proved that atypical variants such as vesicular, scaling, or purpuric-hemorrhagic lesions can occur ¹²². Slight itching, burning or dyesthesia may occur at the site of the EM ¹²³.



Figure 6: a typical EM lesion. Picture used with courtesy of the CDC.

In 30-68% of the cases the EM presents with a flu-like illness ¹²³⁻¹²⁵. The lesion will usually resolve within a month, but may persist for a year. Relapsing lesions may occur ^{126, 127}. Reports of a previous EM in LB are reported in 28-90% of all patients. Multiple EM lesions are more often found in North-American patients and are rare in European individuals ^{113, 114, 128-132}.

Borrelia lymphocytoma

Borrelial lymphocytoma (BL) is a rare cutaneous manifestation of LB that is diagnosed in 3-5% of patients with early Lyme borreliosis in Europe. It is a benign B-cell lymphoproliferative process which appears after about 3 weeks (2 days to 6 months) after a tick bite. It presents as a painless red to bluish papule or nodule with a diameter of up to a few centimeters typically localized usually on the ear lobe of children, on the nipple–areola mammae in adults or other localization such as the scrotum. This lesion can appear in any stage of LB. It is a more common manifestation among children than in adults. Borrelial lymphocytoma can be the only manifestation of the disease or it can be accompanied by other symptoms most frequently by EM, but concomitant ACA can be observed. An untreated BL can persist for several months to more than one year¹³³⁻¹³⁶.

Acrodermatitis chronica atrophicans

ACA is a characteristic late manifestation of LB in Europe. Although ACA rarely has been reported in North America, it may be seen in approximately 10% of European cases of LB^{121, 137, 138}. ACA most often presents in elderly women and is anecdotally reported in children^{121, 139, 140}. ACA is most commonly caused by the genospecies *B. afzelii*, but can sometimes be caused by other genospecies^{141-143, 143}. ACA is usually distributed on the lateral aspects of the distal extremities, but can also be present on the trunk. The disorder is characterized by a chronic, progressing course, beginning with an early inflammatory stage with bluish red discoloration and doughy swelling of the skin without sharp demarcation. Over weeks to months, the disease gradually develops into a late atrophic stage, in which the skin becomes thin, wrinkled, dry, and transparent because of the loss of epidermal and dermal structures. Underlying vessels become visible and multiple telangiectases occur^{121, 140}. Cutaneous neuropathy with abnormal electroneurographic test results can be present in up to 60% of cases and have been elaborately described¹⁴⁴⁻¹⁴⁷. In 10% to 20% of patients with ACA, localized increase of dermal collagen leads to fibrotic nodules, which can lead to limitations of joint movement¹⁴⁸. Upon treatment the infection readily comes to an arrest, but in late stage or prolonged ACA the elaborate atrophy of the skin and peripheral neuropathy will not completely recover¹⁴⁵.

Very often, ACA has already been present for months to years at the time of diagnosis, with a median of 9 months¹⁴⁹, or two years in another study¹¹². This is most likely due to its insidious course and the frequent misinterpretation, especially as chronic venous insufficiency or skin aging, but also as a vascular disorder, deep vein thrombosis, superficial thrombophlebitis, arterial occlusive vascular disease, acrocyanosis or livedo reticularis¹²¹.

Lyme arthritis

Lyme arthritis (LA) can present 12-50 weeks after a tick bite and is considered a late manifestation of LB. Spirochetes disseminate and invade synovial joints, where they induce an inflammatory response in synovial tissue by inducing infiltration of mononuclear cells, neutrophils, immune complexes, complement, and cytokines. The inflammatory response induces synovial hypertrophy and vascular proliferation with eventually cartilage damage¹⁵⁰. LA is a common sign of LB, especially in North America, where it is one of the mayor clinical presentations¹⁵.

In Europe 8- 25% of patients with Lyme disease complain of having arthralgias, often accompanied by other clinical manifestations of LB^{112, 116, 117, 151, 152}. In a European study monoarthritis was observed in 71%, oligoarthritis in 20% and polyarthritis in 10% of the cases¹¹². Manifestations of Lyme arthritis in Europe, as in North America, comprise recurrent attacks or long-lasting objective joint swelling/synovitis sometimes without arthralgia¹⁵³. The joint most commonly affected is the knee (70-95%), but often also elbow, wrist, ankles, shoulders and hip^{15, 112, 116, 153, 154}. Oligoarthritis of the large joints often presents unilateral or symmetric in LA¹⁵³. The recurrent arthritis can persist for months to years. In most cases (79%) the arthritis will eventually resolve without treatment, but in some cases a chronic synovitis will develop. When a patient develops chronic synovitis elaborate destruction of the joint with erosive lesions and permanent joint disability will occur in 50% of the cases¹⁵⁵.

Children with LA are regularly misdiagnosed as suffering from pauciarticular juvenile rheumatoid arthritis or an acute bacterial septic arthritis¹⁵⁶. Differential diagnosis in adults includes rheumatoid arthritis, palindromic arthritis, septic arthritis or a reactive arthritis.

Carditis

Cardiac manifestations in LB rarely appear to be a solitary symptom¹⁵⁷. Most frequently it can be observed in early LB, or in association with neurological symptoms or arthritis. Conduction abnormalities with varying degrees of atrioventricular conduction defects are typical manifestations. In particular, Lyme carditis should be suspected in younger individuals showing conduction abnormalities without other apparent risk factors, and who have a history of recent exposure to ticks. Other rhythm disturbances, endomyocarditis and pericarditis have been infrequently reported¹⁵⁸. Palpitations, bradycardia, or bundle branch block alone are not sufficient for diagnosis and alternative explanations for the cardiac condition presented must be excluded. Temporary

pacemakers are frequently used, but permanent pacemaker implantation is rarely needed^{16, 157, 158}.

Neuroborreliosis

Lyme neuroborreliosis (LNB) is a manifestation of an infection with *B. burgdorferi* sl in up to 20% of cases in Europe and up to 5% in North-America^{112, 122, 159-162}. LNB commonly presents as cranial neuritis, meningitis or radiculoneuritis. The classic triad of radiculitis, peripheral paresis and inflammatory changes of the cerebrospinal fluid (CSF) are also known as Garin-Bujadoux-Bannwarth syndrome.

Headache is a common general symptom, as is fever, though both are more pronounced in children. Nuchal rigidity is not a common symptom and when it is present it is only mild and mainly present in children. Often there is more than one manifestation of LNB present^{111, 114, 163, 164}. Cerebrovascular stroke and transient ischemic attacks are a rare complication of LNB^{111, 114}.

In the presentation of cranial neuritis the nerve most commonly affected is CNVII, also known as the facial nerve. In children, facial nerve paralysis (FNP) often is the first symptom of early LNB. Unilateral FNP in children is thought to be caused by *B. burgdorferi* sl in 35-75% in Lyme endemic countries, with the peak incidence in spring or late summer^{165, 166}. Bilateral FNP in children is caused almost exclusively by *B. burgdorferi* sl^{167, 168}.

In adults the most common cause of FNP is idiopathic and only about 1,5 -2,2% of all FNP in Europe are caused by *B. burgdorferi* sl^{113, 169}. The differential diagnosis of FNP consists of HSV or VZV, trauma/tumor, diabetes mellitus, pregnancy, autoimmune disorders, EBV and HIV¹⁶⁹.

Other cranial nerves can be involved in LNB and in particular involvement of more than one cranial nerve is a reason to suspect Lyme disease. Other cranial nerves commonly (co-)affected are the CNVIII (vestibule-auditory) the CNIII and the CNVI (oculomotor and abducens). CNII (optic nerve) and CNV (trigeminal) are uncommon nerves to be affected^{114, 170-175}.

Radiculitis is a common clinical manifestation of LNB in adults, but is rare in children. It can present one week to months after a recorded tick-bite, but is generally considered a late manifestation¹¹⁴. It starts with a painful radiculitis, eventually leading to focal motor signs. In two-thirds of the patients the pain started at the primary site of the EM. Radicular pain is easily distinguished from back pain as it is severe, burning and deep accompanied with areas of dysesthesia. It has typical nocturnal exacerbations. In about a third of the

untreated patients the symptoms resolve within several months. Neurological examination often reveals involvement of multiple nerve roots ^{111, 114, 176}.

The clinical presentation is sometimes mistaken for herpes zoster neuralgia, cervical or lumbar nerve root compression, visceral disease, brachial plexus neuropathy, Guillain-Barré, polymyalgia rheumatica or kidney concrements.

Progressive encephalomyelitis can manifest in approximately 4-6% of patients with LNB. The disease is often present for a longer duration (6 months to several years) before progressing to this stage. Clinical presentations associated with LNB encephalitis are; an ataxic gait, spastic bladder paresis with progression to spastic para- or tetraparesis, general confusion, opsoclonus-myoclonus, ocular flutter and apraxia ^{111, 114, 177, 178}.

In the differential diagnosis of LNB many diseases can be included, especially auto-immune disorders; Multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), acute disseminated encephalomyelitis (ADEM). In literature many case reports describe misdiagnosis of Lyme disease for the abovementioned diseases, but also visa versa. This is why Lyme disease has been suggested to be the new “great imitator” after neurosyphilis that can also have a spectrum of presentations mimicking other neurologic diseases ¹⁷⁹.

Ocular manifestation

Although ver rare, infections of the eye presenting as uveitis, conjunctivitis, papillitis, episcleritis, retinal vasculitis or keratitis/keratopathy have been described. Ocular involvement usually takes place in the late phases of disease ¹⁸⁰⁻¹⁸².

Other manifestations

Lyme disease has been associated with acute hepatitis and elevated liver enzymes in active infection have frequently been reported ^{111, 183}. Myositis as an additional presentation of Lyme disease has been described on several occasions ¹⁸⁴⁻¹⁸⁷. *B. burgdorferi* infection has been associated in one case with acute disseminated encephalomyelitis (ADEM) ¹⁸⁸.

Host dependent factors

Several animal experiments have shown an increased rate of infectivity and an increased burden with a diminished rate of treatment success in rodents with an immunodeficiency ¹⁸⁹⁻¹⁹². However, in Lyme disease patients treated with immunosuppressive agents, no significant effect on clinical course and

response to treatment was observed^{193, 194}. Treatment of patients with rituximab during the acute infection can lead to seronegative Lyme disease¹⁹⁵. Treatment of Lyme disease patients in pregnancy is not associated with adverse outcome in the mother. One study however showed that an untreated maternal *B. burgdorferi* sl infection may be associated with an adverse outcome of the pregnancy (OR 7) compared to a treated pregnant population. It is therefore advisable to treat the *B. burgdorferi* sl infection during pregnancy^{196, 197}.

1.5 Laboratory diagnosis of Lyme borreliosis

Diagnosing Lyme disease is sometimes troublesome. Definite diagnosis is made by directly detecting living spirochetes, but this is often not possible. The diagnostic methods and their pitfalls in Lyme disease diagnostics are described in this paragraph.

Microscopy

Spirochetes can be detected directly by silver stain like the Warthin-Starry or a modified Steiner's stain^{198, 199}. Indirect immunofluorescence is also possible (see figure 7). Spirochetes can be detected in EM and ACA lesions, in synovial biopsies, (heart)-muscle biopsies and CSF. Literature on these findings is mainly anecdotal^{187, 198-210}. Electron microscopy has been attempted in several cases and detection of spirochetes has been reported. However, in some of these cases presence of active Lyme disease is doubtful²¹¹⁻²¹⁴. For all forms of microscopy the limitation is the low amount of spirochetes in the tissue making it an unsuitable method for diagnosing Lyme disease.

Culture

Culturing *B. burgdorferi* sl has been a tedious task. The first success was by W. Burgdorfer in 1981¹. For years there were attempts to get a good standardized culture medium and modified Barbour–Stoenner-Kelly medium (BSK-H) is now commercially available²¹⁵⁻²¹⁷. However, culturing spirochetes from patient material is labor-intensive, usually slow and not very sensitive.

Culture has been attempted on a large subset of patient materials. Success of blood cultures for *B. burgdorferi* is very much dependent on the volume of blood used for culture. In the early days often only small volumes of blood, serum or plasma were used, yielding a very low sensitivity^{22, 218-223}. Using higher volumes of blood, serum or plasma yielded a higher sensitivity (>40%) in several studies²²⁴⁻²²⁷. Large volumes of plasma have yielded the best results, followed

by serum and then whole blood ^{225, 228}. Culture from blood has a higher positivity rate in patients with cutaneous early manifestations of the disease, like EM. For diagnosing extracutaneous later manifestations blood culture sensitivities vary (5-50 %), but are generally lower than for EM patients ²²⁹⁻²³¹. Most studies that use blood culture are performed in North America. Studies performed in European patients gave significantly less positive findings than in North American patients. A hypothesis is that this is due to the high prevalence of localized *B. afzelii* in the skin lesion, but not in the blood of European patients ^{232, 233}.

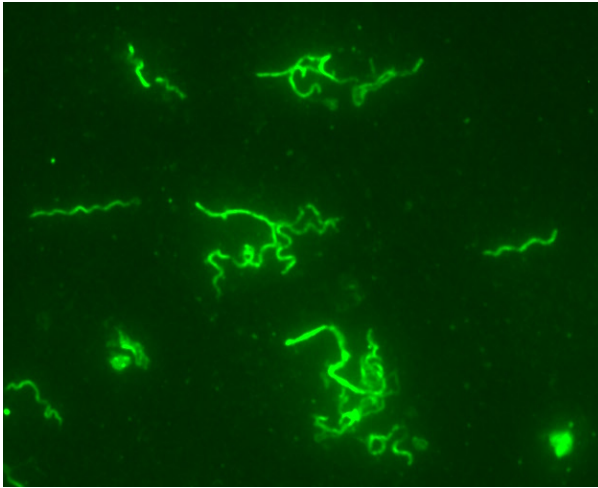


Figure 7: *B. burgdorferi* indirect immunofluorescence stain.

Better results are obtained with cultures from biopsies of EM lesion from European and North American patients (40-88%) ^{234, 235}. There is no significant difference in yield between a biopsy from the center, the edge or beyond the edge of the EM ²³⁶⁻²³⁸.

In biopsies from acrodermatitis chronica atrophicans lesions the sensitivity of culture is 22-60% ^{105, 141, 239}. Even ten years after the primary infection spirochetes can be cultured from an ACA ¹²¹. In a biopsy from a BL the sensitivity of culture ranges from 24 to 33% ^{133, 135}. Cultures from CSF yield a sensitivity of 8-10% ^{235, 240}. From LA synovial fluid or synovial membrane biopsies spirochete recovery is rare ^{241, 242}. After start of effective antibiotic treatment cultures rarely become positive ²³⁵.

Antigen detection

Antigen capture tests for Lyme disease have been developed for urine and CSF. Applicability in a clinical setting has been insufficiently validated, but reports up to now describe a high rate of aspecificity²⁴³⁻²⁴⁶.

T-lymphocyte stimulation assays

Some research has been performed on applying T-lymphocyte stimulation assays on the T-cells of Lyme disease patients. Higher reactivity to *B. burgdorferi* antigens has been shown, but results have been variable. The application of T-lymphocyte stimulating assays does not seem to increase the sensitivity or specificity of the already generally applied assays, like serology²⁴⁷⁻²⁵³. Currently, there is no place for T-lymphocyte stimulation assays in Lyme disease diagnostics, mainly because of concerns regarding the specificity and standardization of the assay²⁵⁴.

Molecular detection

Direct molecular detection of *B. burgdorferi* from patient material has mainly consisted of polymerase chain reaction (PCR)-based methods. Because of the low amount of spirochetes in the tissue correct sample collection, transport and storage is essential for yielding reliable and consistent results. Several PCR targets have been employed; the 16S rRNA gene, p66, flaB, 23S RNA, recA from the chromosome and OspA from the plasmid DNA. Plasmid DNA is believed to be more stable than chromosomal DNA; therefore a plasmid located target has had the preference^{255, 256}. However, for PCR in general the performance of individual in-house PCR's can differ greatly. Currently there is no standard and there are no efforts to develop one. It is therefore difficult to compare the results of performance of PCR from the literature.

DNA isolation protocols are generally also not standardized, but using large volumes of patient material for DNA isolation generally gives a better yield than small volumes^{235, 257}.

Several different PCR techniques have been evaluated, among which: conventional PCR, nested PCR, competitive PCR and real-time-PCR. Overall nested PCR obtained the highest sensitivity, but this technique is very prone to contamination and therefore not generally used in diagnostic practice²⁵⁷.

On skin biopsies from EM and ACA good results have been obtained. In EM lesions the sensitivity ranged from 36-88%, but is generally about 70%. From ACA lesions the sensitivity ranged from 54-100%, generally yielding 80%

sensitivity²³⁵. The sensitivity of PCR on CSF from patients suffering from LNB generally yields 38% sensitivity (range 12-100%)²⁵⁸⁻²⁶⁰. Sensitivity of PCR on synovial fluid and synovial biopsies is much higher than culture for this material, usually detecting DNA in 78% of patients (42-100%). In some studies PCR on biopsies of synovial tissue yields better results than synovial fluid^{255, 256, 261-266}. However, also after adequate treatment with clinical response the synovial tissue can stay positive for borrelia DNA for several months in animal experiments as well as in patients^{267, 268}.

Blood, serum or plasma give low sensitivity in PCR assays (45%). The specificity of PCR on blood has been insufficiently studied²⁵⁷.

Attempts have been made to amplify DNA from urine from Lyme disease patients. The reports on the yield have been highly variable; furthermore there have been reports of nonspecific amplification²⁶⁹⁻²⁷¹. Urine is not an adequate material for PCR for diagnosing Lyme borreliosis.

Due to the fact that PCR can stay positive after treatment for several months in combination with the fact that culture is insensitive, attempts have been made to detect viable spirochetes by detecting 16S mRNA in patient material²⁷². In pre- and post treatment EM lesions it has been shown to give results comparable to that of the DNA PCR. However in EM lesions persistent PCR positivity after treatment is not found.

In patients that have been treated for LA the persistent PCR positivity of synovial fluid has been a diagnostic problem and specifically in this material the pre-treatment mRNA is not highly present. The hypothesis is that DNA of non-viable *Borrelia* is shedded in the synovial fluid from the actual (previous) site of infection²⁶⁸.

Serology

Serology for Lyme disease is the most often performed test to diagnose Lyme disease. The development of serologic tests for Lyme disease has evolved rapidly in the last 3 decades. Many serological techniques have been applied, among which immunofluorescence assays (IFA), enzyme immunoassays (EIA) and Western blots.

IFA is based on (cultured) *B. burgdorferi* fixed onto glass slides. Specific antibodies from patient sera are bound to the *Borrelia* membrane and subsequently detected with fluorescein labeled anti-human IgG. The problems with this technique are the inter-assay variability and the subjectivity of the interpreter^{235, 273}.

In general practice EIAs are more commonly used, advantages are the objective numerical value it yields and the capability of automation. Upon the discovery of *B. burgdorferi* in the late 1970's EIAs were first developed as ELISA's with whole-cell sonicates of *B. burgdorferi* sl. This approach has several technical setbacks. For *B. burgdorferi* sl it is the question which species or strains should be used in the ELISA ²⁷⁴. Several important immunodominant antigens in vivo are not expressed under standard in vitro culture conditions and that some of the immunodominant plasmid-encoded antigens may be lost when the strains are passed multiple times ^{275, 276}. Furthermore, using whole bacteria caused a high rate of aspecific results because of similar antigens shared between *Borrelia* and other bacteria. The EIA gives a cumulative result and it is not possible to distinguish against which antigens the detected antibodies are directed. IgG EIAs are more reproducible than IgM EIAs, because the latter often give false positive results, for instance in patients with autoimmune disorders, or acute infection with other pathogens ^{277, 278}.

In general, the EIA yields a sensitivity of approximately 30-50% in early localized manifestations of Lyme disease. Sensitivity rises to about 70-80% in early disseminated stages of the disease, like LNB. In late manifestations, or prolonged duration of disease the sensitivity can rise to nearly 100%. In early cases a convalescent serum will increase detection of antibodies ^{129, 234, 279-281}. Specificity varies markedly between assays.

In recent years much work has been done on the development of assays of combined purified proteins with more *Borrelia* specific antigens like borrelia flagellin (p41), BmpA (p39), OspA or p83/100. Furthermore the development of EIAs has focused more on immunodominant conserved antigens that are expressed by *B. burgdorferi* sl specifically in early infection in the mammalian host, like OspC, DbpA (p18) and VlsE (IR6/C6-peptide) ²⁸²⁻²⁸⁵. In most assays commercially available now either combinations of these antigens with whole cell sonicate or combinations of one or more recombinant antigens are used. These EIAs often have a comparable sensitivity for detecting anti-borrelia antibodies, but often a higher specificity ^{114, 284, 286-291, 291, 292}.

Another common test for detecting antibodies against *B. burgdorferi* is a Western blot. A Western blot can give a more specific result than an EIA, because antibodies against more than one antigen can be detected separately. The EIA is more sensitive for early manifestations of Lyme disease ²⁹³.

The conventional Western blot is a technique in which a whole-cell sonicate of *B. burgdorferi* sl is separated by SDS-PAGE and blotted on a nitrocellulose membrane. It is important to use a strain that expresses immunodominant epitopes ^{274, 294, 295}. Antibodies from patient serum are incubated with the

nitrocellulose strip. Antibodies are detected by labeled anti-IgG or IgM antibodies. This allows for a more specific determination of the different antibodies against *B. burgdorferi* within the individual patient sera. The difficulty with interpreting a conventional blot is the presence of a high amount of cross-reactive bands, apart from the difference in the quality of reproducibility of the batches of blots²⁹⁶⁻²⁹⁹. Cross-reactive IgM is often a problem in interpreting the blots³⁰⁰. Several algorithms for interpretation of blots have been proposed^{234, 301-303}. The criteria for interpreting Western blots as they are used in North America do not yield a good sensitivity in European patients^{294, 303, 304}. It is important to realize that the prevalence of different genospecies is region specific which can lead to preferential rules for specific geographical regions³⁰³.

In the recent years much research has been done on line-blot, instead of conventional or native blots. Line-blot are produced with purified recombinant proteins which are placed on a nitrocellulose band in a specific concentration. Advantages of this technique are the specific amount of protein that can be applied, the specific proteins expressed in vivo that can be elected and elimination of the background of cross-reactive proteins.

The use of different homologues proteins, from different genospecies can be important to obtain optimal results^{285, 305, 306}. Many of the line-blot commercially available in Europe now use combinations of immunodominant antigens like OspC, flagellin internal conserved fragment, several DbpA homologues, VlsE, p83/100 and BmpA as specific and sensitive *Borrelia* antigens^{234, 306-308}.

Two-tier testing is a common serologic approach for diagnosing LB. Common practice is performing an EIA which is, if positive, followed by an immunoblot as a confirmatory assay. In general the immunoblots yields a higher specificity, but a lower sensitivity in early manifestations of LB^{291, 296, 302, 309-312}. In those cases a convalescent serum taken several weeks later can aid in confirming the diagnosis.

It has been proposed that the use of only one specific recombinant antigen in an EIA alone can give comparable sensitivity and specificity as the two-tier testing strategy^{234, 291, 310, 313, 314}. This strategy is currently not yet generally accepted in many guidelines. A two-tier testing strategy that has been proposed is the use of two EIAs, a whole-cell sonicate EIA and a VlsE/C6-peptide based EIA^{315, 316}.

It is important to realize that serology is an indirect test and development of antibodies can take several weeks, sometimes resulting in negative serologic results in patients presenting with early Lyme disease^{123, 235, 292, 317, 318}. It is

therefore generally advised not to perform serology when a patient presents with a specific early symptom as for instance an EM. If the clinician is in doubt it is advisable to also test a convalescent serum several weeks later, in order to increase sensitivity of antibody detection ²⁹¹.

Furthermore, it is generally not possible to distinguish between active infection from past Lyme disease with serology. In the general population in endemic countries in Europe the prevalence for antibodies against *Borrelia* is 4-7% ^{111, 319-321}. This can be up to 25% in specific populations that have a high exposure rate to ticks ^{320, 322-324}. Most likely the majority of infections in the European population resolve without symptoms, accounting for the high level of seropositivity without clinical manifestation ³²⁵.

Serology after treatment

After treatment anti-*Borrelia* IgG, but also IgM can persist during several years. For IgG this can be lifelong, for IgM persistence has been described for at least one year ^{317, 326-330}. On the other hand, after treatment of early Lyme borreliosis it is possible that the patient will show only very weak or no seroconversion at all, further complicating serologic diagnosis.

When the diagnosis has been made and treatment has been started follow-up serology by Western-blot is not helpful, because in most patients a decline in antibody titer is not detectable ^{329, 331-333}. Furthermore, there is no clear correlation between decline of antibodies after treatment and clinical response ^{290, 334}.

C6, VlsE IR6 Epitope

In recent years the interest in the C6 peptide for diagnosing Lyme borreliosis has increased. C6 is part of a larger protein: VlsE. To maintain chronic infection *Borrelia* can apply antigenic variation. One of these membrane expressed lipoproteins is Variable Major Protein-like sequence, expressed (VlsE). VlsE is encoded on the lp 28-1 where VlsE and its promoter are situated next to a cassette of 15 unexpressed VlsE lipoproteins (See figure 8) ^{57, 335, 336}.

During infection promiscuous recombination events take place from the cassette leading to antigenic variation of the expressed VlsE, potentially leading up to 10^{30} different combinations ⁵⁶. VlsE consists of six variable regions (VR) and six invariable regions (IR). The VR are highly immunogenic and antibodies directed to the VR are able to effectively kill spirochetes that express the corresponding antigens. Spirochetes escape killing by expressing different variable domains as rapid as four days post infection ^{54, 337}. IR are essential for structural conformation of proteins applying antigenic variation and are highly

conserved and highly expressed among *B. burgdorferi* sI complex. Usually IR are not immunogenic, but IR6 of VlsE, is highly immunogenic³³⁸⁻³⁴¹. Incorporated in VlsE on the membrane C6 is not accessible for Abs and Abs to C6 are not bactericidal in vitro³⁴². It has been hypothesized that chronic host exposure to immunodominant C6 diverts the immune system from responding to less antigenic but functionally more important Ags or epitopes, thus serving as a protective strategy for persistent infection³⁴¹. In non-human primates IgG responses are detectable as early as three weeks post inoculation, IgM responses are rarely detectable and do not appear earlier than the IgG response³³⁹. In humans detection of anti-VlsE Abs in an early phase of disease has been described²⁸².

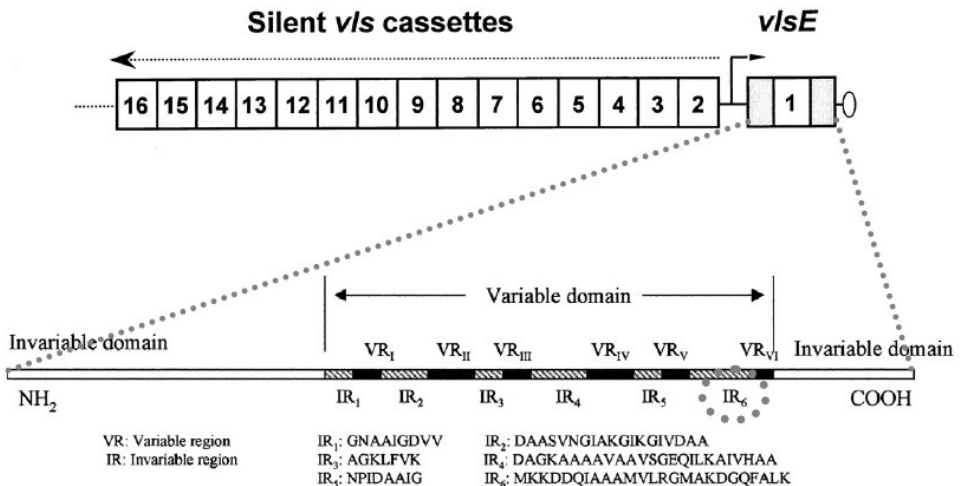


Figure 8: The arrangement of the VlsE gene at the lp28. Picture modified from Liang et al. and Zhang et al.^{56, 338}

Of all of the six IR, IR6 is the most immunogenic and highly conserved among *B. burgdorferi* sI, but can still vary up to 5 amino acids between species^{335, 340, 342-345}. IR6 consists of only 26 amino acids, making it an easy to manufacture and cheap recombinant protein for diagnostic procedures. The IR6 peptide is unique, and hardly any cross reactivity is reported, on GenBank no identical sequence has been described thus far^{339, 341}. Some *B. burgdorferi* strains lack part of the lp28-1 plasmid, but these strains still bear the VlsE cassette and express VlsE³⁴⁶. Only rarely patients do not seem to make an early anti-C6 response, while the cassette is still present and expressed in the infective strain³¹⁰.

Waning of C6-peptide antibodies

It has been proposed that after adequate eradication, the C6 antibody titers wane. In that case, coalescent antibody titer measurements might be an instrument to monitor therapy efficacy as is the case with the VDRL in syphilitic patients. In a study by Philipp et al. 7 rhesus macaques showed rapid anti-C6 decline after infection and subsequent treatment for LB. Week 34 after infection all serological levels dropped to near detection level. The ELISA for anti-p39 did not show such rapid decline. In 15 patients all serum anti-C6 titers dropped more than 4-fold 20 weeks after treatment ³⁴⁷. Philipp et al. followed 105 New York patients after treatment for early localized LB. In all 105 patients C6 levels declined, in 96 (91%) a decline below baseline, or a more than fourfold decrease in C6 titers was found. However, in patients that had multiple EM, and therefore were likely longer exposed to *B. burgdorferi*, the decline was less prominent ³⁴⁸. Anti-VlsE titers can also wane over time, after treatment for EM shown in 67 patients ³⁴⁹

This was also confirmed in a later study in which serum from 11 EM and 34 disseminated LB patients was tested, 80 % showed the more than fourfold decrease in titers after six months. Nine patients did not show the more than fourfold decrease in titers after 6 months, they all belonged to the disseminated disease group ³⁵⁰.

The decline in titer does not mean C6 antibody titers become undetectable in all adequately treated patients. Peltomaa et al. showed in 45 patients (n=24 early disseminated disease, 21 late disseminated disease) titers decreased more than fourfold in 67% of patients with early disseminated disease and only 14% in patients with late disseminated disease. Years (median 11 years) after treatment without signs of relapse 50% of patients with early disseminated and 83% of patients with late disseminated disease still had positive C6 serology ³⁵¹.

In PTLDS patients, no correlation was detected between quality-of-life markers, clinical outcome, response to therapy and anti-C6 presence. The sheer presence of C6 Abs does not necessarily indicate active infection ³⁵².

Diagnosing neuroborreliosis in cerebrospinal fluid

Primarily when a clinician has a LNB in the differential diagnosis several CSF markers will make LNB more probable. The CSF generally shows sign of inflammation; pleiocytosis is usually present, dominated by the presence of mononuclear cells ^{111, 353-355}. Some cases have been described where pleiocytosis was absent and the diagnosis of LNB highly likely due to a combination of a tick bite, a positive antibody index and a clinical picture resembling known LNB manifestations ^{111, 112, 356-358}. Other signs of inflammation in the CSF of a patient

with LNB include; elevated total protein levels, elevated CSF/serum albumin ratio (80-90%), presence of oligoclonal bands (40-50%), elevated lactate (55%) and low glucose (10%)^{111, 354, 357}.

In the CSF antibodies against *Borrelia* can be detected, commonly used EIAs are whole cell sonicate EIAs or a purified flagellin EIA. IgM and/or IgG against *Borrelia* in CSF are invariably positive at time of presentation with a LNB^{356, 359}. Research on the presence of intrathecal anti-C6-peptide yielded conflicting results. The sensitivity in these studies ranged from 61-98%³⁶⁰⁻³⁶². This is remarkable because in the studies applying the C6-peptide EIA in serum an early anti-C6-peptide response is usually present.

Detection of intrathecal anti-*Borrelia* IgG alone is not enough, because passive diffusion from the serum can take place. The way to correct for this phenomenon is to test a paired serum CSF sample and to calculate the antibody index (AI)^{356, 363}. By diluting the serum and CSF to the same concentration of total IgG and performing an ELISA the fraction of specific anti-*Borrelia* IgG can be compared. If the AI index is larger than two, indicating the amount of intrathecal antibodies is relatively twice as high, the AI is positive. It is of minimal relevance whether a whole-cell sonicate or purified flagellin EIA is used and several calculation methods yield identical results^{364, 365}. A second technique is a capture EIA which captures total IgG or IgM and then detects the fraction of anti-flagellin IgG or IgM in serum and CSF samples. The AI is then calculated directly from these results. Advantage of this assay is the lack of necessity to determine amount of total IgG and albumin and the fact that no additional dilution is necessary³⁶⁶.

When the anti-*Borrelia* AI is elevated the diagnosis of LNB is highly probable (89-97%). The *Borrelia* AI can sometimes be positive in patients with relapsing remitting multiple sclerosis³⁶⁷. The sensitivity of a positive AI for diagnosing LNB has been reported to vary from 38-92%, with a median of approximately 70%^{114, 354, 356, 368}. The sensitivity of AI is lower in early manifestations of LNB^{366, 368, 369}. In contrast with this finding, in previous studies it was also demonstrated that patients with LNB can have an earlier response to antigens intrathecally than in serum^{292, 366, 370, 371}. For the clinical manifestation of late LNB in North American patients, it seems that the AI is less sensitive than in European patients^{356, 359}.

IgG is the most reliable immunoglobulin for calculating the AI. In some cases the IgM-AI is more sensitive in early manifestations of LNB^{354, 366, 372}. In LNB patients with a negative AI at diagnostic lumbar puncture a convalescent AI can

be helpful for detecting delayed intrathecal synthesis of IgG, but the AI will stay negative in 33% of previously AI negative cases^{368, 373}.

In most cases the signs of inflammation in the CSF and the AI will decline after treatment. However, a positive AI index can stay positive for decades after effective treatment without any sign of clinical failure^{374, 375}.

Another method for detecting intrathecal production is by Western blot. However the use of immunoblots to determine intrathecal antibody responses is problematic, due to difficulties in interpreting the intensity of bands, variety displayed in individual patients and the lack of good criteria for interpretation^{354, 376-378}.

Biomarkers

Biomarkers for detecting LB have been of interest in Lyme disease diagnostics. It has been proposed that specific cyto- and chemokines play a role in LB. A chemokine of particular interest is CXCL13.

Intrathecal levels of CXCL13 have been suggested as a potential biomarker for diagnosing LNB. CXCL13 is produced by antigen presenting cells and is a selective chemoattractant for B-cells and B-helper T-cells. Toll like receptor 2 is most likely the receptor involved in induction of CXCL13 production in LB³⁷⁹. CXCL13 has been found to be expressed at high levels in pooled cerebrospinal fluid (CSF) from human LNB patients (219ng/g total protein) by a cytokine array, while in pooled CSF from subjects with non-inflammatory neurological disease levels were barely detectable (<1.7ng CXCL13 /g total protein). Patients with multiple sclerosis had a slightly higher level of CXCL13 intrathecally than healthy controls³⁸⁰. In a patient cohort of acute LNB patients CXCL 13 levels were highly elevated in all 37 definite LNB cases and no or minimal elevation was seen in the non LNB controls (n=8)³⁸¹. In another study 28 LNB cases had significantly elevated levels of CXCL13 compared to neurological and infectious controls. Some infectious controls had high levels of CXCL13 intrathecally, but overall sensitivity and specificity for LNB using a cut off of 337ng CXCL13 /g total protein were 96% and 97% respectively³⁸². Another study used a combination of CXCL13 and anti-C6-peptide antibodies for diagnosing LNB in children. This study used a cut-off of 163ng CXCL13/g albumin (equivalent to 142 pg CXCL13/ml CSF), which gave 98% sensitivity (n=124 LNB) and 95% specificity (controls n=92). Adding the detection of antibodies against C6-peptide in CSF increased the specificity for detecting LNB to 97%. This study also included several groups of possible LNB according to EFSN criteria; in the group of serologically antibody positive, CSF antibody negative but pleiocytosis positive group 100% of patients were children and had elevated levels of CXCL13. This was not the case in all pleiocytosis negative, or serologically negative probable LNB³⁷².

A prospective study used CXCL13 to diagnose adult patients with untreated LNB (n=13), controls were 178 patients with pleiocytosis due to other diseases. In this specific study the cut-off was chosen at 1229 pg CXCL13/ml CSF. Patients presenting with an intrathecal lymphoma often had very high levels of CXCL13 intrathecally³⁸³. No consensus on a cut-off has been reached to date. It has been shown that CSF CXCL13 is a biomarker that declines rapidly after treatment for LNB, making it a possible biomarker for evaluating treatment for neuroborreliosis^{382, 383}.

CXCL13 is a chemokine that is produced during presentation of antigens by antigen presenting cells. The question remains whether levels of CSF CXCL13 are specific for LNB or also present in other CNS infections or in other autoimmune diseases in which elevated levels of serum CXCL13 have frequently been reported³⁸⁴⁻³⁸⁸.

CXCL13 is also highly present in *Borrelia* lymphocytoma lesions³⁸⁹. Detecting CXCL13 in serum of LB patients has been attempted but this has been shown to have no diagnostic value for LB^{382, 390}.

1.6 Treatment and prevention strategies

Choice of antibiotics

The first cases of effectively treated LB with penicillin date back to the 1950's^{10, 11}. In vitro, *B. burgdorferi* sl is sensitive to a very large spectrum of antibiotics, including β -lactam antibiotics, tetracyclines and macrolides³⁹¹⁻³⁹⁵. There is a lack of standardized methodology to determine MIC/MBC's³⁹⁶. A standardized microdilution method has been developed³⁹⁷. Aminoglycosides, older generation quinolones and aztreonam are inappropriate antibiotics for *B. burgdorferi* sl^{391, 396, 398, 399}. Antibiotics that have a gram positive spectrum like vancomycin and linezolid surprisingly show activity against *Borrelia* spp in vitro, though it has never been proven that they are adequate therapeutic agents in vivo^{392, 400}. A glycycline (tigecycline) has low MIC/MBC's against *B. burgdorferi* sl in vitro but its effectivity in vivo has been insufficiently studied^{401, 402}. The in the Netherlands commonly applied antibiotics amoxicillin, ceftriaxone and azithromycin have the lowest MIC₉₀'s and MBC's (≤ 2 μ g/ml) in vitro against several *B. burgdorferi* sl tested^{394, 395, 403}.

Despite a low MIC of *B. burgdorferi* sl for macrolides clinical trials have shown failure of the older generation macrolides like erythromycin^{19, 404}. Azithromycin,

however, has shown to be non-inferior or even superior to treatment with doxycycline in early stage LB ⁴⁰⁵⁻⁴⁰⁸

In some studies patients treated with cephalosporines have equal or higher response rates than patients treated with penicillines in late manifestations of LB ⁴⁰⁹⁻⁴¹¹. For the treatment of LNB doxycycline per os (po) was non-inferior to intravenous (iv) ceftriaxone in an European cohort ⁴¹². The data about susceptibility patterns between species differs, but there are some studies that conclude that *B. burgdorferi* ss and *B. afzelii* are more resistant to some classes of antibiotics. Treatment failures have been described for almost any class of antibiotic ³⁹⁶. However, culture confirmed failure of treatment for EM with β -lactams and tetracyclines is rare (<1.7%) ⁴¹³.

Commonly advised antibiotics in the guidelines for treatment of LB in Europe and North America are doxycycline and ceftriaxone. For oral therapy in children doxycycline is usually replaced by amoxicillin ⁴¹⁴⁻⁴¹⁷. In early manifestations oral therapy should be sufficient. For later manifestations intravenous therapy is recommended for specific clinical manifestations.

Duration of treatment

The advised duration of treatment varies mildly between guidelines. For early manifestations of LB duration of treatment of about two weeks (10-21 days) is commonly advised in European and North American guidelines. For later, disseminated manifestations of LB duration of treatment of about 3 weeks (14-30 days) is commonly advised ⁴¹⁴⁻⁴¹⁹.

Early manifestations of Lyme borreliosis

For the treatment of a solitary EM 10 days of treatment is generally sufficient, longer treatment has no effect on outcome ⁴²⁰. In the case of an early disseminated LB most studies on treatment have been performed in North America. Most patients with an early disseminated disease present with multiple EM, but this is not a clinical picture that is prevalent in Europe. In the case of carditis no sufficiently powered study has been done. Generally a treatment of 2 weeks with ceftriaxone iv or 3 weeks of doxycycline po is adequate ⁴²¹.

Acrodermatitis chronica atrophicans

The manifestation of an acrodermatitis chronica atrophicans is rare and mainly present in Europe. There are not many studies that compare duration of treatment in well conducted studies. Longer treatment up to 30 days seems to be indicated for this chronic manifestation of LB. Intravenously or orally

administered β -lactams and doxycycline po seem to be equally effective regimens ^{422, 423}.

Lyme arthritis

LA is a late manifestation of LB. In the studies conducted on the effectivity of antibiotics the success-percentage varies greatly. Treatment regimens of longer duration have a higher response rate than treatment duration of 2 weeks. Intravenously administered β -lactams and doxycycline po are both effective regimens ^{411, 424-427}. After treatment up to 25% of the patients remain with complaints of arthritis or arthralgias for several months, despite the fact that there are no signs of persistent infections ^{153, 268, 428}. In the case of persistent symptoms with no sign of persistent infection expert opinion is to attempt treatment with anti-inflammatory agents or an arthroscopic synovectomy ^{150, 272, 429, 430}.

Lyme neuroborreliosis treatment

In several studies cephalosporines have been compared to penicillin in the treatment of early LNB. Ceftriaxone has been a preferential treatment due to good penetration in the CSF and the favorable pharmacokinetic properties. Penicillin and ceftriaxone seem to be equally effective, though no large randomized study has been performed ^{409, 410, 431-433}. A treatment period of at least 14 days is advisable, because with treatment regimens of 10 days relapses were observed in a small population ⁴³¹.

Treatment with β -lactams is usually intravenous, but an option is the treatment of LNB with orally administered doxycycline. All randomized studies conducted in Europe showed non-inferiority of the doxycycline po compared to ceftriaxone iv in early LNB ^{412, 434}. Many other studies have compared the two treatment regimens unblinded, retrospectively or non-randomized. Mean overall success rate of ten studies with LNB patients treated with a β -lactam antibiotic iv was 95% and for doxycycline po 99%, this difference was not significant. In these studies it can not be excluded that the patients with a milder form of LNB were treated with the oral therapy leading to a selection bias ^{419, 435}.

Research has been performed on addition of corticosteroids for FNP, but the data is not conclusive for Lyme disease and does not significantly favor use of corticosteroids for the recovery of the function of the nerve ⁴³⁶⁻⁴⁴¹. Anecdotal reports suggest that post antibiotic treatment with corticosteroids of other LNB manifestations might reduce residual complaints ^{442, 443}.

In general, many LNB patients after treatment have residual complaints that need months to years to resolve even after adequate treatment. Longer duration of complaints before treatment is associated with slower recovery^{357, 434, 444, 445}. Seventy to eighty percent of patients report complete recovery within 6 months^{446, 447}. About 25% of all LNB patients report neurological problems years after the primary infection, of which half of the patients report it is interfering with daily activities^{437, 448-452}. However, in an age-matched control population the prevalence of neurological complaints and symptoms reported by post treatment LB patients are not significantly different^{450, 453, 454}. In two studies of age-matched adolescents this difference in performance or residual complaints was significant^{437, 448}.

Prolonged treatment for residual complaints

Treatment failure with currently advised regimens is very rare^{124, 420, 455, 456}. It is on the other hand difficult to diagnose persistent active infection after completed treatment, because culture confirmed relapse is rare. In one study it was shown that 22 paired consecutive episodes of EM were due to reinfection rather than relapse⁴⁵⁷.

DNA of spirochetes can persist in joints for several months before it is completely cleared after therapy without clinical failure²⁷². It has also been described that spirochetes can go into a dormant state when the bacteria are in a hostile environment, for instance during treatment. These forms are named cyst form. It has been shown that the cyst forms can be taken up by ticks and transmitted to SCID mice⁴⁵⁸. When SCID mice are infected with *B. burgdorferi* they have extensive infection and inflammation⁴⁵⁹. However, infection with the cyst forms do not give any histological inflammation and the numbers seem to slowly decline in the host concluding that the cyst forms are avirulent and do not cause disease⁴⁶⁰.

The prolonged treatment of patients that have no objective sign of persistent infection is controversial. Studies done on this subject are difficult to interpret due to the difficulties with the definition of post Lyme disease syndrome. Some studies take patients with any kind of residual complaint; other studies only include patients with objective complaints. Several double-blind, randomized, placebo-controlled studies found no effect of prolonged treatment of patients with persistent symptoms after LB⁴⁶¹⁻⁴⁶⁵. Furthermore there have been several observations in which it was clear that antibiotic refractory arthritis does not resolve with prolonged treatment²⁷².

As there is no significant effect of prolonged treatment in placebo controlled trials it is generally advised not to treat patients with persistent symptoms after

treatment according to guidelines ^{414-417, 419}. It is advisable to further examine patients with complaints after LB, because in a significant proportion of the cases another illness can be diagnosed ^{466, 467}.

Correct and evidence-based of treatment of an sufficiently proven LB is important, because the prolonged intravenous treatment is not without risks. Severe complications and deaths due to, the sometimes prolonged, treatment of supposed LB have been described in case reports ^{461, 468-472}.

Vaccination against Lyme disease

Vaccine development has been a hot item in the last few years. Several vaccine proteins have been applied among which OspA, OspB, OspC, DbpA, RevA, BBK32, BB0323 and ACGal ⁴⁷³.

The OspA vaccine using aluminum as adjuvant was approved by the FDA and applied ⁴⁷⁴. However, after wide application it was shown that titers were not sufficient in 5% of the population and that additional boosters were essential for a prolonged effective titer. Furthermore there were some concerns about the safety of the vaccine because of an hypothesis that molecular mimicry between the vaccine antigen and an auto-antigen triggered autoimmunity ⁴⁷⁵. The vaccine was withdrawn from the market due to insufficient sales. Since then new OspA vaccines have been under development ^{476, 477}.

However, an interesting candidate is the OspB vaccine that has shown a complement independent bactericidal effect ^{478, 479}. Combining more than one antigen, for instance OspA/B/C, DbpA or BBK32, in a vaccine has shown promising results ^{480, 481}.

Vaccine research has also been done on tick immunity, which is elicited by repeated feeding on a host. Tick immunity can interfere with transmission of *Borrelia* spp. though it does not give complete protection ⁴⁸²⁻⁴⁸⁵. This strategy however could also give protection to other arthropod borne infections, like *Francisella* spp. ⁴⁸⁶. It is still a long way to developing a suitable vaccine against *Borrelia* spp, but perhaps a strategy combining some of the before mentioned epitopes is going to elicit an effective vaccine ⁴⁷³.

Preventive measures

A highly effective way of preventing an infection with *B. burgdorferi* is preventing the tick bite itself. Wearing light colored, tightly woven clothing, tucking pants into socks and shirt into pants, using insect repellents and frequent checking to remove crawling or attached ticks reduce the risk of tick-borne diseases if conscientiously practiced ⁴⁸⁷⁻⁴⁸⁹.

In North America deer control is one of the most effective measures in diminishing the tick population ⁴⁹⁰. In Europe however this strategy is less effective because of the different tick and host species that are prevalent. The parasitic Ichneumon wasp *Ixodiphagus hookeri* has long been investigated for its potential to control tick populations. It lays its eggs into ticks; the hatching wasps kill their host. Another natural form of control for ticks is the use of the guinea fowl, a bird species which consumes mass quantities of ticks. Just 2 birds can clear 2 acres in a single year ⁴⁹⁰. Furthermore research is being performed on using molds that can infect ticks ⁴⁹¹.

Phenothrin in combination with Methoprene, both acaricides, were a popular topical flea/tick therapy for felines. Acaricides might be applied in an individual setting, but is not effective in reducing the tick population when it is mass applied ^{492, 493}. Furthermore topical (drops/dust) flea/tick medicines may be toxic to animals and humans ⁴⁹⁴.

Prophylaxis after a tick bite

Prophylaxis after a tick bite remains controversial, although some guidelines will propose use of prophylaxis after a tick bite. The risk of contracting Lyme disease after a tick bite of a tick that is not promptly removed is very low, also in the Netherlands (<1-2%) ^{495, 496}. Furthermore, there is no clear evidence on what the prophylaxis should consist of. Some studies apply a 10-day course of antibiotics while others give one dose of 200 mg doxycycline ⁴⁹⁷⁻⁵⁰⁴. On the basis of pharmacodynamic studies one dose of 500mg azithromycin should be as effective as one dose of 200mg doxycycline ⁵⁰⁵. Results of efficacy of the prophylaxis vary greatly between studies. A meta-analysis combining data from North America showed there was a significant reduction in Lyme disease in the population who received prophylaxis consisting of one dose of 200mg doxycyclin. The number needed to treat was 50 to prevent one case of EM in a highly endemic area in North America ⁵⁰⁶.

1.7 Scope of this thesis.

As discussed in the previous section *B. burgdorferi* has a wide variety of strategies to hide from the host immune system. Complement regulatory binding proteins have been described for almost all complement resistant *B. burgdorferi* sl, except for the complement resistant *B. bavariensis*, one of the species that is known to frequently cause Lyme neuroborreliosis.

In **chapter 2** it is attempted to identify CRASP-1 proteins in *B. bavariensis*, formerly known as *B. garinii* OspA serotype 4. Potential CRASP-1 proteins will be cloned and studied for their ability to interact with host derived fluid phase regulators of complement.

The specific role of complement resistance in early effective infection and dissemination of *B. burgdorferi* sl has not been well investigated. Can complement resistance lead to a better and more effective infection and dissemination? In **chapter 3** an in vivo experiment in which the infectivity and dissemination patterns of complement sensitive and complement resistant *B. burgdorferi* sl in a C3 deficient mouse model is described.

After effective transmission from the tick to the host the next challenge in *B. burgdorferi* infection is rapid and accurate detection of the pathogen. Diagnostics of Lyme disease is often compromised due to specific pathogen properties combined with technical shortcomings of bacterial serology.

Two indirect detection methods which can aid in diagnosing patients suffering from Lyme neuroborreliosis were studied. In **chapter 4** the performance of the C6-peptide ELISA for detecting antibodies in CSF in Lyme neuroborreliosis patients is studied. While in **chapter 5** levels of CXCL13 in several patient populations as a potential biomarker for the diagnosis of Lyme neuroborreliosis is studied.

For both indirect markers of presence of *B. burgdorferi* the specificity in clinically resembling and neuroinfectious diseases is of key importance. Several other infectious and inflammatory diseases that have a clinical presentation that can resemble Lyme disease are included in the analysis.

Diagnosing Lyme disease can be difficult in some populations, first because Lyme disease is a relatively rare infection, resembling a large spectrum of other autoimmune and inflammatory diseases. Clinicians could often consider testing for Lyme disease. It is also important to do this in specific preselected populations, because the positive predictive value of a test, but specifically indirect tests such as serology, in a random population, is low.

In **chapter 6** all patients that present with complaints of arthritis at the early arthritis clinic are tested for Lyme arthritis. The prevalence of *B. burgdorferi* seropositivity in this population is studied. Another aim is to identify clinical factors which should urge the doctor to test, or explicitly not test, for Lyme disease in a patient presenting with arthritis in Europe.

In **chapter 7** a case of an HIV positive patient presenting with a meningo-encephalitis caused by *B. burgdorferi* is described. The literature on HIV and Lyme neuroborreliosis co-infections is also reviewed.