Molecular and serological tools for clinical diagnostics of Lyme borreliosis

- can the laboratory analysis be improved?

MALIN LAGER

Molecular and serological tools for clinical diagnostics of Lyme borreliosis

- can the laboratory analysis be improved?

Malin Lager

Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County
SE-551 85 Jönköping, Sweden

Linköping University, Department of Biomedical and Clinical Sciences,
SE-581 85 Linköping, Sweden

Linköping 2020

© Malin Lager, 2020

Published articles have been reprinted with the permission of the copyright holder

Printed in Sweden by LiU-tryck, Linköping 2020

ISSN 0345-0082

ISBN 978-91-7929-833-3

”En droppe droppad i Livets älv,
har ingen kraft att flyta själv.
Det ställs ett krav på varje droppe,
Hjälp till att hålla de andra oppe!”

~ *Tage Danielsson*

ABSTRACT

Lyme borreliosis (LB) is caused by spirochetes within the *Borrelia burgdorferi* sensu lato complex and is the most common tick-transmitted disease in the northern hemisphere. The transmission of the spirochetes to humans in Europe is done by the *Ixodes ricinus* ticks, which can also transmit the relapsing fever species *Borrelia miyamotoi*. LB may cause clinical manifestations in the skin, in the central nervous system, in joints, and in the heart. Diagnosis of LB is mainly based on the patient's medical history, self-described symptoms, and clinical signs in combination with the detection of *Borrelia*-specific antibodies (serological methods). In some cases/issues, detection of *Borrelia*-specific deoxyribonucleic acid (molecular methods) may be used as a complement to serology. All diagnosed LB infections are treated with antibiotics to prevent disease progression, and most patients fully recover without further sequelae. The overall aims of this thesis were to evaluate molecular and serological tools for laboratory diagnosis of LB, with a special focus on Lyme neuroborreliosis (LNB), and to identify potential improvements.

The results presented in this thesis showed that the immunoglobulin (Ig) G assays, currently in use in northern Europe for detection of antibodies in serum, had high diagnostic sensitivity (88 %) together with comparable results both between and within assays. For the IgM assays, the diagnostic sensitivity was lower (59 %) with more heterogeneous results. Small variations in diagnostic performance for IgM and IgG were mainly presented for samples within the borderline zone. These results support the theory that separate testing of IgM antibodies in serum has low diagnostic value. However, simultaneous detection in serum and cerebrospinal fluid (CSF) for both IgM and IgG antibodies was essential for the diagnosis of LNB, at least for certain assays.

So far (to our knowledge), no systematic evaluation and optimisation of the pre-analytical handling of CSF samples before molecular testing has been performed. By use of the precipitate concentrated by moderate centrifugation, extraction of total nucleic acid followed by reverse-transcription to complementary deoxyribonucleic acid, in combination with the absence of

polymerase chain reaction (PCR) inhibitors, detection of *Borrelia garinii*, *Borrelia afzelii*, *Borrelia burgdorferi* sensu stricto, and *B. miyamotoi* was possible. These four species are all known to be pathogenic to humans. The results revealed a high analytical sensitivity and specificity for the optimised pre-analytical conditions. The thesis also presents results showing that the real-time PCR protocols currently used in Scandinavia have high analytical sensitivity, specificity, and concordance. This indicates that the low diagnostic sensitivity for detection of *Borrelia* in CSF was not a result of poorly designed and evaluated PCR protocols, but was possibly due to the low number of spirochetes in the samples. However, to further evaluate the diagnostic performance for detection of *Borrelia* in CSF by PCR, clinical samples need to be evaluated based on our new recommendations for the pre-analytical handling of CSF samples.

In conclusion, this thesis presents results revealing that both molecular and serological tools for detection of *Borrelia* have, in general high sensitivity and specificity with results comparable between different protocols and different laboratories. It also presents recommendations for pre-analytical handling of CSF samples before PCR-analysis, and shows the benefits in diagnostic performance by simultaneous detection of IgM and IgG antibodies in serum and CSF for accurate diagnosis of LNB. Even though the techniques mentioned above have high analytical performance, the ability to discriminate an active infection from a previous one is limited and further studies need to be carried out. These studies need to focus on finding diagnostic tools that can help physicians to determine ongoing infection to ensure adequate treatment. It is also desirable to improve the standardisation of the diagnostic tools and to find methods that can discriminate between different *Borrelia* species.

SAMMANFATTNING PÅ SVENSKA

Borreliosis är den vanligaste fästingöverförda sjukdomen på norra halvklotet och orsakas av bakterier inom *Borrelia burgdorferi* sensu lato gruppen. Överföringen av bakterier till människa i Europa sker via *Ixodes ricinus* fästingar, vilka även överför bakterien *Borrelia miyamotoi* som ger återfallsfeber. Borreliainfektioner uppvisar kliniska uttryck i huden, i det centrala nervsystemet och i leder. En borrelia-diagnos baseras främst på patientens medicinska historia i kombination med kliniska tecken, egenbeskrivna symptom samt påvisning av *Borrelia*-specifika antikroppar (serologiska metoder). Vid vissa frågeställningar kan påvisning av *Borrelia*-bakteriens arvs massa (molekylärbiologiska metoder) användas som komplement till antikroppstester. Alla diagnostiserade borreliainfektioner behandlas med antibiotika för att förhindra utveckling av sjukdomen och merparten av patienterna blir fullt återställda. Det övergripande syftet med avhandlingen var att utvärdera metoder för påvisning av *Borrelia*-specifika antikroppar samt *Borrelia*-specifik arvs massa, men fokus på neuroborreliosis, samt identifiera potentiella förbättringar.

De metoder som används för påvisning av immunoglobulin (IgG)-antikroppar (uppträder sent i en infektion) i serum i norra Europa uppvisar hög känslighet (88 %) med jämförbara resultat både mellan och inom en analysmetod. Vid påvisning av IgM-antikroppar (uppträder tidigt i en infektion) i serum uppvisas lägre känslighet (59 %) och mer olikartade resultat. Små variationer i den diagnostiska förmågan att påvisa IgM och IgG-antikroppar beror till stor del på att flera prover erhållit gränsvärdena vid ett värde som inte kan anses som positivt men inte heller som negativt. Resultaten från denna studie indikerar att påvisning av IgM-antikroppar i serum har lågt värde vid diagnostik av *Borrelia*. Dock bör parallell analys av både IgM och IgG-antikroppar i serum och ryggmärgsvätska utföras vid påvisning av neuroborreliosis.

I dagsläget (till vår kännedom) har ingen systematisk utvärdering och optimering av det pre-analytiska tillvägagångssättet vid påvisning av *Borrelia*-specifik arvs massa i ryggmärgsvätska genomförts. Genom att använda pelleten (bottensatsen som erhålls genom måttlig centrifugering), framrening av total nukleinsyra i kombination med frånvaro av material som

kan påverka PCR-reaktionen på ett negativt sätt (inhibitorer), kan påvisning av *Borrelia*-arterna *Borrelia garinii*, *Borrelia afzelii*, *Borrelia burgdorferi sensu stricto* och *B. miyamotoi* ske. Dessa *Borrelia*-arter är alla patogena för människa. De realtids-PCR protokoll som i dagsläget används i Skandinavien har hög analytisk känslighet, tillförlitlighet och överensstämmelse. Detta tyder på att den låga känslighet som uppvisas vid påvisning av *Borrelia*-specifik arvs massa i ryggmärgsvätska inte beror på dåligt utvärderade och designade PCR-protokoll, utan är troligtvis orsakad av låg bakteriemängd i proverna. För vidare utvärdering av den diagnostiska förmågan att påvisa *Borrelia*-specifik arvs massa i ryggmärgsvätska med PCR, bör kliniska prover samlas in och analyseras utifrån de nya rekommendationerna för pre-analytiskt tillvägagångssätt vid analys av ryggmärgsprover.

Sammanfattningsvis visar resultaten i denna avhandling på generellt hög känslighet och tillförlitlighet samt överensstämmelse mellan olika protokoll/test vid påvisningar av *Borrelia*-specifika antikroppar och *Borrelia*-specifik arvs massa. I avhandlingen presenteras även rekommendationer för pre-analytiskt tillvägagångssätt vid omhändertagande och transport av ryggmärgsvätska till laboratoriet. Resultaten visar även på nyttan i att analysera ryggmärgsvätska och serum parallellt för både IgM och IgG-antikroppar för att erhålla rätt diagnos vid frågeställningen neuroborrelios. Ovan nämnda metoder har trots god prestanda svårt att i alla lägen särskilja en aktiv infektion från en tidigare genomgången, varpå vidare studier krävs. Framtida studier bör fokusera på att finna diagnostiska verktyg som hjälper läkarna att urskilja en pågående infektion så att patienten erhåller passande behandling. Det är också mycket viktigt att arbeta vidare mot en standardisering av de diagnostiska metoderna samt finna metoder som har möjlighet att särskilja mellan olika *Borrelia*-arter.

LIST OF PAPERS

I. **Lager M**, Dessau R, Wilhelmsson P, Nyman D, Jensen G F, Matussek A, Lindgren PE, Anna J, Henningsson A J, The ScandTick Biobank Study Group. Serological diagnostics of Lyme borreliosis: comparison of assays in twelve clinical laboratories in Northern Europe. *Eur J Clin Microbiol Infect Dis* 2019 Aug 09; 38: 1933-1945.

II. Henningsson AJ, **Christiansson M***, Tjernberg I, Löfgren S, Matussek A. Laboratory diagnosis of Lyme neuroborreliosis: a comparison of three CSF anti-Borrelia antibody assays. *Eur J Clin Microbiol Infect Dis* 2013; 33(5):797-803.

III. **Lager M**, Wilhelmsson P, Matussek A, Lindgren PE, Henningsson AJ. *Borrelia burgdorferi* sensu lato and *Borrelia miyamotoi* in cerebrospinal fluid – Is there a potential for improvement in the diagnostics? *Manuscript*

IV. **Lager M**, Faller M, Wilhelmsson P, Kjelland V, Andreassen Å, Dargis R, Quarsten H, Dessau R, Fingerle V, Margos G, Noraas S, Ornstein K, Petersson A, Matussek A, Lindgren PE, Henningsson AJ. Molecular detection of *Borrelia burgdorferi* sensu lato - an analytical comparison of real-time PCR protocols from five different Scandinavian laboratories. *PLOS ONE* 2017 Sep 22; 12 (9):e0185434.

* The author's maiden name is Christiansson

ABBREVIATIONS

ACA	Acrodermatitis chronica atrophicans
AI	Antibody index
BL	Borrelia lymphocytoma
BSK	Barbour-Stoenner-Kelly
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
Cq	Quantification cycle
DNA	Deoxyribonucleic acid
EFNS	European Federation of Neurological Societies
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
EMJH	Ellinghausen-McCullough-Johnson-Harris
Fla	Flagellin peptide
<i>fla</i>	Gene for flagellin peptide
IFA	Immunofluorescent antibody assay
Ig	Immunoglobulin
JRA	Juvenile rheumatoid arthritis
LA	Lyme arthritis
LB	Lyme borreliosis
LC	Lyme carditis

Abbreviations

LD	Lyme disease
LNB	Lyme neuroborreliosis
MKP	Modified Kelly-Pettenkofer
MLST	Multi-locus sequence typing
NA	Nucleic acid
OD	Optical density
Osp	Outer surface protein
<i>osp</i>	Gene for outer surface protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
rRNA	Ribosomal RNA
RT	Room temperature
s.l.	Sensu lato
s.s.	Sensu stricto
SF	Synovial fluid
VlsE	Variable major protein-like sequence E
WB	Western blot

TABLE OF CONTENTS

INTRODUCTION	-1-
BORRELIA BURGDORFERI SENSU LATO	-3-
<i>Structure and morphology of Borrelia burgdorferi sensu lato</i>	-3-
<i>Borrelia proteins and genes – function and expression</i>	-4-
BIOLOGY OF THE TICK	-6-
<i>Tick ecology</i>	-6-
<i>Developmental cycle and hosts for the tick</i>	-7-
<i>Host-seeking behaviour of the ticks</i>	-8-
<i>Blood meal</i>	- 9 -
EPIDEMIOLOGY OF BORRELIA BURGDORFERI SENSU LATO	-11-
<i>Correlation between Borrelia burgdorferi sensu lato species and reservoir host</i>	-11-
<i>Transmission paths for Borrelia burgdorferi sensu lato</i>	-12-
Pathogen transmission – from reservoir host to tick	-13-
Pathogen transmission – from tick to host	-13-
Pathogen transmission – from tick to tick	-14-
<i>Borrelia species and their pathogenicity</i>	-14-
CLINICAL MANIFESTATIONS AND DISEASE EPIDEMIOLOGY	-17-
<i>Incidence of Lyme borreliosis</i>	-17-
<i>Relative proportion of Lyme borreliosis manifestations</i>	-18-
<i>Seroprevalence in the population</i>	-19-
<i>Different clinical manifestations of Lyme borreliosis</i>	-20-
Erythema migrans	-20-
Lyme neuroborreliosis	-22-
Lyme arthritis	-23-
Acrodermatitis chronica atrophicans	-23-
Borrelia lymphocytoma	-25-
Lyme carditis	-26-
Rare manifestations	-26-
Post-treatment Lyme disease syndrome or post-Lyme disease syndrome	-26-
Subclinical seroconversion	-27-
TREATMENT AND PREVENTION	-28-
<i>Therapy for Lyme borreliosis and vaccines</i>	-28-
<i>Risk populations and prevention</i>	-28-
LABORATORY DIAGNOSIS OF LYME BORRELIOSIS	-30-

Table of contents

<i>Borrelia</i> proteins, genes, and their function as diagnostic targets	-31-
<i>Diagnostic tools for direct detection</i>	-32-
Microscopic detection of <i>Borrelia burgdorferi</i> sensu lato	-32-
Detection of <i>Borrelia burgdorferi</i> sensu lato by culture	-33-
Culture medium and conditions	-33-
Diagnostic sensitivity, specificity, and limitations of culture in clinical specimens	-33-
Detection of <i>Borrelia</i> by polymerase chain reaction	-34-
Principle of polymerase chain reaction and real-time polymerase chain reaction	-35-
Polymerase chain reaction	-36-
Real-time polymerase chain reaction	-38-
Diagnostic sensitivity, specificity, and limitations of polymerase chain reaction for analysis of Lyme borreliosis	-39-
<i>Diagnostic tools for indirect detection</i>	-41-
Serological methods for detection of <i>Borrelia</i> -specific antibodies	-41-
Principle for detection of <i>Borrelia</i> -specific antibodies with immunofluorescent antibody assays	-42-
Detection of <i>Borrelia</i> -specific antibodies with immunoblot/Western blot	-43-
Enzyme-linked immunosorbent assay	-44-
Principle for detection of <i>Borrelia</i> -specific antibodies with enzyme-linked immunosorbent assays	-45-
Principle of Luminex technology in the detection of <i>Borrelia</i> -specific antibodies	-46-
Diagnostic sensitivity, specificity and limitations of serological assays	-47-
Difference between various serological methods – limitations, advantages, and how they complement each other	-49-
<i>Detection of biomarker CXCL13</i>	-51-
<i>The situation of today – How do we apply the different diagnostic tools for Lyme borreliosis in clinical samples?</i>	-52-
AIMS OF THE THESIS	-55-
MATERIALS AND METHODS	-57-
MATERIALS	-57-
<i>Subjects and clinical data</i>	-57-
Case definitions (papers I and II)	-57-
Inclusion of study subjects (papers I-IV)	-58-
Collection of clinical data, recruitment of patients, and inclusion of <i>Borrelia</i> species	-60-
Clinical samples from potential LB and LNB patients (papers I and II)	-60-
<i>Borrelia</i> species (papers III and IV)	-61-
<i>Ethics</i>	-62-
METHODS	-63-
<i>Serological methods</i>	-63-
Paper I	-63-
Paper II	-63-
<i>Molecular methods</i>	-65-

Table of contents

Culture methods and conditions for the <i>Borrelia</i> strains (papers III-IV)	-65-
Paper III	-66-
Paper IV	-67-
STATISTICS (PAPERS I-IV)	-68-
RESULTS AND DISCUSSION	-71-
EVALUATION OF SEROLOGICAL METHODS FOR DIAGNOSIS OF LYME BORRELIOSIS IN CLINICAL SAMPLES	-71-
<i>Detection of Borrelia-specific antibodies in serum (paper I)</i>	-71-
<i>Detection of Borrelia-specific antibodies in serum and cerebrospinal fluid (paper II)</i>	-75-
EVALUATION AND OPTIMISATION OF THE MOLECULAR WORKFLOW FOR DETECTION OF <i>BORRELIA</i>	
<i>BURGDORFERI</i> SENSU LATO IN CEREBROSPINAL FLUID BY REAL-TIME POLYMERASE CHAIN REACTION (PAPERS	
III AND IV)	-81-
CONCLUDING REMARKS	-89-
FUTURE PERSPECTIVES	-91-
APPENDIX	-93-
ACKNOWLEDGEMENTS	-95-
REFERENCES	-99-

INTRODUCTION

In October 1975 two mothers from Lyme, Connecticut USA, contacted the Connecticut State Health Department after they noticed an increased number of children with possible juvenile rheumatoid arthritis (JRA) in their neighbourhood ^{1, 2}. Shortly after, a joint investigation directed by Yale University School of Medicine and the Connecticut State Health Department started to evaluate this phenomenon. Dr. Allen Steere, at the Division of Rheumatology at Yale, together with Dr. David Snyderman at the State Health Department started to take extra interest in these cases and put together an active surveillance system in the affected towns ². A total of 39 children together with 12 adults (living in the same area) were diagnosed with swelling and pain in a few large joints, especially the knees. However, other large joints were also involved in the clinical picture. Based on the expected prevalence for JRA, the investigation excluded JRA as a possible reason for the epidemic, since the number of cases was 100 times higher than expected. Instead, Dr. Steere and his co-workers suspected a connection between arthritis and tick bites, since one-quarter of the patients, and often many members within the same family, had developed an expanding red erythematous lesion weeks before the articular manifestations ^{1, 2}. This sort of lesion had previously been described in Sweden in 1909 by the dermatologist Arvid Afzelius ³ who named it erythema migrans (EM) and linked it with the bite of an *Ixodes ricinus* tick. The new disease reported by Dr. Steere and co-workers was named Lyme arthritis (LA), based on the geographical origin and the connection to arthritis. However, the name of the disease was later on changed to Lyme disease (LD) ⁴. In Europe, LD is known as Lyme borreliosis (LB) including not only arthritis but also other clinical manifestations in the skin, the nervous system, and in the heart. In 1981, Dr. Burgdorfer discovered a cluster of spirally shaped bacteria during a microscopical investigation of the midgut content in a dissected tick ⁴. Finally, in 1984 when Dr. Burgdorfer and colleagues found the “same” spirochetes in skin biopsies from EM patients, the connection between tick bites, LD, and the newly discovered spirochete *Borrelia burgdorferi* was established ⁵.

Much research on the subject of *Borrelia*, the different manifestations of LB [EM, Lyme neuroborreliosis (LNB), LA, Acrodermatitis chronica atrophicans (ACA), *Borrelia*

lymphocytoma (BL) and Lyme carditis (LC)] and on the detection of *B. burgdorferi* sensu lato (s.l.) in clinical samples has been performed since these discoveries. This research has led to a deeper knowledge of LB, the *B. burgdorferi* s.l. species, vectors and reservoir hosts, pathogenicity, immunology, clinical diagnosis, prevalence, incidence, genetics, vaccines, and epidemiology. It has also led to the finding of other tick-borne bacteria, viruses, parasites, and the diseases associated with them. However, many questions remain regarding, for instance, the evaluation and investigation of diagnostic tools. The latter is, a problematic issue since 1) serological assays have some biological limitations, which may affect the diagnostic outcome, 2) detection of *Borrelia*-specific deoxyribonucleic acid (DNA) by polymerase chain reaction (PCR) has low diagnostic sensitivity in some clinical sample materials, and 3) the culture of *Borrelia* has low diagnostic sensitivity and is laborious to produce. Another issue is that different countries and laboratories use different serological and molecular methods/protocols for diagnosis of LB and lack of standardisation is a known fact. To make sure that the patient receives the right diagnosis and treatment regardless of country or laboratory, concordance between the methods/protocols together with analytical and diagnostic sensitivity and specificity needs to be further evaluated. Major systemic evaluations between laboratories, where large well-characterised sample panels are used as well as systemic evaluation of the pre-analytical handling of samples before molecular testing are also important issues to investigate more deeply. In previous studies, these issues have been evaluated. However, the evaluations were limited and the results need to be reproduced to strengthen the conclusions previously drawn and new questions in the topic need to be further investigated.

Borrelia burgdorferi sensu lato

The genus *Borrelia* includes spirochetes within two groups, the LB group and the relapsing fever group. Even though the groups have different epidemiological and clinical characteristics, the LB group and the relapsing fever group shares genetic and biological features, which distinguish them in many ways from other spirochetes ⁶. In this thesis, the main focus is on species within the LB group.

Structure and morphology of *Borrelia burgdorferi sensu lato*

LB is caused by spirochetes within the *B. burgdorferi* s.l. complex ⁷. The relapsing fever *Borrelia* species *Borrelia miyamotoi* co-circulates with the *B. burgdorferi* agents, since it is transmitted by the same tick, and is also presenting disease similar to LB ⁸.

The *Borrelia* bacteria is a gram-negative, microaerophilic, mobile, irregular, extracellular, helical shaped spirochete with a size of 10-40 µm in length and 0.2-0.5 µm in diameter, with periplasmic flagella ^{4, 9, 10}. The spirochete can (in vitro) be found in multiple forms e.g. non-uniformly coiled and twisted or intertwined over each other. However, the spirochetes are most frequently found in an aggregated form ^{11, 12}. The *Borrelia* species carries a small linear chromosome, which has a length of around 900-kilobase pair, together with >20 different plasmids, both linear and circular, which usually have a size of 5-62 kilobase pair in length ¹³⁻¹⁵. The major difference between *B. burgdorferi* s.l. species and other gram-negative bacteria is the absence of lipopolysaccharide in the outer membrane ^{14, 16}. Instead the spirochetes exhibit lipoproteins, including major outer surface proteins (Osp) A-F and the variable-major protein-like sequence (Vls) E ¹⁷.

The morphology of the spirochetes shows bundles of flagella, about 7-11 in number, which is implanted into the cytoplasmic membrane near the end of the cell. The flagella extend through the cell wall and into the periplasmic space and twist around the flexible, rod-shaped protoplasmic cylinder of *Borrelia* (Figure 1) ¹⁴. By rotation of the flagella, the spirochetes can move through highly viscous media, which may facilitate for the spirochetes to penetrate the tissues and disperse within ticks or mammals ^{14, 15}.

Figure 1 The morphology of a *Borrelia* spirochete [Reprinted by permission from Springer Nature, Nature Reviews Microbiology, The burgeoning molecular genetics of the Lyme disease spirochaete]

***Borrelia* proteins and genes – function and expression**

As mentioned above *B. burgdorferi* s.l. species lack lipopolysaccharide in the outer membrane. Instead, the bacterium has several immunogenic proteins, lipids, lipoproteins, and carbohydrate antigens placed on its outer membrane, its surface, and inside the cytoplasm¹⁸. The lipoproteins are differently expressed during different stages of the borreliac life-cycle but they may also be expressed at different points during the infection. The surface lipoproteins play an important role in host-pathogen interaction, in virulence, and in maintaining the enzootic cycle of *B. burgdorferi* due to the extracellular nature of the pathogen. Some lipoproteins are important to host transmission, while others are important for the persistence of the spirochetes in the ticks and have also been implicated as important virulence factors¹⁷.

The **OspA** proteins are expressed in high quantities on the surface of spirochetes, when the spirochete is located in the midgut of the tick. OspA serve as adhesins, keeping the spirochetes in the midgut, until the tick starts to feed^{19, 20}. The protein also acts as a significant virulence factor for *B. burgdorferi* s.l. species in colonisation and transmission of the spirochete from tick to the host, but not from the host to the tick^{19, 21}. During a blood meal the OspA is

downregulated and the spirochetes can migrate out of the midgut of the tick and over to the salivary glands, entering the host when the tick feeds ¹⁸⁻²⁰.

The **OspC** is an essential virulence factor in the invasiveness and infectivity of *B. burgdorferi* s. l. species ²². The upregulation of the OspC protein appears parallel to the downregulation of the OspA protein ¹⁹⁻²¹. The change between downregulation of OspA and upregulation of OspC is partly caused by temperature changes. When the warm blood from the host enter the midgut of the tick, the spirochetes are triggered to produce OspC. The upregulation of OspC is done at 32-37 °C but not at lower temperatures ²⁰. OspC assists in the dissemination of spirochetes from the tick to the vertebrate ²¹.

The **VlsE** lipoprotein is located on the surface of the *Borrelia* spirochetes and is thereby likely to come into contact with antibodies during the infection ²³. However, the full biological function of the VlsE protein is still unknown, but it may, like OspC, serve as a protector to the pathogen in the host defence ²⁴. A possible explanation for this host defence, is that the VlsE lipoprotein acts as a shield hiding the epitopes of other surface antigens. This binding of VlsE to other proteins on the membrane surface may block the attachment of antibodies to the lateral surface of VlsE ²⁴. During mammalian infection, large quantities of VlsE are produced at the tick bite site while little or no VlsE is synthesised when the spirochetes are colonising the midgut of a vector tick. The amount of VlsE in the different stages of the infectious cycle can be affected by pH and temperature, where alkaline tick saliva may act as a signal for the synthesis of VlsE during the transmission of the spirochete to the host ²⁵.

Flagellin (Fla) is one of the immunodominant antigens in the *Borrelia* infection. A strong antibody response towards both FlaA and B develops early after a bite from a *B. burgdorferi* s.l. infected tick ²². Flagellin is also the dominant antigen during all the LB stages ²⁶.

A single **16S ribosomal ribonucleic acid (rRNA)** gene and two tandem sets of 23S and 5S rRNA genes are present on each *B. burgdorferi* chromosome ²⁷. By culturing *B. burgdorferi*, the number of 16S rRNA copies are multiplied, becoming 100-1000 times higher than that of gene copies ²⁸. The pattern of transcription and regulation of the 16S and the sets of 23S and 5S rRNA genes are unknown but may be critical for survival and persistence in the host ²⁷.

Biology of the tick

Tick ecology

There are three tick families: the Ixodidae ticks (“hard”), named after the hard sclerotized dorsal plate i.e. scutum, the Argasidae ticks (“soft”), which lack the scutum and have a more flexible cuticle, and the Nuttalliellidae ticks²⁹. The genus of *Ixodes* belongs to the Ixodidae family². The *Borrelia* spirochete (*B. burgdorferi* s.l.) is known to be transmitted by species within the *I. ricinus* species complex. *I. ricinus* and *Ixodes persulcatus* occur in Europe and Asia, *Ixodes scapularis* occurs in eastern and mid-western North America and *Ixodes pacificus* occurs in western North America (Figure 2)^{30, 31}. However, in this thesis, the focus will be on *I. ricinus* (the most common tick in Europe), which is the species that is referred to as “tick” henceforth on in the text.

Figure 2 The global distribution of the vectors of the *Borrelia* spirochetes (all within the *Ixodes ricinus* species complex) [Reprinted by permission from The Lancet, Vol 379, Stanek G, Wormser G P, Gray J, Strle F, Lyme Borreliosis, 461-473, Copyright (2020), with permission from Elsevier] (modified from Stanek et al. (2012))

Developmental cycle and hosts for the tick

I. ricinus has three active developmental stages: larvae, nymph, and adult (male or female) (Figure 3).

Figure 3 The developmental cycle of the tick together with potential hosts. The adult female tick (A) lays eggs in the vegetation (B). The eggs moult to larvae ticks (C), which feed on hosts e.g. small mammals and birds (D). After the blood meal, the larvae moult to nymphal ticks (E), which take a blood meal on medium sized hosts e.g. hares, smaller pets, and humans (F). The nymph further moults into an adult tick (female or male) (G). The adult female tick copulates with an adult male tick, takes another blood meal on a large host e.g. a deer or horse (H), drops to the ground, and lays new eggs before she dies. The adult male tick dies after expending its semen. [Figure created with Biorender.com]

The life span of an *I. ricinus* tick varies from several months to 2-3 years. However, it can last up to six years, depending on environmental conditions e.g. relative humidity, photoperiod, access to blood hosts and temperature^{9, 29}. To moult between the different stages, the tick needs a blood meal from a host³². Ticks in the first developmental stage (larvae) are mostly located in lower vegetation and use smaller host animals e.g. birds, rodents, and hares. Larger ticks like nymphs and adults climb higher in the vegetation using medium-sized and large host

animals e.g. deer, hare, ungulates, pets (cats, dogs, and horses) but also humans ^{32, 33} (Figure 3). If the tick remains attached without removal, the blood meal takes between 2-15 days depending on the developmental stage, type of host, and place of attachment ^{29, 32}. After the blood meal, the tick drops to the ground and digests the meal. Larvae and nymphs moult into a new developmental stage, while the female adult tick copulates with an adult male tick, takes another blood meal, drops to the ground, and lays thousands of eggs. After the male tick has expended its semen it dies, while the female tick dies after oviposit ³⁴.

Host-seeking behaviour of the ticks

During host-seeking, the tick climbs up a blade of grass and waits for a host to pass. Since the ticks lack eyes, they are dependent on sensors (Haller's organ) placed on the top of their front legs. These sensors detect carbon dioxide, different odours produced by the host e.g. butyric acid, lactones and phenols, changes in temperatures associated with warm-blooded animals, ammonia, and movements. When the host is passing the spot where the tick is waiting, the tick grabs on to the host and is carried along with it ^{35, 36}.

Ticks are very sensitive to desiccation during host-seeking since they constantly lose water through the integument of its body surface in dry conditions (transpiratory loss) and through their spiracles in conjunction with respiration during locomotor activity (respiratory loss) ^{37, 38}. To replenish water and restore the fluid content (moisten), the tick returns to the base of the vegetation where it takes up atmospheric water (vapour) ^{38, 39}. Since the larvae are more sensitive to desiccation, they host-seek in the lower parts of the vegetation for shorter periods compared to the adult ticks ^{31, 33, 37}.

The host-seeking behaviour is dependent on the season. Larval ticks in north and central Europe are host-seeking in the early summer, with a peak at midsummer, while adults and nymphs have their peak in early spring and early summer ³¹. However, a second peak of activity may appear in the autumn for adult ticks and nymphs ^{31, 40}. The ticks prefer to host-seek when the temperature is 7-24 °C, but are active down to 4-5°C. Ticks, therefore, prefer warm moist summers followed by warm winters or winters with snow (since the snow constitutes a protective cover for the ticks). During winter the ticks prefer to stay in the upper layer of the soil or in the leaf litter where the temperature is more favourable ^{38, 41, 42}.

Due to climate changes, the periods in which the ticks (but also the hosts) are active have increased. The climate change at higher latitudes and altitudes are also favourable for the geographical distribution and colonisation of the tick population, since potential land areas with a favourable climate for both ticks and hosts have increased ^{38, 42}.

Blood meal

When the tick has found a host, it walks around for hours searching (using their maxillary palps) for a suitable place, preferably with thin skin, before it bites. By cutting the skin (epidermis and dermis) with their mouthparts and by insertion of the hypostome into the skin, various substances can enter the host creating a feeding pool (Figure 4) ⁴⁴. These substances are produced in the salivary glands and include; 1) enzymes, 2) vasodilators, 3) anti-inflammatory, anti-haemostatic and immunosuppressive substances and, 4) cement, which will anchor the mouthparts to the skin. In the first 24-36 hours, the ingestion of blood to the midgut is none or little ²⁹. The feeding time depends on the life stage, where the larvae feed for three to five days, the nymph for three to four days and the female tick for about 14 days. During its entire life cycle, a tick feeds for about 26-28 days ⁴³. Once the tick has fed, it drops to the ground and moults to the next life stage, or if female, lays eggs.

Figure 4 The blood meal and the interactions between the tick, the hosts, and the tick-borne pathogens [Reprinted by permission from Elsevier, Trends in Parasitology, Sialomes and Mialomes: A Systems-Biology View of Tick Tissues and Tick–Host Interactions, J. Chmelař] (modified from Chmelař et al. 2016)

Epidemiology of *Borrelia burgdorferi* sensu lato

Correlation between *Borrelia burgdorferi* sensu lato species and reservoir host

A zoonosis is an infectious disease caused by a pathogen (in this thesis *B. burgdorferi*) that can be transmitted from a non-human animal (usually a vertebrate) to a human. However, the natural infection of *Borrelia* does not include humans as they only inadvertently become infected ¹⁴. The *B. burgdorferi* s.l. complex consists of at least 22 *Borrelia* species ^{45, 46}. Many studies from Europe have recognised *Borrelia afzelii* as the most frequently detected species followed by *Borrelia garinii* and *Borrelia burgdorferi* sensu stricto (s.s.). Other observed species are *Borrelia spielmanii*, *Borrelia valaisiana*, *Borrelia bissettii*, *Borrelia bavariensis*, *Borrelia lusitaniae*, and *B. miyamotoi* ^{45, 47, 48}. *B. miyamotoi* is a relapsing fever species, which is also associated with the *I. ricinus* tick, causing infections in humans presenting mostly with flu-like symptoms such as fever, chills, and severe headache. However, more severe illness involving the central nervous system (CNS) especially in immunosuppressed (but also in cases with immunocompetent) patients has been observed ⁴⁹⁻⁵¹. The different *B. burgdorferi* s.l. species, as well as *B. miyamotoi*, are associated with different reservoir hosts (a host that serves as a source of infection), which are presented in Table 1.

Table 1 The association between different *Borrelia* species and reservoir hosts ^{8,45, 50, 52-54}.

Species	Reservoir host
<i>B. burgdorferi</i> sensu stricto	Small mammals (rodents) and birds
<i>B. garinii</i>	Birds
<i>B. afzelii</i>	Small mammals (rodents)
<i>B. bavariensis</i>	Small mammals (rodents) and birds
<i>B. spielmanii</i>	Small rodents (hazel dormouse)
<i>B. lusitaniae</i>	Lizards
<i>B. bissettii</i>	Small rodents (wood rat)
<i>B. valaisiana</i>	Birds
<i>B. mayonii</i>	Small mammals (rodents)
<i>B. miyamotoi</i>	Small rodents (mice and voles) and birds

B. = *Borrelia*

Transmission paths for *Borrelia burgdorferi sensu lato*

In Figure 5 the transmission path for the *Borrelia* spirochetes is divided into five paths; 1) the pathogen transmission between the reservoir host (animals, both wild and domestic) and the tick (goes both ways), 2) the pathogen transmission between the tick and the host (e.g. humans) and, 3-5) the pathogen transmission between ticks. Each path is presented in more detail in the following sections “Pathogen transmission – from reservoir host to tick” (path 1), “Pathogen transmission – from tick to host” (path 2) and “Pathogen transmission – from tick to tick” (path 3-5).

Figure 5 The transmission paths for the spirochetes between the tick, the reservoir host, and the host; 1) the pathogen transmission between the reservoir host and the tick, 2) the pathogen transmission between the tick and the host, and, 3) the pathogen transmission between ticks stages (transstadial transmission), 4) the pathogen transmission between an adult female tick and the offspring (transovarial transmission) and, 5) the pathogen transmission between ticks by co-feeding [Figure created with Biorender.com]

Pathogen transmission – from reservoir host to tick

The ticks get infected by the *Borrelia* spirochetes through feeding on a *Borrelia*-infected reservoir host, e.g. birds and small mammals (Figure 5, path 1). At least 200 different mammals, birds, and reptiles may serve as hosts for ticks and are thereby potential reservoirs for *Borrelia*^{9, 55}. However, only a few dozen of these animals have been recognized as reservoir host e.g. rodents, birds, hares, hedgehogs and squirrels⁵⁵. Since the *I. ricinus* tick takes two or three blood meals during its developmental cycle, one tick can be infected by multiple species of *Borrelia*, i.e. co-infected⁴⁵. The tick can also be infected by other bacteria, viruses, and parasites e.g. *Anaplasma phagocytophilum*, *Rickettsia* spp., tick-borne encephalitis virus, and *Babesia* spp.⁵⁶.

Pathogen transmission – from tick to host

Since the *I. ricinus* tick only feeds once per stage, bacteria, viruses, and parasites acquired during feeding can only be transmitted to another host (Figure 5, path 2) once the tick has moult to the next stage in the developmental cycle²⁹. The *B. burgdorferi* spirochetes are present in the midgut of the tick, anchoring themselves to the gut epithelium by the receptor for OspA, which is up-regulated by the spirochetes (Figure 4)⁴⁴.

During the blood meal, the *Borrelia* spirochetes start to rapidly multiply within the gut⁵⁷. Hours after the onset of the blood meal the spirochetes start to migrate from the midgut lumen through the epithelial cell wall, reaching the haemolymph and invading the salivary glands from where they will be transmitted to the skin of the host via the saliva (Figure 4)^{44, 58, 59}. This is facilitated by the downregulation of lipoprotein OspA, which will lead to reduced binding capacity of the spirochete to the epithelium, and upregulation of OspC⁵⁷. The transmission of the *B. burgdorferi* spirochete from the midgut to the salivary glands takes between 24 and 48 hours, due to the multiplying in the midgut and transmission to the salivary glands⁶⁰. When the tick has penetrated the dermis, the tick saliva, containing the spirochetes as well as other substances produced by the salivary glands, is injected into the skin²⁹. From the skin of the host/reservoir host, the spirochetes can be further transmitted via the bloodstream to the joints, CNS, heart, and bladder where they establish infections¹⁴. A meta-analysis by Strnad et al. (2017)⁶¹ showed that about 12 % of the total amount of *Ixodes* ticks in Europe are infected with *B. burgdorferi*. However, the amount of infected ticks varies between countries, and

higher numbers, 20-25 %, have been presented ^{47, 60, 62}. However, despite the high frequency of *Borrelia*-infected ticks, the numbers of bites that lead to infection is low and asymptomatic LB in patients with no clinical symptoms associated with LB (documented by seroconversion) is recognised to be frequent in individuals that become infected ⁶³.

Pathogen transmission – from tick to tick

Transmission between ticks or between developmental stages occur (Figure 5, paths 3-5), A) by feeding on a site of a reservoir host (animals) previously infected by *Borrelia*. After a blood meal the spirochetes remain in the deposition site for a few days before it disseminates into the host. During this period an un-infected tick may be infected if it takes a blood meal at this site, B) by co-feeding when several ticks feed closely to each other (sharing the same blood pool), and spread of bacteria (but also viruses and parasites) occurs from one infected tick to an uninfected tick, and C) by transstadial transmission between the different developmental stages ^{29, 45, 64}. For the *B. miyamotoi* bacteria, transovarial transmission occurs between the female tick and the offspring ⁸. However, this type of transmission is rare for the *B. burgdorferi* s.l. species and the prevalence in unfed larvae is less than 1 %, indicating that transmission from the adult female tick to the egg is of minor importance ^{9, 29}.

Borrelia species and their pathogenicity

As mentioned before, the *B. burgdorferi* s.l. complex contains at least 22 *Borrelia* species (Table 2) ^{45, 46}. Of these species, seven are known to be pathogenic to humans (*B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. bavariensis*, *B. spielmanii*, *Borrelia mayonii*, and *B. miyamotoi*). However, only two of these, *B. burgdorferi* s.s. and *B. mayonii*, are present in North America, while six (all but *B. mayonii*) are present in Eurasia ^{51, 65, 66}. This means that only one species causes LB in both Europe and North America namely *B. burgdorferi* s.s. ⁶⁶. Among the other known species, five have pathogenic potential since they have occasionally been detected in humans (Table 2) ^{66, 67}. However, the status of the pathogenicity is still uncertain, since the occurrence of these species in patients is low ^{10, 68}. The other species are currently not known to be pathogenic to humans ¹⁰. Additionally, *B. miyamotoi* (a relapsing fever species, which is transmitted by the *I. ricinus* tick) is a pathogenic species that cause symptoms similar to LB infection in humans. The species is mainly associated with a severe

infection in immunosuppressed persons, while cases of infection in immunocompetent persons have also been described ^{8, 51}. Previous studies have shown correlation between different *B. burgdorferi* s.l. species and clinical manifestations. In Europe, *B. azelii* is known to be associated with skin manifestations e.g. EM and ACA, *B. garinii* with neurological manifestations e.g. LNB and *B. burgdorferi* s.s. with manifestations in joint e.g. LA. However, the different species can occur in most of the clinical manifestations ⁶⁹.

Table 2 *Borrelia* species within the *Borrelia burgdorferi sensu lato* complex together with *Ixodes* species, epidemiological distribution, and pathogenicity in humans

Name (species)	<i>Ixodes</i> species	Epidemiological distribution	Pathogenicity in humans	References
<i>B. burgdorferi sensu stricto</i>	<i>I. ricinus</i> <i>I. scapularis</i> <i>I. pacificus</i>	North America Europe	Pathogenic	10, 66, 70-73
<i>B. garinii</i>	<i>I. ricinus</i> <i>I. persulcatus</i> <i>I. hexagonus</i> <i>I. nipponensis</i>	Eurasia	Pathogenic	10, 66, 70-73
<i>B. afzelii</i>	<i>I. ricinus</i> <i>I. persulcatus</i>	Eurasia	Pathogenic	10, 66, 70-73
<i>B. bavariensis</i>	<i>I. ricinus</i>	Eurasia	Pathogenic	66, 74
<i>B. spielmanii</i>	<i>I. ricinus</i>	Europe	Pathogenic	66, 75
<i>B. japonica</i>	<i>I. ovatus</i>	Japan	Non-pathogenic	66, 68, 70
<i>B. lusitaniae</i>	<i>I. ricinus</i>	North Africa Europe	Pathogenic potential	66, 68, 70, 76
<i>B. sinica</i>	<i>I. ovatus</i>	China	Non-pathogenic	66, 68, 70
<i>B. bissettii</i>	<i>I. ricinus</i> <i>I. pacificus</i> <i>I. spinipalpis</i> <i>I. mino</i> <i>I. scapularis</i>	North America Europe	Pathogenic potential	66, 70, 77-79
<i>B. turdi</i>	<i>I. turdus</i>	Japan	Non-pathogenic	66, 68, 70
<i>B. tunukii</i>	<i>I. tanuki</i>	Japan	Non-pathogenic	66, 68, 70
<i>B. valaisiana</i>	<i>I. ricinus</i> <i>I. columnae</i> <i>I. granulatus</i>	Eurasia	Pathogenic potential	66, 68, 70, 80
<i>B. yangtze</i>	<i>I. granulatus</i>	China	Non-pathogenic	66, 68
<i>B. americana</i>	<i>I. pacificus</i>	North America	Non-pathogenic	66, 68
<i>B. andersonii</i>	<i>I. dentatus</i>	North America	Non-pathogenic	66, 68, 70
<i>B. californiensis</i>	<i>I. pacificus</i> <i>I. jellisonii</i> <i>I. spinipalpis</i>	North America	Non-pathogenic	66, 68
<i>B. carolinensis</i>	<i>I. minor</i>	North America	Non-pathogenic	66, 68, 81
<i>B. kurtenbachii</i>	<i>I. scapularis</i>	North America Europe	Pathogenic potential	66, 68, 82
<i>B. finlandensis</i>	<i>I. ricinus</i>	Finland	Pathogenic potential	67
<i>B. chilensis</i>	<i>I. stilesi</i>	Chile	Non-pathogenic	83
<i>B. mayonii</i>	<i>I. scapularis</i>	North America	Pathogenic	65, 84
<i>B. miyamotoi*</i>	<i>I. ricinus</i>	North America Europe	Pathogenic	85

B. = *Borrelia*, *I.* = *Ixodes*

* belong to the relapsing fever group

Clinical manifestations and disease epidemiology

Incidence of Lyme borreliosis

The most common tick-borne infection in humans in the northern hemisphere is LB. The estimated mean annual incidence in North America is about 330,000 cases (106.6 cases per 100,000 persons per year) ⁸⁶, while Europe has an estimated mean annual incidence of 230,000 cases (22.05 cases per 100,000 persons per year) with a large variation between and within different countries ^{87, 88}. Asia has an estimated mean annual incidence of 3,500 cases (0.008 cases per 100,000 persons per year in Japan) ⁸⁸. Cases of LB are in general rare in Japan and Korea, while the disease is more established in China. However, the incidence is not reported ⁸⁹.

In general, an increasing incidence of LB is reported, which may be associated with different ecological factors ⁹⁰:

- ❖ change in the distribution of tick hosts both in altitude and latitude
- ❖ ecological changes e.g. habitat connectivity and change in land management
- ❖ geographical distribution density and activity of the *I. ricinus* tick, the principal vector for *B. burgdorferi* s.l. species in Europe
- ❖ climatic factors, which contribute to an expansion of the tick's geographic range due to survival, abundance, and seasonal activity
- ❖ anthropogenic induced changes i.e. changes in land management and, agricultural practises, and an increase in hunting, which increases human contact with ticks

The increased incidence of LB over the years may also be a result of raised awareness of the disease, improved reporting practice, or over-diagnosis of the disease ⁸⁷. However, the possibility of under-diagnosis and underreporting (mainly since LB is not a notifiable disease in all countries in Europe) must also be taken into consideration when comparing incidence rates between different countries ^{87, 88}.

The incidence of LB varies over the year and is correlated to differences in tick activity during the year. In many European countries, the incidence is very low in late autumn, winter, and

early spring since the activity of ticks is low during these seasons. The incidence peak of LB in central Europe occurs in June to August as well as September to November ⁹¹⁻⁹³, while the incidence peak in North America is seen in May to August ^{94, 95}. In Sweden, located in Northern Europe, the incidence peaks have been shown to be from April to June and September to October ⁹⁶. The incidence peak of LB is correlated to the host-seeking peak of the tick, where e.g. ticks in the north and central Europe have their peak in early summer and midsummer ³¹. However, the timing for the clinical symptoms and signs of LB differs depending on manifestation, e.g. EM appears earlier compared to LNB ⁹⁷. However, for the majority of LB cases, the onset is in August-September ⁹⁸.

Relative proportion of Lyme borreliosis manifestations

LB is a disease that may affect both genders, and all ages. The proportion of cases in the different clinical manifestations of LB in Sweden ranges from 77 % among patients with EM, down to < 1 % in patients with LC (Table 3) ⁹⁷.

Table 3 The clinical manifestations of Lyme borreliosis and their proportion in Sweden ⁹⁷

Clinical manifestation	Proportion in Sweden (% of the total LB cases)
Erythema migrans (EM)	77
Lyme neuroborreliosis (LNB)	16
Lyme arthritis (LA)	7
Acrodermatitis chronica atrophicans (ACA)	3
Borrelia lymphocytoma (BL)	3
Lyme carditis (LC)	< 1

In North America, the proportion differs in some manifestations. For EM, the number of cases is slightly higher, at least 80 % ^{97, 99}. A higher incidence is also seen in patients with LA in North America where the proportion of cases is more than four times higher (frequency = 30 % of the total LB cases) ^{97, 100}, as well as for LC where the proportion of cases is 4-10 times higher (frequency = 4-10 % of the total LB cases) compared to Sweden ^{100, 101}.

The number of cases with different clinical manifestations differs between different ages and genders. Studies from Sweden have shown that the proportion of LNB cases is higher in young children (<10 years) and in adult patients (>50 years) ^{102, 103}. For LA and BL, cases are more frequently shown in young children (<16 years) compared to adults ^{88, 98, 104, 105}, while ACA appears more often in adult women (> 40 years) compared to men and other age groups ⁸⁸. Only a small number of cases of ACA have been reported in children in the literature and the reason for this is still unclear ¹⁰⁶⁻¹⁰⁹. However, several studies indicate that among children with ACA, more are female ^{106, 109}. On the other hand, LC is more frequently associated with the male sex ¹¹⁰. LB is in most clinical cases not lethal. However, LC is potentially lethal if not treated promptly, and cases with fatal outcomes have been reported ^{110, 111}.

The most common tick bite sites differ between age groups (children and adults), but also between the different developmental stages of the tick. In Sweden and the United Kingdom, nymphs are most often involved in human tick bites and are the main vector of LB in Western Europe ^{112, 113}. Adult humans are bitten by adult ticks and nymphs, while children are mainly bitten by nymphs. The adult ticks and nymphs prefer legs, the skin of the torso/dorsum and arms, on adult humans ¹¹³, while children are more often bitten in the head, neck, and axilla region ^{112, 114}.

Seroprevalence in the population

The seroprevalence between different countries, but also between different areas within countries varies ¹¹⁵⁻¹¹⁹. The numbers may also differ with age (the number of cases increases with age since the probability of having had a *Borrelia* infection increases with age), gender, season, geographical area (the number of cases is higher in highly endemic areas e.g. southern Sweden) and laboratory assays ¹²⁰⁻¹²². In the south-central and eastern parts of Sweden about 20-25 % of the healthy blood donors, without ongoing symptomatic LB, had detectable levels of immunoglobulin (Ig) G antibodies ^{118, 119}. This shows that the seroprevalence, i.e. presence of antibodies in a population without ongoing LB disease, is high. A serological survey from Sweden has shown that young children (<20 years) and adults (> 50 years) show a higher proportion of LB compared to individuals in the age group of 21-50 years. The lowest proportion is shown in the age group of 21-30 years ¹²³. The differences in proportion are probably related to the frequency of outdoor activities and leisure-time behaviour patterns between the different age groups ⁸⁸. A slightly higher number of cases among females has been

reported in most European countries e.g. Austria, Germany, Slovenia, Switzerland, and Sweden ^{88, 122}. The proportion of seroprevalence in an area is something that physicians need to be aware of since a seropositive result is not always a sign of an ongoing infection.

Different clinical manifestations of Lyme borreliosis

B. burgdorferi s.l species are transferred from the tick to the skin during the blood meal. The spirochetes are further spread locally within the skin or disseminate via the bloodstream to other organs, tissues, and body fluids, resulting in nervous system, musculoskeletal, cardiac and ocular manifestations ¹²⁴.

LB is usually described by three clinical stages: early/localised infection (stage I); early/disseminated infection (stage II) and late/long-lasting infection (stage III). However, the different stages may overlap and not all patients undergo all stages ¹²⁵⁻¹²⁷. The first stage involves skin lesions EM and BL. EM often occur at the site of the tick-bite, while BL appears near the tick-bite site. The second stage involves early disseminated infections that affect the neurological system with manifestations of LNB, but it can also involve joints and heart resulting in LA and LC. The third stage involves late skin manifestations such as ACA, late LNB, and LA with long-lasting symptoms. ACA and late LNB are more common in Europe, while LA, often presenting in the knees, is more common in North America ^{125, 126}.

Erythema migrans

The majority of EMs appear in the acute phase of the infection at the site of the tick bite as a round-to-oval, sometimes sharply, demarcated, red to bluish-red macular or papular skin rash (≥ 5 cm). In many lesions, a paler central clearing around the rash followed by an outer red border, a so called bull's-eye lesion, appears (Figure 6A). However, EM can also appear as a homogenous erythema with no central clearing ¹²⁸. The lesion appears when the spirochetes enter the dermis and trigger the innate immune response of the host. The majority of the spirochetes at the centre of the rash are cleared within a week by the innate immune response, while the spirochetes at the edge continue to spread, directing the immune response to follow. This results in two inflammations creating the bulls-eye lesion, one due to the reaction to salivary proteins at the site of the tick bite and one due to the reaction to the moving spirochetes ¹²⁹. The lesion appears days to weeks after the tick bite ^{108, 126, 130}. If the EM remains

untreated, the rash will continue to expand over weeks to months and the diameter of the lesion can range from a few centimetres to more than half a metre ^{7, 93, 130}. The EM rash can appear anywhere on the skin, but the lower extremities are the most common place especially in adults. In children, the face along with the head and neck region, are the most frequent sites ^{97, 128}. The skin lesion may be accompanied by influenza-like symptoms e.g. fatigue, headache, muscle and joint aches, or fever ^{99, 131}. Multiple EMs may occur but are less common than single EMs. However, presentation of multiple EMs is more common in patients from North America compared to European patients, which may be correlated to the higher appearance of *B. burgdorferi* s.s. (the species that is known to be associated with multiple EMs) in North America ¹³². The EM caused by *B. burgdorferi* s.s. in North America is more often accompanied by headache, fatigue, fever, and joint aches ¹³³ and the EM is in general more inflammatory and fast growing than the ones seen in Europe ¹³⁴.

Figure 6 Clinical signs of Lyme borreliosis, A) Erythema migrans, B) Lyme neuroborreliosis (facial palsy), and C) Lyme arthritis [Reprinted from Centers for Disease Control and Prevention (NIH) - Centers for Disease Control and Prevention (NIH), Public Domain (<https://commons.wikimedia.org/w/index.php?curid=29608423>)]

EM in Europe is most often caused by *B. afzelii*, but can also be associated with *B. garinii* and *B. burgdorferi* s.s. ⁶⁹. EM caused by *B. garinii* is more often associated with itching, burning, and pain within the lesion, and the local spreading is faster with a higher proportion of homogenous EMs in comparison to *B. afzelii* and *B. burgdorferi* s.s. infections ^{135, 136}. This may be a result of a stronger and more intense immune response, leading to a more intensive inflammation ¹³⁶. In North America, EM is caused by *B. burgdorferi* s.s. alone ⁷.

Lyme neuroborreliosis

To infect the brain, the circulating spirochetes need to disseminate through the body and cross the blood-brain barrier. This can be done in two ways, either along structures e.g. peripheral nerves or via the bloodstream. However, the exact mechanism for the dissemination and how the spirochetes escape the immune cells in the blood are still unknown. How the spirochetes pass the blood-brain barrier has also not been fully described. Some authors favour penetration of spirochetes between endothelial cells, while others argue for transcellular passage ¹³⁷.

The LNB infections are divided into an early LNB and a late LNB phase. Symptoms and signs of an early LNB have a duration of <6 months, while a late LNB manifestation has a duration of >6 months ^{88, 138}. Most cases (> 95 %) are classified as early LNB and less than 5 % as late LNB ¹³⁸. Among LNB cases in different studies, only 15-40 % of the participants reported signs of EM before or at the presentation of the neurological disease ¹³⁹⁻¹⁴¹.

The most common clinical manifestations of LNB in Europe are painful meningoradiculitis (Bannwarth's syndrome) including headache, fatigue, and radiculitis pain. The manifestation is confined to the peripheral nervous system involving the cranial nerves (nerves connecting the brain to different parts of the head, neck, and trunk), spinal roots (nerve roots connecting the peripheral nervous system to the spinal cord), and peripheral nerves, together with CNS manifestations e.g. meningitis (an acute inflammation of the membranes and fluid surrounding the brain and spinal cord) or myelitis (a chronic inflammation in the spinal cord) ¹³⁸. The Bannwarth's syndrome is often associated with a bite from a *B. garinii* infected tick ^{72, 99}. *B. afzelii* can also cause neurological involvement. However, the clinical manifestations appear to be more diffuse compared to infections caused by *B. garinii* ¹⁴². The most common clinical manifestations of LNB in North America are lymphocytic meningitis with episodic headaches and mild neck stiffness, radiculoneuritis (inflammation in one or more spinal nerves), and cranial neuropathy with damage to one or more cranial nerves causing particularly facial palsy (Figure 6B) ¹²⁶. In late LNB, the patient has suffered from neurological symptoms for > 6 months at the time of diagnosis and may have manifestations from both the peripheral nervous system, e.g. mononeuropathy with damage to a single nerve, and the CNS e.g. cerebral vasculitis with inflammation of the blood vessel wall involving the brain and sometimes also the spinal cord ¹³⁸. Among children, headache, facial nerve palsy, neck pain, and fever are the most common manifestations ^{143, 144}, but unspecific symptoms e.g. nausea and vomiting may also appear in small children ¹⁴⁵. Previous studies have shown a significantly

higher frequency of neurologic manifestations among patients that have been bitten in the head and neck region. This may be an explanation of the higher incidence of LNB among children compared to adults since children are more often bitten in these areas. However, the association is still unclear ^{97, 103}. Late LNB is very uncommon in children, which may be a result of the relatively high percentage of facial nerve palsy in children (55 %), an overt clinical sign, which may lead to earlier contact with health care and earlier diagnosis ¹⁴⁶.

Lyme arthritis

LA affects all age groups and develops, in on average of six months (range: four days to two years) in North America ¹⁴⁷ and in an average of three months (range: 10 days to 16 months) in Europe after a bite from a *Borrelia*-infected tick ¹⁴⁸. Clinical signs of LA are joint swelling and pain primarily in large joints, especially the knee, but it can also affect the elbow, ankle, shoulder, and hip and the involvement is in most cases asymmetrical (Figure 6C) ⁷. However, symmetrical involvement can occur. Monoarticular (involvement of one joint) or oligoarticular (involvement of 2-4 joints) arthritis is a common sign of LA, while polyarticular (involvement of > 5 joints) arthritis is rare ^{7, 147}. The inflammation is often intermittent and lasts a few days to several weeks, but can in some cases last for months to years ^{7, 149}.

As mentioned above, LA occurs less frequently in Europe compared to North America. The difference in disease incidence is probably caused by the prevalence in the appearance of *B. burgdorferi* s.s., which is the species mainly associated with LA, in the two different continents ^{69, 100, 150}. However, *B. burgdorferi* s.s. is not the only *Borrelia* species causing LA in Europe. The species *B. garinii* can also cause inflammation of joints even though it occurs rarely ¹⁵¹.

Acrodermatitis chronica atrophicans

ACA is a slowly progressive bluish-red lesion in the skin, often located outside the joint (extensor surface), starting in one extremity, usually at the hand, foot, knee, or at the joint ^{109, 128}. The ACA manifestation often develops from an acute inflammatory phase to a chronic atrophic phase over weeks to months ¹⁵² and may appear years after the tick bite ^{30, 88}.

Actually, *Borrelia* species can be isolated from ACA lesions >20 years after its first appearance¹²⁶, and if untreated the infection may last for months to years, since an ACA lesion does not heal spontaneously¹⁰⁹. The progressive course, including both inflammatory and atrophic stages, is long and the evolution of lesions is slow¹⁵³. Lesions of ACA may also, apart from the outer extremities, appear on the buttocks, while lesions on the trunk and on the face are rare (Figure 7)¹⁰⁹.

Figure 7 Clinical sign of acrodermatitis chronica atrophicans [Reprinted from The Lancet, Vol 379, Stanek G, Wormser G P, Gray J, Strle F, Lyme Borreliosis, 461-473, Copyright (2020), with permission from Elsevier]

During the progress of the disease, progressive atrophy with transparent, thin skin including visible veins may occur¹⁰⁹. The most common manifestation associated with ACA is peripheral neuropathy, caused by damage to underlying nerves and may result in weakness and pain e.g. in the hands and feet. Peripheral neuropathy is diagnosed in about two-thirds of clinical cases¹⁰⁸. It is not uncommon that patients with ACA also show signs of arthritis in joints adjacent to the skin lesion¹²⁸. ACA may be overlooked, both by patients and physicians due to its slow and insidious development, delayed temporal onset in relation to the tick bite, unfamiliarity of the disease, but also due to the atypical clinical picture. Lesions on the legs can be misdiagnosed as eczema hypostaticum seen in circulatory insufficiency or in lymphedema^{108, 128}. ACA is almost exclusively caused by *B. afzelii*. However, cases of ACA caused by *B. garinii* and *B. burgdorferi* s.s. have been reported¹⁵³. ACA is more common in Europe and only a few cases

have been defined in North America, which can be explained by the correlation between ACA and *B. afzelii*, a species not found in North America ^{106, 154, 155}.

Borrelia lymphocytoma

BL appears as a small skin induration with a solitary bluish-red-purple nodule located on the earlobe or at the areola mammae ¹⁰⁸. However, BL can also appear at other sites e.g. the nose, shoulder, upper arm, and scrotum ⁹⁸. Lesions in adults are often located on the areola mammae, while the earlobes is a more common site among children (Figure 8) ^{98, 105, 108}. The induration slowly enlarges during the infection and can reach a diameter of up to five centimetres ¹⁰⁸.

Figure 8 Clinical sign of a Borrelia lymphocytoma in a child [Reprinted from By Gzzz - Own work, CC BY-SA 4.0, (<https://commons.wikimedia.org/w/index.php?curid=76118879>)]

In addition to the lesion, patients have also reported symptoms like pain, itching, and breast tension ⁹⁸. Most of the BL arise in the vicinity of a previous EM lesion ¹⁰⁸. Still, BL may also appear before or with EM ^{105, 128}, and sometimes it may take 6-10 months after the tick bite before the BL develops ^{98, 128}. BL is often caused by *B. afzelii*, but infections with *B. garinii* may also occur ¹⁰⁵.

Lyme carditis

Cardiac involvement may present in both the acute phase (atrioventricular block and pericarditis) and in the late phase (dilated cardiomyopathy) of a LB infection ¹⁵⁶. LC typically occurs four days to seven months (median 21 days) after a tick bite and is often associated with EM or LNB as primary infection ^{157, 158}. Cardiac involvement can be both asymptomatic and symptomatic, involving clinical manifestations e.g. chest pain and irregular heartbeat ^{159, 160}. However, unspecific symptoms involving fever, headache, and muscles and joint aches, symptoms that also present in LB, are even more common than specific symptoms ¹¹⁰. In Europe, LC has been reported to be caused by different *Borrelia* species e.g. *B. bissettii* ⁷⁹ and *B. afzelii* ¹⁵⁶, while in North America cases with *B. burgdorferi* s.s. have been reported ¹⁶¹.

Rare manifestations

Eye manifestations of LB, apart from cranial nerve palsy causing eye muscle paresis, are rare. However, direct involvement of different tissues of the eye has been described, e.g. conjunctivitis and keratitis ^{162, 163}. Isolated cases of *Borrelia* infections of individual organs or organ systems e.g. the liver, lymphatic system, urinary tract, respiratory tract, and genital organs have also been reported. However, an association with LB has not yet been fully established ⁷.

Post-treatment Lyme disease syndrome or post-Lyme disease syndrome

Most patients get well after antibiotic treatment, but for a small percentage prolonged neurocognitive and somatic symptoms e.g. fatigue, joint and muscle pain, memory impairment, concentration problems, and headache may persist ^{164, 165}. Although, no solid evidence of persisting *Borrelia* spirochetes in patients has been shown ¹⁰¹. This condition is called post-treatment LD syndrome or post-LD syndrome ¹⁶⁶. Unfortunately, the symptoms can persist for months to years after a *Borrelia* infection. However, lengthy antibiotic treatment lacks benefit, and has no better long-term effects than placebo. Most patients recover from persisting symptoms with time ^{164, 166, 167}. Instead, self-diagnosed LB, misdiagnosed LB by physicians, or treatment of suspected LB not meeting the criteria for LB

can lead to serious, sometimes deadly complications as other severe disease may be ignored

¹⁶⁷⁻¹⁶⁹.

Subclinical seroconversion

Individuals without known history of LB subclinical symptoms, have been found to have a positive IgG antibody response towards *Borrelia* in serum. This phenomenon is known as subclinical seroconversion where the detected antibodies are a sign of a previous exposure to *B. burgdorferi* s.l. species, which may have passed asymptotically or with mild, self-limiting symptoms. Subclinical seroconversion is more frequently occurring in highly endemic areas ¹⁷⁰. Asymptomatic seroconversion without the development of LB manifestations has been reported in 3 % of the participants in a study from Sweden (the Tick-Borne Disease-STING study), a number comparable to the incidence of symptomatic LB cases, 2 % (documented by seroconversion), in the same study ¹⁷¹.

Treatment and prevention

Therapy for Lyme borreliosis and vaccines

Most patients treated with antibiotics for EM, LNB, LA, BL, and LC have a good prognosis of full recovery with minimal sequelae, especially if they are treated in the early stage of the infection ^{172, 173}. However, some patients continue to have mild non-specific complaints after EM, and patients with LNB tend to have residual deficits with mild facial nerve palsy ¹⁷². In some exceptional cases implantation of a permanent pacemaker is needed after an LC infection ¹⁵⁹. Patients with LA often recover well, but in some cases (mainly in North America) they continue to have recurrent synovial inflammation for a month or even years after treatment ¹⁷².

LB is treated with different kinds of antibiotics. The guidelines in Europe and North America are mainly in agreement with each other, but minor differences may occur ¹⁷⁴. The recommendations from the Swedish Medical Products Agency for treatment of different manifestations in Sweden are seen in Appendix 1 ⁴².

Currently, there are no available vaccines for LB ⁴². In the 1990s two monovalent recombinant vaccines based on OspA derived from *B. burgdorferi* s.s. were approved in the USA. However, the vaccines were withdrawn by the manufacturers since some studies reported undesired vaccine reactions. Still, these vaccines were only suitable in North America, since they were not able to protect against *B. afzelii* and *B. garinii*. A vaccine with protection against all three human pathogenic species, a polyvalent OspA vaccine, is currently being developed in Europe. However, approval for use is not expected in the near future ^{146, 175}.

Risk populations and prevention

Individuals with the highest risk of getting tick-bites are people residing or working in the forest of endemic areas and consist mainly of forestry workers ^{176, 177} and farmers ^{176, 178}. However, veterinarians ¹⁷⁶, hunters ¹⁷⁹, orienteers ¹⁸⁰, gardeners, and people living near the forest and frequently visiting the area are also more often exposed to ticks and have a higher risk of getting a tick-borne infection ¹⁷⁸.

The most effective method of tick prevention is to use tick-avoiding behaviour such as wearing full-covered and tucked-in clothing, applying repellents, and checking for and removing ticks promptly after visiting environments that are appealing to ticks ^{42, 146, 172, 181}. If a tick is attached to a host it should be removed as soon as possible by pulling the tick out. This can be done by grasping the tick with a tweezer or with fingernails close to the skin surface. If there are parts of the suction organ left in the skin, these can be removed with a needle. However, the remaining part of the head and suction organs will extrude from the skin over time and generally do not cause further discomfort or raised risk of *Borrelia* transfer ^{146, 173}. Persons who remove the tick from the skin later than 24 hours are more likely to develop symptoms, compared to those who remove the tick early, since attachment duration is strongly associated with an increased risk of *Borrelia* transmission ^{171, 181}. Since it takes up to two days, as mentioned before, for the *Borrelia* spirochetes to migrate from the midgut to the salivary glands and over to the skin of the host, it is important to find and remove ticks as early as possible to minimise the risk of getting a *Borrelia* infection ^{42, 59}. In case of a tick bite, it is also advisable to observe the site of the tick-bite for the next weeks and seek medical care if EM or symptoms associated with LB should appear ¹⁴⁶.

Laboratory diagnosis of Lyme borreliosis

Diagnosis of LB is based on the patient's medical history, the history of tick exposure in a disease-endemic area, typical clinical symptoms and signs along with laboratory evidence of a *Borrelia* infection ⁹⁹. To diagnose LB, both direct and indirect detection tools have been developed for a variety of clinical samples. However, most of the direct detection tools have also been used for detection of *B. burgdorferi* s.l. species in ticks and animals. The three most common direct detection methods for diagnosis of LB are; 1) microscopic detection of the spirochetes, 2) culture of the spirochetes, and 3) detection of *Borrelia*-specific DNA by PCR. Although, these methods are not used for diagnosis in clinical specimens from patients with suspected LB in every day clinical practice, except for PCR ¹⁸². Serological assays, which are the most frequently used indirect methods in clinical practice, detect *Borrelia*-specific antibodies, produced as a response to the *Borrelia* infection in clinical specimens ¹⁸³. Some rapid tests (for home-use) intended for testing the tick for the presence of *Borrelia* are on the market, which is worrying. These tests have not been evaluated and standardized by external quality assessment schemes and are therefore of questionable reliability. However, the most important issue is that the detection of a pathogen in a tick does not imply that the pathogen has been transmitted to the host, which may lead to unnecessary worry for the patient and unnecessary antibiotic treatment ⁵⁰.

Validation of detection methods for clinical laboratory diagnosis is divided into the assessment of analytical performance and the assessment of diagnostic performance. Analytical performance determines the possibility for an instrument or method to measure the analyte of interest e.g. antibodies and DNA. Diagnostic performance determines the ability of a test to distinguish between diseased and non-diseased individuals. Both concepts are related to each other, and a method or instrument with poor analytical performance will most likely generate poor diagnostic performance. Diagnostic performance should therefore not be evaluated unless the analytical performance is fully characterised with reference intervals or clinical decision threshold ¹⁸⁴.

***Borrelia* proteins, genes, and their function as diagnostic targets**

All of the proteins and the genes mentioned in the section “*Borrelia* proteins and genes – function and expression” can be used as targets for a wide array of serological and molecular assays¹⁸. The most commonly used ones are the lipoproteins Osp A and C, the lipoprotein VlsE, the Fla protein (41-kDa) together with various heat shock proteins¹⁸. In many serological assays, several antigens from the human pathogenic *Borrelia* species are combined, since the proteins are expressed at different stages of the infection⁵⁰. Below are listed some commonly used proteins along with genes coding for the protein, however; other proteins (cytokines and chemokines) can also be used as diagnostic biomarkers in the diagnosis of LNB and analysed by flow cytometric analysis¹⁸⁵⁻¹⁸⁷.

OspA is a lipoprotein known to be expressed late in the infection and previous studies have shown that IgG antibodies to OspA correlate with LA. Early IgM and IgG responses of OspA also appear, however with fast disappearance followed by re-development of an IgM response to OspA months to years later¹⁸⁸. The *ospA* gene is also a commonly used target for PCR-analysis. However, previous studies have shown that protocols for detecting *ospA* are unable to detect the species *B. spielmanii* and *B. miyamotoi*, both of which are known to be pathogenic to humans¹⁸⁹.

OspC is a lipoprotein that is expressed early in the infection. This makes it useful as a target in early infections. The expression of OspC (especially IgM) declines over time and OspC may become undetectable in the later infection stages¹⁹⁰.

VlsE, which also is expressed early in the infection has in previous studies been shown to be the most sensitive target for IgG antibodies, but also for IgM^{191, 192}. In a study by Schulte-Spechte et al. (2003)¹⁹³, the use of VlsE as a diagnostic target in a recombinant immunoblot assay revealed an increased diagnostic sensitivity (86 %) compared to whole-cell lysate immunoblot (53 %), without loss in diagnostic specificity. In the early stage of LB the prevalence of antibodies against VlsE ranges from 20-50 %, in the second stage from 70-90 %, and in the third stage it has reached 100 %²³.

Flagellin is the most dominate antigen during all LB stages and is used as a diagnostic target for LB (especially FlaA and B for IgM)^{26, 146}. Unfortunately, the flagellin antigen is highly cross-

reactive to other bacteria, but also to antigens found in mammalian tissues from the joints, heart, and nerves. Assays based in flagellin should therefore preferably be used as a recombinant truncated internal fragment ^{22, 50}. The *flaB* gene is also used as a target in PCR-analysis and has been proven to detect all pathogenic strains ¹⁸⁹.

16S rRNA sequences are widely used to infer the relationship between bacteria, since they are shared by all organisms including all pathogenic *Borrelia* strains, contain both conserved and more variable regions and have a sequence length that is suitable for sequencing. However, in cases of closely related species, the resolution of the 16S rRNA gene is too low, and whole genome sequence analyses are required ¹⁹⁴.

Diagnostic tools for direct detection

Microscopic detection of *Borrelia burgdorferi* sensu lato

In general, microscopy is mainly used for detection of *B. burgdorferi* s.l. spirochetes in the midgut or in the salivary glands of the tick. However, the *Borrelia* spirochetes can also be visualised directly in animal tissue by dark-field microscopy or after immunological or non-specific staining of specimens. The use of microscopy for detection of *Borrelia* in tissues or body fluids from patients is rarely possible, since; 1) dark-field microscopy is only useful in specimens with a large number of spirochetes, 2) the spirochete density is low in clinical samples, and 3) great experience is required to distinguish spirochetes from other structures in the samples since the morphology of the spirochetes is variable regarding length and structure ^{22, 195}. Therefore, the diagnostic value of microscopy-based methods is limited ¹⁹⁵. In a previous study, Laane et al. (2013) ¹⁹⁶ described a microscopic method for detection of live spirochetes in blood. However, this method was later questioned by Aase et al. (2016) ¹⁹⁷, since the method had not been evaluated against a reference method using relevant patients and controls. Aase et al. (2016) ¹⁹⁷ argued that detection of spirochetes in blood by microscopy cannot be recommended for identifying *Borrelia*-infected patients.

Detection of *Borrelia burgdorferi* sensu lato by culture

Culture medium and conditions

The *B. burgdorferi* s.l. spirochete requires a special medium for cultivation since the small genome of the bacteria is unable to direct synthesis of nucleotides, amino acids, fatty acids, enzyme co-factors, and most of the cellular building blocks ^{2, 15}. This explains the need for serum-supplemented mammalian cell culture medium for growth of *B. burgdorferi* s.l. in vitro ¹⁵. The most frequently used medium for cultivation of *B. burgdorferi* s.l. spirochetes is the Barbour-Stoenner-Kelly (BSK) II medium ¹¹, a medium first mentioned by Kelly in 1971 for cultivation of *Borrelia hermsii* ¹⁹⁸. This medium was further developed by Stoenner in 1974 ¹⁹⁹ and by Barbour in 1981 ¹¹. Another commonly used cultivation medium based on the Kelly-medium is the modified Kelly-Pettenkofer (MKP) medium ²⁰⁰. The main ingredients in BSK II medium are CMRL-1066, a standard medium used for cultivation of many different mammalian cells containing; N-acetylglucosamine, a precursor for bacterial cell wall biosynthesis; bovine serum albumin fraction V, a protein- and lipid-rich source that also stabilises pH; citrate; rabbit serum and pyruvate ². All three *Borrelia* species, *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*, known to be pathogenic to humans, grow well in BSK II medium ²⁰¹. The optimal temperature for reaching a peak multiplication in 4-6 days is 30-34°C ²⁰², and microaerophilic conditions are preferable ²². The incubation time for culture of *B. burgdorferi* s.l. spirochetes is much longer than for most other human bacterial pathogens, which is partly due to the prolonged generation time (7-20 hours or longer) for the spirochetes during log-phase growth ^{11, 202}. For detection of growth, the cultures are periodically examined for the presence of spirochetes by phase-contrast microscopy or by dark-field microscopy and fluorescence microscopy after staining with acridine orange or fluorescence-labelled antibodies ^{18, 203}.

Diagnostic sensitivity, specificity, and limitations of culture in clinical specimens

Culture is not a method that is used in every day clinical practice for diagnosis of LB, since the method has low diagnostic sensitivity in many sample materials, is time-consuming and require special cultivation medium and expertise. However, culture is important in LB

research, where it is essential to be able to culture and isolate *B. burgdorferi* s.l. spirochetes. Therefore, the method remains gold standard to confirm the microbiological diagnosis ¹⁸².

B. burgdorferi s.l. spirochetes can be cultured from skin biopsies (primarily EM, ACA, and BL), synovial fluid (SF), blood and cerebrospinal fluid (CSF) ²². In some individual cases, culture from the heart muscle and biopsy from the iris may be carried out. However, this is rare ⁵⁰. The diagnostic sensitivity of culture from blood (<10 %) and CSF (10-30 %) in early LNB is low ^{22, 138} and the cultures are only very rarely positive in SF ²⁰⁴. The low recovery rate in blood and CSF may reflect the small number of viable spirochetes in these locations ¹⁸, while the low diagnostic sensitivity in SF may be a result of the non-presence of viable spirochetes in the sample material, which makes it impossible to re-culture. However, the viable *B. burgdorferi* s.l. cells seem to be present in the joint outside the SF compartment, whereupon synovial tissue may be a more suitable sample material for culture ²⁰⁵. Why viable cells do not enter the synovial cavity are not entirely clear. However, PCR-analysis of SF has high diagnostic sensitivity since the method is able to detect non-viable spirochetes. The diagnostic sensitivity in cultures of spirochetes from skin biopsies is higher (>50 % and up to 88 % in EM) ^{72, 206} and skin biopsies from EM are the most useful material for culture in early LB. However, visual recognition of the characteristic lesion is usually sufficient for an accurate diagnosis, whereupon no laboratory testing is necessary ¹⁷². The diagnostic sensitivity of culture from skin biopsies from ACA (< 60 %) ^{22, 207}, from BL (< 25 %), ¹⁰⁸, and samples from heart tissue in suspected LC is low ²⁰⁸.

Due to these limitations, culture is not used in everyday clinical practice and has a very low diagnostic yield in patients with LB ^{204, 209}. However, in a minority of the cases, with atypical rash or in patients unaware of tick exposure in a highly endemic LB area, culture can be used as a complement ²²

Detection of *Borrelia* by polymerase chain reaction

The most commonly used diagnostic tool for detection of *Borrelia*-specific DNA in clinical practice is use of real-time PCR (for more information regarding real-time PCR see section “Principle of polymerase chain reaction and real-time polymerase chain reaction”. For classification and characterisation of *Borrelia* species techniques e.g. ribotyping, sequencing

of 16S rRNA and other conserved genes, species-specific PCR, multi-locus sequence typing (MLST)/multi-locus sequence analysis, and whole genome sequencing may be used ¹⁰.

Both chromosomal targets (e.g. *fla*, 16S rRNA, 23S rRNA, 5S-23S and 16S-23S intergenic spacers) and plasmid targets (e.g. *ospA*, *ospC* and *vlsE*) can be used for molecular detection of *B. burgdorferi* s.l. Plasmid targets are present in multiple copies within each bacterium compared to chromosomal target, which only occur as a single copy in each bacterium. Detection of a plasmid target e.g. *ospA* may therefore theoretically result in a higher sensitivity compared to the detection of chromosomal targets, e.g. 16S rRNA, and *fla* genes ^{18, 210}. Plasmid target genes have high variability both within and between species, which can lead to false-negative results, while chromosomal target genes are more conserved and may have problem discriminating between different *B. burgdorferi* species ⁵⁰. To study the bacterial phylogeny of *B. burgdorferi* s.l., genotyping by sequencing of target gene 5S-23S intergenic spacer, a genomic region with a high degree of variability between species, can be performed ⁴⁷.

Detection of *B. burgdorferi* by PCR is not used as a primary diagnostic tool, but can be used for; 1) confirmation in uncertain cases where serological results are inconclusive ²¹¹, 2) identification/typing of spirochetes in clinical samples or culture, and 3) detection of co-infections by *B. burgdorferi* and other pathogens associated with tick-borne infections ². Detection of *Borrelia*-specific DNA by PCR may be applied to multiple sources including skin biopsies, blood, serum, plasma, SF, CSF, urine, and ticks. However, the diagnostic sensitivity when used with different sources varies ²⁰⁴.

Principle of polymerase chain reaction and real-time polymerase chain reaction

By PCR-amplification billions of DNA molecules can be synthesised, resulting in large amounts of copies specific to the microorganism of interest. During each cycle, the amount of DNA copies doubles, leading to an exponential increase. This means that only a few target sequences of DNA can generate large amounts of amplified DNA (copies) in a few hours. PCR in general detection of viruses and bacteria is easy to perform, and has high diagnostic sensitivity and specificity as well as high efficiency ²¹².

Polymerase chain reaction

The principle for PCR using Taqman polymerase is shown in Figure 9.

Figure 9 Principle of polymerase chain reaction-analysis. During the denaturation step, the double-stranded DNA helix is separated into two DNA strands. In the annealing step, primers, complementary to the two DNA strands, are binding to the single strands. Finally, during the extension step, two new double-stranded DNA sequences (complementary to the original strand) are established by Taq polymerase and single nucleotides (dNTP = deoxynucleotide triphosphate, DNA = deoxyribonucleic acid) [Figure created with Biorender.com]

In the first step, the denaturation step, double-stranded DNA will be separated by heating, resulting in single-stranded DNA. In the second step, the annealing step, the temperature is decreased to one optimised for the primers (small complementary sequences of synthetic DNA) to hybridise by hydrogen bonds to the single-stranded DNA sequence ²¹³. Each primer usually contains 20-30 bases and are designed to be complementary to the sites next to the region of interest. Since the primers determine the specificity, they are the most critical components in the PCR and essential for successful reactions. Conditions like primer length,

primer sequence (% GC), primer melting temperature, and secondary structures affect the binding of the primer to the target DNA sequence and need to be taken into consideration when designing primers ²¹⁴. Once the primers are attached to the target DNA sequence, an enzyme called DNA polymerase (in Figure 9 known as Taq polymerase) starts to synthesise a new double-stranded DNA sequence starting at the primer. By use of single deoxynucleotide triphosphates, [cytosine (C), thymine (T), adenine (A), guanine (G)], the entire target DNA sequence on both strands is copied ²¹³. This is called the extension step and starts once the temperature is raised to 72 °C, a temperature which is optimum for Taq polymerase. The entire process is called a cycle and by applying several cycles repeating the three steps, multiple copies of DNA molecules can be produced. In a standard PCR, analysis of the PCR products is carried out at the plateau phase of the reaction (endpoint analysis) (Figure 10) and the PCR-products are usually visualised using gel electrophoresis ²¹⁴.

Figure 10 Amplification plot showing the phases of a PCR-reaction. For traditional PCR the measure is carried out when the plateau phase is reached, while the measure for a real-time PCR is performed in the exponential phase of the reaction (Cq = Quantification cycle) [Figure created with Biorender.com]

Real-time polymerase chain reaction

A real-time PCR reaction includes both primers and probes. Probes are single-stranded DNA oligos complementary to a specific sequence on the target region of the PCR template ²¹⁴. By the use of a fluorescent marked probe generating a signal when it is digested, the growing target copy numbers can be displayed, and data can be collected in real-time (i.e. while the PCR-reaction is ongoing). This shortens the laboratory time since there is no need for post-PCR methods (e.g. gel electrophoresis) in the final phase (the plateau phase) of the reaction. It is also possible to apply a quantitative standard to the real-time PCR-analysis to quantify the amount of starting material in an unknown sample. The measure is carried out in the exponential phase of the reaction. During the exponential phase the amount of PCR product present in the reaction tube is directly proportional to the amount of emitted fluorescence and the amount of the initial target sequence (Figure 10) ²¹⁴.

Two types of real-time PCR techniques, SYBR Green and Taqman, use different dyes and different ways for the dye to bind to the DNA strand. The SYBR Green method utilizes the dye SYBR Green, which binds to all double-stranded DNA in the samples, including non-specific regions amplified by the PCR. To distinguish between different PCR-products or between non-specific products like primer-dimers, the PCR-analysis must be followed by a melting curve analysis, indirectly analysing the sequence variation of the amplified fragments. Each product has a characteristic melting temperature, depending on the composition of the strand (GC content, length). During the melting curve analysis, double-stranded DNA with high fluorescence is denatured by heat to single-stranded DNA. The fluorescein dye is released leading to loss in absorbance intensity. The melting temperature for each product is obtained when 50 % of the double-stranded DNA becomes single-stranded ^{215, 216}. The Taqman technology (Figure 11), which is used in this thesis, is more specific, utilising probes designed to match the target DNA sequence and to generate fluorescence. The probe is composed of a single-stranded DNA sequence, complementary to the target DNA sequence, and chemically modified in the 3'-end so that the polymerase cannot extend it. At the 5'-end the probe has a fluorescent dye (the reporter) and at the 3'-end a non-fluorescent dye (the quencher). Once the extending strand reaches the probe, the probe is degraded by the Taq polymerase's 5'-nucleotide activity, and release of the reporter from the vicinity of the quencher results in a fluorescence signal ²¹⁴. By use of a quantification cycle (Cq) value for each reaction, also known as the cycle threshold value, it is possible to detect how many cycles it took before the PCR product could be detected above the background signal, the threshold (Figure 10) ²¹⁷.

Figure 11 Principle for Taqman real-time polymerase chain reaction (PCR) technology. A double-stranded deoxyribonucleic acid (DNA) helix is, during the denaturation step (A), separated (by heat) into single-stranded DNA sequences. During the polymerisation step (B) primers and probe, complementary to the target DNA target sequence, are attached to the strand. The probe contains two dyes, one fluorescent dye (R = reporter) at the 5'-end of the probe and one non-fluorescent dye (Q = quencher) at the 3'-end of the probe. During the polymerisation step of the DNA, the exonuclease activity of the Taqman polymerase digests the probe and extends the primers (C). By the release of the reporter from the vicinity of the quencher a fluorescence signal is created (D), which is measured in real-time [Figure created with Biorender.com]

Diagnostic sensitivity, specificity, and limitations of polymerase chain reaction for analysis of Lyme borreliosis

A drawback to the use of molecular methods for detection of *B. burgdorferi* is the limitation in diagnostic sensitivity in some clinical samples and the absence of standardisation of methods. To optimise the diagnostic sensitivity, recommendations regarding selection of clinical samples, elution volumes, molecular targets, and analytical conditions are needed ⁵⁰. Even though the analytical sensitivity and specificity for detection of *B. burgdorferi* s.l. in CSF with PCR are high ¹⁸⁹, the diagnostic sensitivity in CSF but also blood, the two most common sample materials, is low. The diagnostic sensitivity and specificity for different specimens for detection of *B. burgdorferi* s.l. by PCR are listed in Table 4.

Table 4 Meta-analysis of diagnostic sensitivity and specificity in different specimens for detection of *Borrelia*-specific DNA in both Europe and North America ²¹⁰

Clinical specimens	Sensitivity (%)	Specificity (%)
Skin biopsy		
Overall*	76	100
EM	71	
ACA	84	
Plasma/serum&	29	100
CSF		
Overall#	24	100
Non-chronic	21	
SF*	65	99
Urine&	74	99

EM = erythema migrans, ACA = acrodermatitis chronica atrophicans, CSF = cerebrospinal fluid, SF = synovial fluid

* Sum of EM and ACA samples

& from patients with LB infection

Sum of CSF from LNB patients with early and long-lasting neurologic involvement (≥ 6 months)

* SF from patients with LA

The highest diagnostic sensitivity has been shown in skin biopsies from patients with EM or ACA, where fresh or fresh-frozen biopsies are preferable ⁵⁰. However, in general, PCR-analysis of EM is not necessary in clinical practice, since the typical EM lesion is regarded as characteristic for LB and needs no further laboratory diagnosis ². Nevertheless, in more diffuse rashes with suspicion of LB, PCR may serve as a diagnostic tool. The diagnostic sensitivity in SF is also high (with probable detection of dead spirochetes) and for LA patients, PCR is a suitable diagnostic tool as a complement to serology (Table 4) ²¹⁰. The lowest diagnostic sensitivity is seen in blood, which may be a reflection of the small number of spirochetes in this specimen ²¹⁸, the lack of spirochetemia or transient spirochetemia, and/or the presence of PCR inhibitors such as haemoglobin in erythrocytes and lactoferrin in leukocytes in the host blood ^{22, 219}. The diagnostic sensitivity in CSF is also low, which may be a result of the lack of spirochetes in the specimens, CSF white cell count, and disease duration ²². However, the diagnostic sensitivity in CSF is higher in patients with suspected LNB with a disease duration of < 2 weeks and in patients with CSF pleocytosis. To increase the diagnostic sensitivity, CSF samples should preferably be centrifuged to concentrate the cells, before analysis ²¹¹. In the case of a suspected *B. miyamotoi* infection, PCR detection in serum and CSF has high diagnostic sensitivity, and the method is, therefore, suitable for diagnosis of *B. miyamotoi* disease ²²⁰. Detection of *Borrelia* DNA in myocardial biopsy specimens in patients with suspected LC by PCR may help to establish the diagnosis, but cannot be used as a primary tool

¹⁵⁹. The same is the true for the use of PCR for the detection of *B. burgdorferi* in patients with BL, where PCR-analysis only catches about two-thirds of cases ¹⁰⁸. However, PCR may be used as a complement to serology and pathological-anatomical diagnosis in ambiguous cases of BL ²²¹. Detection of *Borrelia* in urine by PCR is not a suitable method due to the presence of PCR inhibitors in the samples and absence of correlation with clinical disease ^{210, 222}.

Nevertheless, the diagnostic specificity of PCR is high and more homogenous, regardless of the clinical sample type (Table 4) ²¹⁰. Due to the limited diagnostic sensitivity, detection of *B. burgdorferi* with PCR is not widely used in everyday clinical practice but can be used as a supplement to other diagnostic tools ²²³.

Finally, PCR does not have the ability to distinguish between active and recent infection, since DNA may be detected from both viable and non-viable spirochetes ²²⁴. Conclusion of suspected therapy failure after antibiotic treatment could not be drawn due to the presence of residual DNA, where a positive PCR result (after antibiotic treatment) without clinical manifestations has no clinical relevance ⁵⁰.

Diagnostic tools for indirect detection

Indirect detection of *B. burgdorferi* s.l. infections normally entails to serological methods, i.e. antibody detection, although cellular tests for the T cell response have been used for research purpose ^{225, 226}. The IgM antibodies against different targets e.g. lipoprotein Osp A and C, VlsE, and the Fla protein all mentioned in the section “*Borrelia* proteins and genes – function and expression”, normally appear within the first weeks ¹⁸. Most LB patients develop IgG antibodies one month after symptom on-set. These antibodies can remain for years after the LB symptoms have resolved ^{18, 227}, and the highest titers are in general seen in patients with LA ²²⁷.

Serological methods for detection of *Borrelia*-specific antibodies

Serological assays, which detect *Borrelia*-specific antibodies is the main tool for diagnosis of LB in clinical laboratories for most clinical manifestations (except EM). Many countries in Europe follow a two-step strategy, involving a screening test [e.g. enzyme-linked immunosorbent assay (ELISA)], which in the case of reactive or equivocal results is followed by immunoblot [e.g. Western blot (WB)]. Recent research recommends use of a single test

strategy employing modern ELISAs based on recombinant or synthetic antigens e.g. C6 peptides, since both diagnostic sensitivity and specificity are sufficient. However, since there are multiple pathogenic species in Europe with variability in immunodominant antigens and because the diagnostic specificity in single tests is somewhat lower, successful application of the single-test approach may be limited ¹²⁴. In the United States, a two-tier strategy based on two ELISA methods (one whole-cell sonicated ELISA followed by an ELISA based on recombinant or synthetic antigens e.g. C6 peptides) has been presented with comparable or even better diagnostic performance compared to standard two-tier testing ²²⁸.

Principle for detection of *Borrelia*-specific antibodies with immunofluorescent antibody assays

The immunofluorescent antibody assay (IFA) technique can be used for both direct and indirect detection of a pathogen (Figure 12).

Figure 12 Principle for direct and indirect immunofluorescence assay [Figure created with Biorender.com]

By direct IFA, the labelled antibodies react directly with the antigen on the microorganism, while an indirect IFA is based on antibody detection by an antigen-antibody complex. In an IFA a solid phase (e.g. microscopic glass) is coated with antigens. In direct IFA, antibodies labelled by a covalently attached fluorescent, e.g. fluorescein isothiocyanate, bind directly to the antigen in the solid phase. In indirect IFA, an unmarked primary antibody in the sample is

bound to the antigen attached in the solid phase, followed (after washing off unbound material) by binding of a secondary fluorophore marked antibody. Fluorescein isothiocyanate can be detected microscopically, by fluorescence microscopy, when it emits a bright fluorescent colour after it is illuminated with light of particular wavelengths ^{212, 213}. The colour of the emitted fluorescent light is usually yellow-green or red-orange, depending on which dye is used ²¹². The fluorescent molecules can absorb ultraviolet light and then release light in the visible range ²¹³. Fluorescent labelled antibodies are widely used in both diagnostic and research assays.

An advantage in using fluorescent antibodies in diagnostics is that they permit direct identification of a microorganism in patient serum (in situ) and no isolation or cultivation is needed ²¹². However, the use of IFA is not recommended in everyday clinical practice since the method is subjective, time-consuming and requires well-trained staff ²². Therefore, the method is mainly used in research to detect *B. burgdorferi* in tissue samples ²²⁹. The disadvantages of IFA are the same as for all serological assays and include delayed antibody response, cross-reactivity, and persistence of antibodies after an infection, which is further discussed in the section “*Diagnostic sensitivity, specificity and limitations of serological assays*”. Nowadays, IFAs are rarely used in laboratory diagnosis of LB since they have generally been replaced by the ELISA technique, which is more suitable for large-scale testing.

Detection of *Borrelia*-specific antibodies with immunoblot/Western blot

In the earlier ELISA methods based on whole-cell sonicates, the diagnostic specificity was low due to cross-reactivity. To improve the diagnostic specificity, a second diagnostic assay with several *Borrelia* antigens, an immunoblot/WB, was added to the algorithm in order to increase the diagnostic specificity. The immunoblot/WB technique can be used for both direct and indirect detection. However, in the following section, the focus is on indirect detection, i.e. antibody detection. The principle for indirect immunoblot is presented in Figure 13.

Figure 13 Principle for indirect immunoblot/Western blot for confirmation of the presence of *Borrelia*-specific antibodies [Figure created with Biorender.com]

Immunoblotting is used to detect antibodies in a patient's serum. *Borrelia*-specific proteins (antigens) are separated by size and charge by gel electrophoresis on a sodium dodecyl sulfate polyacrylamide gel. The protein bands are transferred from the gel to a nitrocellulose membrane by blotting. At this point, the bands are not visible. When the patient's serum is added to the nitrocellulose membrane, *Borrelia*-specific antibodies (if present in the sample) will bind to the bands (*Borrelia*-specific antibodies referred to as primary antibody in Figure 13). After incubation, unbound antibodies are washed off and then radiolabelled or enzyme-linked polyclonal or monoclonal anti-human antibodies are added for detection (secondary antibody) ^{230, 231}. The most common procedure uses enzyme-linked antibodies and visualisation is made possible by the addition of a chromogenic substrate, which produces a coloured product and the appearance of coloured bands ²³². WB can be used in many applications but is often used to test serum samples for the presence of antibodies ²³⁰.

Enzyme-linked immunosorbent assay

The first generation of ELISA for detection of *Borrelia*-specific antibodies was based on whole-cell sonicates (with a large number of antigens) from cultured *B. burgdorferi* s.l. strains. Unfortunately, this resulted in poor diagnostic specificity caused by cross-reactivity of antibodies directed against similar antigens from other bacteria ^{193, 233, 234}, but also to tissue

components (autoantibodies) ¹⁸. To improve the diagnostic specificity, the second generation of antibody assays were based on purified native proteins such as the flagellum protein. The third generation of antibody assays is based on recombinant antigens (e.g. VlsE) and synthetic peptides (e.g. the C6 peptide derived from VlsE) ²³⁵. By including VlsE in both the ELISA and WB the performance of the assays can be improved ²³⁶.

Principle for detection of *Borrelia*-specific antibodies with enzyme-linked immunosorbent assays

In general ELISA assays can be categorised into four different groups, 1) direct ELISA assays, 2) indirect ELISA assays, 3) capture/"sandwich" ELISA assays, and 4) competitive ELISA assays. However, only indirect ELISA and capture/"sandwich" ELISA assays will be presented in the text below, since these are the ones used in this thesis. The principles for indirect ELISA and capture/"sandwich" ELISA assays are shown in Figure 14.

Figure 14 Principle for indirect enzyme-linked immunosorbent (ELISA) assays and capture/"sandwich" ELISA assay [Figure created with Biorender.com]

In an indirect ELISA, a solid phase, in most cases a well on a microtiter plate e.g. polystyrene microplates or shallow wells ²¹³, is coated with antigens or antibodies (depending on method). In the following description, antigens are bound to the solid phase. When the patient's sample is applied to the well, specific antibodies (if present in the sample) find the matching antigen

and connect to it (referred to primary antibody in Figure 14). After washing of unbound material, a secondary antibody containing a covalently attached enzyme is added (referred to enzyme-linked secondary antibody in Figure 14), which binds to the first unlabelled primary antibody²¹². After a second washing step a substrate suitable for the enzyme is added, resulting in the formation of color. The colour intensity is measured spectrophotometrically. The amount of antibodies in the sample is proportional to the optical density (OD) of the colour produced^{212, 213}.

In a capture/"sandwich" ELISA assay a capture antibodies are attached to the solid phase (in most cases a well in a microtiter plate). When the sample is added, complementary antigens bind to the antigen-binding site of the capture antibody. After washing of unbound material, a secondary enzyme-linked antibody is added, which will bind to the antigen, forming a "sandwich" of antibodies and antigen. After a second wash, a substrate suitable for the linked enzyme is added, and a visible colour is produced. The colour intensity is measured spectrophotometrically. The amount of antigens in the sample is promotional to the amount to the OD of the colour produced^{212, 231}.

Principle of Luminex technology in the detection of *Borrelia*-specific antibodies

Luminex technology is based on paramagnetic or polystyrene beads (microspheres) dyed with infrared and red fluorophores of different proportions. Each sample is applied to a microwell containing a mixture of colour-coded beads that are pre-coated with *Borrelia*-specific antigens. After incubation of patient samples and microspheres, the wells are washed. By adding biotinylated anti-human antibodies, an antigen-primary antibody-secondary antibody complex is formed. Phycoerythrin-conjugated streptavidin is attached to the biotinylated detection (secondary) antibodies. The beads are washed a second time and detected on a dual-laser flow-based instrument, for example the Luminex instrument. The Luminex instrument contains two lasers, where one laser classifies the beads and the analyte that is detected, while the second laser defines the magnitude of the phycoerythrin-derived signal. The signal is proportional to the amount of bound antibodies^{237, 238}. The principle for Luminex technology is shown in Figure 15.

Figure 15 Principle for Luminex technology [Figure created with Biorender.com]

Diagnostic sensitivity, specificity and limitations of serological assays

Results from a meta-analysis by Leeflang et al. (2016) ²³⁹ showed that the diagnostic accuracy of ELISA (but also immunoblots) for the detection of LB varies in Europe. In many countries, two-tier testing (including a sensitive ELISA followed by a more specific immunoblot) is used and recommended. However, both diagnostic sensitivity and specificity have been increased since the two-tier strategy was adopted in 1995 ²⁴⁰, and the benefit in use of the strategy has been questioned. Results from the same study also ²³⁹ show that two-tier testing does not outperform single testing. However, other authors have raised doubts regarding the approach of single testing in Europe, mainly due to the presence of several pathogenic *B. burgdorferi* species with variability in immunodominant antigen, which can make it harder to design an appropriate assay that fits all species ^{50, 124}. The diagnostic sensitivity and specificity for serological methods differ between different clinical samples, as presented in Table 5.

Table 5 Meta-analysis of diagnostic sensitivity and specificity in different specimens for detection of *Borrelia*-specific antibodies in Europe ²³⁹

Clinical specimens	Sensitivity (%)*	Specificity (%)*
Skin biopsy		
EM	50	95
ACA	98	94
LNB		
Serum	76	89
CSF	74	96
AI	83	95
LA [#]	96	94

EM = Erythema migrans, ACA = Acrodermatitis chronica atrophicans, LNB = Lyme neuroborreliosis, CSF = cerebrospinal fluid, AI = antibody index, LA = Lyme arthritis

*median value and no meta-analysis

[#]based on results from enzyme-linked immunosorbent assays, Western blot assays, and calculated antibody indexes

The diagnosis of BL with serological analysis of serum by two-tier testing, shows a positive result in 70–95 % of the cases, mainly in the IgG response ¹⁰⁸.

The diagnostic accuracy of serological assays depends on different factors; 1) the timing of collection of specimens in relation to disease onset, 2) the selection of suitable target peptides or antigens, 3) antibody expansion to a certain infectious agent and, 4) the methodology of the assay ²³³. Even though the serological methods have acceptable diagnostic sensitivity and specificity, some limitations are still currently caused by biological limitations. These drawbacks are due to:

1) **Slow formation of antibodies**, which can result in false-negative results and low diagnostic sensitivity especially for patients seeking medical care for suspected LB in the early stage of the infection, since it takes weeks for the antibodies to develop ^{18, 182}. As mentioned before the IgM antibodies often occur early in the infection and disappear more quickly than the IgG antibodies. However this varies greatly between individuals and the presence or absence of IgM antibodies cannot be used to determine the timing of infection ¹⁷³. Generally, IgG antibodies for LB are expressed in >99 % of patients after six to eight weeks ²²³.

2) **Cross-reactivity** of antibodies to other pathogens e.g. parvovirus ²⁴¹, human granulocytic anaplasmosis ²⁴², Epstein-Barr virus ²⁴³, *Treponema* bacteria ²⁴⁴, *Helicobacter pylori* ¹⁸, and *Lepthospira* bacteria ¹⁸, but also to autoantibodies in autoimmune diseases e.g. rheumatoid arthritis and systemic lupus erythematosus ²⁴⁵. The cross-reactivity is shown especially for IgM

assays resulting in lower diagnostic specificity. This may lead to misdiagnosis, improper clinical management, and mistreatment of patients and therefore it has been argued that the IgM test should not be ordered in patients with duration of symptoms >1 month ¹⁷³.

3) **High seroprevalence** (IgG) in highly endemic areas and populations engaged outdoor activities e.g. forest workers and hunters may indicate false-positive results due to former *Borrelia* infections or asymptomatic exposure since the serological assays are not able to distinguish between a previous exposure and a new one. Often the patient is unaware of the infection, since it has been resolved spontaneously or was treated with relevant antibiotics due to another infection. Therefore the physicians need to be aware of the seroprevalence in the local area and should not order borrelia serology in patients without distinct clinical symptoms (not EM) ^{223, 246}.

4) **Persistence of antibodies** after infection and the inability of the assays to distinguish between an active infection and a previous one. Patients with no additional tick bites and no signs of active infection, such as EM and flu-like symptoms, can have both IgM and IgG antibodies in serum 10-20 years after the initial infection despite adequate antibiotic treatment ^{182, 227}.

Difference between various serological methods – limitations, advantages, and how they complement each other

The various serological tools used in clinical practice have different advantages and disadvantages. However, all serological tools have the same biological limitations, which are described in the section “*Diagnostic sensitivity, specificity and limitations of serological assays*”. The following section will focus on the differences between the serological techniques and how they complement each other.

The first developed serological assay for detection of *Borrelia*-specific antibodies was IFA. Over the years, this method has been replaced by enzyme immune assays including ELISA and immunoblot for routine analysis at clinical laboratories, since IFA is cumbersome and includes subjectivity in reading, and requires fluorescence microscopy and experienced staff ^{2, 22}.

ELISA is the most commonly used assay today since it is easy to perform, is relatively cheap, and has high diagnostic sensitivity ²¹². Another benefit in using ELISA compared to IFA is the

objective determination by use of quantitative values (OD, index) for the antibody concentration in serum and CSF, and the possibility of automation and high throughput in clinical laboratories. The quantitative values can be compared to different controls, and objective cut-off values can be established ²⁴⁶.

However, even if the ELISA methods are the most frequently used assays for detection of LB, differences in the diagnostic accuracy of ELISA occur due to lack of standardisation ²². This may be a result of the various antigens used in different assays (whole-cell sonicates, purified native proteins, or recombinant/synthetic peptides), which is a limitation of the ELISA methods. The diagnostic sensitivity also depends on the clinical manifestation and the infection stage. For example, the diagnostic sensitivity of the ELISA method is relatively high in LNB, and high in LA and ACA compared to EM, where it is low. Studies have also shown that recombinant antigens generate both high diagnostic sensitivity and specificity for LNB, while assays based on a whole-cell antigen mixture generated high diagnostic sensitivity but lower diagnostic specificity due to cross-reactivities to other diseases and pathogens ^{239, 247}. Whole-cell antigen assays may therefore be used as screening assays (followed by additional tests e.g. immunoblot), while recombinant assays are more suitable as single tests in diagnostic practice ²⁴⁷. Although the serological methods have improved in both diagnostic sensitivity, and especially diagnostic specificity, over the years, the problem with diagnostic specificity in IgM in detection of LB remains, especially in serum samples. IgM is less specific than the IgG assays and for patients with symptoms > 4 weeks, only IgG antibody testing is of substantial value ¹⁸.

The ELISA methods are sometimes complemented by immunoblot to raise the diagnostic specificity (two-tier testing) ¹⁸. By use of the immunoblot technique after the initial ELISA assay, different *Borrelia* antigens can be separated. The presence of different bands (representing different antigens), which provides a band pattern, can be used to divide the immune response into two stages, early and late. It may also provide a better correlation between the clinical symptoms and laboratory results ²²³. However, the immunoblot assay also has limitations including visual scoring and subjective interpretation of band intensity, false-positive results (especially for IgM), cost, lengthy hands-on-time, and the variation in band pattern in patients with the same manifestation of LB ²². Immunoblot is mainly used for diagnosis of LB in serum and is not required for the diagnosis of LNB in CSF samples due to a lower risk of cross-reactivity issues ²²³.

Recently, development of the traditional ELISA has been established by use of Luminex technology. This technology has in general a larger measurement range than ELISA, requires a smaller sample volume, and is able to detect several *Borrelia* antigens at the same time. However, the assay is expensive and requires special equipment ^{237, 238}.

Detection of biomarker CXCL13

CXCL13 is a B cell-attracting chemokine. When the *Borrelia* spirochetes invade the subarachnoid space, monocytes start to release CXCL13 into the CSF, and B cells are recruited to the infection site. This will further lead to the characteristic B cell-enriched CSF pleocytosis ²⁴⁸. The chemokine CXCL13 serves as a chemoattractant of plasma cells and B lymphocytes into the CNS ²⁴⁹. CXCL13 is detected in high concentrations in CSF samples from patients with LNB ^{223, 249}.

Detection of the chemokine CXCL13 may be used as a complement to serology to strengthen the diagnostic performance of laboratory analysis in LNB ²²³, especially in children with possible LNB, who often present with symptoms early in the disease course ^{250, 251}. The test is often used in early cases (<2 weeks), when the antibody index (AI) may still be negative ¹⁴⁶. CXCL13 is strongly up-regulated in the CSF in early LNB and rarely reaches so high levels due to other aetiologies of CNS inflammation ²⁴⁹.

During antibiotic treatment of LNB, the levels of CXCL13 decline rapidly, which makes it useful as a marker to follow the outcome of treatment ^{186, 223, 249}. CXCL13 can also be used as a marker for active disease and to distinguish acute LNB from other CNS disorders ¹⁸⁶. However, CSF CXCL13 is not elevated only in patients with LNB, but can also be found in other diseases e.g. neurosyphilis and HIV, autoimmune diseases (multiple sclerosis), malignancies (CNS lymphoma), and tubercular meningitis ^{146, 223, 249}. However, a shortcoming of CXCL13 as a diagnostic tool is the different cut-off values used in different studies, even when the same ELISA assay has been used. Standardisation of cut-off values is therefore required to establish the diagnostic value of CXCL13 for LNB patients ^{146, 186, 249}.

However, CXCL13 is not the only biomarker of interest for the diagnosis of LNB. Previous studies have shown promising results for the use of other biomarkers (cytokines and chemokines e.g. CXCL1, GM-CSF, IL-7, and IL-10 and IL-17A) to discriminate LNB patients from non-LNB patients, but also as possible biomarkers for prognosis and time to recovery

after treatment^{185, 187}. However, further evaluations is needed to determine the future possible usefulness of these biomarkers for the laboratory diagnosis of LNB.

The situation of today – How do we apply the different diagnostic tools for Lyme borreliosis in clinical samples?

First, it is important to point out that a diagnosis of LB is not solely determined by laboratory results, but is always based on the patient's medical history and, clinical signs as well as laboratory results. However, diagnosis of EM is in most cases based on clinical appearance only, since the diagnostic sensitivity in serology in the early phase of the disease is low and the EM lesion is highly characteristic of LB²². For serology to be useful, appropriate clinical judgement when the sample is ordered and during the interpretation of the results is required²⁴⁶. When the likelihood of LB is low and the case definition criteria are not met, laboratory diagnosis for LB should not be performed to avoid reports of irrelevant or possible false-positive results, and other possible diagnoses should be taken into account²²³.

Currently, there are various diagnostic tools on the market for the diagnosis of LB. The different tools have their own inherent advantages and disadvantages (which have been discussed earlier in the section "*Laboratory diagnosis of Lyme borreliosis*"). Due to the biology of the *B. burgdorferi* s.l. spirochetes (with the addition of *B. miyamotoi*), the different clinical manifestations, and different infection stages, various tools are used for different purposes. So far there has been a lack of standardised procedures between laboratories and countries regarding methods, assays, protocols and algorithms; therefore, further development of recommendations for interpretations and reporting is warranted to create more consistent laboratory diagnosis.

In a study by Eldin et al. (2019)²⁵², 16 guidelines from seven countries in Europe and America have been compared (Table 6). The results revealed that most of the guidelines for diagnosis of different manifestations of LB were comparable. For most of the clinical manifestations, serological two-tier testing is the most commonly used diagnostic tool. However, for some clinical manifestations, culture and/or PCR may be used as a complement²⁵². Neither serology nor PCR can discriminate between an active and a previous infection in all cases¹⁸². Evaluation and if possible optimisation of the diagnostic tools are needed, due to the lack of

standardisation of both serological and molecular methods, since it is highly important that the patients receive equivalent medical care regardless of laboratory or country.

In addition to the traditional diagnostic tools, the literature describes several diagnostic techniques including lymphocyte transformation tests, immunochemical detection in biopsies and antigen in urine and blood, detection of cystic bacterial forms, circulating immunocomplexes, and visual contrast sensitivity test. However, these tests are not recommended due to their poor diagnostic sensitivity, specificity, and reproducibility ⁵⁰.

Table 6 Application of the different diagnostic tools for the detection of *Borrelia burgdorferi* sensu lato in different clinical samples. The table is based on two review articles presenting current guidelines in Europe and America ^{22, 25*}. For more information regarding numbers for diagnostic sensitivity and specificity in serological and molecular biological analysis see the sections “Diagnostic sensitivity, specificity and limitations of culture in clinical samples”, “Diagnostic sensitivity, specificity, and limitations of polymerase chain reaction for analysis of Lyme borreliosis” and “Diagnostic sensitivity, specificity and limitations of serological assays”.

Clinical manifestation	Diagnostic tool	Advantages	Disadvantages
Erythema migrans (single/multiple)			
Typical	* Clinical diagnosis	* Lesion is often typical for EM	* Low diagnostic sensitivity in serological detection especially in early infection*
Atypical	* Culture	* Relatively high diagnostic sensitivity in culture	* Moderate diagnostic sensitivity in PCR
	* PCR	* High diagnostic specificity for PCR	
Lyme neuroborreliosis			
Early	* CSF cell count and protein	* Intrathecal production of specific antibody is the gold standard	* Serological detection has low diagnostic sensitivity in very early LNB
	* Serology in CSF and blood (calculation of intrathecal AI)	* Detection of AI is more specific than detection of antibodies in CSF and blood alone	* PCR in CSF has low diagnostic sensitivity but may be useful in some cases (early LNB)
	* PCR	* CXCL3 may be a useful tool but needs further investigation	* The diagnostic sensitivity for PCR in blood is very low and the method may not be appropriate to use
	* Culture		* Culture has low diagnostic sensitivity
	* CXCL3		
Late	* CSF cell count and protein	* Intrathecal production of specific antibodies is the gold standard	* PCR-analysis in CSF has low diagnostic sensitivity
	* Serology in CSF and blood (calculation of intrathecal AI)	* Detection of AI is more specific than detection of antibodies in CSF and blood alone	* The diagnostic sensitivity for PCR in blood is very low and the method may not be appropriate to use
	* PCR	* CXCL3 may be a useful tool but needs further investigation	* Culture has low diagnostic sensitivity
	* CXCL3		
Lyme arthritis	* Two-tier serology	* High diagnostic sensitivity in serological detection of IgG but lower for IgM	* Detection of <i>B. burgdorferi</i> s.l. in SF by culture is low
	* PCR	* Diagnostic sensitivity of detection by PCR in SF is moderately high and PCR may be used as a complement to serology	* Clinical signs may be misinterpreted as rheumatological diseases
		* High diagnostic specificity in PCR	
Acrodermatitis chronica atrophicans	* Clinical diagnosis	* High diagnostic sensitivity in serological detection of IgG but lower for IgM	* Clinical signs may be misinterpreted as a circulatory disorder e.g. due to atypical clinical picture
	* Two-tier serology	* Diagnostic specificity in PCR is high and the method may be used in ambiguous cases	* Diagnostic sensitivity in culture is low
	* PCR		
	* Culture		
Borrelia lymphocytoma	* Two-tier serology	* High diagnostic sensitivity in serological detection especially for IgG	* Diagnostic sensitivity in culture is low
			* Diagnostic sensitivity in PCR is moderate (2/3 of the cases)
Lyme carditis	* Two-tier serology	* Most patients with LC are positive in serological detection at the time of presentation	* Diagnostic sensitivity in culture is low

EM = erythema migrans, LNB = Lyme neuroborreliosis, LC = Lyme carditis, AI = antibody index, CSF = cerebrospinal fluid, SF = synovial fluid, PCR = polymerase chain reaction, *B. burgdorferi* = *Borrelia burgdorferi*
 *American patients are more often diagnosed as seropositive, compared to European patients ²⁷

AIMS OF THE THESIS

The overall aim of this Ph.D. thesis was to evaluate molecular and serological tools for laboratory diagnosis of LB with a special focus on LNB, and to identify possible needs for improvement. The more specific aims were to:

I. Evaluate and compare the diagnostic sensitivities and specificities along with the concordance (both between and within assays) of several serological methods currently used in clinical laboratories in northern Europe, for diagnosis of LB and LNB.

II. Evaluate and optimize the workflow for PCR-analysis of CSF samples. This was carried out by systematic optimisation of the pre-analytical steps for molecular detection of *B. burgdorferi* s.l. by real-time PCR and by evaluation of different PCR protocols currently in use in Sweden, Norway and Denmark. The protocols were compared with respect to analytical sensitivity, specificity, and concordance.

MATERIALS AND METHODS

Materials

Subjects and clinical data

Case definitions (papers I and II)

Definite LNB (papers I and II): Patients classified according to the European Federation of Neurological Societies (EFNS) guidelines¹³⁸ with neurological symptoms of LNB (e.g. headache, neck pain and radiating pain, sensory and motor nerve dysfunction, vertigo and cranial nerve palsy) without other plausible reasons, pleocytosis in CSF (total cell count $\geq 5 \times 10^6 \text{ L}^{-1}$) and intrathecal anti-*Borrelia* antibody production (IgM and/or IgG).

Possible LNB (papers I and II): Patients with neurological symptoms of LNB according to the EFNS guidelines¹³⁸ without other plausible reasons and with pleocytosis in CSF (total cell count $\geq 5 \times 10^6 \text{ L}^{-1}$).

Suspected LB patients (paper I): Patients with possible disseminated LB, not classified as LNB, ACA, LA, EM, BL or LC. The patients had symptoms not explained by any other disease together with significantly elevated IgG antibody titer in serum. Intrathecal antibody production in CSF was noted for some cases, but no CSF pleocytosis or increased levels of CXCL13 ($<20 \text{ pg} \cdot \text{mL}^{-1}$) in CSF were reported. The patients were from an endemic area, but only some had observed a previous tick-bite. All patients responded well to antibiotic treatment (amoxicillin or doxycycline). Within the group of patients with suspected LB, some patients with previous LB were included. These patients presented with intrathecal production in CSF, but without signs of pleocytosis (total cell count $\geq 5 \times 10^6 \text{ L}^{-1}$) or raised CXCL13 in CSF ($<20 \text{ pg} \cdot \text{mL}^{-1}$). The patients had not been treated with antibiotic. Finally, the group of suspected LB patients also included some patients who were not classified, since they had not been lumbar punctured, therefore no CSF analysis was performed.

Other diseases (paper I): Patients with neurological symptoms who did not meet the criteria for definite LNB, possible LNB, ACA, LA, or EM but with a diagnosis of other CNS illness (e.g. tick-borne encephalitis and enterovirus meningitis) or no CNS illness.

Blood donors (papers I and II): Blood donors had stated no current signs or symptoms of disease and had also completed a health declaration.

ACA (paper I): Patients with clinical signs compatible with ACA, *Borrelia*-specific IgG antibodies in serum, and detected *Borrelia*-specific DNA in skin biopsy.

LA (paper I): Patients with clinical signs of arthritis, *Borrelia*-specific IgG antibodies in serum, and *Borrelia*-specific DNA detected in SF.

EM (paper I): Patients with a recent tick bite and characteristic skin rash >5 cm in diameter (assessed by a physician).

Pleocytosis in CSF for other reasons (paper II): Non-LNB patients diagnosed with other diseases (viral meningitis, multiple sclerosis, cerebral tumour, *Mycoplasma pneumoniae* encephalitis, endocarditis lenta, drug intoxication, Bell's palsy, migraine and sciatica, suspected autoimmune diseases and subarachnoid haemorrhage), had pleocytosis in CSF (total cell count $\geq 5 \times 10^6 \text{ L}^{-1}$) and serum anti-*Borrelia* antibodies (IgM and/or IgG). Not necessarily all these signs were shown in the same patient.

No CSF pleocytosis (paper II): Non-LNB patients with normal CSF count, no detectable anti-*Borrelia* antibodies in serum or CSF, and a normal CSF:serum albumin ratio.

Inclusion of study subjects (papers I-IV)

Paper I: Among the 396 samples included in the study, 59 were sera from clinically and laboratory well-characterised patients with LB manifestations of definite LNB, ACA, LA, or EM (named as LB patients), 24 were sera from patients with suspected LB and 112 were sera from patients who had sought medical care due to symptoms suspected to be associated with LB but with no objective evidence supporting the diagnosis (named as patients with other diseases). The patients in the suspected LB group did not fulfil the criteria for any of the LB groups and these patients were mainly referred to the specialist clinics for evaluation of suspected LB due to their seropositivity. However, these patients were not included in the statistical analysis,

since they were difficult to evaluate and classification was uncertain. Moreover, the study also included 201 sera from blood donors.

Serum, and in the cases of suspected LNB, CSF were collected from the patients as they were referred to the specialist clinics at the Department of Infectious Diseases, County Hospital Ryhov, Region Jönköping County, Sweden (2013-2017), the Department of Medicine, Åland Central Hospital and Bimelix Laboratory, Mariehamn, Åland, Finland (2014-2017), and at the Department of Neurology, Sørlandet Hospital, Kristiansand, Norway (2015-2017) for suspected LB manifestations. The samples were analysed in line with the local standard procedure at the respective laboratory at the recruiting hospital. Analysis involved CSF cell count, detection of *Borrelia* specific-antibodies, and calculation of AI. The serological assays used at the recruiting hospitals were; the IDEIA Lyme Neuroborreliosis test (Oxoid, Hampshire, UK) and Enzygnost *Borrelia* Lyme IgM/IgG (Siemens/DADE Behring, Marburg, Germany) in Sweden, Immunogenics® C6 LYME ELISA™kit, IgM/IgG (Immunetics, Inc., Boston, MA) and RecomWell *Borrelia* IgM/IgG (Mikrogen GmbH, Neuried, Germany) on the Åland Islands, and Enzygnost *Borrelia* Lyme IgM/IgG (Siemens/DADE Behring) in Norway. For manifestations of ACA and LA, confirmation was carried out by detection of *Borrelia*-specific DNA, while EM was diagnosed solely by the physician's clinical assessment. All samples were taken before antibiotic treatment.

Paper II: Of the 175 individuals included in the study, 52 were patients classified as definite LNB and four were classified as possible LNB according to the EFNS guidelines ¹³⁸. The non-LNB group contained 119 patients, of which 29 had CSF pleocytosis but no elevated anti-*Borrelia* AI and most of them received other specific diagnoses e.g. viral meningitis, multiple sclerosis, migraine, or sciatica. The remaining 90 patients had a normal CSF cell count and CSF:serum albumin ratio, with no detectable anti-*Borrelia* antibodies in CSF or serum. Finally, serum samples from 90 healthy blood donors were used to compare the serum results from the non-LNB group to determine the degree of seroprevalence in the population.

Papers III & IV: Anonymised CSF samples were collected at the Division of Clinical Chemistry, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden from patients without clinically suspected LNB, under investigation for other diseases not correlated to tick-borne infections. Samples with high erythrocyte levels ($>6 \times 10^6 \text{ L}^{-1}$) were excluded from the study to minimise the risk of PCR inhibition. The samples were pooled together creating a homogenous CSF volume. In paper IV, CSF samples without pleocytosis in CSF (mononuclear

cell count $>5 \times 10^6 \text{ L}^{-1}$) were used, while paper III included two CSF pools, one pool contained samples with pleocytosis and one had samples without pleocytosis.

Collection of clinical data, recruitment of patients, and inclusion of *Borrelia* species

Clinical samples from potential Lyme borreliosis and Lyme neuroborreliosis patients (papers I and II)

Paper I: The patients in paper I were prospectively included in the study and retrospectively classified into groups based on both laboratory results and by a review of their medical charts. The classification was made by specialist physicians in infectious disease, clinical microbiology, or neurology.

The patients were recruited at three specialist clinics (for more information see section “*Inclusion of study subjects (paper I)*”). The Norwegian patients were recruited by Åslaug R. Lorentzen at the Department of Neurology, Sørlandet Hospital Trust, Kristiansand, and the Norwegian National Advisory Unit on Tick-Borne Diseases, Arendal, Norway (2015-2017); the Swedish patients by Anna J. Henningsson at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden (2013-2017); and the patients from Åland by Marika Nordberg, Clara Nyberg and Linda Perander at Åland Central Hospital and Bimelix Laboratory, Mariehamn, Åland, Finland (2014-2017). The samples were classified by Randi Eikeland at the Department of Paediatric, Sørlandet Hospital Trust, Arendal, and the Norwegian National Advisory Unit on Tick-Borne Diseases, Arendal, Norway; by Anna J. Henningsson at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden; and by Dag Nyman, Marika Nordberg, Clara Nyberg, and Linda Perander at Åland Central Hospital and Bimelix Laboratory, Mariehamn, Åland, Finland. Ram Dessau, Department of Clinical Microbiology, Slagelse Hospital, Slagelse, Denmark was also involved in the classification. The samples from Norway were sent to the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden by Guro F. Jensen at the Department of Medical Microbiology, Sørlandet Hospital, Kristiansand, Norway, and the samples from Åland by the staff at Bimelix, Mariehamn, Åland. The healthy controls (blood donors) included in the study were recruited at the Division of Transfusion Medicine, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden (2016-2017). All blood

donors were included prospectively in the study and were all urged to answer health declaration regarding e.g. previous tick-bites or tick-related diseases.

Paper II: Serum and CSF samples were retrospectively selected from clinically well-characterised individuals previously investigated for suspected LNB in Jönköping County, Sweden between 2003 and 2007. The LNB patients were clinically evaluated with regards to symptoms, course of the disease, and response to therapy by review of medical records according to a standardised protocol and by laboratory tests. The samples were collected at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden. Sample inclusion and classification of the samples was carried out by Anna J. Henningsson. The samples from healthy blood donors were collected at the Division of Transfusion Medicine, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden (2016-2017). The blood donors were retrospectively included anonymously, regardless of gender and sex.

***Borrelia* species (papers III and IV)**

Paper III: The third paper was divided into two parts, one evaluation part and one part where the optimised pre-analytical steps from the first part were applied to two *B. burgdorferi* s.l. species and one *B. miyamotoi* species. In the evaluation part, *B. garinii* Lu 59 was used to spike i.e. apply a known amount of cells to each sample of pooled CSF. In the second part of the paper, the three human pathogenic species, *B. afzelii* Lu81, *B. burgdorferi* s.s. B31 and *B. miyamotoi* H31 were used to evaluate the analytical specificity. The *B. burgdorferi* s.l. strains were kindly provided by Sven Bergström, Umeå University, Umeå, Sweden and the *B. miyamotoi* strain was provided by Joppe Hovius, Center for Experimental and Molecular Medicine, Amsterdam Multidisciplinary Lyme Borreliosis Center, University Medical Center, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.

Paper IV: The fourth study was divided into three parts. In the first and second parts of the paper, *B. afzelii* Lu81, *B. garinii* Lu59, and *B. burgdorferi* s.s. B31 were used to spike water or CSF. The strains were kindly provided by Sven Bergström, Umeå University, Umeå, Sweden. In the third part, samples containing DNA extracted from 15 cultured *B. burgdorferi* s.l. strains (*B. burgdorferi* s.s. strains B31 and PBre, *B. afzelii* strains PKo and PVPM, *B. garinii* strains PBr, PHei, PWudII, PRef and PLa, *B. spielmanii* PSigII, *B. bavariensis* PBi, *B. bissettii* PGeb,

B. lusitaniae Poti B2, *B. valaisiana* VS116 and *Borrelia japonica*) and five specific controls including samples from *B. hermsii*, *B. miyamotoi*, *Treponema phagedenis*, and *Leptospira* were included. The samples in part three of the study were all a part of the INSTAND programme (Instand, Germany). The strains were kindly provided to us as DNA extracted from cultured *Borrelia* species by Volker Fingerle, German National Reference Centre for *Borrelia*, Bavarian Health and Food Safety Authority, Oberschleißheim, Germany.

Ethics

Paper I was approved by the Regional Ethical Review Board, Linköping, Sweden (M83-05, T91-08 and 2012/246-31) in accordance with the 1964 Helsinki declaration and its later amendments. Informed consent was obtained and all participants agreed to participate in the study. **Paper II** was approved by the Regional Ethical Review Board, Linköping, Sweden (2013/238-31, 2014/326-32, and 2016/211-31) and the permission to read the patient's medical records was given by the medical director at each participating clinic. In **Papers III and IV** no ethical approval was needed since the studies include strains from microorganisms and pooled CSF samples, which could not be traced back to a specific patient.

Methods

Serological methods

Paper I

Paper I included 12 laboratories situated in Sweden (n=6), Norway (n=4), Denmark (n=1), and on the Åland Island, Finland (n=1). The laboratories were included in the study mainly based on their nationality and their willingness to participate.

The serological analyses were performed at each participating laboratory according to their routine procedure and the samples were analysed as blinded. Five different ELISA assays were included; 1) Liaison Borrelia IgM/Liaison Borrelia IgG (DiaSorin, Saluggia, VC, Italy) (n=5), 2) Enzygnost Borrelia Lyme IgM/Enzygnost Borrelia Lyme link VlsE/IgG (Siemens/DADE Behring) (n=3), 3) Anti-Borrelia ELISA (IgM)/Anti-Borrelia plus VlsE ELISA (IgG) (EUROIMMUN AG, Lübeck, Germany) (n=1), 4) RecomWell Borrelia IgM/RecomWell Borrelia IgG (Mikrogen GmbH) (n=1), and 5) Immunogenics® C6 LYME ELISA™ kit (Immunetics, Inc.) (n=2). The results together with the method description including assay, manufacturer, cut-off values/reference intervals and units for measurement were sent to the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden, for compilation. Samples detected as borderlines were regarded as positive in the statistical analysis. The laboratory participants were asked to establish the qualitative results (positive, borderline, or negative) as well as to report the quantitative values as numerical data for each sample.

Paper II

In paper II three serological assays for LNB diagnostics were compared. Two of the assays were third generation assays and one was a second generation assay. The assays in the study were selected based on the question regarding increase in diagnostic sensitivity and specificity for the detection of LNB by use of newly developed third generation assays including several recombinant antigens.

The initial serological detection of antibodies in serum and CSF samples in paper II was performed with the Lyme Borreliosis ELISA kit 2nd Generation assay (Dako Cytomation A/S,

Glostrup, Denmark). The assay is based on purified native flagellum from *B. burgdorferi* and was used prior to the study period as a routine method at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden. The *Borrelia*-specific AI was calculated according to Peter (1990) ²⁵³ using the formula $[(\text{Borrelia-specific IgG in CSF (OD)}/\text{Borrelia-specific IgG in serum (OD)})/(\text{Rubella-specific IgG in CSF (OD)}/\text{Rubella-specific IgG in serum (OD)})]$.

In the comparison, three different assays were used; 1) IDEIA Lyme Neuroborreliosis (Oxoid) an enzyme immunoassay (EIA) based on purified native flagellum from *B. afzelii* strain DK1, 2) VIDAS Lyme IgG (bioMérieux, Marcy, l'Étoile, France), a random access enzyme-linked fluorescent assay based on the recombinant *Borrelia* proteins VlsE, decorin-binding protein A and OspC and, 3) recomBead *Borrelia* IgM and IgG assay (Mikrogen GmbH), a multiplex bead assay using recombinant *Borrelia* antigens (OspA from *B. afzelii*, p100 from *B. afzelii*, VlsE fusion protein representing different species, OspC from *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*, p39 from *B. afzelii*, p58 from *B. garinii*, p18/decorin-binding protein A from *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. bavariensis*, and *B. spielmanii*). For the IDEIA assay, no correction of passive transudation of serum antibodies over the blood-brain barrier was needed compared to the VIDAS and recomBead assays. The VIDAS assay only measured anti-*Borrelia* antibodies of IgG class in CSF specimens and *Borrelia*-specific AI was calculated for all *Borrelia* antigens as a group in relation to [CSF:serum albumin ratio] or [CSF:serum total IgG ratio] ²⁵⁴. The measurement for the VIDAS assay was performed using the VIDAS automated immunoanalyser, while the recomBead assay was performed by using Luminex xMAP technology, in this study with a Bio-Plex 200 System (BioRad Laboratories, Inc., Hercules, CA). For the recomBead assay the *Borrelia*-specific intrathecal AI for each *Borrelia* antigen was calculated according to Reiber and Peter (2000) ²⁵⁵ $[(\text{IgG}_{\text{spec}} (\text{CSF})/\text{IgG}_{\text{spec}} (\text{serum}))]/[(\text{IgG}_{\text{total}} (\text{CSF})/\text{IgG}_{\text{total}} (\text{serum}))]$. Cut-off levels and interpretation criteria were applied according to the manufacturer for each test, and equivocal results were considered as positive. Albumin, total IgM, and total IgG used in AI calculation for the recomBead *Borrelia* IgM and IgG assay were measured in serum and CSF with the Immage 800 instrument, using rate nephelometry (Beckman Coulter, Fullerton, CA). All serological analyses in paper II were performed at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden.

Molecular methods

Culture methods and conditions for the *Borrelia* strains (papers III-IV)

Paper III: A total of 1 mL of the relapsing fever strain *B. miyamotoi* H31 was cultured in MKP medium with Fetal Calf Serum (pH 7.5) at 37°C for five days at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden. The strain was kindly provided to us by Joppe Hovius, Center for Experimental and Molecular Medicine, Amsterdam Multidisciplinary Lyme Borreliosis Center, University Medical Center, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands. The spirochetes were counted and checked for viability in a phase-contrast microscopy and a 10-fold dilution series ranging from 2000 to 0.2 cells μL^{-1} was prepared.

Papers III and IV: 1 mL of each clinical *Borrelia* strain; *B. afzelii* Lu81, *B. garinii* Lu59, and *B. burgdorferi* s.s. B31 were cultured in 14 mL BSK II medium supplemented with 6 % rabbit serum (Sigma Aldrich, St. Louis, Missouri, US) at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden. The *B. afzelii* Lu81 strain was cultured at 35 °C for eight days, the *B. garinii* Lu59 strain at 37 °C for seven days, and the *B. burgdorferi* s.s. B31 strain at 35 °C for six days. The three strains were kindly provided by Sven Bergström, Umeå University, Umeå, Sweden. The spirochetes were counted and checked for viability in a phase-contrast microscopy and a 10-fold dilution series ranging from 2000 to 0.2 cells μL^{-1} was prepared.

Paper IV: The strains of *B. lusitaniae* Poti B2, *B. valaisiana* VS116, and *B. japonica* were all derived from ticks, while the other *B. burgdorferi* s.l. strains were from clinical samples. All strains of *B. burgdorferi* s.l. together with the strain of *B. hermsii* were cultured in MKP medium at 33 °C and harvested at a density of 10^7 cells mL^{-1} . The strains of *Leptospira* were cultured in Ellinghausen-McCollough-Johnson-Harris (EMJH) medium (Leptospira Medium Base EMJH BD, Difco™ and Leptospira Enrichment EMJH Difco™, New Jersey, USA) at 28 °C, while the *Treponema phagedenis* was cultured in Fluid Thioglycollate Medium (bioMérieux) with 10 % rabbit serum (C. C. Pro GmbH, Germany) at 37 °C. Both strains were harvested at a density of 10^7 cells mL^{-1} . Finally, the *B. miyamotoi* strain was cultured in a 6 % CO_2 atmosphere in MKP medium enriched with 50 % human serum. The strains were kindly

provided to us as DNA by Volker Fingerle, German National Reference Centre for *Borrelia*, Bavarian Health and Food Safety Authority, Oberschleißheim, Germany.

Paper III

Paper III was divided into two parts: 1) optimisation of the pre-analytical steps before PCR analysis and, 2) implementation of the new optimised pre-analytical steps on three human pathogenic species. The different parameters e.g. centrifugation time and speed, sample volumes, extraction methods, were selected based on a literature search and compilation of previously used protocols of pre-analysis before PCR-analysis.

In the first part, different parameters were investigated regarding their influence on the analytical sensitivity for detection of *B. burgdorferi* s.l. in CSF. The parameters that were investigated were: 1) extract from precipitate versus supernatant, 2) centrifugation time and speed, 3) extraction of DNA versus extraction of total nucleic (NA), 4) sample volume, 5) storage and transportation conditions, 6) the influence of blood in CSF due to needle stick injury and, 7) the influence of pleocytosis in CSF. The optimised pre-analytical steps were further evaluated for reproducibility, both between two separate real-time PCR-analysis and within one real-time PCR-analysis where one sample was analysed in multiple wells. For DNA extraction, the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) was used and for extraction of total NA with no DNase treatment the EZ1 RNA Tissue Mini Kit (Qiagen) was used. Both extractions were carried out on an EZ1 advanced XL instrument (Qiagen). The reverse-transcribed NA synthesis was performed by use of the Illustra™ Ready-to-Go RT-PCR bead kit (GE Healthcare Life Science, Chicago, IL). In the part where DNA extraction was compared to extraction of total NA, the entire amount of total NA (three complementary deoxyribonucleic acid (cDNA) syntheses pooled into one sample) was used for the reverse-transcription of NA. This means that 100 % of the initial amount (assuming that 100 % NA was extracted) of the spirochetes per sample was detected. In the entire optimization cultured *Borrelia* spirochetes of the strain *B. garinii* Lu59 were used to spike CSF samples in a concentration range of 10^4 - 10^0 cells per sample extraction.

In the second part of the study, the determined recommendations were evaluated on three human pathogenic species namely *B. afzelii* Lu81, *B. burgdorferi* s.s. B31 and *B. miyamotoi* H31.

Paper IV

The study in paper IV was divided into three parts, to compare the analytical sensitivity and specificity: 1) between different target genes when cDNA was used as the template, 2) between different extracted materials (cDNA and DNA) and, 3) between different PCR protocols when DNA was used as the template.

The first part of the study included cDNA samples (n=20) extracted from water spiked with *Borrelia* spirochetes in the dilution range of 10^4 - 10^0 cells mL⁻¹ for each species (*B. afzelii* Lu81, *B. garinii* Lu59 and *B. burgdorferi* s.s. B31). The study also included five negative controls containing *Escherichia coli* J1 or RNase free water (GE Healthcare Life Science). The extraction was carried out using the MagAttract® RNA Tissue Mini M48 kit (Qiagen) and BioRobot M48 Workstation (Qiagen) according to the manufacturer's instructions. Reverse-transcribed NA synthesis was performed by using the Illustra™ Ready-to-Go RT-PCR beads kit (GE Healthcare Life Science). In this first part, the laboratory participants were asked to analyse the samples with their own PCR protocol/protocols for detection of *B. burgdorferi* s.l.

In the second part pooled CSF samples, obtained from patients without CSF pleocytosis and clinically suspected LNB, were used. The pooled CSF samples were spiked with known concentrations of *B. afzelii* Lu81, *B. garinii* Lu59, and *B. burgdorferi* s.s. B31 in the dilution range of 10^4 - 10^0 cells mL⁻¹ for each species. In this part of the study, the laboratory participants were asked to extract the samples and analyse them with the protocol/protocols used at each laboratory to detect *B. burgdorferi* s.l.

Finally, the third part, contained DNA samples of known concentration in the dilution range of 10^4 - 10^{-1} cells in 5 µL. The DNA was extracted from 15 cultured *B. burgdorferi* s.l. strains and five specific controls. The extraction was carried out using the Maxwell® 16 Instrument (Promega, Madison, WI) according to the manufacturer's recommendations. The DNA concentration was measured with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Massachusetts, USA). The samples in part three of the study were all a part of the INSTAND programme (Instand, Germany) and the laboratory participants were asked to analyse the samples with their own PCR protocol/protocols for detection of *B. burgdorferi* s.l.

The results, along with the method descriptions, were reported to the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden for compilation.

Statistics (papers I-IV)

Paper I: For statistical analysis and graphics, the statistical software R was used ²⁵⁶. In the quantitative comparison, the results are based on the numerical values of each sample from each participating laboratory, while the qualitative comparison is based on the interpretation of results established by the participating laboratories (positive, borderline, or negative). By use of receiver operating characteristic (ROC) curves (used for both quantitative and qualitative comparison), the performance of the diagnostic assays and the connection between the diagnostic sensitivity and specificity was shown graphically for LB patients, blood donors and patients with other diseases. For estimation of ROC curves, analyses were performed using package pROC and mada, a tool implementing the “Reitsma” method for meta-analysis of bivariate diagnostic accuracy. For bivariate comparison (used for quantitative analysis) of diagnostic sensitivity and specificity, the summary ROC approach was applied. The confidence region of 95 % was used and results outside were considered as statistically significant. For statistical comparison of the area under the curve, the command roc-test with the default “Delong” algorithm was used. By using area under the curve the ability of a parameter to distinguish between two diagnostic groups was shown.

Paper II: For the statistical analysis, SPSS version 15.0 (SPSS Inc., Chicago, IL), was used. Pairwise comparison between the serological assays was carried out by using McNemar’s test with Yate’s correction. Two-tailed p-values <0.05 were considered as significant. McNemar’s test is used to compare the diagnostic sensitivity and specificity between two serological assays analysed on the same group of patients. Yate’s correction is used to prevent overestimation of statistical significance in small data sets.

Paper III: Most of the results were based on descriptive statistics since the number of samples in each panel was low and calculation could have given misleading statistical results. For the continuous variables, the paired T-test was used to assess the significance of the differences between mean Cq values. All statistical analyses were evaluated at a significance level of 0.05, and the 95 % binomial confidence interval was used to estimate how far away the sample mean was from the actual population mean. The analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com).

Paper IV: Most of the results (mainly the analytical sensitivity) were based on descriptive statistics, since the number of samples in each panel was low and calculation could have led to

misleading statistical results. The analytical specificity was calculated to establish how well the methods could detect the pathogens. For calculation of the 95 % binominal confidence interval, to estimate how far away the sample mean was from the actual population mean, the R-software ²⁵⁶ was used by applying the command `binom.confint` in package `binom` with the Wilson method. The Wilson method was used since it is recommended when confidence intervals are close to the limit of zero or one.

RESULTS AND DISCUSSION

Evaluation of serological methods for diagnosis of Lyme borreliosis in clinical samples

Currently, there is a lack of standardized recommendations for detection of *Borrelia*-specific antibodies in serum and CSF. Evaluation of assays is important to establish diagnostic sensitivity, specificity, and concordance between different assays. In this thesis different serological assays have been compared for diagnosis of serum alone (used for all manifestations of LB) and for serum and CSF together (used for LNB patients).

Detection of *Borrelia*-specific antibodies in serum (paper I)

Different assays, used for detection of antibodies in serum in patients with suspected LB were evaluated by use of a large serum panel (n=396). The diagnostic sensitivity, specificity, and concordance among five different serological assays used at 12 clinical laboratories in northern Europe, including Sweden, Norway, Denmark, and the Åland islands, were evaluated.

Is there a difference in diagnostic sensitivity, specificity, and concordance within and between different serological assays for detection of IgM and IgG antibodies in serum?

Five different assays, where three were used at more than one laboratory, were compared. The diagnostic sensitivity, specificity, and concordance for IgM and IgG were compared both within assays and between assays.

The average diagnostic sensitivity for IgG (qualitative comparison) was 88 % (range 85–91 %), both between and within assays (Figure 16), while the IgM results presented lower diagnostic sensitivity, 59 % (ranging 50–67 %), with more heterogeneous results both between and within assays (Figure 16).

Positive IgM and IgG results

Figure 16 Numbers of positive samples (IgM and IgG) for the entire study population as well as for the three groups, LB patients, patients with other diseases, and blood donors, separately. For laboratories 11 and 12, the result is an overall result since IgM and IgG are detected in the same assay for Immunogenic® C6 Lyme ELISA™ assay.

The diagnostic specificity between IgM and IgG also differed, where IgM presented a lower diagnostic specificity. This was not unexpected since it is known that IgM antibodies are less specific than IgG antibodies, which may result in false-positive IgM reactions due to cross-reactivity to other pathogens closely related to *Borrelia*, but also to other genera or diseases ¹⁸. The comparison between assays showed that the Anti-Borrelia plus VlsE IgG (EuroImmun AG) assay (based on whole-cell lysate) had a slightly lower diagnostic specificity; however, with no major advantage in diagnostic sensitivity compared to the other assays. These results support a previous study showing that the diagnostic sensitivity and specificity are higher in assays based on recombinant antigens compared to whole-cell lysates ²⁵⁷. A previous study by Marangoni (2008) ²⁵⁸, which is in line with the result in this study, indicates that IgG assays including recombinant VlsE antigens from all three human pathogenic *B. burgdorferi* species [used in the Enzygnost Borrelia Lyme link VlsE/IgG assay (Siemens/DADE Behring)] yield improved diagnostic specificity with persistent diagnostic sensitivity.

Detection of IgM antibodies in serum– when and why?

The results generated for the IgM assays showed heterogeneous results with low diagnostic specificity and no obvious advantage in diagnostic sensitivity. This is a known problem and the diagnostic value of IgM in serum has been questioned, especially in late stages of LB ^{223, 259}.

Enzygnost Borrelia Lyme IgM (Siemens/DADE Behring) generated the highest diagnostic sensitivity and specificity among the five assays included in the study. This in fact was shown in both control groups (blood donors and patients with other diseases). These results are also presented in a previous study by Marangoni (2008) ²⁵⁸ comparing Enzygnost Borrelia Lyme IgM (Siemens/DADE Behring) and Liaison Borrelia IgM (Diasorin).

IgM antibodies are developed early in the infection, before IgG antibodies, and in some cases, the measurable IgM antibody response fades away in the foreseeable future. However, IgM antibodies can persist for years generating positive results not correlated to a recently developed *Borrelia* infection or a persisting *Borrelia* infection ²²⁷. When using IgM for diagnosis of LB it is important to be aware that IgM is relevant to analyse only in early infections and that the clinical relevance is low in late LB. In patients with long-lasting disease, i.e. late LNB, ACA, and LA, IgM assays are not optimal to use to determine an ongoing infection. Actually, an IgM response in suspected late stages of LB e.g. ACA and LA, probably

indicates for persisting antibodies rather than an ongoing infection. However, IgM may be of clinical use in young children who have not been highly exposed to ticks, and in disseminated LB with symptom duration < 6-8 weeks ²²³

Detection of IgM, especially in serum, may also lead to false-positive reactions, which may lead to misdiagnosis and over-diagnosis of LB followed by unnecessary antibiotic treatment and worried patients ²⁶⁰. In particular, unspecific IgM reactivities constitute a serious problem in cases with unspecific and vague symptoms and low clinical probability of LB. In an unpublished study ²⁶¹, patients sampled for *Borrelia*-specific antibodies in serum in Jönköping county during 2017 were evaluated with focus on the number of cases where borrelia serology for IgM was ordered based on the right indications. The results showed that many patients were not tested according to current European recommendations and that positive IgM results in serum have limited clinical value ^{124, 138}.

By use of a highly sensitive IgG assay including VlsE or C6 peptides as antigens, which can often be detected as early as IgM antibodies, the IgM assays may be excluded since they have no additional value and may reduce the diagnostic specificity in early infections. However, this depends on the antigen mix in the assay ^{146, 262}. Our results support the view that detection of IgM in serum in adult patients gives no or very limited added diagnostic information. However, detection of IgM in CSF in LNB may have a diagnostic value.

What is an optimal group to use as negative controls and which one should we use (paper I)?

Different studies include different sorts of control groups. A control group can consist of healthy volunteers, blood donors, or patients with other diseases. This thesis included two control groups, one containing patients with other diseases and one containing serum from healthy blood donors.

By use of blood donors, higher diagnostic specificity was shown compared to the use of patients with other diseases. This was probably due to the fact that patients with other diseases included patients referred to specialised centres for lumbar puncture due to symptoms that could be attributed to a tick-borne infection and concomitant seropositivity. However, the seropositivity was not correlated to an active infection or disease. The presence of antibodies, especially IgG, was also high in the group with blood donors (average 22 %) probably mirroring the high degree of *Borrelia* exposure in this tick-endemic area. The knowledge of the seroprevalence in

the population of interest is something to take into consideration in the study design and in the choice of control group. According to Leeflang et al. (2016) ²³⁹ diagnostic accuracy studies should preferably be carried out in settings where the test will be used in practice. This indicates that the most preferable study design is based on planned cross-sectional studies with samples prospectively collected at the health care centre where the patients first sought care for suspected LB, in order to avoid selection bias due to seropositivity. However, this may be hard for practical reasons, since this would involve a large number of clinics, and would necessitate the inclusion of a very large number of patients as only a minor proportion of the patients will actually have an ongoing *Borrelia* infection. So for practical reasons, case-control studies seem to be the most realistic way forward.

Detection of *Borrelia*-specific antibodies in serum and cerebrospinal fluid (paper II)

Among the different manifestations of LB, LNB is considered the most important one to diagnose at an early stage. It is also the second most common LB manifestation in Europe. Therefore, we wanted to focus on evaluation and comparison of different assays used for the detection of intrathecal production of *Borrelia*-specific antibodies. The issues we aimed to investigate were; 1) is there a difference in diagnostic sensitivity and specificity between a second generation ELISA assay, currently in use at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden, and two third generation ELISA assays combining several recombinant antigens for laboratory diagnosis of LNB in serum and CSF?, 2) is there a difference between the two third generation ELISA assays and what is the value of pairwise diagnosis of serum and CSF followed by calculation of AI? and, 3) is there a difference in diagnostic value in detection of the intrathecal IgM antibodies index compared to detection of IgM in serum alone in patients with suspected LNB?

When comparing new tests to previous ones it is important to have both clinically and laboratory well-characterised patient material, since the classification of the patients is partly based on the current assay used at the laboratory. Classification based solely on previous serological results can lead to bias and problems in the interpretation of the test results.

Is the diagnostic sensitivity and specificity improved in pairwise diagnosis of serum and cerebrospinal fluid by use of third generation antibody assays?

The results showed no significant difference in overall diagnostic sensitivity and specificity (IgM and IgG taken together) between the three assays when analysing CSF and serum simultaneously followed by calculation of AI (Figure 17).

Figure 17 Number of positive and negative results for the four groups detecting *Borrelia* IgM and IgG antibodies in both serum and cerebrospinal fluid. LNB = Lyme neuroborreliosis, IDEIA = IDEIA Lyme neuroborreliosis, VIDAS = VIDAS Lyme IgG, recomBead = recomBead *Borrelia* IgM/IgG

The highest diagnostic sensitivity was shown for the recomBead *Borrelia* IgM/IgG assay (100 %), while the diagnostic specificity in all three assays was comparable ranging from 97 to 99 %. However, since the IDEIA Lyme Neuroborreliosis assay uses the same antigen as the Lyme Borreliosis ELISA kit 2nd generation assay, which was used to select the patient groups, the diagnostic specificity for the IDEIA Lyme Neuroborreliosis assay cannot be reliably assessed.

In a comparison (IgM and IgG taken together for the IDEIA Lyme neuroborreliosis and recomBead *Borrelia* IgM/IgG assays) between patients included in the definite LNB group, the IDEIA Lyme Neuroborreliosis assay (n = 48, diagnostic sensitivity= 88 %) and the VIDAS Lyme IgG assay (n = 45, diagnostic sensitivity = 86 %) presented fewer samples with positive results compared to the recomBead *Borrelia* IgM/IgG assay (n = 52, diagnostic sensitivity = 100 %). This differences is statistically significant [IDEIA Lyme Neuroborreliosis assay versus

recomBead Borrelia IgM/IgG assay ($p=0.016$) and recomBead Borrelia IgM/IgG assay versus VIDAS Lyme IgG assay ($p=0.008$)]. However, there was no significant difference between the IDEIA Lyme Neuroborrelios assays and the VIDAS Lyme IgG assay ($p=1.0$). The recomBead Borrelia IgM/IgG assay was the only assay detecting *Borrelia*-specific antibodies in all samples in the LNB group (including possible LNB) as positive ($n=56$, diagnostic sensitivity = 100 %) (Figure 17). This is an advantage for the recomBead Borrelia IgM/IgG assay since the patients in the possible LNB group are the most challenging ones. In a study by Skogman et al. (2017)²⁶³ evaluating the recomBead Borrelia IgM/IgG assay in children, the overall diagnostic sensitivity (IgM and IgG taken together) was lower (74 %) than the results obtained in paper II, while the diagnostic sensitivity (97 %) was in line with the results from the present study. However, the study by Skogman et al.²⁶³ showed an advantage for the recomBead Borrelia IgM/IgG assay in the group with possible LNB compared to the IDEIA Lyme neuroborreliosis assay, which is in line with this study. A meta-analysis by Leeflang et al. (2016)²³⁹ showed that assays with recombinant antigens have an advantage in both diagnostic sensitivity and specificity for detection of LNB.

All three assays presented some positive AI results in the patient group classified as non-LNB, especially in patients with pleocytosis in CSF for other reasons than LNB. This may be a result of unspecific antibody reactivity, but could also be a result of corrected AI, since the classification of the patients was established on the older second generation Lyme Borreliosis ELISA assay (Dako Cytomation A/S). Potential negative results from the old assays may have influenced the clinical diagnosis, resulting in the classification of the patients in the group with other diseases. However, none of the patients showed positive AI in more than one of the assays.

Is there a difference in diagnostic sensitivity and specificity between the two third generation assays and is there a difference between pairwise detection of serum and cerebrospinal fluid compared to the detection of serum samples alone?

Comparison (IgM and IgG were taken together) of analyses of serum alone for the VIDAS Lyme IgM/IgG assay and the recomBead Borrelia IgM/IgG assay showed comparable numbers of positive results ($n = 48$ respective $n = 45$) for the patients in the LNB group (Figure 18).

Figure 18 Number of positive and negative results for the four groups detecting IgM and/or IgG antibodies in serum. LNB = Lyme neuroborreliosis, IDEIA = IDEIA Lyme neuroborreliosis, VIDAS = VIDAS Lyme IgG, RecomBead = recomBead Borrelia IgM/IgG

The negative results for the patients in the non-LNB group were also comparable (n=81 respective n=79). However, the diagnostic sensitivity was higher for the VIDAS Lyme IgM/IgG assay (93 %) compared to the recomBead Borrelia IgM/IgG assay (86 %), while the diagnostic specificity was comparable, 86 % versus 85 % respectively. The diagnostic sensitivity for detection of serum alone was lower compared to simultaneous analyses of CSF and serum followed by calculation of AI. These results underline the importance of paired testing of serum and CSF in LNB diagnosis.

The seropositivity (S-IgM and S-IgG taken together) in the 90 healthy blood donors was 11 % for both the recomBead Borrelia IgM/IgG assay and the VIDAS Lyme IgM/IgG assay, while the seropositivity in the non-LNB group was 14 % for the VIDAS Lyme IgM/IgG test (S-IgM and/or S-IgG) and 15 % for the recomBead Borrelia IgM/IgG test (S-IgM and/or S-IgG). These numbers are in line with other studies including blood donors in the same geographical area ²⁶⁴.

Is there a difference in diagnostic value between detection of IgM in serum in Lyme neuroborreliosis patients and detection of intrathecal IgM antibody index in Lyme neuroborreliosis patients?

As mentioned in the section “Detection of IgM antibodies in serum– when and why?” the diagnostic specificity for IgM in serum is low and the diagnostic value is questioned. Unlike

other LB patients, the laboratory diagnosis of LNB patients is based on the detection of antibodies in serum and CSF simultaneously, followed by calculation of an intrathecal *Borrelia*-specific AI. This is done to make sure that the antibodies detected in CSF are produced intrathecally and not as a result of a damaged blood-brain-barrier with leakage of serum antibodies over to CSF ²⁵⁵.

According to the EFNS, LNB is diagnosed based on neurological symptoms, detection of pleocytosis ($>6 \times 10^6 \text{ mL}^{-1}$) in CSF in combination with an elevated intrathecal *Borrelia*-specific AI (CSF/serum) ¹³⁸. The determination of AI is more specific than the detection of antibodies in serum or CSF alone. Unlike IgM in serum, the diagnostic specificity in IgM AI is almost comparable to IgG AI in patients with LNB. The difference in patients presenting IgM AI, IgG AI or both, is probably a result of biological variance in the immune response, the duration of the neurological symptoms, or the use of different assays and the antigens included. The diagnostic sensitivity for IgM based on an intrathecal *Borrelia*-specific AI is higher than for IgM in serum alone. This means that the diagnostic value of evaluating IgM AI in LNB patients with early infection is higher than for IgM evaluation in serum samples ^{255, 263, 265}. However, the diagnostic sensitivity and specificity of the LNB assays are, as well as the serum assays, dependent on the antigen composition in the assay, which is discussed in the section “*Detection of IgM antibodies in serum– when and why?*”. To determine an active LNB, additional signs of CSF pleocytosis, which is a sign of CSF inflammation, need to be presented. Detection of CSF pleocytosis is therefore a suitable complementary diagnostic tool for diagnosis of LNB ²²³. Still, shortly after the onset of the disease intrathecal antibodies in CSF may not be detectable and CSF pleocytosis may be absent ^{7, 146}. Under these circumstances, CXCL13, which also is a marker for inflammation, may be of value in the assessment of certain cases ²⁴⁸.

Interpretation of serological results in current everyday practice for diagnosis of Lyme neuroborreliosis

Conclusions drawn from studies included in this thesis have partly changed the algorithm for diagnosis of LNB at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden (Figure 19). Currently, samples with negative results in the IDEIA Lyme Neuroborreliosis assay, but with pleocytosis in CSF are further analysed with the recomBead Borrelia assay. The algorithm is also complemented with the analysis of CXCL13, to further improve the diagnostic sensitivity.

Figure 19 The algorithm used for diagnosis of Lyme neuroborreliosis at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden. CSF = cerebrospinal fluid, LNB = Lyme neuroborreliosis

Evaluation and optimisation of the molecular workflow for detection of *Borrelia burgdorferi* sensu lato in cerebrospinal fluid by real-time polymerase chain reaction (papers III and IV)

Molecular methods are not used as primary diagnostic tools in everyday practice due to their low diagnostic sensitivity in clinical samples e.g. CSF and blood. However, the method can be used as a complement in certain cases of atypical EM and in SF samples from suspected LA, since the diagnostic sensitivity is higher in these sample materials. PCR may also serve as a supplement to serological analysis for certain conditions, as in early LNB. To date, there is a lack of standardised protocols for both pre-analytical handling of samples before molecular testing and PCR protocols where different target genes and primers/probes are used in different studies. This limits the usage of the PCR in everyday routine practice. To make sure that the real-time PCR protocol used in the third paper in this thesis was stable and comparable to other protocols, the comparison study between different PCR-protocols in Scandinavia (paper IV) was carried out before optimization of the pre-analytical steps (paper III). However, in this thesis, the results are presented according to the diagnostic workflow with pre-analysis followed by PCR-analysis.

What are the optimal conditions for transportation of samples and how should the samples be stored on arrival at the laboratory (paper III)?

In order for laboratories to receive samples that are in good condition, proper transportation of the samples and correct storage on arrival at the laboratories are essential to reach high diagnostic sensitivity.

In a minor study (performed before study IV), the viability of *Borrelia* spirochetes in BSK II medium stored at room temperature (RT) and the possibility of re-cultivate the spirochetes after one to seven days, was examined. The results showed that the spirochetes were viable up to seven days at RT and that re-cultivation was possible. This information is important in regard to the transportation of samples before cultivation, and not that important when it comes to detection of *Borrelia*-specific DNA since PCR can detect non-viable spirochetes. However, for some conditions both PCR and cultivation are carried out on the same sample, and therefore it is important to transport the samples at optimal conditions for culture.

The results obtained in paper III regarding storage and transportation showed that the analytical sensitivity decreased over time for storage at both RT (0-7 days) and +2-8 °C (0-30 days). However, the decrease between one and three days was less than 1.2 PCR cycles, indicating that transportation may be carried out at RT or +2-8 °C if the samples are placed in the refrigerator upon arrival. According to this study, for short-term storage (0-7 days), the samples should be stored at +2-8 °C or -20 °C. For long-term storage (0-30 days), a temperature of -20 °C yields the lowest Cq values with values stable over time. The highest Cq values for both short and long-term storage were shown for storage at -80 °C. This is surprising, since many papers but also manufacturers of commercial kits recommend storage at -80 °C. However, these recommendations may be based on the knowledge that the Cq values remain stable over time for storage in low-temperature freezers, which is desirable for long-term storage. Still, the results show that storage at -20 °C also fulfils these requirements, at least up to 30 days.

What can we do to optimise the pre-analytical handling of cerebrospinal fluid samples before molecular analysis (paper III)?

Seven different experimental setups (in this thesis known as setups) were used to evaluate the different parameters, which may influence the sensitivity for detection of *B. burgdorferi* s.l. species in CSF. The results showed that three of the different parameters had a greater influence on the analytical sensitivity compared to the others namely; 1) concentration (by centrifugation) and use of the precipitate for extraction, 2) extraction of total NA followed by reverse-transcribed NA synthesised to cDNA and, 3) use of samples without the inclusion of erythrocytes.

The use of precipitate (obtained by centrifugation) before further extraction generated a 100 times higher analytical sensitivity based on the evaluation of Cq values, with cycles values about eight cycles lower for the extracts from the precipitate. The extracts from precipitates showed detection down to 100 cells per sample extraction compared to 1000 cells per sample extraction for the extracts from the supernatant. In previous studies different centrifugation times and speeds have been used ^{211, 266-268}. In two of these studies, high speed and long centrifugation time have been used to increase the diagnostic sensitivity. However, the results in this study show that a more moderate time and speed (3 000 * g for 10 min) resulted in comparable results regarding analytical sensitivity, which may be more favorable in everyday

clinical practice since it decreases the turn-around time as well as spare the fragile spirochetes in the samples.

By extraction of total NA followed by reverse-transcribed NA synthesised to cDNA the analytical sensitivity was increased by three to ten times (one to three cycles). The extraction methods and the advantage of extraction of total NA versus DNA is further presented in section “*What is important to think about regarding the choice of target gene in correlation to extraction method and issue (paper IV)?*”.

Another important issue to take into consideration is the influence of possible inhibitors in different samples. Different components in blood e.g. haemoglobin and lactoferrin, components in erythrocytes, and leukocytes, are known to have an inhibiting effect on the PCR outcome ²¹⁹. The results in this study showed higher Cq values for the samples containing erythrocytes compared to the ones that did not. The samples with erythrocytes detected down to 100 cells per sample extraction, while the samples without erythrocytes detected down to 10 cells per sample extraction. Needlestick injuries may theoretically cause leakage of erythrocytes over to the CSF during a lumbar puncture, a problem that both physician and laboratory staff need to take into consideration when analysing and reporting results since inhibition can lead to false-negative results. However, it would be interesting to determine at which erythrocyte concentration this decrease in analytical sensitivity appears, to find out potential false-negative results.

The optimisation of the pre-analytical steps was finally applied to different *Borrelia* species, to evaluate the outcome of the optimisation. All species were detected down to 10 cells per sample extraction. Figure 20 demonstrates a flowchart for the optimised pre-analytical steps. Even though the analytical sensitivity is increased by use of the new optimised pre-analytical steps, further evaluation of patient samples needs to be carried out to determine if it is relevant to change the workflow for PCR-analysis. This would preferably be performed on fresh samples that have not been stored at RT, in a freezer, or a refrigerator (-20°C) for more than three days from sampling, since the analytical sensitivity decreases with time.

Figure 20 A flowchart for the optimised pre-analytical steps for detection of *Borrelia* specimens in cerebrospinal fluid. cDNA = complementary deoxyribonucleic acid [Figure created with Biorender.com. Picture of the van is from <https://www.vecteezy.com/members/GraphicsRF>]

What is important to think about regarding the choice of target gene in correlation to extraction method and issue (paper IV)?

Another important pre-analytical step before real-time PCR is the extraction method. The most frequently used extraction methods in published studies are the extraction of DNA ^{211, 266-268}. Currently, few studies are based on samples extracted for total NA and to our knowledge no systematic comparison with spiked CSF samples has been carried out regarding analytical sensitivity in DNA compared to analytical sensitivity in total NA. The results in this study showed differences in Cq values between the two extraction kits, in favour of the extraction of total NA. Extraction of total NA followed by synthesis to cDNA generated a three to ten times (one to three PCR cycles) higher analytical sensitivity.

The extraction of total NA followed by synthesis to cDNA may theoretically result in higher analytical sensitivity, since the samples extracted for total NA were composed of both RNA and DNA. A study by Ornstein and Barbour (2006) ²⁶⁹ showed that there is (theoretically) a 100-fold higher number of copies per cell for rRNA. However, the number of rRNA copies in a cell depends on the replication status of the cells ²⁶⁹. However, results obtained by Lager et al. (2017) ¹⁸⁹ showed that an increase in analytical sensitivity is correlated to the use of different target genes. For example, for increased analytical sensitivity in samples extracted for total NA the primers and probes need to be aimed to the target gene *16S* rRNA. By use of, for example, *ospA* and *flaB*, the increase in sensitivity is not observed. rRNA molecules, but also *fla* are highly conserved genes, presumably subject to a very low mutation frequency among *B. burgdorferi* s.l. species and present in large quantities within a cell ^{270, 271}. By using *16S* rRNA, stable primers and probes distinguishing *B. burgdorferi* s.l. species from other *Borrelia* species, can be designed ²⁷⁰. Analytical sensitivity with detection down to 10 cells per extraction sample for all three human pathogenic species was presented when *16S* rRNA was used as the target, while protocols detecting the target genes *ospA* and *flaB* showed lower analytical sensitivity. However, the analytical specificity was high regardless of the choice of target gene.

In the new improved real-time PCR assay presented in paper III detection of the highly conserved target gene *16S* rRNA is used, detecting both *B. burgdorferi* s.l. species and *B. miyamotoi*. This PCR protocol widely detects both human pathogenic and non-pathogenic *Borrelia* species. Within the framework of the National Reference Laboratory for LB, the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden uses this newly improved real-time PCR method for detection of LB in CSF, SF, skin biopsies, and serum (in cases of a suspected *B. miyamotoi* infection). If the real-time PCR-analysis generates a positive result with the suspicion of *B. miyamotoi*, a complemented *B. miyamotoi*-specific real-time PCR-analysis detecting target gene *flaB* is done ⁵¹. The preferred diagnostic tool for detection of *B. miyamotoi* is PCR, which generates higher diagnostic sensitivity in both serum and CSF compared to the detection of *B. burgdorferi* s.l. species ^{220, 272}.

Finally, it is highly important to combine the right target gene with the right sample material (cDNA or DNA) of interest when creating protocols for detection of spirochetes by real-time PCR. However, it is also important to take into consideration clinical issues like *B. miyamotoi* and other potential human pathogenic species when designing new PCR protocols or updating already existing ones.

Detection of Borrelia burgdorferi sensu lato in Scandinavia by real-time polymerase chain reaction – are the results comparable and are the protocols able to detect different Borrelia species (paper IV)?

Three different panels containing spiked CSF, cDNA, and DNA were analysed by eight different PCR protocols currently in use in Sweden, Norway, and Denmark. The protocols detecting three different target genes (*ospA*, *flaB*, and *16S* rRNA), were based on five different published primer and probe pairs, and altogether the laboratories used five different PCR instruments.

In panel I, cDNA samples transcribed from total NA from water spiked with a known amount of *Borrelia* spirochetes were used. The results from panel I presented full concordance between the protocols (targeting *16S* RNA, *flaB* and *ospA*) down to 1000 cells per sample extraction. However, the concordance between the protocols using *16S* rRNA as the target gene was even higher with comparable results down to 10 cells per sample extraction. These results show the importance of using the right combination of primers and probes based on the target gene, which is discussed in the section “What is important to think about regarding the choice of target gene in correlation to extraction method and issue (paper IV)?”.

In panel II, CSF samples spiked with known amount of *B. burgdorferi* s.l. spirochetes were used. In this part of the study, some laboratories extracted total NA and other DNA. The results from panel II showed that the protocols extracting DNA had comparable analytical sensitivity with detection down to 100 cells per sample extraction for all three *Borrelia* species, irrespective of the target genes. For the protocols extracting total NA the analytical sensitivity was lower, with detection down to 1000 cells per sample extraction.

Panel III contained DNA from 15 different *Borrelia* species from nine different *Borrelia* specimens together with three different species closely related to *Borrelia*. The results showed high specificity and concordance between the different protocols without correlation to the target gene. However, all protocols were not able to detect *B. japonica*, *B. spielmanii*, and *B. lusitaniae*. This is a short-coming for these protocols since *B. spielmanii* is considered to be pathogenic to humans while for *B. lusitaniae* there is a suspicion of pathogenicity to humans²⁷³. All but one protocol, detecting target gene *ospA*, was able to detect *B. miyamotoi*, a newly discovered relapsing fever *Borrelia* species, which can be transferred to humans by *I. ricinus* ticks⁸. The cross-reactivity to other species closely related to *Borrelia* was low, showing that the analytical specificity was high.

As mentioned above, one of the protocols (detecting *ospA*) was not able to detect *B. miyamotoi*. This is probably a result of the fact that *B. burgdorferi* s.l. species possess Omps and VlsE proteins, while relapsing fever, and thereby *B. miyamotoi*, possesses variable large and variable small proteins and not OmpA^{274 274}. So, if the PCR protocol used is based on target gene *ospA*, a complementary assay specific for *B. miyamotoi* needs to be performed, since *ospA* is not detectable in *B. miyamotoi*.

The conclusion of paper IV is presented in the fact box in Figure 21.

- The PCR protocols in Scandinavia have high analytical sensitivity, specificity, and concordance
 - The analytical sensitivity of cDNA as a template is correlated to the use of primers and probes aimed at 16S rRNA as the target gene
 - The cross-reactivity to other species closely related to *Borrelia* is low
 - The PCR protocols need to be controlled and updated continuously to detect new pathogens, which may be pathogenic to humans
 - The low diagnostic sensitivity in CSF samples is not a result of poorly designed PCR protocols

Figure 21 Fact box containing the results from paper IV

CONCLUDING REMARKS

Based on the results presented in this thesis the following conclusions were drawn regarding the diagnosis of LB and LNB in clinical practice by serological and molecular methods:

The IgG assays currently used in northern Europe have comparable diagnostic sensitivity both within and between assays, while the IgM assays presented lower diagnostic specificity and less comparable results.

Separate detection of IgM in serum in adult patients provides no additional diagnostic value in the diagnosis of LB when modern IgG assays using VlsE antigens from the main pathogenic species are used. However, IgM AI in patients with suspected LNB has a higher diagnostic value and is more specific compared to IgM detection in diagnosis of serum.

Parallel analysis of anti-*Borrelia* antibodies in CSF and serum followed by calculation of AI is highly important to receive an accurate diagnosis of LNB.

Use of the recomBead *Borrelia* IgM/IgG assay (a third generation assay) for parallel detection of serum and CSF showed improved diagnostic sensitivity. However, the diagnostic specificity of the IDEA Lyme Neuroborreliosis assay, the VIDAS Lyme IgG assay, and the recomBead *Borrelia* IgM/IgG assay were comparable showing that the diagnostic specificity was not improved by use of third generation assays.

The analytical sensitivity and specificity of real-time PCR for detection of *B. burgdorferi* s.l. species are high after optimisation of the pre-analytical steps, where the concentration step (by centrifugation) and use of precipitate, the extraction of total NA followed by cDNA synthesis, and the absence of inhibitors (erythrocytes) in CSF, were the most important parameters.

The analytical sensitivity, specificity, and concordance are high between the real-time PCR protocols currently in use for detection of *B. burgdorferi* s.l. species in Scandinavian laboratories and the cross-reactivity to other microorganisms (e.g. *Leptospira*) and diseases is low. These results indicate that the low diagnostic sensitivity in CSF is not associated with poorly evaluated and established real-time PCR protocols, but may be due to absence of spirochetes in CSF or lack of systematic optimisation of the pre-analytical steps.

Even though the analytical sensitivity and specificity are high between the different real-time PCR protocols, it is important to combine the accurate target gene (16S rRNA, *ospA*, or *flaB*) with the optimal type of template (cDNA/DNA), when designing protocols, primers and probes. It is also important to continuously control and optimise the primers and probes to detect new species and mutations, but also new species that may be pathogenic to humans. Currently, the PCR methods used should at least be able to detect, *B. afzelii*, *B. garinii*, and *B. burgdorferi* s.s., but it is also desirable that the protocols can detect *B. spielmanii*, *B. miyamotoi*, and *B. lusitaniae* since there are indications that these species also have pathogenicity to humans.

Even though the pre-analytical steps used before PCR-analysis as well as the PCR protocols used in Scandinavia, presented high analytical sensitivity and specificity, further evaluations need to be carried out, and PCR-analysis is still only suitable as a supplementary method to serology.

FUTURE PERSPECTIVES

When this thesis was initiated it was well known that there was a lack of standardization in both molecular and serological methods used for laboratory diagnosis of LB in clinical samples. It was also known that the diagnostic sensitivity and specificity among the serological assays differed (partly due to different generations of assays) and that the diagnostic sensitivity for molecular detection of *B. burgdorferi* s.l. by PCR in CSF was low. The results presented in this thesis show that the lack of standardisation is still of major concern, even though the results showed comparable performance (however, not fully) both between and within assays for both the molecular and serological methods. Further research needs to be performed to standardize the serological (but also the molecular) methods to obtain concordant results at different laboratories in different countries (not necessarily based on the same serological assay or molecular protocol) and to ensure that patients receive the correct diagnosis and the same treatment.

Since the diagnostic specificity in the ELISA assays has become higher, there have been recent attempts to change the serological strategy from two-tier testing to a single-test strategy. By integration of new antigens, e.g. VlsE or C6 peptides, promising results with improved diagnostic sensitivity and specificity have been shown. However, this change from a two-tier strategy to a single-test strategy may be more applicable in North America where there are fewer pathogenic species compared to Europe ⁵⁰. Therefore, two-tier testing based on two ELISA assays instead of a combination of ELISA and immunoblot may be more suitable in Europe. However, this idea must be further evaluated ²³³. Still, the most challenging part of laboratory diagnosis of LB with serology is that many samples are ordered on the wrong indications without appropriate clinical assessment.

One of the major drawbacks in all forms of laboratory diagnosis of LB is the inability to distinguish between an active and a previous infection and methods need to be further evaluated and optimised to meet these challenges. Hopefully, new techniques with combinations of multiple antigens, but possibly also other biomarkers than CXCL13 will help in differentiating between active and past infection, and will also help in diagnosis of early

infection. The development of biomarkers, which can detect active infection, the response to therapy, and the possibility of using accurate point-to-care tests would constitute a major advance for the diagnosis of LB ¹⁸².

The results presented in this thesis regarding the molecular methods have shown that the protocols generated comparable analytical sensitivity, specificity, and concordance. Based on the knowledge that the PCR-protocols were stable regardless of country (concerning the target gene in combination with the template), the pre-analytical steps were further evaluated and optimised. The optimisation of the pre-analytical conditions showed a promising improvement of analytical sensitivity and specificity but needs to be further evaluated on a well-characterised body of patient material to determine the gain in the change of the molecular work-flow for detection of *B. burgdorferi* in CSF. The samples used should preferably be fresh and should be analysed as soon as they arrive at the laboratories. Hopefully, this is a project that can be started during the next tick season.

To evaluate which species have generated positive results in the routine samples analysed at the Division of Clinical Microbiology, Laboratory Medicine, Region Jönköping County, Jönköping, Sweden, it would be interesting to establish MLST at our laboratory. This would help us to acquire deeper knowledge and understanding of *B. burgdorferi* epidemiology in our geographical region. The MLST method makes it possible to distinguish between different species in correlation to different clinical manifestations. To go even deeper into the division of different species, whole genome sequencing must be used, and future studies will be aimed at working against a stable workflow for this kind of analysis. However, this requires more DNA, i.e. more spirochetes, which will require enrichment by culture from CSF, SF, and skin biopsies to get suitable sample material.

APPENDIX

Appendix 1: The Swedish treatment recommendations for Lyme borreliosis ⁴²

Diagnosis	Antibiotic treatment	Dose	Duration (days)
<i>Erythema migrans</i> (solitary)	Phenoxymethylpenicillin ^A	1 g x 3 ¹	10
	Doxycycline (in case of penicillin allergy) ^{2, A}	100 mg x 2	10
	Azithromycin ^{3, A}	500 mg x 1 (day 1) 250 mg x 1 (day 2-5)	5
	Phenoxymethylpenicillin ^C Azithromycin p.o. (in case of penicillin allergy) ^C	25 mg/kg x 3 10 mg/kg x 1 (day 1) 5 mg/kg x 1 (day 2-5)	10 5
<i>Erythema migrans</i> (multiple) ⁴	Doxycycline ^{2, A}	100 mg x 2	10
	Ceftriaxone (in case of pregnancy) ^A	2 g x 1 i.v.	10
	≥8 years: Doxycycline p.o. ^C	4 mg/kg x 1	14
	<8 years: Amoxicillin p.o. ^C	15 mg/kg x 3	14
<i>Acrodermatitis chronica atrophicans</i>	<8 years: Azithromycin p.o. (in case of penicillin allergy) ^C	10 mg/kg x 1 (day 1) 5 mg/kg x 1 (day 2-5)	5
<i>Lyme neuroborreliosis</i>	Doxycycline ^{2, A}	100 mg x 2	21
	Phenoxymethylpenicillin ^A	2 g x 3	21
	Doxycycline ^{2, A}	200 mg x 1	14
	Ceftriaxone ^A	200 mg x 2	10
<i>Borrelia lymphocytoma</i>	≥8 years: Doxycycline p.o. ^C	2 g x 1 i.v.	14
	<8 years: Amoxicillin p.o. ^C	4 mg/kg x 1	10
	<8 years: Azithromycin p.o. (in case of penicillin allergy) ^C	50-100 mg/kg x 1	10
<i>Lyme arthritis</i>	Doxycycline ^{2, A}	100 mg x 2	14
	Phenoxymethylpenicillin ^A	1 g x 3	14
	≥8 years: Doxycycline p.o. ^C	4 mg/kg x 1	14
	<8 years: Amoxicillin p.o. ^C	15 mg/kg x 3	14
<i>Lyme carditis</i>	<8 years: Azithromycin p.o. (in case of penicillin allergy) ^C	10 mg/kg x 1 (day 1) 5 mg/kg x 1 (day 2-5)	5
	Doxycycline ^{2, A}	100 mg x 2	14
	Ceftriaxone ^A	2 g x 1 i.v.	14
<i>Lyme carditis</i>	≥8 years: Doxycycline p.o. ^C	4 mg/kg x 1	21
	<8 years: Amoxicillin p.o. ^C	15 mg/kg x 3	21

¹ 2 g x 3 for 10 days for pregnant women's

² not recommended in the 2nd and 3rd trimester of pregnancy

³ not recommended in the 1st trimester of pregnancy

⁴ Erythema migrans with fever and for children with erythema migrans in the head and neck region

^A adult, ^C children

i.v. = intravenous, p.o. = oral intake

ACKNOWLEDGEMENTS

Anna J Henningsson, min huvudhandledare och vän. Utan att överdriva har jag haft den bästa av de bästa med mig på denna resa. Tack för att du 2014 tog dig an uppgiften som min handledare och att du genom hela min doktorandtid gjort det med entusiasm och glädje. Du har varit ett enormt stöd genom år, peppat och pushat mig att blir mitt bästa jag och varit ett lysande exempel på att man kommer långt med ödmjukhet. Din dörr har alltid stått öppen och trots att du haft både mailkorg och kalender full har du alltid funnit tid när jag behövt dig och dina goda råd. Jag hoppas att vi kan fortsätta vårt fina samarbete många år till.

Peter Wilhelmsson, min bihandledare, vän och grabben med STING. Tack för att du gjort min doktorandtid lite roligare, för att du alltid ställer upp och för trevligt resesällskap. Stor guldstjärna till dig. Jag ser fram emot att fortsätta vårt goda samarbete genom nya spännande och "fästliga" projekt.

Per-Eric Lindgren, min bihandledare. Stort tack för att du har delat med dig av din gedigna kunskap och att du stötta mig under denna resa.

Andreas Matussek, min bihandledare. Tack för gott samarbete under min doktorand tid och för många goda synpunkter och idéer.

Jan Söderman, kollega och tidigare chef. Tack för att du en gång öppnade dörren till forskningens förlovade land, att du fick mig att tänka själv, ta egna initiativ och att du gav mig chansen.

Pia Kjellander, **Linda Wass**, **Paula Gyllemark** och **Mattias Waldeck** mina doktorandkollegor. Ni har gjort min tid som doktorand väldigt trevlig med spännande diskussion och konferenser. Jag hoppas att vi kan fortsätta våra diskussioner i framtiden och skapa ny spännande projekt tillsammans.

Stort tack till Fästing-gruppen i Jönköping, Linköping, Kalmar, Falun, Malmö and Åland för intressanta samtal och fina samarbeten. Extra tack till **Anna Angel** och **Malin Wiberg**,

fästinggruppen Jönköping, för att ni gör mitt arbete extra roligt och för alla trevliga samtal vi har på kontoret. Tack Anna för att du alltid är så glad och positiv och Malin för att du är en fin vän.

Ram Dessau, medförfattare. Stort tack för din hjälp, din kunskap och ditt tålamod. Det har varit mycket lärorikt att samarbeta med dig och jag hoppas att vi får chans att göra det mer i framtiden.

Stort tack till alla **medförfattare**, för att ni orkat ta er an mina paneler med stor entusiasm, och för intressanta samtal gällande diagnostik. Extra tack till **Randi Eikeland, Guro F. Jensen, Vivian Kjelland, Åslaug R. Lorentzen, Dag Nyman, Katharina Ornstein, Ann-Cathrine Peterson, Hanne Quarsten** och **Ivar Tjernberg**. Jag hoppas vi kan få till många nya spännande studier i framtiden. Även stort tack till **Andrew Jenkins** för att du tog dig tid att läsa min avhandling och för värdefulla synpunkter och till **Barbro H. Skogman** för spännande projekt vid sidan av mitt doktorerande.

Pia Forsberg, the STING Queen. Tack för att du fick mig att ta klivet in som doktorand, för din entusiasm och driv och för att du är ett fint bevis på att störst inte alltid är starkast. Även stort tack för att du läste min avhandling och för värdefull feedback.

Stort tack till alla **kollegor på Laboratoriemedicin** i samband med provinsamling och för att ni gör så att det alltid är roligt att gå till jobbet. Tack till **"Forskargänget"** för stöd och glada hejarop utmed vägen. Extra tack till **Hanna Gustafsson Bragde** och **Linda Berglind**, mina fina trygga kollegor och vänner som alltid har ett öra eller en kram till övers när det behövs. Även stort tack till **Hanna Odén Poulsen** för stöd och pepp.

Emma och Daniel Linder, Sofia och Robban Blomqvist samt Angelica och Andreas Swenlert, kära Mellogång. Tack för lång och härlig vänskap med skratt, mellokvällar, spontanbokade resor, bubbel och temakvällar. Med er har man aldrig tråkigt.

Stina Dittschlag, Marcus Larsson, Tessan Granberg, Danne Skaar, Moni och Martin Lager, kompisar och innebandy/handbollssupportrar. Stort tack för att jag får dela min vardag med er och era barn och att ni alltid sätter guldkant på den. Genom framgång och motgång följs vi åt och genom skatt och gråt förenas vi. Tack för att ni alltid stöttar och hejar och för allt kul vi hittar på tillsammans.

Ingemar Christiansson, min älskade far från vilket jag ärvt mitt humör, min envishet och min orangea man. Tack för att du alltid tror på mig, försöker läsa mina publikationer trots att de är svåra att förstå och för att du är världens bästa pappa och morfar. Love U!

Hanna Vänerö, söstra mi. Tack för att du inte enbart är min syster utan även en av mina bästa vänner. Du har en stor plats i mitt hjärta.

Ella och **Sigge**, min älsklingar, utan er vore livet bra trist. Ni får mig att skratta, gråta, gnata, tjata, men framför allt gör ni mig till världens stoltaste mamma. Fortsätter vara de trygga, goa, glada, envisa och målmedvetna individer som ni är. Jag älskar er från månen och tillbaka.

Marcus, min man och bästa vän. Tack för att du älskar mig för den jag är, för att du alltid finns där när jag behöver dig som bäst och för din positiva inställning till det mesta. Tack för att du låter mig utvecklas, för att du stöttar och för att du påminner mig om vad som är viktigt i livet. Kort sagt "Vad vore jag utan dig" (~ *Håkan Hellström* ~).

Solveig Christiansson, älskad mamma och ängel. Precis som Pippi tror jag att du nu sitter på ditt moln, ler och tittar ner på mig. Och jag vet att du är stolt och att du hade velat dela detta med mig. I mitt hjärta delar vi fortfarande allt.

This thesis was supported by grants from Futurum – the Academy for Health and care, Region Jönköping County, the Interreg IVA Program ScandTick and the Interreg V program ScandTick Innovation.

REFERENCES

1. Steere AC, Malawista SE, Snyderman DR, Shope RE, Andiman WA, Ross MR, et al. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities. *Arthritis Rheum.* 1977;20(1):7-17.
2. JD, Samuels DS. *Borrelia : molecular biology, host interaction, and pathogenesis.* Norfolk, UK: Caister Academic Press; 2010. xii, 547 p., 4 p. of plates p.
3. Afzelius A. Verhandlungen der Dermatologischen Gesellschaft zu Stockholm. *Arch Derm Syph.* 1910;101, 404.
4. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP. Lyme disease- a tick-borne spirochetosis? *Science.* 1982;216(4552):1317-9.
5. Burgdorfer W. Discovery of the Lyme disease spirochete and its relation to tick vectors. *Yale J Biol Med.* 1984;57(4):515-20.
6. Margos G, Gofton A, Wibberg D, Dangel A, Marosevic D, Loh SM, et al. The genus *Borrelia* reloaded. *PLoS One.* 2018;13(12):e0208432.
7. Stanek G, Strle F. Lyme borreliosis. *Lancet.* 2003;362(9396):1639-47.
8. Cutler S, Vayssier-Taussat M, Estrada-Pena A, Potkonjak A, Mihalca AD, Zeller H. A new *Borrelia* on the block: *Borrelia miyamotoi* - a human health risk? *Euro Surveill.* 2019;24(18).
9. Anderson JF, Magnarelli LA. Epizootiology of Lyme disease-causing borreliae. *Clin Dermatol.* 1993;11(3):339-51.
10. Wang G, Liveris D, Mukherjee P, Jungnick S, Margos G, Schwartz I. Molecular Typing of *Borrelia burgdorferi*. *Curr Protoc Microbiol.* 2014;34:12C 5 1-31.
11. Barbour AG. Isolation and cultivation of Lyme disease spirochetes. *Yale J Biol Med.* 1984;57(4):521-5.
12. Kurtti TJ, Munderloh UG, Johnson RC, Ahlstrand GG. Colony formation and morphology in *Borrelia burgdorferi*. *J Clin Microbiol.* 1987;25(11):2054-8.
13. Ferdows MS, Barbour AG. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. *Proc Natl Acad Sci U S A.* 1989;86(15):5969-73.
14. Rosa PA, Tilly K, Stewart PE. The burgeoning molecular genetics of the Lyme disease spirochaete. *Nat Rev Microbiol.* 2005;3(2):129-43.
15. Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, et al. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature.* 1997;390(6660):580-6.
16. Takayama K, Rothenberg RJ, Barbour AG. Absence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect Immun.* 1987;55(9):2311-3.
17. Kenedy MR, Lenhart TR, Akins DR. The role of *Borrelia burgdorferi* outer surface proteins. *FEMS Immunol Med Microbiol.* 2012;66(1):1-19.
18. Reed KD. Laboratory testing for Lyme disease: possibilities and practicalities. *J Clin Microbiol.* 2002;40(2):319-24.

19. Schwan TG, Piesman J. Temporal changes in outer surface proteins A and C of the lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. *J Clin Microbiol.* 2000;38(1):382-8.
20. Schwan TG, Piesman J, Golde WT, Dolan MC, Rosa PA. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. *Proc Natl Acad Sci U S A.* 1995;92(7):2909-13.
21. Schwan TG. Temporal regulation of outer surface proteins of the Lyme-disease spirochaete *Borrelia burgdorferi*. *Biochem Soc Trans.* 2003;31(Pt 1):108-12.
22. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of lyme borreliosis. *Clin Microbiol Rev.* 2005;18(3):484-509.
23. Krzemiński PJ. Role of VlsE/C6 antigen as a marker for early Lyme borreliosis diagnosis and monitoring the effectiveness of its treatment. *Health Problems of Civilization.* 2017;11(2):87-92.
24. Bankhead T. Role of the VlsE Lipoprotein in Immune Avoidance by the Lyme Disease Spirochete *Borrelia burgdorferi*. *For Immunopathol Dis Therap.* 2016;7(3-4):191-204.
25. Bykowski T, Babb K, von Lackum K, Riley SP, Norris SJ, Stevenson B. Transcriptional regulation of the *Borrelia burgdorferi* antigenically variable VlsE surface protein. *J Bacteriol.* 2006;188(13):4879-89.
26. Noppa L, Burman N, Sadziene A, Barbour AG, Bergstrom S. Expression of the flagellin gene in *Borrelia* is controlled by an alternative sigma factor. *Microbiology.* 1995;141(Pt 1):85-93.
27. Bugrysheva JV, Godfrey HP, Schwartz I, Cabello FC. Patterns and regulation of ribosomal RNA transcription in *Borrelia burgdorferi*. *BMC Microbiol.* 2011;11:17.
28. Radulović M, Milutinović M, Tomanović S, Mulenga A. Detection of *Borrelia*-specific 16S rRNA sequence in total RNA extracted from *Ixodes ricinus* ticks. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia.* 2010;62:862-7.
29. Parola P, Raoult D. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clin Infect Dis.* 2001;32(6):897-928.
30. Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. *Lancet.* 2012;379(9814):461-73.
31. Gray JS, Kahl O, Lane RS, Levin ML, Tsao JI. Diapause in ticks of the medically important *Ixodes ricinus* species complex. *Ticks Tick Borne Dis.* 2016;7(5):992-1003.
32. Kjelland V, Korslund L, A. S. Fakta om flått: Kagge forlag AS; 2014.
33. Mejlom HA, Jaenson TGT. Seasonal prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* in different vegetation types in Sweden. *Scand J Infect Dis.* 1993;25(4):449-56.
34. Kiszewski AE, Matuschka FR, Spielman A. Mating strategies and spermiogenesis in ixodid ticks. *Annu Rev Entomol.* 2001;46:167-82.
35. Leonovich SA. Phenol and lactone receptors in the distal sensilla of the Haller's organ in *Ixodes ricinus* ticks and their possible role in host perception. *Exp Appl Acarol.* 2004;32(1-2):89-102.
36. Suss J. Epidemiology and ecology of TBE relevant to the production of effective vaccines. *Vaccine.* 2003;21 Suppl 1:S19-35.
37. Randolph SE, Storey K. Impact of microclimate on immature tick-rodent host interactions (Acari: Ixodidae): implications for parasite transmission. *J Med Entomol.* 1999;36(6):741-8.
38. Herrmann C, Gern L. Search for blood or water is influenced by *Borrelia burgdorferi* in *Ixodes ricinus*. *Parasit Vectors.* 2015;8:6.

39. Kahl O, Knulle W. Water vapour uptake from subsaturated atmospheres by engorged immature ixodid ticks. *Exp Appl Acarol.* 1988;4(1):73-83.
40. Kubiak K, Dziekonska-Rynko J. Seasonal activity of the common European tick, *Ixodes ricinus* (Linnaeus, 1758), in the forested areas of the city of Olsztyn and its surroundings. *Wiad Parazytol.* 2006;52(1):59-64.
41. Duffy DC, Campbell SR. Ambient air temperature as a predictor of activity of adult *Ixodes scapularis* (Acari: Ixodidae). *J Med Entomol.* 1994;31(1):178-80.
42. Läkemedelsverket. Läkemedelsbehandling av borreliainfektion – ny rekommendation. [Available from: <https://www.lakemedelsverket.se/globalassets/dokument/behandling-och-forskrivning/behandlingsrekommendationer/behandlingsrekommendation/behandlingsrekommendation-borreli.pdf>].
43. Taylor MA, Coop RL, Wall R. *Veterinary parasitology.* Oxford; Ames, Iowa: Blackwell Pub.; 2007.
44. Chmelar J, Kotal J, Karim S, Kopacek P, Francischetti IMB, Pedra JHF, et al. Sialomes and Mialomes: A Systems-Biology View of Tick Tissues and Tick-Host Interactions. *Trends Parasitol.* 2016;32(3):242-54.
45. Waindok P, Schicht S, Fingerle V, Strube C. Lyme borreliæ prevalence and genospecies distribution in ticks removed from humans. *Ticks Tick Borne Dis.* 2017;8(5):709-14.
46. Cutler SJ, Ruzic-Sabljic E, Potkonjak A. Emerging borreliæ - Expanding beyond Lyme borreliosis. *Mol Cell Probes.* 2017;31:22-7.
47. Wilhelmsson P, Fryland L, Borjesson S, Nordgren J, Bergstrom S, Ernerudh J, et al. Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. *J Clin Microbiol.* 2010;48(11):4169-76.
48. Hvidsten D, Stordal F, Lager M, Rognerud B, Kristiansen BE, Matussek A, et al. *Borrelia burgdorferi sensu lato*-infected *Ixodes ricinus* collected from vegetation near the Arctic Circle. *Ticks Tick Borne Dis.* 2015;6(6):768-73.
49. Wagemakers A, Jahfari S, de Wever B, Spanjaard L, Starink MV, de Vries HJC, et al. *Borrelia miyamotoi* in vectors and hosts in The Netherlands. *Ticks Tick Borne Dis.* 2017;8(3):370-4.
50. Lohr B, Fingerle V, Norris DE, Hunfeld KP. Laboratory diagnosis of Lyme borreliosis: Current state of the art and future perspectives. *Crit Rev Clin Lab Sci.* 2018;55(4):219-45.
51. Henningsson AJ, Asgeirsson H, Hammas B, Karlsson E, Parke A, Hoornstra D, et al. Two Cases of *Borrelia miyamotoi* Meningitis, Sweden, 2018. *Emerg Infect Dis.* 2019;25(10):1965-8.
52. Becker NS, Margos G, Blum H, Krebs S, Graf A, Lane RS, et al. Recurrent evolution of host and vector association in bacteria of the *Borrelia burgdorferi sensu lato* species complex. *BMC Genomics.* 2016;17(1):734.
53. Eisen L, Eisen RJ, Mun J, Salkeld DJ, Lane RS. Transmission cycles of *Borrelia burgdorferi* and *B. bissettii* in relation to habitat type in northwestern California. *J Vector Ecol.* 2009;34(1):81-91.
54. Richter D, Schlee DB, Matuschka FR. Reservoir competence of various rodents for the lyme disease Spirochete *Borrelia spielmanii*. *Appl Environ Microbiol.* 2011;77(11):3565-70.
55. Gern L. *Borrelia burgdorferi sensu lato*, the agent of lyme borreliosis: life in the wilds. *Parasite.* 2008;15(3):244-7.

56. Heyman P, Cochez C, Hofhuis A, van der Giessen J, Sprong H, Porter SR, et al. A clear and present danger: tick-borne diseases in Europe. *Expert Rev Anti Infect Ther.* 2010;8(1):33-50.
57. Schwan TG, Piesman J. Vector interactions and molecular adaptations of lyme disease and relapsing fever spirochetes associated with transmission by ticks. *Emerg Infect Dis.* 2002;8(2):115-21.
58. Gern L, Zhu Z, Aeschlimann A. Development of *Borrelia burgdorferi* in *Ixodes ricinus* females during blood feeding. *Ann Parasitol Hum Comp.* 1990;65:2:89-93.
59. Ribeiro JM, Mather TN, Piesman J, Spielman A. Dissemination and salivary delivery of Lyme disease spirochetes in vector ticks (Acari: Ixodidae). *J Med Entomol.* 1987;24(2):201-5.
60. Piesman J. Dynamics of *Borrelia burgdorferi* transmission by nymphal *Ixodes dammini* ticks. *J Infect Dis.* 1993;167(5):1082-5.
61. Strnad M, Honig V, Ruzek D, Grubhoffer L, Rego ROM. Europe-Wide Meta-Analysis of *Borrelia burgdorferi* Sensu Lato Prevalence in Questing *Ixodes ricinus* Ticks. *Appl Environ Microbiol.* 2017;83(15).
62. Wilhelmsson P, Lindblom P, Fryland L, Ernerudh J, Forsberg P, Lindgren PE. Prevalence, diversity, and load of *Borrelia* species in ticks that have fed on humans in regions of Sweden and Aland Islands, Finland with different Lyme borreliosis incidences. *PLoS One.* 2013;8(11):e81433.
63. Fryland L, Wilhelmsson P, Lindgren PE, Nyman D, Ekerfelt C, Forsberg P. Low risk of developing *Borrelia burgdorferi* infection in the south-east of Sweden after being bitten by a *Borrelia burgdorferi*-infected tick. *Int J Infect Dis.* 2011;15(3):e174-81.
64. Gern L, Rais O. Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks (Acari: Ixodidae). *J Med Entomol.* 1996;33(1):189-92.
65. Pritt BS, Mead PS, Johnson DKH, Neitzel DF, Respicio-Kingry LB, Davis JP, et al. Identification of a novel pathogenic *Borrelia* species causing Lyme borreliosis with unusually high spirochaetemia: a descriptive study. *Lancet Infect Dis.* 2016;16(5):556-64.
66. Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH, Jr. Updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. *Ticks Tick Borne Dis.* 2011;2(3): 123-8.
67. Casjens SR, Fraser-Liggett CM, Mongodin EF, Qiu WG, Dunn JJ, Luft BJ, et al. Whole genome sequence of an unusual *Borrelia burgdorferi* sensu lato isolate. *J Bacteriol.* 2011;193(6):1489-90.
68. Chomel B. Lyme disease. *Rev Sci Tech.* 2015;34(2):569-76.
69. Balmelli T, Piffaretti JC. Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. *Res Microbiol.* 1995;146(4):329-40.
70. Masuzawa T. Terrestrial distribution of the Lyme borreliosis agent *Borrelia burgdorferi* sensu lato in East Asia. *Jpn J Infect Dis.* 2004;57(6):229-35.
71. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti JC, Assous M, et al. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. *Int J Syst Bacteriol.* 1992;42(3):378-83.
72. van Dam AP, Kuiper H, Vos K, Widjojokusumo A, de Jongh BM, Spanjaard L, et al. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin Infect Dis.* 1993;17(4):708-17.
73. Baranton G, De Martino SJ. *Borrelia burgdorferi* sensu lato diversity and its influence on pathogenicity in humans. *Curr Probl Dermatol.* 2009;37:1-17.

74. Margos G, Vollmer SA, Cornet M, Garnier M, Fingerle V, Wilske B, et al. A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. *Appl Environ Microbiol.* 2009;75(16):5410-6.
75. Maraspin V, Ruzic-Sabljic E, Strle F. Lyme borreliosis and *Borrelia spielmanii*. *Emerg Infect Dis.* 2006;12(7):1177.
76. Collares-Pereira M, Couceiro S, Franca I, Kurtenbach K, Schafer SM, Vitorino L, et al. First isolation of *Borrelia lusitaniae* from a human patient. *J Clin Microbiol.* 2004;42(3):1316-8.
77. Margos G, Lane RS, Fedorova N, Koloczec J, Piesman J, Hojgaard A, et al. *Borrelia bissettae* sp. nov. and *Borrelia californiensis* sp. nov. prevail in diverse enzootic transmission cycles. *Int J Syst Evol Microbiol.* 2016;66(3):1447-52.
78. Postic D, Garnier M, Baranton G. Multilocus sequence analysis of atypical *Borrelia burgdorferi* sensu lato isolates--description of *Borrelia californiensis* sp. nov., and *genospecies 1* and *2*. *Int J Med Microbiol.* 2007;297(4):263-71.
79. Rudenko N, Golovchenko M, Mokracek A, Piskunova N, Ruzek D, Mallatova N, et al. Detection of *Borrelia bissetti* in cardiac valve tissue of a patient with endocarditis and aortic valve stenosis in the Czech Republic. *J Clin Microbiol.* 2008;46(10):3540-3.
80. Ryffel K, Peter O, Rutti B, Suard A, Dayer E. Scored antibody reactivity determined by immunoblotting shows an association between clinical manifestations and presence of *Borrelia burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. valaisiana* in humans. *J Clin Microbiol.* 1999;37(12):4086-92.
81. Rudenko N, Golovchenko M, Grubhoffer L, Oliver JH, Jr. *Borrelia carolinensis* sp. nov., a novel species of the *Borrelia burgdorferi* sensu lato complex isolated from rodents and a tick from the south-eastern USA. *Int J Syst Evol Microbiol.* 2011;61 (Pt 2):381-3.
82. Margos G, Hojgaard A, Lane RS, Cornet M, Fingerle V, Rudenko N, et al. Multilocus sequence analysis of *Borrelia bissetti* strains from North America reveals a new *Borrelia* species, *Borrelia kurtenbachii*. *Ticks Tick Borne Dis.* 2010;1(4):151-8.
83. Huang W, Ojaimi C, Fallon JT, Travisany D, Maass A, Ivanova L, et al. Genome Sequence of *Borrelia chilensis* VA1, a South American Member of the Lyme Borreliosis Group. *Genome Announc.* 2015;3(1).
84. Wilhelmsson P, Lindgren PE. Detection of a novel Lyme borreliosis pathogen. *Lancet Infect Dis.* 2016;16(5):511-2.
85. Krause PJ, Narasimhan S, Wormser GP, Rollend L, Fikrig E, Lepore T, et al. Human *Borrelia miyamotoi* infection in the United States. *N Engl J Med.* 2013;368(3):291-3.
86. Nelson CA, Saha S, Kugeler KJ, Delorey MJ, Shankar MB, Hinckley AF, et al. Incidence of Clinician-Diagnosed Lyme Disease, United States, 2005-2010. *Emerg Infect Dis.* 2015;21(9):1625-31.
87. Sykes RA, Makiello P. An estimate of Lyme borreliosis incidence in Western Europedagger. *J Public Health (Oxf).* 2017;39(1):74-81.
88. Hubalek Z. Epidemiology of lyme borreliosis. *Curr Probl Dermatol.* 2009;37:31-50.
89. Stone BL, Tourand Y, Brissette CA. Brave New Worlds: The Expanding Universe of Lyme Disease. *Vector Borne Zoonotic Dis.* 2017;17(9):619-29.
90. Medlock JM, Hansford KM, Bormane A, Derdakova M, Estrada-Pena A, George JC, et al. Driving forces for changes in geographical distribution of *Ixodes ricinus* ticks in Europe. *Parasit Vectors.* 2013;6:1.

91. Stanek G, Flamm H, Groh V, Hirschl A, Kristoferitsch W, Neumann R, et al. Epidemiology of borrelia infections in Austria. *Zentralbl Bakteriol Mikrobiol Hyg A*. 1987;263(3): 442-9.
92. Wilske B, Steinhuber R, Bergmeister H, Fingerle V, Schierz G, Preac-Mursic V, et al. [Lyme borreliosis in South Germany. Epidemiologic data on the incidence of cases and on the epidemiology of ticks (*Ixodes ricinus*) carrying *Borrelia burgdorferi*]. *Dtsch Med Wochenschr*. 1987;112(45):1730-6.
93. Strle F, Videcnik J, Zorman P, Cimperman J, Lotric-Furlan S, Maraspin V. Clinical and epidemiological findings for patients with erythema migrans. Comparison of cohorts from the years 1993 and 2000. *Wien Klin Wochenschr*. 2002;114(13-14):493-7.
94. Orloski KA, Hayes EB, Campbell GL, Dennis DT. Surveillance for Lyme disease--United States, 1992-1998. *MMWR CDC Surveill Summ*. 2000;49(3):1-11.
95. CDC. From the Centers for Disease Control and Prevention - Lyme Disease 2017 [Available from: <https://www.cdc.gov/lyme/datasurveillance/charts-figures-recent.html>].
96. Bennet L, Halling A, Berglund J. Increased incidence of Lyme borreliosis in southern Sweden following mild winters and during warm, humid summers. *Eur J Clin Microbiol Infect Dis*. 2006;25(7):426-32.
97. Berglund J, Eitrem R, Ornstein K, Lindberg A, Ringer A, Elmrud H, et al. An epidemiologic study of Lyme disease in southern Sweden. *N Engl J Med*. 1995;333(20):1319-27.
98. Strle F, Pleterski-Rigler D, Stanek G, Pejovnik-Pustinek A, Ruzic E, Cimperman J. Solitary borrelial lymphocytoma: report of 36 cases. *Infection*. 1992;20(4):201-6.
99. Steere AC. Lyme disease. *N Engl J Med*. 2001;345(2):115-25.
100. Strle F, Stanek G. Clinical manifestations and diagnosis of lyme borreliosis. *Curr Probl Dermatol*. 2009;37:51-110.
101. Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AC, Klemperer MS, et al. The clinical assessment, treatment, and prevention of lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. *Clin Infect Dis*. 2006;43(9):1089-134.
102. Dahl V, Wisell KT, Giske CG, Tegnell A, Wallensten A. Lyme neuroborreliosis epidemiology in Sweden 2010 to 2014: clinical microbiology laboratories are a better data source than the hospital discharge diagnosis register. *Euro Surveill*. 2019;24(20).
103. Sodermark L, Sigurdsson V, Nas W, Wall P, Trollfors B. Neuroborreliosis in Swedish Children: A Population-based Study on Incidence and Clinical Characteristics. *Pediatr Infect Dis J*. 2017;36(11):1052-6.
104. Sood SK. Lyme disease in children. *Infect Dis Clin North Am*. 2015;29(2):281-94.
105. Maraspin V, Nahtigal Klevisar M, Ruzic-Sabljic E, Lusa L, Strle F. Borrelial Lymphocytoma in Adult Patients. *Clin Infect Dis*. 2016;63(7):914-21.
106. Maraspin V, Mrvic T, Ruzic-Sabljic E, Jurcic V, Strle F. Acrodermatitis chronica atrophicans in children: Report on two cases and review of the literature. *Ticks Tick Borne Dis*. 2019;10(1):180-5.
107. Zalaudek I, Leinweber B, Kerl H, Mullegger RR. Acrodermatitis chronica atrophicans in a 15-year-old girl misdiagnosed as venous insufficiency for 6 years. *J Am Acad Dermatol*. 2005;52(6):1091-4.
108. Mullegger RR, Glatz M. Skin manifestations of lyme borreliosis: diagnosis and management. *Am J Clin Dermatol*. 2008;9(6):355-68.

109. Asbrink E. Acrodermatitis chronica atrophicans. *Clin Dermatol.* 1993;11(3):369-75.
110. Yoon EC, Vail E, Kleinman G, Lento PA, Li S, Wang G, et al. Lyme disease: a case report of a 17-year-old male with fatal Lyme carditis. *Cardiovasc Pathol.* 2015;24(5):317-21.
111. Cary NR, Fox B, Wright DJ, Cutler SJ, Shapiro LM, Grace AA. Fatal Lyme carditis and endodermal heterotopia of the atrioventricular node. *Postgrad Med J.* 1990;66(772):134-6.
112. Robertson JN, Gray JS, Stewart P. Tick bite and Lyme borreliosis risk at a recreational site in England. *Eur J Epidemiol.* 2000;16(7):647-52.
113. Wilhelmsson P, Lindblom P, Fryland L, Nyman D, Jaenson TG, Forsberg P, et al. Ixodes ricinus ticks removed from humans in Northern Europe: seasonal pattern of infestation, attachment sites and duration of feeding. *Parasit Vectors.* 2013;6:362.
114. Skogman BH, Ekerfelt C, Ludvigsson J, Forsberg P. Seroprevalence of Borrelia IgG antibodies among young Swedish children in relation to reported tick bites, symptoms and previous treatment for Lyme borreliosis: a population-based survey. *Arch Dis Child.* 2010;95(12):1013-6.
115. van Beek J, Sajanti E, Helve O, Ollgren J, Virtanen MJ, Rissanen H, et al. Population-based Borrelia burgdorferi sensu lato seroprevalence and associated risk factors in Finland. *Ticks Tick Borne Dis.* 2018;9(2):275-80.
116. Wilking H, Fingerle V, Klier C, Thamm M, Stark K. Antibodies against Borrelia burgdorferi sensu lato among Adults, Germany, 2008-2011. *Emerg Infect Dis.* 2015;21(1):107-10.
117. Rigaud E, Jaulhac B, Garcia-Bonnet N, Hunfeld KP, Femenia F, Huet D, et al. Seroprevalence of seven pathogens transmitted by the Ixodes ricinus tick in forestry workers in France. *Clin Microbiol Infect.* 2016;22(8):735 e1-9.
118. Johansson M, Manfredsson L, Wistedt A, Serrander L, Tjernberg I. Significant variations in the seroprevalence of C6 ELISA antibodies in a highly endemic area for Lyme borreliosis: evaluation of age, sex and seasonal differences. *APMIS.* 2017;125(5):476-81.
119. Lager M, Dessau RB, Wilhelmsson P, Nyman D, Jensen GF, Matussek A, et al. Serological diagnostics of Lyme borreliosis: comparison of assays in twelve clinical laboratories in Northern Europe. *Eur J Clin Microbiol Infect Dis.* 2019;38(10):1933-45.
120. Lernout T, Kabamba-Mukadi B, Saegeman V, Tre-Hardy M, de Laveleye M, Asikainen T, et al. The value of seroprevalence data as surveillance tool for Lyme borreliosis in the general population: the experience of Belgium. *BMC Public Health.* 2019;19(1):597.
121. Dehnert M, Fingerle V, Klier C, Talaska T, Schlaud M, Krause G, et al. Seropositivity of Lyme borreliosis and associated risk factors: a population-based study in Children and Adolescents in Germany (KiGGS). *PLoS One.* 2012;7(8):e41321.
122. Bennet L, Stjernberg L, Berglund J. Effect of gender on clinical and epidemiologic features of Lyme borreliosis. *Vector Borne Zoonotic Dis.* 2007;7(1):34-41.
123. Gustafson R, Forsgren M, Gardulf A, Granstrom M, Svenungsson B. Clinical manifestations and antibody prevalence of Lyme borreliosis and tick-borne encephalitis in Sweden: a study in five endemic areas close to Stockholm. *Scand J Infect Dis.* 1993;25(5):595-603.
124. Stanek G, Fingerle V, Hunfeld KP, Jaulhac B, Kaiser R, Krause A, et al. Lyme borreliosis: clinical case definitions for diagnosis and management in Europe. *Clin Microbiol Infect.* 2011;17(1):69-79.

125. Brouqui P, Bacellar F, Baranton G, Birtles RJ, Bjoersdorff A, Blanco JR, et al. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. *Clin Microbiol Infect.* 2004;10(12):1108-32.
126. Steere AC, Strle F, Wormser GP, Hu LT, Branda JA, Hovius JW, et al. Lyme borreliosis. *Nat Rev Dis Primers.* 2016;2:16090.
127. Schoen RT. Challenges in the Diagnosis and Treatment of Lyme Disease. *Curr Rheumatol Rep.* 2020;22(1):3.
128. Asbrink E, Hovmark A. Early and late cutaneous manifestations in Ixodes-borne borreliosis (erythema migrans borreliosis, Lyme borreliosis). *Ann N Y Acad Sci.* 1988;539:4-15.
129. Griffin AH. The Bulls-Eye Rash of Lyme Disease: Investigating the Cutaneous Host-Pathogen Dynamics of Erythema Migrans 2018 [Available from: <https://asm.org/Articles/2018/April/going-skin-deep-investigating-the-cutaneous-host-pj>].
130. Oksi J, Marttila H, Soini H, Aho H, Uksila J, Viljanen MK. Early dissemination of *Borrelia burgdorferi* without generalized symptoms in patients with erythema migrans. *APMIS.* 2001;109(9):581-8.
131. Coulter P, Lema C, Flayhart D, Linhardt AS, Aucott JN, Auwaerter PG, et al. Two-year evaluation of *Borrelia burgdorferi* culture and supplemental tests for definitive diagnosis of Lyme disease. *J Clin Microbiol.* 2005;43(10):5080-4.
132. Egberts F, Moller M, Proksch E, Schwarz T. Multiple erythema migrans--manifestation of systemic cutaneous borreliosis. *J Dtsch Dermatol Ges.* 2008;6(5):350-3.
133. Wormser GP. Clinical practice. Early Lyme disease. *N Engl J Med.* 2006;354(26):2794-801.
134. Jones KL, Muellegger RR, Means TK, Lee M, Glickstein LJ, Damle N, et al. Higher mRNA levels of chemokines and cytokines associated with macrophage activation in erythema migrans skin lesions in patients from the United States than in patients from Austria with Lyme borreliosis. *Clin Infect Dis.* 2008;46(1):85-92.
135. Carlsson SA, Granlund H, Jansson C, Nyman D, Wahlberg P. Characteristics of erythema migrans in *Borrelia afzelii* and *Borrelia garinii* infections. *Scand J Infect Dis.* 2003;35(1):31-3.
136. Logar M, Ruzic-Sabljic E, Maraspin V, Lotric-Furlan S, Cimperman J, Jurca T, et al. Comparison of erythema migrans caused by *Borrelia afzelii* and *Borrelia garinii*. *Infection.* 2004;32(1):15-9.
137. Rupprecht TA, Koedel U, Fingerle V, Pfister HW. The pathogenesis of lyme neuroborreliosis: from infection to inflammation. *Mol Med.* 2008;14(3-4):205-12.
138. Mygland A, Ljostad U, Fingerle V, Rupprecht T, Schmutzhard E, Steiner I, et al. EFNS guidelines on the diagnosis and management of European Lyme neuroborreliosis. *Eur J Neurol.* 2010;17(1):8-16, e1-4.
139. van Burgel ND, Bakels F, Kroes AC, van Dam AP. Discriminating Lyme neuroborreliosis from other neuroinflammatory diseases by levels of CXCL13 in cerebrospinal fluid. *J Clin Microbiol.* 2011;49(5):2027-30.
140. Backman K, Skogman BH. Occurrence of erythema migrans in children with Lyme neuroborreliosis and the association with clinical characteristics and outcome - a prospective cohort study. *BMC Pediatr.* 2018;18(1):189.
141. Bierman SM, van Kooten B, Vermeeren YM, Bruintjes TD, van Hees BC, Bruinsma RA, et al. Incidence and characteristics of Lyme neuroborreliosis in adult patients with facial palsy in an endemic area in the Netherlands. *Epidemiol Infect.* 2019;147:e160.

142. Strle F, Ruzic-Sabljic E, Cimperman J, Lotric-Furlan S, Maraspin V. Comparison of findings for patients with *Borrelia garinii* and *Borrelia afzelii* isolated from cerebrospinal fluid. *Clin Infect Dis*. 2006;43(6):704-10.
143. Skogman BH, Croner S, Nordwall M, Eknefelt M, Ernerudh J, Forsberg P. Lyme neuroborreliosis in children: a prospective study of clinical features, prognosis, and outcome. *Pediatr Infect Dis J*. 2008;27(12):1089-94.
144. Broekhuijsen-van Henten DM, Braun KP, Wolfs TF. Clinical presentation of childhood neuroborreliosis; neurological examination may be normal. *Arch Dis Child*. 2010;95(11):910-4.
145. Huisman TA, Wohlrab G, Nadal D, Boltshauser E, Martin E. Unusual presentations of neuroborreliosis (Lyme disease) in childhood. *J Comput Assist Tomogr*. 1999;23(1): 39-42.
146. Rauer S, Kastenbauer S, Hofmann H, Fingerle V, Huppertz H, Hunfeld K, et al. Guidelines for diagnosis and treatment in neurology – Lyme neuroborreliosis *GMS Ger Med Sci* 2020;18(Doc03).
147. Steere AC, Schoen RT, Taylor E. The clinical evolution of Lyme arthritis. *Ann Intern Med*. 1987;107(5):725-31.
148. Herzer P. Joint manifestations of Lyme borreliosis in Europe. *Scand J Infect Dis Suppl*. 1991;77:55-63.
149. Steere AC, Angelis SM. Therapy for Lyme arthritis: strategies for the treatment of antibiotic-refractory arthritis. *Arthritis Rheum*. 2006;54(10):3079-86.
150. Nocton JJ, Dressler F, Rutledge BJ, Rys PN, Persing DH, Steere AC. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *N Engl J Med*. 1994;330(4):229-34.
151. Limbach FX, Jaulhac B, Puechal X, Monteil H, Kuntz JL, Piemont Y, et al. Treatment resistant Lyme arthritis caused by *Borrelia garinii*. *Ann Rheum Dis*. 2001;60(3):284-6.
152. Weger W MR. Histopathology and immunohistochemistry of dermatoborreliosis. *Acta Dermatoven APA*. 2001;10:135-42.
153. Picken RN, Strle F, Picken MM, Ruzic-Sabljic E, Maraspin V, Lotric-Furlan S, et al. Identification of three species of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) among isolates from acrodermatitis chronica atrophicans lesions. *J Invest Dermatol*. 1998;110(3):211-4.
154. Ohlenbusch A, Matuschka FR, Richter D, Christen HJ, Thomssen R, Spielman A, et al. Etiology of the acrodermatitis chronica atrophicans lesion in Lyme disease. *J Infect Dis*. 1996;174(2):421-3.
155. Ruzic-Sabljic E, Strle F, Cimperman J, Maraspin V, Lotric-Furlan S, Pleterski-Rigler D. Characterisation of *Borrelia burgdorferi* sensu lato strains isolated from patients with skin manifestations of Lyme borreliosis residing in Slovenia. *J Med Microbiol*. 2000;49(1):47-53.
156. Hidri N, Barraud O, de Martino S, Garnier F, Paraf F, Martin C, et al. Lyme endocarditis. *Clin Microbiol Infect*. 2012;18(12):E531-2.
157. Steere AC, Batsford WP, Weinberg M, Alexander J, Berger HJ, Wolfson S, et al. Lyme carditis: cardiac abnormalities of Lyme disease. *Ann Intern Med*. 1980;93(1):8-16.
158. McAlister HF, Klementowicz PT, Andrews C, Fisher JD, Feld M, Furman S. Lyme carditis: an important cause of reversible heart block. *Ann Intern Med*. 1989;110(5):339-45.
159. Scheffold N, Herkommer B, Kandolf R, May AE. Lyme carditis--diagnosis, treatment and prognosis. *Dtsch Arztebl Int*. 2015;112(12):202-8.

160. Fish AE, Pride YB, Pinto DS. Lyme carditis. *Infect Dis Clin North Am.* 2008;22(2): 275-88, vi.
161. Paim AC, Baddour LM, Pritt BS, Schuetz AN, Wilson JW. Lyme Endocarditis. *Am J Med.* 2018;131(9):1126-9.
162. Kauffmann DJ, Wormser GP. Ocular Lyme disease: case report and review of the literature. *Br J Ophthalmol.* 1990;74(6):325-7.
163. Mikkila HO, Seppala IJ, Viljanen MK, Peltomaa MP, Karma A. The expanding clinical spectrum of ocular lyme borreliosis. *Ophthalmology.* 2000;107(3):581-7.
164. Cairns V, Godwin J. Post-Lyme borreliosis syndrome: a meta-analysis of reported symptoms. *Int J Epidemiol.* 2005;34(6):1340-5.
165. Steere AC, Coburn J, Glickstein L. The emergence of Lyme disease. *J Clin Invest.* 2004;113(8):1093-101.
166. Scieszka J, Dabek J, Cieslik P. Post-Lyme disease syndrome. *Reumatologia.* 2015;53(1):46-8.
167. Marks CM, Nawn JE, Caplow JA. Antibiotic Treatment for Chronic Lyme Disease-Say No to the DRESS. *JAMA Intern Med.* 2016;176(12):1745-6.
168. Strizova Z, Patek O, Vitova L, Horackova M, Bartunkova J. Internet-based self-diagnosis of Lyme disease caused death in a young woman with systemic lupus erythematosus. *Joint Bone Spine.* 2019;86(5):650-1.
169. Patel R, Grogg KL, Edwards WD, Wright AJ, Schwenk NM. Death from inappropriate therapy for Lyme disease. *Clin Infect Dis.* 2000;31(4):1107-9.
170. Steere AC, Sikand VK, Schoen RT, Nowakowski J. Asymptomatic infection with *Borrelia burgdorferi*. *Clin Infect Dis.* 2003;37(4):528-32.
171. Wilhelmsson P, Fryland L, Lindblom P, Sjowall J, Ahlm C, Berglund J, et al. A prospective study on the incidence of *Borrelia burgdorferi* sensu lato infection after a tick bite in Sweden and on the Åland Islands, Finland (2008-2009). *Ticks Tick Borne Dis.* 2016;7(1):71-9.
172. Nadelman RB, Wormser GP. Lyme borreliosis. *Lancet.* 1998;352(9127):557-65.
173. Hu LT. Lyme Disease. *Ann Intern Med.* 2016;164(9):ITC65-ITC80.
174. O'Connell S. Recommendations for diagnosis and treatment of Lyme borreliosis: guidelines and consensus papers from specialist societies and expert groups in Europe and North America [Poster]. [updated 2020-05-04. Available from: https://www.aldf.com/pdf/ECCMID_Poster_4.22.10.pdf].
175. Wressnigg N, Barrett PN, Pollabauer EM, O'Rourke M, Portsmouth D, Schwendinger MG, et al. A Novel multivalent OspA vaccine against Lyme borreliosis is safe and immunogenic in an adult population previously infected with *Borrelia burgdorferi* sensu lato. *Clin Vaccine Immunol.* 2014;21(11):1490-9.
176. De Keukeleire M, Robert A, Kabamba B, Dion E, Luyasu V, Vanwambeke SO. Individual and environmental factors associated with the seroprevalence of *Borrelia burgdorferi* in Belgian farmers and veterinarians. *Infect Ecol Epidemiol.* 2016;6:32793.
177. De Keukeleire M, Robert A, Luyasu V, Kabamba B, Vanwambeke SO. Seroprevalence of *Borrelia burgdorferi* in Belgian forestry workers and associated risk factors. *Parasit Vectors.* 2018;11(1):277.
178. Zajac V, Pinkas J, Wojcik-Fatla A, Dutkiewicz J, Owoc A, Bojar I. Prevalence of serological response to *Borrelia burgdorferi* in farmers from eastern and central Poland. *Eur J Clin Microbiol Infect Dis.* 2017;36(3):437-46.

179. Tokarska-Rodak M, Plewik D, Michalski AJ, Kolodziej M, Melgies A, Panczuk A, et al. Serological surveillance of vector-borne and zoonotic diseases among hunters in eastern Poland. *J Vector Borne Dis.* 2016;53(4):355-61.
180. Zhioua E, Gern L, Aeschlimann A, Sauvain MJ, Van der Linden S, Fahrner H. Longitudinal study of Lyme borreliosis in a high risk population in Switzerland. *Parasite.* 1998;5(4):383-6.
181. Tijssen-Klasen E, Jacobs JJ, Swart A, Fonville M, Reimerink JH, Brandenburg AH, et al. Small risk of developing symptomatic tick-borne diseases following a tick bite in The Netherlands. *Parasit Vectors.* 2011;4:17.
182. Marques AR. Laboratory diagnosis of Lyme disease: advances and challenges. *Infect Dis Clin North Am.* 2015;29(2):295-307.
183. Schutzer SE, Body BA, Boyle J, Branson BM, Dattwyler RJ, Fikrig E, et al. Direct Diagnostic Tests for Lyme Disease. *Clin Infect Dis.* 2019;68(6):1052-7.
184. Flatland B, Friedrichs KR, Klenner S. Differentiating between analytical and diagnostic performance evaluation with a focus on the method comparison study and identification of bias. *Veterinary clinical pathology.* 2014;43(4):475-86.
185. Skogman BH, Lager M, Brudin L, Jenmalm MC, Tjernberg I, Henningsson AJ. Cytokines and chemokines in cerebrospinal fluid in relation to diagnosis, clinical presentation and recovery in children being evaluated for Lyme neuroborreliosis. *Ticks Tick Borne Dis.* 2020;11(3):101390.
186. Henningsson AJ, Gyllemark P, Lager M, Skogman BH, Tjernberg I. Evaluation of two assays for CXCL13 analysis in cerebrospinal fluid for laboratory diagnosis of Lyme neuroborreliosis. *APMIS.* 2016;124(11):985-90.
187. Gyllemark P, Forsberg P, Ernerudh J, Henningsson AJ. Intrathecal Th17- and B cell-associated cytokine and chemokine responses in relation to clinical outcome in Lyme neuroborreliosis: a large retrospective study. *J Neuroinflammation.* 2017;14(1):27.
188. Kalish RA, Leong JM, Steere AC. Early and late antibody responses to full-length and truncated constructs of outer surface protein A of *Borrelia burgdorferi* in Lyme disease. *Infect Immun.* 1995;63(6):2228-35.
189. Lager M, Faller M, Wilhelmsson P, Kjelland V, Andreassen A, Dargis R, et al. Molecular detection of *Borrelia burgdorferi sensu lato* - An analytical comparison of real-time PCR protocols from five different Scandinavian laboratories. *PLoS One.* 2017;12(9):e0185434.
190. Wagner B, Freer H, Rollins A, Garcia-Tapia D, Erb HN, Earnhart C, et al. Antibodies to *Borrelia burgdorferi* OspA, OspC, OspF, and C6 antigens as markers for early and late infection in dogs. *Clin Vaccine Immunol.* 2012;19(4):527-35.
191. Goettner G, Schulte-Spechtel U, Hillermann R, Liegl G, Wilske B, Fingerle V. Improvement of Lyme borreliosis serodiagnosis by a newly developed recombinant immunoglobulin G (IgG) and IgM line immunoblot assay and addition of VlsE and DbpA homologues. *J Clin Microbiol.* 2005;43(8):3602-9.
192. Magnarelli LA, Lawrenz M, Norris SJ, Fikrig E. Comparative reactivity of human sera to recombinant VlsE and other *Borrelia burgdorferi* antigens in class-specific enzyme-linked immunosorbent assays for Lyme borreliosis. *J Med Microbiol.* 2002;51(8): 649-55.
193. Schulte-Spechtel U, Lehnert G, Liegl G, Fingerle V, Heimerl C, Johnson BJ, et al. Significant improvement of the recombinant *Borrelia*-specific immunoglobulin G immunoblot test by addition of VlsE and a DbpA homologue derived from *Borrelia*

- garinii for diagnosis of early neuroborreliosis. *J Clin Microbiol.* 2003;41(3):1299-303.
194. Coenye T, Vandamme P. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol Lett.* 2003;228(1):45-9.
195. Aberer E, Duray PH. Morphology of *Borrelia burgdorferi*: structural patterns of cultured borreliæ in relation to staining methods. *J Clin Microbiol.* 1991;29(4):764-72.
196. Laane MM, Mysterud I. A simple method for the detection of live *Borrelia spirochaetes* in human blood using classical microscopy techniques. *Biological and Biomedical Reports.* 2013;3(1):15-28.
197. Aase A, Hajdusek O, Oines O, Quarsten H, Wilhelmsson P, Herstad TK, et al. Validate or falsify: Lessons learned from a microscopy method claimed to be useful for detecting *Borrelia* and *Babesia* organisms in human blood. *Infect Dis (Lond).* 2016;48(6):411-9.
198. Kelly R. Cultivation of *Borrelia hermsi*. *Science.* 1971;173(3995):443-4.
199. Stoenner HG. Biology of *Borrelia hermsii* in Kelly medium. *Appl Microbiol.* 1974;28(4):540-3.
200. Preac-Mursic V, Wilske B, Schierz G. European *Borrelia burgdorferi* isolated from humans and ticks culture conditions and antibiotic susceptibility. *Zentralbl Bakteriell Mikrobiol Hyg A.* 1986;263(1-2):112-8.
201. Ruzic-Sabljic E, Strle F. Comparison of growth of *Borrelia afzelii*, *B. garinii*, and *B. burgdorferi sensu stricto* in MKP and BSK-II medium. *Int J Med Microbiol.* 2004;294(6):407-12.
202. Preac-Mursic V, Wilske B, Reinhardt S. Culture of *Borrelia burgdorferi* on six solid media. *Eur J Clin Microbiol Infect Dis.* 1991;10(12):1076-9.
203. Wormser GP, Bittker S, Cooper D, Nowakowski J, Nadelman RB, Pavia C. Comparison of the yields of blood cultures using serum or plasma from patients with early Lyme disease. *J Clin Microbiol.* 2000;38(4):1648-50.
204. Wilske B. Microbiological diagnosis in Lyme borreliosis. *Int J Med Microbiol.* 2002;291 Suppl 33:114-9.
205. Wormser GP, Nadelman RB, Schwartz I. The amber theory of Lyme arthritis: initial description and clinical implications. *Clin Rheumatol.* 2012;31(6):989-94.
206. Berger BW, Johnson RC, Kodner C, Coleman L. Cultivation of *Borrelia burgdorferi* from erythema migrans lesions and perilesional skin. *J Clin Microbiol.* 1992;30(2):359-61.
207. Picken MM, Picken RN, Han D, Cheng Y, Ruzic-Sabljic E, Cimperman J, et al. A two year prospective study to compare culture and polymerase chain reaction amplification for the detection and diagnosis of Lyme borreliosis. *Mol Pathol.* 1997;50(4):186-93.
208. Haddad O, Gillinov M, Fraser T, Shrestha N, Pettersson GB. Mitral Valve Endocarditis: A Rare Manifestation of Lyme Disease. *Ann Thorac Surg.* 2019;108(2):e85-e6.
209. Wallach FR, Forni AL, Hariprasad J, Stoeckle MY, Steinberg CR, Fisher L, et al. Circulating *Borrelia burgdorferi* in patients with acute Lyme disease: results of blood cultures and serum DNA analysis. *J Infect Dis.* 1993;168(6):1541-3.
210. Dumler JS. Molecular diagnosis of Lyme disease: review and meta-analysis. *Mol Diagn.* 2001;6(1):1-11.
211. Gooskens J, Templeton KE, Claas EC, van Dam AP. Evaluation of an internally controlled real-time PCR targeting the *ospA* gene for detection of *Borrelia burgdorferi sensu lato* DNA in cerebrospinal fluid. *Clin Microbiol Infect.* 2006;12(9):894-900.

212. Madigan MT, Martinko JM, Brock TD. Brock biology of microorganisms. Upper Saddle River, NJ: Pearson Prentice Hall; 2006.
213. Batzing BL. Microbiology: An Introduction: Brooks/Cole; 2002.
214. Buckingham L. Molecular Diagnostics: Fundamentals, Methods, and Clinical Applications : F.A. Davis Company; 2012.
215. Mommert S, Gutzmer R, Kapp A, Werfel T. Sensitive detection of *Borrelia burgdorferi* sensu lato DNA and differentiation of *Borrelia* species by LightCycler PCR. *J Clin Microbiol.* 2001;39(7):2663-7.
216. Life Technologies. Real-time PCR handbook 2012 [Available from: <https://www.gene-quantification.de/real-time-pcr-handbook-life-technologies-update-flr.pdf>].
217. Murphy JaB, S.A. qPCR, Theory, Reliability and Use in Molecular Analysis. *Molecular Analysis and Genome Discovery*2011. p. 43-55.
218. Wormser GP, Bittker S, Cooper D, Nowakowski J, Nadelman RB, Pavia C. Yield of large-volume blood cultures in patients with early Lyme disease. *J Infect Dis.* 2001;184(8):1070-2.
219. Al-Soud WA, Radstrom P. Purification and characterization of PCR-inhibitory components in blood cells. *J Clin Microbiol.* 2001;39(2):485-93.
220. Mayo clinic laboratories. *Borrelia miyamotoi* Detection PCR, Blood [Available from: <https://www.mayocliniclabs.com/test-catalog/Clinical+and+Interpretive/649702020>]
221. Hofmann H, Fingerle V, Hunfeld KP, Huppertz HI, Krause A, Rauer S, et al. Cutaneous Lyme borreliosis: Guideline of the German Dermatology Society. *Ger Med Sci.* 2017;15:Doc14.
222. Rauter C, Mueller M, Diterich I, Zeller S, Hassler D, Meergans T, et al. Critical evaluation of urine-based PCR assay for diagnosis of Lyme borreliosis. *Clin Diagn Lab Immunol.* 2005;12(8):910-7.
223. Dessau RB, van Dam AP, Fingerle V, Gray J, Hovius JW, Hunfeld KP, et al. To test or not to test? Laboratory support for the diagnosis of Lyme borreliosis - Author's reply. *Clin Microbiol Infect.* 2018;24(2):211-2.
224. Li X, McHugh GA, Damle N, Sikand VK, Glickstein L, Steere AC. Burden and viability of *Borrelia burgdorferi* in skin and joints of patients with erythema migrans or lyme arthritis. *Arthritis Rheum.* 2011;63(8):2238-47.
225. Jin C, Roen DR, Lehmann PV, Kellermann GH. An Enhanced ELISPOT Assay for Sensitive Detection of Antigen-Specific T Cell Responses to *Borrelia burgdorferi*. *Cells.* 2013;2(3):607-20.
226. Callister SM, Jobe DA, Stuparic-Stancic A, Miyamasu M, Boyle J, Dattwyler RJ, et al. Detection of IFN-gamma Secretion by T Cells Collected Before and After Successful Treatment of Early Lyme Disease. *Clin Infect Dis.* 2016;62(10):1235-41.
227. Kalish RA, McHugh G, Granquist J, Shea B, Ruthazer R, Steere AC. Persistence of immunoglobulin M or immunoglobulin G antibody responses to *Borrelia burgdorferi* 10-20 years after active Lyme disease. *Clin Infect Dis.* 2001;33(6):780-5.
228. Branda JA, Linskey K, Kim YA, Steere AC, Ferraro MJ. Two-tiered antibody testing for Lyme disease with use of 2 enzyme immunoassays, a whole-cell sonicate enzyme immunoassay followed by a VlsE C6 peptide enzyme immunoassay. *Clin Infect Dis.* 2011;53(6):541-7.
229. Crossland NA, Alvarez X, Embers ME. Late Disseminated Lyme Disease: Associated Pathology and Spirochete Persistence Posttreatment in Rhesus Macaques. *Am J Pathol.* 2018;188(3):672-82.

230. Murphy K, Weaver C. Janeway's immunobiology 2017.
231. Tortora GJ, Funke BR, Case CL. Microbiology - an introduction. Ninth edition ed. Benjamin Cummings, San Francisco: Pearson Education, Inc. ; 2007.
232. Goldsby RA, Kindt TJ, Osborne BA, Kuby J. Kuby immunology. 2000.
233. Theel ES. The Past, Present, and (Possible) Future of Serologic Testing for Lyme Disease. *J Clin Microbiol.* 2016;54(5):1191-6.
234. Gomes-Solecki MJ, Dunn JJ, Luft BJ, Castillo J, Dykhuizen DE, Yang X, et al. Recombinant chimeric *Borrelia* proteins for diagnosis of Lyme disease. *J Clin Microbiol.* 2000;38(7):2530-5.
235. Wilske B, Fingerle V, Schulte-Spechtel U. Microbiological and serological diagnosis of Lyme borreliosis. *FEMS Immunol Med Microbiol.* 2007;49(1):13-21.
236. Branda JA, Aguero-Rosenfeld ME, Ferraro MJ, Johnson BJ, Wormser GP, Steere AC. 2-tiered antibody testing for early and late Lyme disease using only an immunoglobulin G blot with the addition of a VlsE band as the second-tier test. *Clin Infect Dis.* 2010;50(1):20-6.
237. R&D systems. Luminex Assay Principle 2019 [Available from: <https://www.rndsystems.com/resources/technical/luminex-assay-principle>].
238. Thermo Fisher Scientific. How Luminex® Technology Works 2019 [Available from: <https://www.thermofisher.com/se/en/home/references/protein-analysis-guide/multiplex-assays-luminex-assays/how-luminex-technology-works.html>].
239. Leeflang MM, Ang CW, Berkhout J, Bijlmer HA, Van Bortel W, Brandenburg AH, et al. The diagnostic accuracy of serological tests for Lyme borreliosis in Europe: a systematic review and meta-analysis. *BMC Infect Dis.* 2016;16:140.
240. CDC. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. *MMWR Morb Mortal Wkly Rep.* 1995;44(31):590-1.
241. Tuuminen T, Hedman K, Soderlund-Venermo M, Seppala I. Acute parvovirus B19 infection causes nonspecificity frequently in *Borrelia* and less often in *Salmonella* and *Campylobacter* serology, posing a problem in diagnosis of infectious arthropathy. *Clin Vaccine Immunol.* 2011;18(1):167-72.
242. Wormser GP, Horowitz HW, Dumler JS, Schwartz I, Aguero-Rosenfeld M. False-positive Lyme disease serology in human granulocytic ehrlichiosis. *Lancet.* 1996;347(9006):981-2.
243. Pavletic AJ, Marques AR. Early Disseminated Lyme Disease Causing False-Positive Serology for Primary Epstein-Barr Virus Infection: Report of 2 Cases. *Clin Infect Dis.* 2017;65(2):336-7.
244. Magnarelli LA, Miller JN, Anderson JF, Riviere GR. Cross-reactivity of nonspecific treponemal antibody in serologic tests for Lyme disease. *J Clin Microbiol.* 1990;28(6):1276-9.
245. Hsieh YF, Liu HW, Hsu TC, Wei JC, Shih CM, Krause PJ, et al. Serum reactivity against *Borrelia burgdorferi* OspA in patients with rheumatoid arthritis. *Clin Vaccine Immunol.* 2007;14(11):1437-41.
246. Moore A, Nelson C, Molins C, Mead P, Schrieffer M. Current Guidelines, Common Clinical Pitfalls, and Future Directions for Laboratory Diagnosis of Lyme Disease, United States. *Emerg Infect Dis.* 2016;22(7).
247. Kodym P, Kurzova Z, Berenova D, Picha D, Smiskova D, Moravcova L, et al. Serological Diagnostics of Lyme Borreliosis: Comparison of Universal and *Borrelia* Species-Specific Tests Based on Whole-Cell and Recombinant Antigens. *J Clin Microbiol.* 2018;56(11).

248. Rupprecht TA, Manz KM, Fingerle V, Lechner C, Klein M, Pfirrmann M, et al. Diagnostic value of cerebrospinal fluid CXCL13 for acute Lyme neuroborreliosis. A systematic review and meta-analysis. *Clin Microbiol Infect.* 2018;24(12):1234-40.
249. Remy MM, Schobi N, Kottanattu L, Pfister S, Duppenenthaler A, Suter-Riniker F. Cerebrospinal fluid CXCL13 as a diagnostic marker of neuroborreliosis in children: a retrospective case-control study. *J Neuroinflammation.* 2017;14(1):173.
250. Tjernberg I, Henningsson AJ, Eliasson I, Forsberg P, Ernerudh J. Diagnostic performance of cerebrospinal fluid chemokine CXCL13 and antibodies to the C6-peptide in Lyme neuroborreliosis. *J Infect.* 2011;62(2):149-58.
251. Henningsson AJ, Lager M, Brannstrom R, Tjernberg I, Skogman BH. The chemokine CXCL13 in cerebrospinal fluid in children with Lyme neuroborreliosis. *Eur J Clin Microbiol Infect Dis.* 2018;37(10):1983-91.
252. Eldin C, Raffetin A, Bouillier K, Hansmann Y, Roblot F, Raoult D, et al. Review of European and American guidelines for the diagnosis of Lyme borreliosis. *Med Mal Infect.* 2019;49(2):121-32.
253. Peter J. Use and interpretation of tests in clinical immunology. Omaha: Interstate Press. 1990.
254. Tibbling G, Link H, Ohman S. Principles of albumin and IgG analyses in neurological disorders. I. Establishment of reference values. *Scand J Clin Lab Invest.* 1977;37(5):385-90.
255. Reiber H, Peter JB. Cerebrospinal fluid analysis: disease-related data patterns and evaluation programs. *J Neurol Sci.* 2001;184(2):101-22.
256. TeamRDC. R Development Core Team, R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria 2015. p. [Available from:<http://www.R-project.org/>].
257. Dickeson DJ, Chen SC, Sintchenko VG. Concordance of four commercial enzyme immunoassay and three immunoblot formats for the detection of Lyme borreliosis antibodies in human serum: the two-tier approach remains. *Pathology.* 2016;48(3):251-6.
258. Marangoni A, Moroni A, Accardo S, Cevenini R. Borrelia burgdorferi VlsE antigen for the serological diagnosis of Lyme borreliosis. *Eur J Clin Microbiol Infect Dis.* 2008;27(5):349-54.
259. Bremell D, Jacobsson G. Minska antalet borreliaserologier. *Läkartidningen.* 2018;115:E46E.
260. Seriburi V, Ndukwe N, Chang Z, Cox ME, Wormser GP. High frequency of false positive IgM immunoblots for Borrelia burgdorferi in clinical practice. *Clin Microbiol Infect.* 2012;18(12):1236-40.
261. Hillerdal H, Henningsson AJ. Serodiagnosis of Lyme borreliosis - is IgM in serum more harmful than helpful? Poster presented a International Symposium on Tick-Borne Pathogens and Diseases, ITPD; Vienna, Austria (8-11 September) 2019.
262. Dessau RB. Diagnostic accuracy and comparison of two assays for Borrelia-specific IgG and IgM antibodies: proposals for statistical evaluation methods, cut-off values and standardization. *J Med Microbiol.* 2013;62(Pt 12):1835-44.
263. Skogman BH, Lager M, Henningsson AJ, Tjernberg I. The recomBead Borrelia antibody index, CXCL13 and total IgM index for laboratory diagnosis of Lyme neuroborreliosis in children. *Eur J Clin Microbiol Infect Dis.* 2017;36(11):2221-9.
264. Tjernberg I, Kruger G, Eliasson I. C6 peptide ELISA test in the serodiagnosis of Lyme borreliosis in Sweden. *Eur J Clin Microbiol Infect Dis.* 2007;26(1):37-42.

265. Henningsson AJ, Christiansson M, Tjernberg I, Lofgren S, Matussek A. Laboratory diagnosis of Lyme neuroborreliosis: a comparison of three CSF anti-Borrelia antibody assays. *Eur J Clin Microbiol Infect Dis*. 2014;33(5):797-803.
266. Barstad B, Quarsten H, Tveitnes D, Noraas S, Ask IS, Saeed M, et al. Direct Molecular Detection and Genotyping of *Borrelia burgdorferi* Ssensu Lato in Cerebrospinal Fluid of Children with Lyme Neuroborreliosis. *J Clin Microbiol*. 2018;56(5).
267. Cerar T, Ogrinc K, Cimperman J, Lotric-Furlan S, Strle F, Ruzic-Sabljic E. Validation of cultivation and PCR methods for diagnosis of Lyme neuroborreliosis. *J Clin Microbiol*. 2008;46(10):3375-9.
268. Picha D, Moravcova L, Vanousova D, Hercogova J, Blechova Z. DNA persistence after treatment of Lyme borreliosis. *Folia Microbiol (Praha)*. 2014;59(2):115-25.
269. Ornstein K, Barbour AG. A reverse transcriptase-polymerase chain reaction assay of *Borrelia burgdorferi* 16S rRNA for highly sensitive quantification of pathogen load in a vector. *Vector Borne Zoonotic Dis*. 2006;6(1):103-12.
270. Marconi RT, Lubke L, Hauglum W, Garon CF. Species-specific identification of and distinction between *Borrelia burgdorferi* genomic groups by using 16S rRNA-directed oligonucleotide probes. *J Clin Microbiol*. 1992;30(3):628-32.
271. Panelius J, Lahdenne P, Saxen H, Heikkila T, Seppala I. Recombinant flagellin A proteins from *Borrelia burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii* in serodiagnosis of Lyme borreliosis. *J Clin Microbiol*. 2001;39(11):4013-9.
272. Boden K, Lobenstein S, Hermann B, Margos G, Fingerle V. *Borrelia miyamotoi*-Associated Neuroborreliosis in Immunocompromised Person. *Emerg Infect Dis*. 2016;22(9): 1617-20.
273. Raileanu C, Moutailler S, Pavel I, Porea D, Mihalca AD, Savuta G, et al. *Borrelia* Diversity and Co-infection with Other Tick Borne Pathogens in Ticks. *Front Cell Infect Microbiol*. 2017;7:36.
274. Stone BL, Brissette CA. Host Immune Evasion by Lyme and Relapsing Fever *Borreliae*: Findings to Lead Future Studies for *Borrelia miyamotoi*. *Front Immunol*. 2017;8:12.

Papers

The papers associated with this thesis have been removed for copyright reasons. For more details about these see:

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-170740>

FACULTY OF MEDICINE AND HEALTH SCIENCES

Linköping University Medical Dissertation No. 1744, 2020
Department of Biomedical and Clinical Sciences

Linköping University
SE-581 83 Linköping, Sweden

www.liu.se