Risk assessment and treatment of Lyme borreliosis in southern Norway

Examination of *Ixodes ricinus* ticks for prevalence and genotypes of Lyme disease and *Borrelia* species in southern Norway.

Terese Stave Marcussen

*Degree Thesis in Pharmacy 30 ECTS*
*Master’s Programme in Pharmaceutical Science*
*Report passed: May 6, 2013*
*Supervisor: Sven Bergström, Tobias Jakobsson*
**Abstract**

During the summer of 2012, host-seeking *Ixodes ricinus* ticks were collected from four different locations in southern Norway in order to determine the prevalence and treatment potential of *Borrelia burgdorferi* sensu lato (s.l.). Almost 30% of the 785 ticks collected were investigated for *Borrelia* by culture and polymerase chain reaction (PCR) amplification of the intergenic spacer sequence (IGS) rRNA gene. Cultured *B. garinii* and *B. afzelii* was further applied in an attempted in vitro antibiotic study where penicillin, tetracycline, azithromycin and cefotaxime were used to determine the minimum inhibition concentration (MIC) and minimal bactericidal/borreliacidal concentration (MBC). The overall result showed that 7.9% (7/89) of the nymphs and 21.1% (28/133) of the adults were infected by *B. burgdorferi* s. l. resulting in an infection rate of 15.8% (35/222). An underestimation of the infection rate is seen as 41% of the ticks were dead when examined. Therefore, a new calculation of the infection rate on living ticks was performed and found to be 23.7% (31/131). The most prevalent *B. burgdorferi* genospecies identified was *B. afzelii* 66.7% (4/6), followed by *B. burgdorferi* s. s. and *B. garinii* both with an infection rate of 16.7% (1/6). MIC and MBC was not determined as a result of practical problems in the experiment.
# Table of contents

Abstract ................................................................................................................................... I
Table of contents ....................................................................................................................... IV
1. Introduction ............................................................................................................................ 1
   1.1 History ............................................................................................................................... 1
   1.2 Biology .............................................................................................................................. 1
   1.3 Ticks in Norway ............................................................................................................... 2
   1.4 NorTick research ............................................................................................................. 3
   1.5 Lyme borreliosis ............................................................................................................... 4
      1.5.1 Dermatological manifestations ................................................................................. 4
      1.5.2 Nervous system manifestations .............................................................................. 5
      1.5.3 Cardiac manifestations ............................................................................................ 5
      1.5.4 Lyme arthritis ......................................................................................................... 5
   1.6 Diagnostic and treatment of Lyme borreliosis ................................................................. 5
   1.7 Vaccine against Lyme borreliosis .................................................................................... 7
   1.8 Aim of the master thesis ................................................................................................. 8
2. Methods ................................................................................................................................. 8
   2.1 Field site ........................................................................................................................... 8
   2.2 Tick collections ............................................................................................................... 9
   2.3 Cultivating *Borrelia* from ticks ........................................................................................ 9
   2.4 DNA extraction from ticks ............................................................................................. 9
   2.5 Polymerase chain reaction (PCR) .................................................................................... 10
   2.6 DNA sequencing ............................................................................................................ 10
   2.7 Cloning *Borrelia* DNA ................................................................................................. 10
   2.8 Antibiotic treatment and resistance .............................................................................. 10
   2.9 Statistical analysis .......................................................................................................... 11
3. Results ................................................................................................................................. 11
   3.1 Ticks in southern Norway .............................................................................................. 11
   3.2 Prevalence ....................................................................................................................... 12
   3.3 Genospecies .................................................................................................................... 13
   3.4 Treatment and resistance ............................................................................................... 13
4. Discussion ............................................................................................................................ 14
   4.1 Ticks in southern Norway .............................................................................................. 14
4.2. Prevalence ................................................................................................................................. 14
4.3. Genospecies ............................................................................................................................... 15
4.4. Treatment and resistance ........................................................................................................... 15
5. Conclusions .................................................................................................................................. 16
6. Acknowledgement ....................................................................................................................... 16
7. References ................................................................................................................................... 17
8. Appendix ..................................................................................................................................... 21
8.1. Terms and abbreviations ........................................................................................................... 1
1. Introduction

1.1 History
The history of Lyme borreliosis (LB) started in 1883 when a physician named Buchwald described Acrodermatitis chronicum atrophicans (ACA), a late skin manifestation caused by an ongoing infection of *Borrelia* (Buchwald, 1883). In 1902 Herxheimer and Hartman were able to clearly characterized ACA and many of their patients with the condition remembered having been bitten by *Ixodes ricinus* known as sheep tick (Herxheimer & Hartman, 1902). Some years later Dr. Afzelius described a new type of rash called erythema migrans (EM) at a dermatologic meeting in Stockholm, where EM is the rash appearing at the initial stage of Lyme borreliosis (LB). Dr. Afzelius suggested that the rash was a result from a tick bite or another insect (Afzelius, 1921). Many colleagues were unconvinced by his hypothesis, never to know that LB would be the most common vector-borne zoonosis, i.e. animal diseases that can be transmitted to humans or other animals through a vector (Bolzoni *et al.*, 2012).

The first reported case of EM with nervous system involvement in Norway was in 1950 leading to an increased focus on tick borne diseases (Bjornstad & Mossige, 1955). In 2008 the NorTick research network was established, as a result of an increased notified incidence of LB during the last two decades.

1.2. Biology
*Borrelia* is a bacterium in the class of spirochaetes where the most notable characteristic of them is the coiled appearance (Figure 1). Variations can occur between the genospecies but they are generally less than 1 µm thick and up to 30 µm long. *Borrelia* is only able to live within a tick or vertebrate host and has a generation time of 12-24 hours *in vitro* (Barbour, 1984). Culturing *Borrelia* from a tick or host is therefore a challenge since the need of an extremely nutritional medium, no oxygen and a long incubation time (up to several weeks).

![Figure 1](image1.png)

Figure 1: Microscopic picture of *Borrelia afzelii* cultivated from tick in Southern Norway.

The *Ixodes ricinus* tick specie is the primary vector responsible for spreading *Borrelia* in Norway. *Ixodes ricinus* has four life-stages: egg, larva, nymph and adult.
shown in figure 2. Ticks have a life cycle of 2-4 years depending on the geographic range thus in warmer areas the expected life time can be 2 years (Sonenshine, 1991). The tick feed three times during their life, once at each non-egg life stage (Radolf et al., 2010). The females lay several thousand of eggs, and the eggs are uninfected by Borrelia (Sonenshine, 1991). When the larva feeds on an infected host it becomes almost always infected with Borrelia. After detachment of the host, the larva hides for several months to develop into nymphs. When an infected tick consumes blood from a new host, it results in a metabolic reaction that prepares the spirochaetes for the transmission. It is unclear if the transfer is through active migration or passive transfer. The spirochaetes are in the lumen of the tick gut after 24 hours and are found in the entire gut tissue by 72 hours. After 48 hours of feeding the transmission occurs, therefore removing ticks within 24 hours is an efficient way to prevent infection (Pal & Fikrig, 2010).

Figure 2: Life-stages of Ixodes ricinus from egg to adult.

1.3. Ticks in Norway

Nine different tick species are found in Norway where Ixodes ricinus (figure 3) known as “skogflåtten” is the most common (Skarpaas, 2002). Borrelia burgdorferi sensu lato (s.l) consist of 12 different genospecies where B. garinii, B. afzelii and B. burgdorferi sensu stricto (s.s.) are most commonly found among Ixodes ricinus ticks in Norway. All three are human pathogens and known as LB-causing genospecies (Kjelland et al., 2010). Each genospecies is associated with different reservoir hosts: B. afzelii with rodents, B. garinii with birds and less frequently rodents, while B. burgdorferi s.s. is not so common and is therefore less seen in birds and rodents (Comstedt, 2008).
Figure 3: *Ixodes ricinus* adult female.

Ticks are found in areas where there are more than 175 days/year with a temperature above 5 °C, resulting in high prevalence in southern Norway. As a result to climatic changes the abundance of ticks in Northern Europe has increased, leading to higher notified incidence of LB (Danielová et al., 2010). In 2010, 288 cases of LB were reported to the Norwegian institute of public health (table 1). As much as 70% of the reported cases were in the time frame June-October and 77% of the incidences resulted in admission to hospital. Only cases fulfilling specific criteria’s as disseminated and chronic forms of LB are reported, leading to an underestimation of LB incidences and making it difficult to compare the extent between countries (Folkehelseinstituttet, 2012). For instance, in Sweden around 10000 people are diagnosed each year with LB as a result of erythema migrans fulfilling the Swedish criteria’s. EM is not reported in Norway as it is not a disseminated or chronic form of LB.

Table 1: Cases of tick-borne disease in Aust-Agder (58° 27’ 34” N, 8°46’ 0” E) and Norway from 2004-2011 (Folkehelseinstituttet, 2012).

<table>
<thead>
<tr>
<th>Lyme Borreliosis</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aust–Agder</td>
<td>34</td>
<td>27</td>
<td>17</td>
<td>32</td>
<td>26</td>
<td>15</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Norway</td>
<td>255</td>
<td>280</td>
<td>316</td>
<td>328</td>
<td>345</td>
<td>274</td>
<td>288</td>
<td>247</td>
</tr>
</tbody>
</table>

### 1.4. NorTick research

The increase in ticks and tick borne diseases in Southern Norway led to an initiative from the health service in Arendal to establish NorTick research in 2008. NorTick network consists of a group of specialized researcher with scientific experience in different fields such as, microbiology, bacteriology, virology, veterinary medicine and both human and community medicine. The aim of the network is to initiate and conduct medical research within ticks and tick borne diseases. This includes new knowledge about treatments and diagnosis, tick distribution, life-cycle, habitat, transfer of the disease to humans and animals, and the frequency of infection in ticks (NorTick research). NorTick has co-operation and contacts with Danish and Swedish research networks. The main project currently ongoing is the tick-study where tick-
bitten individuals voluntarily can deliver the tick to health centers linked to the study. The study has its base in Linköping, Sweden, and Arendal is the only place in Norway where this project is present. The aim with that study is to collect a total of 10,000 ticks, including blood samples from people bitten by tick taken at the time of tick removal and three months later to analyze the prevalence of Lyme borreliosis, TBE-virus and Anaplasmosis. The working name of this study is «Stingstudien».

1.5. Lyme borreliosis

Lyme borreliosis is a multisystemic infectious disease where various clinical manifestations occur in which Erythema migrans (EM) is the most common sign. In a study performed in Germany on 313 cases with LB, 89% of the patients had EM, 5% presented with arthritis, 3% had early neurological manifestations, 2% had borreial lymphocytoma, 1% had ACA and less than 1% had cardiac manifestations. None of the cases presented with late neurological LB (Huppertz et al., 1991).

*B. garinii, B. afzelii and B. burgdorferi s.s.* are all known to cause LB in Norway, although *B. garinii* and *B. afzelii* infections accounts for most cases. Infections by *B. afzelii* is known to cause skin manifestations such as EM and ACA, *B. garinii* seems to be more related to neurotropic symptoms while infections by *B. burgdorferi s.s.* are associated with arthritic symptoms (Stanek et al., 2012).

1.5.1 Dermatological manifestations

There are three types of skin manifestations of LB. EM, borreial lymphocytoma (BL), and ACA (Müllegger & Glatz, 2008).

Erythema migrans appears at the initial stage of infection by *B. burgdorferi* s.l. and is the most common clinical manifestation of LB (Stanek et al., 2012). EM occurs as an expanding, annular, erythematous, red or bluish-red skin rash where the lesion appears 7-14 days after detachment of the tick. The rash is characteristics and known as a bull’s eye rash. It expands around 20 cm² per day, due to migration of the spirochaetes away from the bite, resulting in a local inflammatory response (Radolf et al., 2010). Without treatment, the rash can reach 1 m in diameter (Helsedirektoratet, 2009). The EM lesion can appear everywhere on the body in children and is mostly seen under the waist in adults, as a result of children crawling around in the grass. Other symptoms of EM can be flu-like with low-grade fever and chills, also fatigue, headache and malaise are seen, and the symptoms can vary from non to mild (Radolf et al., 2010 p. 508). EM is diagnosed by the clinical picture and not by lab results since fewer than 50% of patients rarely produce antibodies so early in the stage of the disease. One exception is when EM has persisted for more than six weeks, then the sensitivity of a skin biopsy result would be 50-70% (Helsedirektoratet, 2009).

Acrodermatitis chronica atrophicans is known as a late skin manifestation caused by an ongoing infection by *B. afzelii* and is typically seen 10 years after the tick bite (Radolf et al., 2010). The characteristic symptom of ACA is the presences of inflammation of the skin with a bluish red discoloration with swelling and edema (Chodynicka, 2012). Leaving ACA untreated will result in the lesion becoming atrophic or sclerotic (Radolf et al., 2010 p. 510). It is mandatory to take serologic and histologic tests to confirm the diagnosis (Helsedirektoratet, 2009).
Borrelial lymphocytoma is a rare subacute lesion that may occur simultaneously with the infection or months later (Radolf et al., 2010). BL often last longer than EM and the characteristic with it is the site where the lesion is seen, as the most common site is the ear lobe in children and nipple areas in adults. BL has also been seen in the genital areas in adults (Strle et al., 1996). BL is as EM a manifestation that is diagnosed by the clinical picture (Helsedirektoratet, 2009).

1.5.2. Nervous system manifestations
When *Borrelia* spirochaetes migrates from the tick bite it can spread to various organs that includes the heart, joint, peripheral and central nervous system (CNS). The invasion of spirochaetes into the peripheral and CNS leads to neurological complication. The spirochaetes has to invade the human immune system and also cross the blood-brain barrier. How the spirochaetes manage this is not clarified in detail (Rupprecht et al., 2008). In a study done in North America 15 % of patients with untreated EM developed meningitis or cranial neuritis (Radolf et al., 2010 p. 510). Antibodies must be found in the spinal fluid to confirm the LB diagnosis (Helsedirektoratet, 2009).

1.5.3. Cardiac manifestations
The incidence of Lyme carditis has decreased dramatically as a result of greater attention to the disease. Most patients make a complete recover, and the disease is benign. The manifestation rarely results in death (Pinto, 2002). Patients with Lyme carditis develops acute onset of varying degree of atrioventricular heart block (Radolf et al., 2010 p. 511). Serologic and histologic tests are required to confirm diagnosis (Helsedirektoratet, 2009).

1.5.4. Lyme arthritis
Lyme arthritis (LA) is the most common manifestation from an ongoing infection by *B. burgdorferi* s.s. (Radolf et al., 2010). A previous study showed that 34 out of 55 patients who presented with EM and not treated with antibiotics developed LA (Steere et al., 1986). LA is episodes of inflammation in the joints that can result in swellings but rarely give any pain. The knee is the most affected joint (Radolf et al., 2010). Earlier studies has showed that most patients who have LA have no recollection of being bitten by a tick nor showed any sign of early clinical manifestation of LB (Stanek et al., 2012). It is mandatory to take serologic and histologic tests to confirm the diagnosis in Norway (Helsedirektoratet, 2009).

1.6. Diagnostic and treatment of Lyme borreliosis
In Europe and North America there are specific criteria’s that require supportive laboratory findings to confirm the diagnostic of LB. The direct detection of spirochaetes is considered as diagnostic golden standard. The usefulness of this in clinical practice is however small as culture and PCR has high sensitivity in skin biopsy for EM (up to 80%) and a low sensitivity in urine and cerebrospinal fluid for Lyme neuroborreliosis 7% and 17%, respectively (Lebech et al., 2000). Since EM and BL are the only manifestations diagnosed by the clinical picture the need for laboratory findings are small. In Norway the detection of specific antibodies against *B. burgdorferi* s.l. is the first choice of methods used in the diagnostics of LB, as antibodies are the result of complex immune responses when spirochaetes are infecting humans. Although, anti-Lyme disease *Borrelia* antibodies provide evidence
of the infection, it can also represent antibodies from a prior infection. As a result to low sensitivity and specificity for laboratory findings a 100% certain diagnosis will never be defined (Helsedirektoratet, 2009). There are many different methods used in Norway to detect antibodies from *Borrelia* infection, however these methods will not be discussed here. For further reading the annual rapport from “helsedirektoratet” concerning diagnostic and treatment of Lyme borreliosis 2009 is recommended.

It is important to determine the most clinically effective regimen as treatment failures occur in 5 to 10% of EM patients (Hunfeld *et al.*, 2005). Antibiotics have been an effective treatment since LB was first identified as a disease in 1982, however reports of treatment failure have been made for almost every suitable antimicrobial agent used. The mistreatment of antibiotics may cause resistances or development of late stage manifestations of LB. The current understanding about *Borrelia* and antibiotics resistance is limited and is largely based on vitro studies (Hunfeld & Brade, 2006). *Borrelia* is known to be sensitive to tetracycline, most penicillin's, many second- and third-generations cephalosporins and macrolides. It has shown resistance to specific fluoroquinolones, rifampicin and first-generation cephalosporins (Stanek *et al.*, 2012). In 2005 an in vitro susceptibility study showed that 1.7% of the patients with culture-confirmed EM had persistent borrelia infection after antibiotic treatment. If this is due to resistance is not clarified, however the speculations are supported by the generation of specific genetic mutation in *parC* and *16S-rDNA* (Hunfeld *et al.*, 2005). Many studies has been performed to determine the minimum inhibitory concentration (MIC) and minimum borreliacidal/bactericidal concentration (MBC) of antimicrobial agents for *Borrelia*, but as a result to differences in test conditions a wide variability of published MIC and MBC values are seen. For instance the reported MIC of penicillin G varies from 0.003µg/ml to 8µg/ml and the MBC from 0.05µg/ml to >50µg/ml (Hunfeld & Brade, 2006).

The Norwegian recommendation for treatment of different manifestations of Lyme borreliosis is shown in table 2. Azithromycin can be used as an alternative when the patients treated are under 8 years of age, are pregnant, or are allergic to penicillin (Helsedirektoratet, 2009).

Table 2: Treatment of different manifestations of Lyme Borreliosis (Helsedirektoratet, 2009).
Controversy exists regarding the diagnosis and treatment of LB in Norway, as many patients with persistent symptoms after standard (2-4 weeks) antibiotic therapy are denied new treatment. Patients with these persisting symptoms seek advice from private health care where doctors are prescribing long-term use of antibiotics. The prolonged use has many side effects where the most serious is the development of resistance against certain antibiotics. A randomized trial was performed in 2001 where patients with persisting symptoms after the standard therapy were treated with long-term use of antibiotics. The findings from the study showed that treatment with oral and intravenous antibiotics for 90 days did not improve the symptoms more than placebo. The study was discontinued after the first 107 patients as a result of no differences seen between the placebo group and the group treated (Klempner et al., 2001).

1.7. **Vaccine against Lyme borreliosis**

The most efficient way to prevent against LB is to avoid tick bites. This is, as many know, not always possible, especially in high risk areas. In 1998 the company SmithKline Beecham in USA produced a vaccine against LB known as LYMErix (Meltzer et al., 1999).

*Borrelia* spirochaetes contain several lipoproteins in the cell wall, one of them is the outer surface protein A (OspA). The spirochaetes express OspA on their surface when grown in culture and within the tick vector. The recombinant OspA Lymerix vaccine when introduced into vertebrate hosts, including humans, will evoke an immunological response and create anti-OspA antibodies. Subsequently, when the tick feeds on the host the antibodies kills the spirochaetes in the tick gut, and therefore prevents transmission by killing the spirochetes before they migrate to the salivary gland and into the host (Bergström & Zückert, 2010). The vaccine was

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Disease</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>Uncomplicated Erythema migrans</td>
<td>•Fenoxymethypenicillin 1.3 g/2 MIE x3 for 10-14 days.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregnant ➔ Fenoxymethypenicillin 1.3 g x4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Allergi ➔ Doxycycline 200 mg x1 for 10-14 days.</td>
</tr>
<tr>
<td>Multi Erythema migrans</td>
<td></td>
<td>•Doxycycline 200 mg x1 (or 100 mg x2) for 14 days.</td>
</tr>
<tr>
<td>Lymphoedema</td>
<td></td>
<td>•Doxycycline 200 mg x1 (or 100 mg x2) for 14 days.</td>
</tr>
<tr>
<td>Acrodermatitis chronic atrophicans</td>
<td></td>
<td>•Doxycycline 200 mg x1 for 3 weeks</td>
</tr>
<tr>
<td>Neurologic disease</td>
<td>Early neuroborreliosis</td>
<td>•14 days of treatment with oral doxycycline (200 mg x 1), IV ceftriaxone (2g x 1), IV Penicillin (5 million IU x 4) or IV cefotaxine (2g X 3). If symptoms of central nervous system ➔ IV ceftriaxone</td>
</tr>
<tr>
<td></td>
<td>Late neuroborrellosis</td>
<td>•Three weeks of treatment with oral doxycycline (200 mg x 1) or IV ceftriaxone (2g x 1). If symptoms of central nervous system ➔ IV ceftriaxone (2g x 1)</td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>Carditis</td>
<td>•Not specified therapy recommendation</td>
</tr>
<tr>
<td>Joint, muscle</td>
<td>Arthritis</td>
<td>•Doxycycline 200 mg x1 for 3 weeks</td>
</tr>
</tbody>
</table>
effective but protected only infection by *B. burgdorferi s.s.* After four years on the market the vaccine was withdrawn. The reasons were poor sales and high cost per year compared to antibiotic treatment. The vaccine was also not recommended for children under age 15 (Meltzer *et al.*, 1999). Although, the main reason was probably the fear of lawsuits, as a direct result from suspected cases of side effect from the vaccine, especially autoimmune arthritis. This has however never been proven (Silverman, 2002).

Today there is no prophylactic vaccine for humans against Lyme disease on the market, then again many scientists are trying to develop new vaccines and several studies are currently ongoing in Europe.

### 1.8. Aim of the master thesis
The aim of this project was to examine the prevalence of *Borrelia* infection in ticks and identify the different genospecies of *Borrelia burgdorferi* s.l in different areas in Southern Norway. Also, the *Borrelia* found in ticks in Norway was tested against different classes of antibiotics to determine the best treatment.

### 2. Methods

#### 2.1. Field site
*Ixodes ricinus* nymphs and adults were collected from four different locations in the area Aust-Agder in southern Norway. From 08.07.12 to 05.11.12, seven periodic samplings were done. Area N2 was replaced by area N4 due to high presence of adder and a low prevalence of ticks. Figure 4 presents the area and the collection sites, while figure 5 shows the vegetation at the different locations. The four locations are separated, apart from N2 and N4, which are on the same island. Roe deer, badger, fox, hare and small rodents has been observed on all four locations, as well as moose at location N3 (Personal communication, local hunter 2012).

![Figure 4: Map of southern Norway on left, where the red circle indicates the area, and the selected locations on right. N1 (black) = Dahl, N2 (red) = Tromsøya, N3 (blue) = Godderstad and N4 (green) = Tromsøya.](image-url)
Figur 5: Vegetation at the collection site N1= Dahl, N2= Tromøya, N3= Godderstad and N4 =Tromøya.

2.2. Tick collections

In July, August, October and November of 2012, host seeking ticks were collected by drag sampling method, as drag sampling has been shown the best method to maximize number of ticks (Falco & Fish, 1992). Flannel sheets were dragged for 10 meters and turned over for collection of ticks. The size of the study area differed as a result to the prevalence of ticks, but the same route was performed each time. The time spent at the locations was from 40 to 45 min. The sampling was only preformed under dry weather conditions, resulting in variation of sampling due to suddenly rain. Every collection was done between 10 a.m. to 16 p.m. Temperature ranged from 16-21˚C in July and August, 8-10˚C in October and 5˚C in November. The ticks were transferred to 50 ml tubes and stored away at 4˚C until the analyses were done in Umeå at the end of November 2012. Tubes were opened once a week for new air supply and Kleenex paper was added to prevent condense. The ticks were not identified under the microscope to establish the species, but the main type of tick in Norway is Ixodes ricinus.

2.3. Cultivating Borrelia from ticks

Ticks were washed in 70% ethanol for removal of bacteria on the surface and cut longitudinally in two halves using a sterile blade. One half was stored at -20˚C for DNA extraction, while the other half was used for cultivation of Borrelia. BSKII medium supplemented with 7% rabbit serum (RS) (Barbour, 1984) was transferred to a 2 ml test tube containing the half tick. BSKII 7% RS was further supplemented with two types of antibiotic; Phosphomycin (P) and Sulfamethoxazol (S), and a fungicide Amphoterecin B (Am) to prevent contamination of other bacteria and fungus. The tubes were incubated at 35˚C for several weeks and examined for viable Borrelia under the microscope. The negative tubes were stored away for later examinations while the positive tubes were transferred to larger tubes with fresh BSKII 7% RS medium. When the Borrelia in the positive tubes had grown to a fine culture they were stored away in the freezer with 10% glycerol at -83˚C for the antibiotic resistant part.

2.4. DNA extraction from ticks

DNA was extracted from 222 ticks using DNeasy blood and tissue kit (Qiagen) and stored at -20˚C until PCR were done. The extraction procedure was carried out according to the manufacturer’s instructions.
2.5. Polymerase chain reaction (PCR)

DNA extracted from ticks is in small amounts. PCR were used to amplify DNA into larger quantities in a few hours. Specific forward and reverse primers for a *Borrelia* target gene were used, including taq polymerase, 10x PCR buffer, 10xdNTP-mix and positive and negative controls to detect any errors. The positive control contained *Borrelia burgdorferi* B31. After the first PCR new forward and reverse primers were added to do a nested PCR to amplify the DNA further. Both PCR and nested PCR amplified the IGS (intergenic spacer sequence) region between the rrs (16s) and rrl (23s) *Borrelia* rRNA gene (Postic D et al., 1994). The forward (F), reverse (R), forward-nested (Fn), and reverse-nested (Rn) primers and reaction condition used are as follows:

F, 5′-GTATGTTTAGTGAGGGGGGTG-3′, R, 5′-GGATCATAGCTCAGGTGGTTAG-3′, Fn, 5′-AGGGGGTGAAGTCGTAACAAG-3′, Rn, 5′-GTCTGATAAACCTGAGGTCGGA-3′.

PCR: 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 75°C for 2 min, ending with 74°C for 2 min.

PCR nested: 95°C for 5 min, followed by 39 cycles of 94°C for 30 s, 60°C for 30 s, 74°C for 2 min, ending with 74°C for 7 min.

TBE (Tris/Borate/EDTA) buffer with 1% agarose was used as a separation gel to examine the PCR nested product for the presence of *Borrelia* DNA as TBE and agarose allows DNA to pass the gel. Molecular weight (MW) ladder was used to identify the band size of the DNA while the positive control showed *B. burgdorferi* B31 compared to the molecular weight ladder. The bands from a co-infected tick were separated by using 3% agarose gel as the gel becomes less viscous.

2.6. DNA sequencing

The samples that showed presence of DNA on the agarose gel were purified with two different methods depending on if the tick was co-infected or not. GeneJet gel extraction kit (Fermentas) was used when the DNA was collected from the agarose gel, while the high pure PCR product purification kit (Roche) was used on the PCR nested product. The purification procedure was carried out according to the manufacturer’s instructions. The purified products were prepared by BigDye and delivered in for sequencing, and a program called BLAST was used to identify the sequence of the different genospecies.

2.7. Cloning *Borrelia* DNA

DNA was cloned when the DNA sequencing failed to detect any DNA in the sample. The cloning was prepared using the purified nested PCR product with Thermo scientific Instaclone PCR cloning kit (Thermo). The products were added to competent *Escherichia coli* cells and electro eluted. The cells were then transferred to petri dishes and incubated at 37°C overnight. The white colonies obtained were transferred to tubes containing MQ (purified water) and heat shocked. PCR was done on the sample to amplify the DNA and further added on agarose gel where obtained bands indicated successful cloning. The PCR products that were successfully cloned were prepared and delivered in for DNA sequencing as mentioned above.

2.8. Antibiotic treatment and resistance

*B. garinii* and *B. afzelii* cultivated from ticks were tested against four different types of antibiotics: penicillin, tetracycline, azithromycin and cefotaxime, all of them
known to be an effective treatment against infection by *Borrelia* (Hunfeld et al., 2000). The method used was a modified version from a previously publication by Hunfeld, et al., 2000. The antibiotics used were diluted in the following concentrations: 1:2, 1:4, 1:8, 1:64, 1:256, 1:1024 and 1:5096. The plate was incubated at 35°C with 1% CO₂ for three days with measurements taken after 0, 24, 48 and 72 hours. The procedure was carried out to determine the minimal inhibitory concentrations (MIC) by measuring the colour changes in the wells resulting from spirochaetes metabolizing after 72 hours of incubation. The lowest concentration of antibiotic where no colour changes was observed was determined as MIC. The wells that showed no change in absorbance were transferred to fresh BSKII 7% RS medium and incubated in the same conditions as above for 3 weeks. The cultivations were then examined under the microscope for the presence or absence of viable spirochaetes to determine the minimal borreliacidal concentration (MBC) that is defined as the lowest concentration of antibiotics where no viable spirochaetes is seen (Hunfeld K P, et al 2000)

2.9. Statistical analysis

Chi-square is a method used to test the likelihood that the observed results are due to a change or not. Probability (p) can be further calculated by the chi-square value. These tests are used to calculate the prevalence of *B. burgdorferi* s.l. in adults and nymphs and to see if the result from this study is a significant result.

3. Results

3.1. Ticks in southern Norway

Host-seeking *Ixodes ricinus* ticks were investigated for the prevalence of *B. burgdorferi* s.l. by two different methods: cultivation and PCR. Altogether 785 ticks were collected from four different locations: Dhal (N1), Tromøya (N2), Godderstad (N3) and Tromøya (N4). The ticks collected comprised two different groups, 54.3% (426/785) nymphs and 45.7% (359/785) adults. The larvae were excluded from this study. At each location 3-4 periodic samplings were performed. The area with largest amount of ticks was at location N1 where 283 ticks were collected. The other areas showed an occurrence of 111 ticks at N2, 156 at N3 and 235 at N4. In total 49.5% (211/426) of the nymphs were collected from N1, while 47.6% (171/359) of all the adults were collected from N4. The periodic sampling started in July and continued in periods until early November. Due to variations in periodic sampling at the four areas, calculations of the infection rate at different sample times were not possible. Table 3 shows the prevalence of nymphs and adults at the different areas at specific dates.
Table 3: Number ticks collected, the area and the date.

<table>
<thead>
<tr>
<th>Area</th>
<th>08.07.12</th>
<th>17.07.12</th>
<th>21.07.12</th>
<th>05.08.12</th>
<th>15.08.12</th>
<th>11.10.12</th>
<th>05.11.12</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nymphs</td>
<td>22</td>
<td>105</td>
<td></td>
<td>77</td>
<td>7</td>
<td></td>
<td></td>
<td>211</td>
</tr>
<tr>
<td>N1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adult</td>
<td>6</td>
<td>58</td>
<td></td>
<td>7</td>
<td>1</td>
<td></td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>N2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nymphs</td>
<td>20</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>N2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adult</td>
<td>30</td>
<td>26</td>
<td>12</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>N3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nymphs</td>
<td>21</td>
<td>34</td>
<td>60</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>124</td>
</tr>
<tr>
<td>N3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adult</td>
<td>17</td>
<td>5</td>
<td>5</td>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td>32</td>
</tr>
<tr>
<td>N4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nymphs</td>
<td></td>
<td></td>
<td></td>
<td>21</td>
<td>24</td>
<td>19</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>N4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
<td></td>
<td>63</td>
<td>79</td>
<td>29</td>
<td></td>
<td>171</td>
</tr>
</tbody>
</table>

3.2. Prevalence

Only 16.8% (131/785) of the ticks were still alive when the laboratory project started in Umeå at the end of November 2012. In total, 24 nymphs and 107 adults were examined for presence of *B. burgdorferi* s.l. through cultivations. Of the ticks that were culture positive 106 were collected from area N4, 12 from N1, 9 from N2 and 4 from N3, leading to an uneven distribution of ticks/area. In total, 16.03% (21/131) of the cultures showed presence of viable *Borrelia* when examined under the microscope, two nymphs and 19 adults. This results in an infection rate of 8.3% (2/24) for the nymphs and 17.8% (19/107) for the adults. Each area had presence of infected ticks where 8.3% (1/12) of the ticks at N1 was infected, 66.7% (6/9) at N2, 25% (1/4) at N3 and 12.3% (13/106) at N4. However a significance prevalence calculation could not be done, due to the uneven distribution of the ticks/area. These results can therefore not be considered as the real infection rate/area.

A total of 222 ticks were tested by PCR to detect the prevalence of *Borrelia* even though only 59% (131/222) of the ticks were alive when the DNA extraction was performed. As much as 47.7% (106/222) of the ticks were collected from area N4, 24.8% (55/222) from N3, 23.4% (52/222) from N1 and 4.1% (9/222) from N2. The PCR showed that 19 out of 222 ticks were positive for *Borrelia*, resulting in an infection rate of 8.6% (19/222). When looking at the ticks that were alive a much higher value 11.5% (15/131) was presented. Figure 6 shows the result from the first five ticks. The figure includes the MW ladder to determine the size of the DNA band, the five first samples, and the positive and negative control to detect any errors in the PCR. Samples 1, 4 and 5 show the presence of *Borrelia* DNA, where sample 1 has three different bands indicating different DNA fragment size, probably as a result of a tick being co-infected by different genospecies of *B. burgdorferi* s.l. Sample one was an adult and the only co-infected tick detected in this project. It was not possible to identify the genospecies from two of the three bands. The band in the middle was identified by DNA sequencing, but it was not possible to determine if the tick was infected with *B. afzelii* or *B. garinii.*
Figure 5: Presence of *Borrelia* DNA in sample 1, 4 and 5. Sample 1 shows 3 different bands, indication co-infection by different genospecies of *Borrelia*.

The results from PCR and cultivations from all the 222 ticks examined showed a prevalence of 7.9% (7/89) for the nymphs and 21.1% (28/133) for the adults presenting a total infection rate of 15.8% (35/222). Calculating the prevalence of the 133 ticks alive, a much higher prevalence was presented where 12.5% (3/24) of the nymphs and 26.2% (28/107) of the adults were infected leading to a total infection rate of 23.7% (31/131).

### 3.3. Genospecies

Only 6 out of 35 positive samples was identified as an specific genospecies where the most prevalent genospecies identified was *B. afzelii* 66.7% (4/6), followed by *B. burgdorferi s.s* and *B. garinii* both with an infection rate of 16.7% (1/6).

Unfortunately, 34.3% (12/35) of the ticks were undefined, as a result of that BLAST could not identify the genospecies by the DNA sequencing from the purified nested PCR product. As much as 48.5% (17/35) of the ticks that were positive for *Borrelia* was not identified to any genospecies by sequencing. The DNA sequencing results from PCR and cultivation is shown in table 4.

Table 4: *B. burgdorferi* s.l. genospecies in *Ixodes ricinus* ticks in southern Norway. Left table shows the result from all the ticks examined, while the right table shows the result from the 131 live ticks examined.

<table>
<thead>
<tr>
<th></th>
<th>Nymphs</th>
<th>Adults</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. ticks tested</td>
<td>89</td>
<td>133</td>
<td>222</td>
</tr>
<tr>
<td>No. positive</td>
<td>7</td>
<td>28</td>
<td>35</td>
</tr>
<tr>
<td>No. % positive</td>
<td>7.9</td>
<td>21.1</td>
<td>15.8</td>
</tr>
<tr>
<td>Genospecies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. garinii</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>B. afzelii</em></td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>B. burgdorferi s.s.</em></td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>3</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>Not found</td>
<td>3</td>
<td>14</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Nymphs</th>
<th>Adults</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. ticks tested</td>
<td>24</td>
<td>107</td>
<td>131</td>
</tr>
<tr>
<td>No. positive</td>
<td>3</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>No. % positive</td>
<td>12.5</td>
<td>26.2</td>
<td>23.7</td>
</tr>
<tr>
<td>Genospecies</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. garinii</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>B. afzelii</em></td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>B. burgdorferi s.s.</em></td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Indeterminate</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Not found</td>
<td>1</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

### 3.4. Treatment and resistance

It was not possible to determine MIC or MBC by the findings of the in vitro antibiotic experiment as the result obtained showed viable spirochaetes in all the wells.
containing penicillin, tetracycline and cefotaxime, and as the opposite occurred for azithromycin where no viable spirochaetes were observed.

4. Discussion

4.1. Ticks in southern Norway
This study presented a finding of an uneven distribution of ticks/area/time of collection. One major problem was that the majority of the ticks were dead upon arrival to the laboratory. As many as 81% of the ticks that survived was collected from the same location leading to problems performing statistical analysis, hence similar distribution of ticks is required. The likely reason for the death is low humidity in the test tubes when storing the ticks, as relative humidity below 80% will lessen their chances to survive (Knap et al., 2009). As condense was observed in the tubes during storage of the live ticks this is a likely explanation for the large amount of dead ticks.

One of the aims with the study was to calculate the infection rate at different sample times and areas. Unfortunately, it was not possible to perform statistical analyses as a result to the dead ticks since similar distribution is required. As a result to this the found infection rate presenting in the study is the sum of all four locations in Arendal.

4.2. Prevalence
The overall result showed that 7.9% of the *Ixodes ricinus* nymphs and 21.1% of the *Ixodes ricinus* adults were infected by *B. burgdorferi* s.l. leading to an infection rate of 15.8%. The difference between the infection rate of nymphs and adults is explained by the spirochaetes being transmitted through blood meals, and where the risk of infection is increased with the number of blood meals from different hosts. Several studies have been performed in Norway to determine the prevalence of *Borrelia* infected ticks. In 2001 Jenkins et al., studied two different sites in southeastern Norway and found that 15% of the nymphs and 16% of the adults were infected. In 2008 Paulauskas et al., studied nine locations at different parts of Norway and found that 13% of adults and 5.2% of nymphs were infected. The most recent study was done in 2010 by Kjelland et al. where they studied four different locations in southern Norway, one of the location was at the same island, Tromøya, as in the present study. The findings from Tromøy showed that 22.1% of the nymphs and 28.9% of the adults were infected resulting in an overall infection rate of 22.1% (Kjelland et al., 2010). The measured prevalence of *B. burgdorferi* s.l. in Kjelland et al., study is thought to reflect the true infection rate due to precautions taken at every step of the analysis. The studies by Jenkins and Paulauskas et al. were done in other areas in Norway where the density of ticks is less than seen in southern Norway. Since the study done by Kjelland et al. is the most recent study and also performed at the same location as the present study there should be more similarities. Observed differences can be a result of practical problems in the present study. For starters, the ticks collected were not examined for *Borrelia* directly, but stored for a couple of months resulting in the death of many ticks. By calculating the prevalence of the 131 ticks that were alive the result showed a much higher value that more easily can be related to the study done by Kjelland et al.. Results from the analysis of live ticks in the present
study show that 12.5% of the nymphs and 26.2% of the adults were infected, leading to an overall infection rate of 23.7%. If the prevalence rate is calculated from results obtained by analysis of dead tick the infection rate is as low as 4.4% (4/91). These calculations show that the dead ticks leads to an underestimation of the prevalence of *Borrelia* infections in ticks. A suggestion to this can be that when any living organism dies metabolic reactions occur where nuclease and protease degrades DNA nucleotides. This is only speculations and was not verified in this study. The difference seen between the infection rate of the dead and alive can also be a result of that the live ticks were examined for the prevalence of *Borrelia* by two different methods, PCR and cultivation, while the dead were only examined by PCR. Cultivation is an accurate method in the way that the ticks are positive when viable *Borrelia* is seen under the microscope. However, the problem with cultivation is that the method is time consuming and sensitive to errors due to the spirochaetes need of a nutrient rich medium that easily becomes contaminated by other microbes. PCR on the other hand is a faster method where presence of *Borrelia* DNA is visualized clearly in the agarose gel after one day of work. However, probes and primers can confuse the result and a specific amount of *Borrelia* DNA is needed to present a result. Some ticks were determined positive in PCR and not by the cultivation and vice versa. Only five of all 31 positive samples were positive in both methods, resulting in that the two methods complement each other.

### 4.3. Genospecies

Only 17.1% (6/35) of the positive samples were identified to a specific genospecies, were the dominant was *B. afzelii* with a percentage of 66.7% (4/6). The observation can relate well to the literature where *B. afzelii* is associated with dermatoborreliosis symptoms and EM is the most common manifestation of LB in Norway. The result cannot be considered as significant without statistical calculations, but by comparing the result with findings made by Kjelland *et al.*, 2010 a clear similarity is observed. As much as 69.3% (61/88) of the identified genospecies at Tromøya summer 2007 was determined to be *B. afzelii*, hence the most common genospecies. Personal communication with author Kjelland by e-mail showed a similarity between temperature and relative humidity during tick collection summer 2007 and 2012, and the only notable difference was a higher mean temperature during October 2012.

As much as 82.9% of the positive samples were not identified to a specific genospecies, although several procedures were performed to identify these unknown samples. New PCR and nested PCR with higher amount of the extracted DNA were used, IGS primers were replaced with 16S gene primers, DNA was cloned to amplify the amount, and DNA was extracted from the positive cultivations. Unfortunately the positive cultivations had been incubated for 2 months resulting in no visible *Borrelia* presence after DNA extraction.

### 4.4. Treatment and resistance

*B. afzelii* and *B. garinii* cultivated from ticks in Southern Norway were applied in an in vitro study to determine MIC and MBC, but also for the resistance against antibiotics. The result of this experiment was not possible to evaluate, since the majority of the tubes containing penicillin, tetracycline and cefotaxime showed presence of viable spirochaetes after three weeks of incubation. The conclusion from
this finding is that the spirochaetes are resistant to the above-mentioned antibiotics, leading to a less likely result since previous studies have found spirochaetes sensitive to all four classes of antibiotics. A study from 2000 by Hunfeld et al. showed findings using cefotaxime and azithromycin that displayed lowest MIC, and azithromycin displaying best borreliacidal activity. The MIC and MBC value for penicillin in the previous study is not included here as different types of penicillin were used in the present and previous studies.

There are many sources of errors for the failed experiment, as caution regarding stability, calculations of the different concentrations used of the antibiotics and right preforming of the dilutions is important. For many aqueous drug solutions degradation can occur as a result to stress conditions such as pH, light, temperature and oxidizing atmosphere (Kommanaboyina & Rhodes, 1999). Tetracycline is known to be sensitive to light as photons degrades the structure (Chen et al., 2012), penicillin degradation is due to hydrolysis of the beta-lactam ring, while pH, temperature, and storage time is crucial for azithromycin stability (Moreno et al., 2009). Cefotaxime was the only antibiotic used in the experiment that is only slightly less stable than the main derivate (Fabre et al., 1984) and should therefore have given a result if the only error was stability. Cautions were made throughout the experiment in some degree but stress conditions were not inevitable. However, similar studies have been performed previously where results were obtained leading to thoughts whether wrong calculations or dilutions were made. The answer will not be found in the present, since there was no time left at the practical work to repeat the experiment. Thus, further studies are recommended, as this is a relevant topic since right treatment is crucial for the resistance and prevention of late stage manifestations of Lyme borreliosis.

5. Conclusions
Findings from this study showed that examination of dead ticks may cause an underestimation of the infection rate. The overall infection rate was determined to be 15.8% (35/222), however the real infection rate was found to be as much as 23.7% (31/131) when excluding the dead ticks. The most prevalent B. burgdorferi genospecies identified was B. afzelii 66.7% (4/6), followed by B. burgdorferi s. s. and B. garinii both with an infection rate of 16.7% (1/6). Unfortunately, it was not possible to determine MIC and MBC as a result of practical problems with the experimental set up.

6. Acknowledgement
I want to give a special thanks to my supervisors Sven Bergström and Tobias Jakobsson for giving me the opportunity to work at the laboratory in Umeå and making the study possible. Ingela, for all the help and good advices. Shahab and Mukunda for fun times and moral support. Mari, Maria, Patrick, Michaela and Jim for making me feel like one in your group. Last but not least my dad Odd Marcussen for his help collecting ticks with me throughout the summer.
7. References


Buchwald A. Ein fall von diffuser idiopathischer. Archives of dermatological research. 1883;553-556.


Hunfeld KP, Brade V. Antimicrobial susceptibility of Borrelia burgdorferi sensu lato: what we know, what we don’t know, and what we need to know. Wien Klin Wochenschr. 2006 Nov;118(21-22):659-68.


Kjelland V, (Dr/researcher). Sørlandet Sykehus HF/UiA. Personal communication, [2013-03-06].


Marcussen O, (local hunter). Personal communication, [2012-09-13].


Stingstudien. Tick study at Lindköpings university. Taken from: http://www.stingstudien.se [cited 2013-01-12].


8. Appendix
### 8.1. Terms and abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>acrodermatitis chronica atrophicans</td>
</tr>
<tr>
<td>BL</td>
<td>borrelial lymphocytoma</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>EM</td>
<td>erythema migrans</td>
</tr>
<tr>
<td>IGS</td>
<td>intergenic spacer sequence</td>
</tr>
<tr>
<td>LA</td>
<td>lyme arthritis</td>
</tr>
<tr>
<td>LB</td>
<td>lyme borreliosis</td>
</tr>
<tr>
<td>LNB</td>
<td>lyme neuroborreliosis</td>
</tr>
<tr>
<td>MBC</td>
<td>minimal borreliacidal/bactericidal concentration</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentrations</td>
</tr>
<tr>
<td>OspA</td>
<td>outer surface protein A</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>s.l</td>
<td>sensu lato “in the wide or broad sense”</td>
</tr>
<tr>
<td>s.s</td>
<td>sensu stricto “in the strict sense”</td>
</tr>
</tbody>
</table>

**Lyme Borrelia** genospecies: The 12 genospecies transmitted by *Ixodes ricinus* tick

LB-causing genospecies: *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s