BIRDS AND BORRELIA

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ABSTRACT

BIRDS AND BORRELLA

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The Lyme disease causing spirochaete Borrelia burgdorferi sensu lato is transmitted by ticks within the genus Ixodes. These ticks are liberal host seekers and parasitise mammals, birds and reptiles.

Prior to this study, the distribution of I. ricinus ticks and Lyme disease was thought to be restricted to the southern half of Sweden. On the island Norrbyskär, located in the Bothnian Gulf, there were reports of a high incidence of tick infestation on humans. To investigate the occurrence of B. burgdorferi s.l. in these ticks and to characterise presumptive isolates at the molecular level we sampled a number of I. ricinus ticks. Three different isolates were obtained from two different ticks, NBS16 from a nymph and NBS23a and NBS23b from an adult female tick.

The seabird associated tick I. uriae is circumpolar distributed in both hemispheres. On the island Bonden, which house one of the largest seabird colonies in the Baltic Sea, I. uriae were collected and surveyed for spirochaetes. One isolate of B. burgdorferi s.l. was obtained. This B. burgdorferi s.l. isolate is identical to the Lyme disease Borrelia strain NBS16 isolated from Norrbyskär.

To investigate the role of seabirds in the epidemiology of B. burgdorferi s.l., I. uriae were collected from seabird colonies in the southern and northern hemispheres. Borrelia DNA was extracted from the ticks and from cultured spirochaetes. Sequence analysis of the flagellin gene revealed that the DNA obtained was from B. garinii, regardless of the geographical origin of the sample. Identical fla gene fragments in ticks collected in both hemispheres indicate a transhemispheric exchange of B. garinii. A marine ecological niche and epidemiological route for Lyme disease Borrelia are proposed.

The prevalence of B. burgdorferi s.l. infected ticks on migrating passerine birds was studied. A total of 22,998 birds were caught and examined for ticks. The presence of spirochaetes in the 967 collected ticks was determined by DNA amplification by PCR on all ticks. To determine which B. burgdorferi s.l. species were present, classification was performed by DNA amplification using species-specific 16S rDNA primers and by DNA sequencing. Flagellin gene sequences of all species of B. burgdorferi s.l. previously recorded in Europe were found. B. garinii was the most prevalent. These data support the notion that passerine birds are at least partly responsible for the distribution of Lyme disease Borrelia spirochaetes in Europe.

To elucidate the distribution of B. burgdorferi s.l. in subarctic regions, strains isolated from I. ricinus and I. uriae ticks found on islands in the northern Atlantic and Baltic Sea were characterised molecularly. All isolates were verified as B. garinii by 16S-rRNA gene analysis and immunoblotting using monoclonal antibodies specific for the outer surface proteins A and C. Three ribotypes (RT’s) of B. garinii were found. The I. ricinus associated RT1 is phenotypically the most heterogeneous. RT2 is restricted to the islands in the northern Baltic Sea, whereas RT3 was also recovered from ticks found on islands in the North Atlantic. The heterogeneity of the B. garinii population in the Baltic Sea might be influenced by two geographically opposite directions, North Atlantic (RT3) and Euroasia (RT1).

Key words: Borrelia, Ixodes ticks, Birds, Epidemiology.
BIRDS AND *BORRELIA*

by

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and Infectious Diseases

Umeå 1995
Front cover: Razorbills (*Alca torda*)

Photo: Björn Olsen
This thesis is dedicated to Harry Hoogstraal. His extensive work on birds and ticks has been a source of inspiration for me.
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PAPERS 1-5
ABSTRACT

The Lyme disease causing spirochaete *Borrelia burgdorferi sensu lato* is transmitted by ticks within the genus *Ixodes*. These ticks are liberal host seekers and parasitise mammals, birds and reptiles.

Prior to this study, the distribution of *I. ricinus* ticks and Lyme disease was thought to be restricted to the southern half of Sweden. On the island Norrbyskär, located in the Bothnian Gulf, there were reports of a high incidence of tick infestation on humans. To investigate the occurrence of *B. burgdorferi s.l.* in these ticks and to characterise presumptive isolates at the molecular level we sampled a number of *I. ricinus* ticks. Three different isolates were obtained from two different ticks, NBS16 from a nymph and NBS23a and NBS23b from an adult female tick.

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To investigate the role of seabirds in the epidemiology of *B. burgdorferi s.l.*, *I. uriae* were collected from seabird colonies in the southern and northern hemispheres. *Borrelia* DNA was extracted from the ticks and from cultured spirochaetes. Sequence analysis of the flagellin gene revealed that the DNA obtained was from *B. garinii*, regardless of the geographical origin of the sample. Identical *fla* gene fragments in ticks collected in both hemispheres indicate a transhemispheric exchange of *B. garinii*. A marine ecological niche and epidemiological route for Lyme disease *Borrelia* are proposed.

The prevalence of *B. burgdorferi s.l.* infected ticks on migrating passerine birds was studied. A total of 22,998 birds were caught and examined for ticks. The presence of spirochaetes in the 967 collected ticks was determined by DNA amplification by PCR on all ticks. To determine which *B. burgdorferi s.l.* species were present, classification was performed by DNA amplification using species-specific 16S rDNA primers and by DNA sequencing. Flagellin gene sequences of all species of *B. burgdorferi s.l.* previously recorded in Europe were found. *B. garinii* was the most prevalent. These data support the notion that passerine birds are at least partly responsible for the distribution of Lyme disease *Borrelia* spirochaetes in Europe.

To elucidate the distribution of *B. burgdorferi s.l.* in subarctic regions, strains isolated from *I. ricinus* and *I. uriae* ticks found on islands in the northern Atlantic and Baltic Sea were characterised molecularly. All isolates were verified as *B. garinii* by 16S-rRNA gene analysis and immunoblotting using monoclonal antibodies specific for the outer surface proteins A and C. Three ribotypes (RT’s) of *B. garinii* were found. The *I. ricinus* associated RT1 is phenotypically the most heterogeneous. RT2 is restricted to the islands in the northern Baltic Sea, whereas RT3 was also recovered from ticks found on islands in the North Atlantic. The heterogeneity of the *B. garinii* population in the Baltic Sea might be influenced by two geographically opposite directions, North Atlantic (RT3) and Euroasia (RT1).
This thesis is based on the following articles which are referred to in the text by their Arabic numbers 1-5.


INTRODUCTION

1. GENERAL BACKGROUND

1.1. History

For a long time studies on human borreliosis dealt only with the relapsing fever spirochaetes. Their ecological complexity attracted much attention, especially in the first half of this century (Felsenfeld 1971). In 1977 a new disease was identified in the area of Lyme, Connecticut, USA (Steere 1977, Burgdorfer et al. 1982). Lyme disease, or Lyme borreliosis, is caused by the spirochaetal species group *Borrelia burgdorferi sensu lato* and transmitted by several species of ticks of the *Ixodes ricinus* complex. Within *B. burgdorferi* s.l. are the three main species associated with human disease, *B. burgdorferi sensu stricto*, *B. garinii* and *B. afzelii*. This tick-transmitted zoonosis is widespread in temperate regions of the northern hemisphere. Lyme disease has become the most commonly reported arthropod-borne disease in the United States and Europe. The main vertebrate reservoirs of *B. burgdorferi* are small mammals such as rodents.

In October 1975, two mothers, living in Lyme, Connecticut, whose children had recently been diagnosed with juvenile rheumatoid arthritis, notified the Connecticut State Health Department of several cases of arthritis in the area. The epidemiological investigation, by Drs. Allen C. Steere and David Snydman, revealed an incidence of arthritis much higher than might be expected. The uneven distribution of cases was enigmatic, most victims were living in wooded areas and only a few in town centers. The disease was obviously not contagious and individuals
of the same family often contracted the disease in different years. About 25% of the patients remembered having a strange skin rash prior to the onset of the arthritis. On the basis of these findings Steere and colleagues concluded that the disease was caused by an unknown virus or bacteria transmitted by an unknown arthropod (Habicht et al. 1987). However, testing sera from Lyme disease patients for the presence of antibodies against a number of arthropode borne diseases gave no positive results.

The rash, or erythema, of the Lyme patients was reminiscent of that described by the Swedish dermatologist Arvid Afzelius in 1909 (Afzelius 1910). Afzelius had named it erythema chronicum migrans (ECM) and suspected that it was caused by a tick bite.

In 1977 some patients with Lyme disease remembered having been bitten by ticks at the site of the rash. One tick was saved and was identified as *Ixodes dammini*, a species closely related to *I. ricinus*. Despite the finding of a possible vector, no Lyme disease causing pathogen was found. In the fall of 1981, Willy Burgdorfer examined several *I. dammini* collected on Shelter Island, New York. By phase contrast microscopy, he found spirochaete-like bacteria in some of the ticks. He knew that *I. dammini* was the likely vector of Lyme disease and realised that these spirochaetes could be the causative agent of the disease.

Alan G. Barbour at the Rocky Mountain Laboratories was able to grow the spirochaetes in a special medium, BSKII (Barbour 1984). Serum samples from Lyme disease patients were tested for the presence of antibodies against the newly found agent. The sera showed a pronounced antibody response to the bacteria. The etiologic agent of
Lyme disease was thereby discovered and the spirochaete was named *Borrelia burgdorferi* (Steere *et al.* 1983). Further characterisation of the agent was possible with the development of the method to grow them in culture (Barbour 1984).

1.2. Clinical manifestations

Lyme disease is a multisystemic disorder which may involve several organ systems including the skin, nervous system and joints (Steere *et al.* 1977, Steere 1989, 1991). Like spirochaetal infections as syphilis, the clinical course of Lyme disease or Lyme borreliosis can be separated into stages. The first sign of infection is usually a small homogeneous erythema at the site of the tick bite, often accompanied by regional lymph gland enlargement. The erythema called erythema chronicum migrans (ECM) increases in size over a few weeks with a central bleaching (Åsbrink 1991). ECM is usually localised at the site of the tick bite. Occasionally, patients present multiple erythema after a single tick bite. This is probably due to a rapid dissemination of the spirochaete with satellite foci formation. Another symptom, sometimes seen in early but more often in late infection, is lymphadenosis benigna cutis which is characterised by a bluish red tumour-like cutaneous and subcutaneous infiltrate (Åsbrink *et al.* 1984, Åsbrink 1991).

After a few weeks, usually not more than 2-3 months after infection, symptoms from the nervous system, the heart and/or the musculoskeletal system can arise due to dissemination of the spirochaetes. Both central and peripheral nervous system manifestations occur, including meningitis, Bells palsy, meningoradiculitis.
(Bannwarth's syndrome) and meningoencephalitis. Moreover, the patients often develop nonspecific symptoms such as headache, nausea and fatigue (Stiernstedt et al. 1988). The meningoencephalitis is usually mild in nature and may resolve without antimicrobial treatment.

Lyme arthritis is usually mono- or oligo-articular and primarily afflicts the large joints, often intermittently (Herzer 1991, Steere 1991). There seems to be a preponderance of arthritis in response to B. burgdorferi s.s., whereas infection by B. garinii and B. afzelii more often results in neurological or late cutaneous manifestations, respectively (Assous et al. 1993, Canica et al. 1993, van Dam et al. 1993). Patients with carditis present rhythm disturbances and aberrant conductance can be seen in electro cardiograms. Cardiac manifestations occur in less than 10% of patients with disseminated disease (Stechenberg 1988).

The third stage of Lyme borreliosis ensues several months to years after the tick bite. The main manifestations of this stage of the disease are acrodermatitis chronica atrophicans (ACA), chronic arthritis often with an intermittent course, and central nervous system disturbances such as depression, chronic encephalitis and peripheral neuropathy (Åsbrink et al. 1986).

1.3. The etiological agent

B. burgdorferi s.l. which includes the main species B. burgdorferi s.s, B. garinii, B. afzelii and B. japonica, belongs to the genus Borrelia within the order of Spirochaetales. They are thin, elongated, motile, helical-shaped bacteria, 20-30 elongated μm long (Figure 1). The most
recently recognised species, *B. japonica*, was isolated from the Japanese tick *I. ovatus* and has not yet been shown to cause human disease (Postic *et al.* 1993).

*B. burgdorferi* *s.l.* has a low guanine and cytosine content of 28-30.5 mole % (Schmid *et al.* 1984, Bergström *et al.* 1989). The genome of the *Borrelia* is organised in one linear maxichromosome with additional linear minichromosomes (linear plasmids) and a few circular supercoiled plasmids (Hyde *et al.* 1986, Barbour and Garon 1987, Simpson *et al.* 1990, Bergström *et al.* 1992).

The protoplasmic cylinder of *Borrelia* spirochaetes is surrounded by a fluid outer cell membrane which consists of 45-62% protein, 23-50% lipid and 3-4% carbohydrate (Holt 1978). Unlike other bacterial flagella, those of the spirochaetes are endoflagella, located between the protoplasmic cylinder and the outer cell membrane and attached to the poles of the bacterial cell. *B. burgdorferi* has 7-20 endoflagellae, coiled around the cell (Holt 1978). The gene encoding the flagellar protein is located on the chromosome (Casjens and Huang 1993).

More than 100 polypeptides have been identified in the outer surface membrane of *B. burgdorferi* *s.l.* (Luft *et al.* 1989). The proteins most extensively studied are the so called Osps (Outer Surface Proteins). To date, six Osps have been identified, OspA-F. They are all surface exposed (Barbour *et al.* 1984) and anchored by a lipid moiety to the fluid outer surface membrane (Brandt *et al.* 1990). When various *B.*
*B. burgdorferi* s.l. isolates are compared, the Osp proteins show considerable heterogeneity within as well as among the different species. According to analysis with monoclonal antibodies the OspA protein is homogeneous in East American *B. burgdorferi* s.l. isolates but more heterogeneous among *B. burgdorferi* s.l strains from Europe (Wilske et al. 1993). When compared with regard to apparent molecular size and reactivity with monoclonal antibodies the OspB protein is generally more heterogeneous than the OspA protein, (Bundoc and Barbour 1989, Lane and Pascocello 1989). Both OspA and OspB are encoded by the same operon on a 49-kb linear plasmid (Bergström et al. 1989, Barbour 1988).

There is an inverse relationship between the expression of OspA and OspC in vitro: strains with a low expression of OspC have strong expression of OspA and vice versa (Margolis and Rosa 1993). During tick feeding the spirochaete alters its expression of Osps. In non-engorged ticks the spirochaete expresses and produces OspA and probably OspB on the surface. The production of these proteins decreases at the time of feeding, and instead the spirochaete promotes production of OspC (Schwan et al. 1995). OspC is encoded on a 27-kb circular plasmid. Recent studies suggest that the expression of the ospC gene is influenced, in an inverse relationship, by the expression of the ospAB genes (Sadziene et al. 1993, Jonsson and Bergström 1995). OspC shows a high degree of polymorphism (Wilske et al. 1995).

OspA and OspB play important roles in the pathogenesis in Lyme disease, being involved in adherence to and penetration of host cells. Anti- OspA monoclonal antibodies inhibit adhesion of *B. burgdorferi* to endothelial cell surfaces (Comstock et al. 1993). During subculturing in
BSKII medium the spirochaetes lose OspB and their infectivity (Schwan and Burgdorfer 1987, Schwan et al. 1988). Furthermore, OspB-less mutants do not penetrate into endothelial cells and their infectivity is 30-300 fold lower than that of wild-type spirochaetes (Sadziene et al. 1993).

1.4. Molecular characterisation of B. burgdorferi s.l.

The low DNA homology and the low guanine and cytosine content separate the *Borrelia* from the genera of *Treponema* and *Leptospira* (Hyde and Johnson 1984).

After the discovery of the connection between ticks and the relapsing fever *Borrelia*, classification was based on the different vectors involved in the transmission of the spirochaetes. A "one tick vector species-one *Borrelia* species" concept was introduced, but has now been abandoned since many exceptions have been reported (Barbour and Hayes 1986, Felsenfeld 1971). For example, at least five *Borrelia* species, including *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* have been isolated from *I. ricinus*. Moreover the vector preference for some *B. burgdorferi* s.l. seems to be liberal. *B. garinii* has been isolated from at least four tick species: *I. ricinus*, *I. persulcatus*, *I. ovatus* and *I. hexagonus* (Aeschlimann et al. 1986, Kryuchkechnikov et al. 1988, Kawabata et al. 1987, Gern et al. 1991).

Compared to *B. burgdorferi* s.l. strains from North America, isolates from Eurasia are more heterogeneous both at the genotypic and the phenotypic levels (Wilske et al. 1986, 1988). After analysis of rRNA gene restriction patterns, protein electrophoresis patterns and
differences in reactivity to specific murine monoclonal antibodies, the Lyme disease *Borrelia* was separated into at least four species; *B. burgdorferi* s.s., *B. garinii*, *B. afzelii* and *B. japonica* (Baranton et al. 1992, Marconi and Garon 1992a, b, Canica *et al.* 1993, Kawabata *et al.* 1993, Postic *et al.* 1993). As a consequence, the common name of these four species is *B. burgdorferi* s.l.

The species defining process is ongoing. *B. burgdorferi* s.l. associated strains have been isolated from a variety of potential tick vectors (Assous *et al.* 1994, Postic and Baranton 1994). Recently, phylogenetic analyses support the notion that a *B. burgdorferi* s.l. isolated from the rabbit tick *I. dentatus* should be classified as a separate species, *B. andersonii* (Marconi *et al.* 1995).

To date, at least eight genomic species have been distinguished within *B. burgdorferi* s.l. (Postic and Baranton 1994). Whether all of these genomic species are human pathogens is not yet clear.

1.4.1. Genotypic characterisation

Various genes have been studied in order to delineate and characterise *B. burgdorferi* s.l. Particularly the 16SrRNA gene, the flagellin gene and the genes encoding the major outer surface protein OspA have been examined.

1.4.1.1. DNA/DNA hybridisation

DNA/DNA hybridisation is one of the reference methods for species delineation (Wayne *et al.* 1987). In 1983-84, *B. burgdorferi* was described as a new species. The description was mainly based on
genotypic, (DNA/DNA hybridisation) but also phenotypic characteristics such as reactivity of the monoclonal antibody H5332 to OspA (Barbour 1984, Johnson et al. 1984).

Besides the four distinctive species of *B. burgdorferi s.l.*, at least four other genomic species are delineated by DNA/DNA hybridisation (Postic and Baranton 1994). Six of these eight species are present in Eurasia. In North America three species exist with different ecological predominance and molecular characteristics (Postic and Baranton 1994, Marconi et al. 1995).

### 1.4.1.2. The 16S rRNA gene

rRNAs are currently the most commonly used molecular chronometer. They show a high degree of constancy, which assures good clock-like behaviour (Woese 1987).

Based on variation in the 16S rRNA gene, as disclosed by sequencing of the gene and fragment length polymorphism, *B. burgdorferi s.l.* can be classified into different ribotypes (RT’s).

Analysis of fragment length polymorphisms and sequencing of rRNA genes have been developed for phylogenetic studies of spirochaetes including *B. burgdorferi s.l.* (Grimont and Grimont 1986, Paster et al. 1991, Adam et al. 1991, Ralph et al. 1993). Marconi and Garon (1992c), investigated *B. burgdorferi s.l.* isolates from various sources and geographical regions and found that certain 16S rRNA gene signatures corresponded to the three known disease causing species of *B. burgdorferi s.l.* They constructed primers for DNA amplification unique to each species. The 16S rRNA genes from the relapsing fever
spirochaetes \textit{B. hermsii} and \textit{B. coriaceae} and the avian borreliosis agent, \textit{B. anserina}, did not cross amplify in spite of 16S rRNA gene sequence similarity values of 96.7, 96.6 and 96.4\% respectively, with that of \textit{B. burgdorferi} s.s. B31. The primer sets did not either cross amplify within the three main species of \textit{B. burgdorferi} s.l. Several 16S rRNA gene sequence studies demonstrate that human Lyme disease causing isolates from North America are a homogeneous group, with most isolates, belonging to \textit{B. burgdorferi} s.s.. The European isolates exhibit a more heterogeneous pattern, but have still been included in each of the three species, \textit{B. burgdorferi} s.s., \textit{B. garinii} and \textit{B. afzelii} (Marcon and Garon 1992a, Marcon and Garon 1992b, Marcon and Garon 1992c, Marcon et al. 1992, Marcon et al. 1995).

\textbf{1.4.1.3. The OspA gene.}

The majority of Lyme disease spirochaetes express the major linear plasmid encoded protein OspA (Barbour and Schrumpf 1986). The open reading frame of \textit{ospA} genes consists of 819-825 nucleotides (Bergström et al. 1989, Jonsson et al. 1992). Sequence analysis of the \textit{ospA} gene of \textit{B. burgdorferi} s.s., \textit{B. afzelii} and \textit{B. garinii} has revealed that the genotypic signatures correlate with the species specific reactivity of monoclonal antibodies against OspA (see below \textbf{1.4.2.1}, Will et al. 1995). The \textit{ospA} gene of \textit{B. burgdorferi} s.s. and \textit{B. afzelii} shows a high degree of homology at the intraspecies level, whereas \textit{B. garinii} itself can be delineated into at least four major subspecies (Wallich et al. 1992, Will et al. 1995).
1.4.1.4. The flagellin gene

The flagellin of *Borrelia* has been studied extensively with the aim of developing diagnostic tools for Lyme disease, both by immunological detection and PCR-amplification methodology. Moreover, molecular analysis of the *fla* gene and its gene product have been used for phylogenetic studies of *Borrelia* and to classify the different Lyme disease causing species (Rosa and Schwan 1989, Rosa *et al.* 1991, Picken 1992, Assous *et al.* 1994). Assous and coworkers (1994) amplified and subsequently hybridised a species variable part of the flagellin gene from 46 strains, 36 representatives from the four known Lyme disease *Borrelia* species and 10 atypical strains. According to the distinct reactivity in the hybridisation system these could be separated into six different genomic groups, retrospectively confirming previous results and the origin of the isolates (Picken 1992, Assous *et al.* 1994).

1.4.2. Phenotypic characterisation

1.4.2.1. OspA

Wilske and coworkers (1993) could clearly correlate species specific 16S rRNA signatures with specific OspA phenotypic markers. A panel of monoclonal antibodies was obtained to differentiate the spirochaetes at an inter and intra species-specific level. To date seven different OspA serotypes, 1-7, have been found. *B. burgdorferi s.s.* and *B. afzelii* are strongly associated with serotype 1 and 2, respectively. In contrast *B. garinii* isolates show a more heterogeneous immunological reactivity pattern, belonging to serotypes 3-7 (Wilske *et al.* 1993).
1.4.2.2. **OspC**

Using a panel of monoclonal antibodies directed against OspC, Wilske and coworkers analysed 38 *B. burgdorferi s.l.* strains of different origins representing the three major species of Lyme disease *Borrelia*. Thirteen different OspC serotypes were detected, of which three were represented in North American and 10 in Eurasian strains (Wilske *et al.* 1995). These results agree with a study by Schwan *et al.* (1993), indicating that OspC in North American *B. burgdorferi s.l.* strains is more conserved than in European strains. The different OspC types correlate well to species and OspA serotype classification (Wilske *et al.* 1995). However, the 13 different OspC variants were observed among only six different OspA serotypes, indicating that OspC is more heterogeneous than OspA.

1.5. **The vectors**

1.5.1. **Ticks as vectors of pathogenic microorganisms**

Ticks are vectors of more types of microorganisms than any other single arthropod taxon, including mosquitoes (Hoogstraal 1985). There are several reasons for the potency of ticks in the spread of diseases to man and animals; 1) they are persistent bloodsuckers that attach firmly while feeding and are not easily removed, 2) they are usually slow feeders which permits enough time for effective transfer of pathogens, 3) many species have a wide host range which ensures more certain sources of blood and opportunities to acquire and transmit pathogens, 4) they are long lived which enhances the chances of acquiring and
transmitting pathogens, 5) some pathogens may be transmitted transovarially and this impacts infectivity to some members of the next generation, 6) they are relatively free of natural enemies and 7) the reproductive potential among ticks is great, some species deposit as many as 18 000 eggs (Arthur 1968).

Ticks lack a distinct head, but have a headlike structure known as the capitulum. In ixodids, or hard ticks, the dorsum of the adult male is largely or totally covered by a plate called the scutum. In immature and adult female ixodids the scutum covers only the anterior part of the dorsum. A pair of simple eyes can be located on the lateral margins of the scutum in hard ticks. However, many species are eyeless, instead they have photosensitive areas located where eyes are found in eyed species (Sonenshine 1991).

The pathogenic microorganisms transmitted by ticks to man include rickettsiae (Rocky Mountain spotted fever and fièvre boutonneuse), protozoa (babesiosis spp.), viruses (Tick-Borne Encephalitis, Russian Spring and Summer Encephalitis, Crimean-Congo and Omsk hemorrhagic fever) and spirochaetes (tick-borne relapsing fevers, and of course Lyme disease).

1.5.2. Tick taxonomy

Tick taxonomy is controversial in the literature. According to the taxonomy suggested by Hoogstraal and Aeschlimann (1982), ticks are arthropods belonging to the subclass Acari. The three families, Argasidae, Nuttalliellidae and Ixodidae constitute the superfamily Ixodoidea which includes about 800 species. The Argasidae or soft
ticks includes 140 species of four genera. The Ixodidae or hard ticks is the dominant family with about 650 species arranged in 5 subfamilies and 13 genera. The hard ticks are morphologically characterised by the scutum and a terminal capitulum, they are sexually dimorphic as adults and the female is capable of enormous expansion during feeding. The genus *Ixodes* is the largest, most ancient and widespread genus of the family and includes some 250 species. (Sonenshine 1991, Oliver 1989).

1.5.3. Tick ecology

Some species within the family Ixodidae are one-host ticks, i.e. highly adapted to a particular vertebrate species and may complete their whole life cycle on the same host. Most ixodids, however, require three different individual hosts, and these hosts are not necessarily of the same species.

In contrast to the soft ticks the hard ticks are slow feeders (James and Harwood, 1969) and the bite is usually not painful. During its life cycle, the hard ticks undergo three stages of development. After hatching from the egg, the six legged larvae need a successful blood meal to transform into an eight legged nymph. For the further development into an adult tick the nymph needs an additional blood meal. Larval and nymphal ixodids feeding on warm-blooded hosts usually require 3-7 and 4-8 days, respectively. Mating usually takes place on a host prior to or during the final blood-meal. Female ixodids increase their weight 80-120 times while feeding (Oliver 1989). After insemination and the last blood meal she deposits 500-5000 eggs, usually in the soil in one prolonged oviposition (Arthur 1968, James and...
Harwood 1969). The incubation period of the eggs varies, from a few weeks to several months, depending on the environmental temperature and humidity (Arthur 1968, James and Harwood 1969).

1.5.4 The *I. ricinus* complex

The *I. ricinus* complex, sometimes referred to as the *I. persulcatus* complex, consists of several species of ixodids with morphological and ecological similarities. In Europe and Asia *I. ricinus* (Figure 2) and *I. persulcatus* and in North America *I. dammini*, *I. scapularis* and *I. pacificus* play the most important roles in the transmission of *B. burgdorferi s.l.* between different hosts including man. All these species feed on a wide range of hosts including birds, small rodents, insectivores and intermediate sized and large mammals.

In the Palearctic, *I. ricinus* has a more western distribution than *I. persulcatus*, ranging over the whole of Europe, except for the Arctic region. The range of *I. persulcatus* is from Eastern Europe to the Pacific Ocean (Dekondenko et al. 1988). The overlapping zone between *I. ricinus* and *I. persulcatus* is along the eastern part of the Baltic Sea and further south along that longitude into middle Europe (Korenberg 1994). Where the two species overlap there are microclimatic conditions separating their distribution (Korenberg 1994). *I. ricinus* occurs mainly in pasture habitat, whereas *I. persulcatus* inhabits more barren areas near primary coniferous forests. This latter condition is commonly referred to as taiga, and *I. persulcatus* is known as the taiga tick. Compared with other Palearctic tick species
*I. persulcatus* is more flexible and less sensitive to hydrothermal changes in the environment (Korenberg 1994).

**Figure 2.** Dorsal view of an unfed *I. ricinus* nymph, the main vector of *B. burgdorferi* s.l. in Europe.  
**Photo.** Gary Wife and Thomas Jaenson
In Japan, *I. persulcatus* is a vector of *B. garinii* and *B. afzelii* (Nakao et al. 1994). *B. japonica* is restricted to *I. ovatus*, a tick species associated with insectivorous mammals. No human cases of Lyme disease caused by *B. japonica* have been confirmed in Japan (Kawabata et al. 1993, Nakao et al. 1994).

In North America six species of ixodes ticks are known to be involved in *B. burgdorferi s.l.* enzootic cycles. The three tick species known to be the primary vectors of *B. burgdorferi s.s.* to humans in the USA are *I. daminii* (by many considered as a synonym of *I. scapularis*) in the north and north-east, *I. scapularis* in the south-east and *I. pacificus* in the west (Rich et al. 1995, Lane et al. 1991). A fourth vector, the rabbit tick *I. dentatus* found in eastern North America, is primarily associated with the cotton tail rabbit (*Sylvilagus floridanus*) and occasionally birds (Anderson et al. 1989). In California, the one host tick, *I. neotomae* maintain Lyme borreliosis in the dusky footed wood-rat (*Neotoma fuscipes*) population (Brown and Lane 1992). An analogous enzootic cycle in northern Colorado, involving *I. spinipalpis* and the Mexican wood-rat (*Neotoma mexicana*) was recently described (Maupin et al. 1994) The risk of humans contracting Lyme disease from *I. dentatus*, *I. neotomae* and *I. spinipalpis* is low since these vectors are restricted to their own hosts including a few vertebrate species but not humans.

The Australian tick *I. holocyclus* is not a memeber of the subgenus *Ixodes*, and it is unclear whether this tick is a competent vector of *B. burgdorferi s.l.* There are serological findings indicating the presence of Lyme disease in Australia (Barry et al. 1994), but *B. burgdorferi s.l* has not been isolated from this continent. When more than 12,000
*I. holocyclus* were examined by either direct microscopy or PCR, no spirochaetes were detected (Russel *et al.* 1994).

Other species of ixodid ticks have been found infected with spirochaetes, not necessarily Lyme disease *Borrelia; Amblyomma americanum* (Schulze *et al.* 1984), *Dermacentor variabilis* (Anderson *et al.* 1985), *Haemaphysalis* (Anderson and Magnarelli 1984), *Rhipicephalus* (Rawlings 1986) and *I. hexagonus* (Gern *et al.* 1991). However, there is no evidence to date that these tick species serve as vectors of *B. burgdorferi s.l.* to humans.

Normally the life cycle of *I. ricinus* in northern Europe, including Sweden, is completed in three years. In central and southern Sweden all active stages, larvae, nymphs, adults are active from late spring through early autumn (Jaenson 1991, Mejlon and Jaenson 1993). The seasonal host-seeking activity pattern of *I. ricinus* larvae and nymphs in southern Sweden is often bimodal with peaks in May-June and August-September (Mejlon and Jaenson 1993). Both larvae and nymphs utilise a broad host range, and attach to a variety of small mammals and birds.

The infection prevalence of host seeking larvae vary from 0% in some regions (Mejlon and Jaenson 1993) up to 4.8% in others (Doby *et al.* 1990). Transovarial transmission of *B. burgdorferi s.l.* is rare and has, in general, little or no effect on the infection prevalence in host seeking nymphs. Spirochaete transmission during larval feeding depends on the access of infected reservoir competent animals in a specific area, which influences the prevalence of spirochaete infected nymphs the following spring (Donahue *et al.* 1987). It is possible that the difference in infection prevalence of immature ticks between different geographical areas is associated with a difference in seasonal
occurrence of larval and nymphal ticks. Even if there is a greater
prevalence of the infection in adult ticks, which is related to the
numbers of blood meals ingested and to the efficient transstadial
transmission of the spirochaete (Aeschlimann et al. 1986), most human
cases of Lyme disease result from bites of nymphal ticks. The nymphs
are small and difficult to find, many are infected with spirochaetes, and
their host seeking peak in the late summer and early autumn coincide
with the recreation period of humans (Jaenson 1991). The adult I.
ricinus tend to have a more prolonged seasonal activity without any
distinct peaks (Mejlon and Jaenson 1993).

1.5.5. I. uriae

The tick I. uriae (Figure 3) has one of the most extensive geographic
distributions of any tick species in the world. It is a common
ectoparasite of seabirds and has a unique circumpolar distribution in
both hemispheres (Main 1972, Clifford 1979, Chastel 1988). This
bihemispheric distribution probably reflects dispersal by migrating
seabirds (Zumpt 1952). Its taxonomic position has been the subject of
much controversy; some scientists give I. uriae generic status
(Ceratixodes) (Filippova 1977), while others refer to it as a subgenus of
Ixodes (Clifford 1979, Mehl and Traavik 1983).

Although, the principal hosts of I. uriae in the northern hemisphere
are thought to be members of the family Alcidae such as the Guillemot
(Uria aalge) and Razorbill (Alca torda), it may in fact infest any
available bird. More than 50 species of seabirds colonising high
latitudes in both hemispheres have been reported to be infested by

**Figure 3.** Engorged female of *I. uriae*.

*Photo.* Thomas Jaenson

Depending upon the microclimate and availability of hosts, the life cycle can be completed in 4-5 years, with the majority of the population having a 4 year life cycle (Eveleigh and Threlfall 1974). At warmer latitudes the life cycle can be completed in two years, while under extreme conditions at higher latitudes it may take up to 8 years to complete (Lvov *et al.* 1975).
In contrast to *I. ricinus* ticks, *I. uriae* exhibit a more active host-finding strategy, the tick actually crawls towards its host (personal observation).

The effects of *I. uriae* on its seabird hosts have been poorly investigated. However, high tick infestation has resulted in chick mortality and sometimes in nest and even colony desertion. (Duffy 1983). Together with a high density of ectoparasites, the various pathogens that *I. uriae* may transmit can be of importance either for the individual seabird or play a role in the population dynamics of seabirds. A growing number of arboviruses has been isolated from *I. uriae*, including viruses within the Reo-, Flavi- and Bunyaviridae (Nuttall 1984, Chastel 1988, Oprandy *et al.* 1988).

1.6. **Vertrebrate-*Ixodes* tick-*Borrelia* interaction**

1.6.1. **Mammalian reservoirs and blood hosts**

Not all spirochaete-infected hosts are competent to serve as reservoirs for the spread of these bacteria. To establish whether or not a particular host species is a competent reservoir, evaluation of host infectivity is necessary. Under experimental conditions tick xenodiagnosis is the optimal method for assessment of reservoir competence of a specific animal. In field studies, when it is difficult to use xenodiagnostic methods, the evaluation of host infectivity is often based on the rate of infected larvae collected from a specific animal. (About transovarial transmission see under 1.5.4.) If the prevalence of
infected larvae exceeds the prevalence found on unfed, host-seeking larvae it is likely that the animal serves as a competent reservoir.

In Europe, several species of rodents and insectivores are competent reservoirs and are involved in the epizootology of *B. burgdorferi s.l.* (Aeschlimann *et al.* 1986, Vittoz *et al.* 1990, Humair *et al.* 1993, Matuschka *et al.* 1992, Tälleklint and Jaenson 1994). In one report, after feeding on the yellow necked field mouse (*Apodemus flavicollis*) and the wood mouse (*A. sylvaticus*) *I. ricinus* moulted into *B. burgdorferi* infected nymphs (Aeschlimann *et al.* 1986). Together with the bank vole (*Clethrionomys glareolus*) these rodents are probably the main tick hosts and infective reservoirs of *B. burgdorferi* in Europe (Aeschlimann *et al.* 1986, Hovmark *et al.* 1988, Humair *et al.* 1990, Tälleklint and Jaenson 1994). The yellow necked field mouse and the wood mouse have been found to be efficient sources of infection to *I. ricinus* immatures for long periods, up to two years in the laboratory (Vittoz *et al.* 1990). In Sweden, reservoir competency by the varying hare (*Lepus timidus*) has been shown on islands not inhabited by rodents (Tälleklint and Jaenson 1993).

The main reservoir for *B. burgdorferi s.s.* in eastern North America is the white-footed mouse (*Peromyscus leucopus*) (Levine *et al.* 1985, Donahue *et al.* 1987, Lane *et al.* 1991). This rodent is commonly found in the same habitat as *I. dammini* and is an important host for this ticks larval and nymphal stages. After obtaining the infection from an infected nymph (Mather *et al.* 1991) the white-footed mouse can remain infective to feeding ticks for at least six months (Donahue *et al.* 1987).

The infection prevalence in the black-legged tick *I. pacificus* in western North America is far lower (1-4%) compared to the infection
rates in *I. dammini* populations in the northeast (up to 60%) (Brown and Lane 1992, Piesman 1989, Schwan *et al.* 1993). This difference probably depends on the host association of immature *I. pacificus* ticks with the western fence lizard (*Sceloporus occidentalis*), which is not a competent reservoir for *B. burgdorferi s.s.* (Lane *et al.* 1991).

The rabbit tick *I. dentatus* acquires *B. burgdorferi s.l.* (suggested by Marconi *et al.* 1995 to be a distinct species, *B. andersonii*) while blood feeding on infected cottontail rabbits (*Sylvilagus floridanus*). The ticks maintain the enzootic cycle by transmitting it back to noninfected rabbits. *I. dentatus* shows a high degree of host specificity but can also be found attached to birds (Telford and Spielman 1989). Humans are rarely bitten and the role of this tick as a vector of *B. burgdorferi s.l.* is probably minute.

Of great importance for the total population of ticks are the various species of larger mammals that primarily serve as blood hosts, but not reservoirs for spirochaetes, for adult ticks. In northern Europe there are several species that primarily have a role as blood hosts such as the roe deer (*Capreolus capreolus*) and the moose (*Alces alces*) (Jaenson and Tälleklint 1992, Tälleklint and Jaenson 1994).

In Eastern North America the white tailed deer (*Odocoileus virginianus*) have increased in numbers and geographical distribution in recent years. This increased availability of blood hosts for adult ticks in the suburban and rural areas is probably the most important factor influencing the total number of ticks. However, ticks detaching from non infective blood reservoirs will have a diluting effect on the total infection prevalence in the area (Telford *et al.* 1988).
The spirochaete-host relationship is characterised by the benign nature of *B. burgdorferi* s.l. infection in its natural hosts. Animals infected with *B. burgdorferi* s.l. show few signs of disease. The white-footed mouse and the deer mouse (*Peromyscus maniculatus*), are, however, reported to show symptoms similar to those of dogs, cats, horses and humans; lethargy, weight loss, lameness and arthritis (Bosler *et al.* 1988, Greene 1989, Magnarelli *et al.* 1990, Wright and Nielsen 1990). It should be noted, however, that the apparent lack of symptoms may reflect an inability to recognise and interpret symptoms in wild animals.

### 1.6.2. Passerine birds as reservoirs of *B. burgdorferi* s.l.

The perching birds, known as passerines, (Figure 4) form the largest order of the class Aves, representing over half (about 5300) of the total living species. The passerines are a diverse group of small to medium sized land birds, mainly arboreal but also terrestrial and aerial. Passerines are cosmopolitan, but with one exception, they do not occur in Antarctica. Some of the passerine species are long distant migrants.

There are only a few reports of the effects of *B. burgdorferi* s.l. on birds, wild or domestic. The few challenge studies performed (on non-passerine birds), revealed that the *B. burgdorferi* infection *per se* has no negative impact on the bird's health (Burgess 1989, Isogai *et al.* 1994). However, it is well known that defence mechanisms against infections in birds, and of course mammals, can be modulated by environmental factors such as nutrition, stress and age, and by intrinsic
factors such as hormonal fluctuations i.e. during breeding and migration (Dreent and Daan 1980).

Since *B. burgdorferi s.l.* has its *in vitro* growth optimum at 34°-37° C, (Barbour 1984), the high body temperature of passerine birds, 39°-42° C, (Welty and Baptista 1988) has been one reason to neglect their importance as *Borrelia* reservoirs. On the other hand, the body temperature in birds is not uniform, both in a temporal and a spatial manner. During long-term flight the temperature increases compared to that at rest. The skin and the air sacs hold a lower temperature than do internal organs (Welty and Baptista 1988).

In Europe, the migratory routes of birds are more diverse than in North America, with both south-north and east-west components (Alerstam 1982). A common strategy of migrating birds is to use

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**Figure 4.** This Mistle Thrush (*Turdus viscivorus*) is a passerine bird. **Photo.** Björn Olsen
different stop-over sites along their routes. At these locations, where birds feed and rest, ticks and other ectoparasites may attach, and later detach along the migration routes or in breeding areas. New foci of tickborne diseases have been suggested to be created in this way (Anderson et al. 1986, Mehl et al. 1984).

Several investigations have been performed in Europe and the Middle East concerning the role of birds as carriers of ticks (Hoogstraal and Kaiser 1961, Hoogstraal et al. 1961, Hoogstraal et al. 1963, Mehl et al. 1984) and tick-associated arboviruses (Brinck et al. 1965). From those results it is clear that migratory birds play an important role as distributors of ticks within and between continents.

In the United States avian reservoirs of Lyme disease Borrelia have been suggested since B. burgdorferi s.s has been isolated from the liver of a Veery (Catharus fuscescens) (Anderson et al. 1986), from the blood of a Song Sparrow (Melospiza melodia) (McLean et al. 1993) and from I. dammini larvae attached to birds (Anderson et al. 1990, Weisbrod and Johnson 1989, Magnarelli et al. 1992).

Not all passerines seem to be competent reservoirs of Lyme disease Borrelia. In North America, the Gray Catbird (Dumetella carolinensis) is regarded as an incompetent reservoir of B. burgdorferi s.s. (Mather et al. 1989). Matuschka and Spielman (1992) showed that ticks infected with B. burgdorferi s.l. lost their infection in the course of feeding on European Blackbirds (Turdus merula) and failed to transmit the spirochaetes to other blackbirds. These results, however, were based on only two wild-caught and two laboratory reared birds, and therefore need confirmation.
Based on differences in Restriction Fragment Length Polymorphism pattern, RFLP, between bird and mammal isolates of Lyme disease *Borrelia* there are suggestions that certain ribotypes of *B. burgdorferi* s.l. have a preference for birds (Nakao et al. 1994). These author hypothesise that there are two enzootic transmission cycles, one bird-tick cycle and one rodent-tick cycle, in nature which maintain *B. burgdorferi* s.l. On the other hand, Anderson and coworkers (1990), have shown that *B. burgdorferi* s.s. isolates from bird-feeding *I. dammini* larvae were infectious to Syrian hamsters. It is therefore likely that at least *B. burgdorferi* s.s. can be transmitted between birds and rodents by subadult *Ixodes* ticks.

### 1.6.3. Seabirds as reservoirs of *B. burgdorferi* s.l.

The seabirds (Figure 5) consist of approximately 300 species of birds that feed mainly at sea (Harrison 1984). Their role as blood hosts for seabird associated ticks is well documented (Clifford 1979, Mehl et al. 1983) but the role of seabirds as reservoirs of *B. burgdorferi* s.l. has not been investigated.

An important biological and ecological characteristic shared by all marine birds is their obligation to return to land to breed, where they congregate in large, frequently mixed, colonies, numbering several thousands to millions of individuals. During the relatively short breeding season, the close contact that occurs between birds favours the exchange of endo and ectoparasites (Clifford 1979).

Many species of seabirds are fast, long distance migrants both between and within the hemispheres. Species within the order
*Procellariiformes* (albatrosses, shearwaters and stormpetrels) are extremely mobile, with many species undertaking long, complex migrations of several thousand kilometres, in which the whole population may be engaged. Some species breeding during the south polar summer (October-March), spend "their winter" in the North Atlantic and the North Pacific Ocean. This coincides with the breeding season for the seabirds in the northern hemisphere.

![Razor bills](image)

**Figure 5.** Razorbills (*Alca torda*)

**Photo.** Björn Olsen

Several species within the order *Procellariiformes* and penguins have body temperatures as low as 36°-38° C (Warham 1990, Cockrem 1990) which are theoretically suitable for *B. burgdorferi s.l.* growth.
1.7. Distribution of Lyme Disease

The geographical distribution of Lyme disease correlates with the known distribution of the *B. burgdorferi s.l.* ixodid vectors.

In Europe, Lyme disease occurs from the Mediterranean area to the central part of Europe, including the British Isles, to southern Scandinavia (Stanek *et al.* 1992). In Scandinavia, cases have been recorded from all four countries in areas corresponding to the known distribution of *I. ricinus*. In the northern part of Sweden *I. ricinus* ticks and Lyme disease have been thought to be virtually absent, possibly due to climatological conditions.

In a study by Dekonenko and coworkers (1988) Lyme disease was found from the European-Asian boundary to the Pacific coast in the former Soviet Union. The disease is also prevalent in Japan, Korea and in the northern part of China (Kawabata *et al.* 1987, Park *et al.* 1993, Zhe Fu 1994).

In the United states, Lyme disease is presently known to occur in 41 states. The distribution is closely tied to the presence of the three main vectors, *I. dammini, I. scapularis* and *I. pacificus*. Three different areas in the US are the most affected, the east, the north central region and the Pacific coast (Steere and Malavista 1979, MMWR 1989).

Of particular interest are the reports of possible Lyme disease cases in Australia and South Africa, although no Lyme disease causing spirochaete has yet been isolated from these regions (Stanek *et al.* 1986, Barry *et al.* 1994, Russel *et al.* 1994).

Thus, *B. burgdorferi s.l.* infected ticks and Lyme disease appear to be widely distributed over temperate regions in the northern hemisphere. Whether the Lyme disease agent occurs in human
associated tick vectors in the southern hemisphere needs further elucidation.
AIMS OF THIS THESIS

To investigate whether Lyme disease *Borrelia* occur in northern Sweden, and to characterise eventual spirochaetal isolates.

To evaluate the role of migrating birds in the spread of *B. burgdorferi* s.l.

To examine the possible role of seabirds and *I. uriae* in the distribution of Lyme disease *Borrelia*.

To determine whether Lyme disease *Borrelia* enzootic foci can be maintained in the absence of mammals.

To study whether different types of *B. garinii* coexist in the same ecological niche in the Baltic Sea.
SUMMARY AND DISCUSSION OF THE RESULTS

2.1. Characterisation of Lyme disease *Borrelia* in Northern Sweden. (Paper 1)

The main occurrence of *I. ricinus* ticks in the northern part of Sweden is restricted to a narrow zone along the coast of the Baltic Sea (Jaenson *et al.* 1994). Except for a few small areas, the inland climate is probably not suitable for ticks. Consistent with this view, there are no reports of Lyme disease acquired in the inland of Northern Sweden. Prior to this study, this was true also for the coast of Northern Sweden. In the late 1980s there were anecdotal reports of a high incidence of tick bites on humans and dogs on the island of Norrbyskär (Figure 1, Paper 1). It was unknown whether these ticks might be infected with Lyme disease *Borrelia*. Our aim was, therefore, to investigate the occurrence of *B. burgdorferi* s.l. in ticks collected from Norrbyskär and to characterise presumptive isolates at the molecular level.

By dragging a flannel cloth over the ground (flagging), a total of 85 *I. ricinus* ticks were collected. After identification, the idiosoma of each tick was aseptically excised onto a glass slide in a drop of phosphate buffered saline and examined at 400X or 500X by phase contrast microscopy. Half of the tick idiosoma was then placed into BSKII medium and the culture was incubated at 34° C for three weeks. Three different isolates were obtained from two different ticks, NBS16 from a nymph and NBS23a and NBS23b from an adult female tick.

From the other half of the tick, total DNA was extracted and subjected to PCR amplification using *fla* and *ospA* specific primer pairs. In 16/85 (19%) of the ticks *B. burgdorferi* DNA was successfully
amplified. From this data, the prevalence of *B. burgdorferi* in the tick population on Norrbyskär was concluded to be similar to what is found in endemic areas in south-central Sweden (Jaenson 1991).

Characterisation of the three strains using genus and species specific monoclonal antibodies in an immunoblot analysis revealed that the isolated spirochaetes were *B. burgdorferi s.l.* Using the anti-flagellum monoclonal antibody H9724 (Barbour *et al.* 1986) all three strains showed an identical reactivity pattern. All three isolates gave different protein profiles in SDS-PAGE (Figure 2, Paper 1). The strain NBS23b expressed a protein of about 22 kDa (OspC) which was not seen in NBS23a. More recently, however, phenotypic analysis with monoclonal antibodies against OspA and OspC as well as genotypic characterisation, have shown identity between NBS23a and NBS23b indicating that they may represent the same strain (data not shown). The reason for the apparent isolate difference is not known. Our hypothesis is that it is due to variable gene expression rather than to an occurrence of two populations of *B. burgdorferi s.l.* in the same tick. The species specific monoclonal antibody H5332, recognised OspA in all NBS strains. However, in the NBS16 strain the protein reacting with H5332 exhibited a slightly larger molecular weight than the reactive proteins in NBS23a and NBS23b (Figure 3, Paper 1). None of the strains reacted with a monoclonal antibody directed against the OspB protein. This lack of reactivity is probably due to epitope differences between different isolates, i.e. heterogeneity (Shoberg *et al.* 1994) although a loss of OspB during culturing *in vitro* (Schwan and Burgdorfer 1987) cannot be excluded.
To further characterise the isolated *Borrelia*, partial sequencing of the flagellin and OspA encoding genes were performed. This confirmed the identification as *B. burgdorferi* s.l. When compared to the *fla* and *ospA* genes of *B. burgdorferi* strains B31 and *B. afzelii* ACA1 the nucleotide sequence of both the flagellin and *ospA* genes showed considerable difference. Later, by 16S rRNA gene analysis, the NBS strains were identified as *B. garinii* (Paper 4).

These isolates may be clinically relevant. A 10-year-old boy experienced a typical erythema after a one-week-stay on Norrbyskär. One month later the boy presented with a headache, neck stiffness and fever and was therefore admitted to the pediatric clinic. The cerebrospinal fluid (CSF) from the patient showed meningitis with a preponderance of mononuclear cells. Both the serum and CSF reacted strongly in immunoblot analysis with whole cell extracts from the three different NBS isolates, but not with *B. burgdorferi* s.s. or *B. afzelii* (Figure 4, Paper 1), suggesting that the infection had been acquired on Norrbyskär.

In conclusion, the distribution of *B. burgdorferi* s.l. infected ticks is more extensive than previously thought. Although rare, Lyme disease must be considered in the northern part of Sweden.
2.2. The seabird tick *I. uriae* infected with *B. burgdorferi* s.l. (Paper 2)

The finding of *B. burgdorferi* s.l. infected ticks on the island of Norrbyskär raised several questions. Was the occurrence due to dispersal of infected ticks by migratory birds? Could bird species other than passerines possibly be involved?

During bird ringing in the summer of 1991 on the island Bonden, situated 12 km from the Swedish mainland in the Bothnian Gulf, we found some ticks attached to Razorbill and Guillemot feet. The ticks were identified as *I. uriae*, a species known to be strongly associated with seabirds. This was only the second report of *I. uriae* in Sweden.

When the idiosomas of the ticks were examined by phase contrast microscopy and by an indirect immunofluorescence assay using the antiflagellum monoclonal antibody H9724 (Barbour *et al.* 1986), spirochaetes were detected (Figure 1, Paper 2). Cultivation attempts were however, unsuccessful. The following year the survey and collection of ticks were more extensive. In three of 37 engorged and in one of 100 nonengorged *I. uriae* ticks, spirochaetes were detected. From two of the collected ticks spirochaetes were successfully isolated by in vitro culture. The DNA of the spirochaete infected ticks was purified and subjected to amplification by PCR using oligonucleotides corresponding to gene fragments of the *ospA* and *fla* genes of *B. burgdorferi* (Gassman *et al.* 1989, Bergström *et al.* 1989). In 5 of 37 of the ticks collected in 1992, *B. burgdorferi* DNA could be amplified. The amplified *fla* gene fragments were sequenced and compared to *fla* sequences from different *B. burgdorferi* s.l. strains and to other *Borrelia* species such as *B. anserina*, *B. hermsii* and *B. crocidurae*. 

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The nucleotide sequences of the *fla* gene fragments showed similarity to the different *B. burgdorferi s.l.* isolates but less similarity to *B. anserina* and the relapsing fever spirochaetes.

When comparing the *B. burgdorferi s.l.* strain NBS16 (Figure 2, Paper 2) and the *I. uriae* isolate an identical *fla* sequence was revealed. To confirm these results, a 150 bp gene fragment from the *ospA* gene was sequenced from the two organisms and found to be identical. Moreover, the protein pattern on SDS-PAGE and the reactivity against the monoclonal antibodies H5332 and H9724 on immunoblots were essentially identical. The strain was designated IUB18 (*I. Uriae Bonden tick no. 18*). Later, both NBS16 and IUB18 were shown to belong to *B. garinii* by 16S rRNA gene sequence analysis (Paper 4).

These findings raised additional questions: i) Were there any small mammals, such as rodents, that could maintain the enzootic cycle on Bonden? This seemed unlikely since a capture test with 15 mouse traps yielded no mammals over one month of exposure. ii) Could the Razorbills and Guillemots be the reservoir for *B. burgdorferi* on Bonden? To investigate this, we sampled biopsies from Razorbill fledgling’s feet. From one of the samples we were able to amplify *B. burgdorferi* DNA. The amplified flagellin DNA was identical in size to the amplified *fla* gene fragment from the cultured IUB18 strain. Thus, it seemed likely that the birds at Bonden were infected with this spirochaete.

To summarise, since Razorbills and Guillemots spend most of the year far away from land where no mammals are found, it is likely that these birds are, together with *I. uriae*, the major components of a *B. burgdorferi s.l.* enzootic cycle on Bonden. If so, this cycle would be
less complex than the terrestrial cycles previously described (Figure 6) (Jaenson 1991). The spirochaetes infecting *I. uriae* on Bonden showed nucleotide sequence identity to one of the strains from the island of Norrbyskär located near the mainland (NBS16 see Paper 1), indicating a close relationship between these populations of *B. burgdorferi s.l.*

*Figure 6. The major components of the terrestrial and marine enzootic cycles of *B. burgdorferi s.l.**
2.3. Transhemispheric exchange of Lyme disease *Borrelia* (Paper 3)

Could *I. uriae* populations in seabird colonies other than Razorbills and Guillemots be infected with *B. burgdorferi* s.l.? Is there a predominance of a certain *Borrelia* species? Could we find any evidence or indications for exchange of *B. burgdorferi* s.l. between different seabird colonies within and between the hemispheres? To address these questions, seabird-ringing groups throughout the world were contacted and asked for ticks collected from different seabird species. A total of 523 *I. uriae* were collected from three seabird colonies in the southern and six seabird colonies in the northern hemisphere (Figure 7).

![Figure 7](image_url)

*Figure 7.* Location of seabird colonies where *Ixodes uriae* ticks were collected.
All three active developmental stages (larvae, nymphs and adults) were represented with a predominance of nymphs and adults. The idiosoma of all live ticks were examined by phase contrast microscopy and/or by an immunofluorescence assay (IFA). In 43, (one from the southern and 42 from the northern hemisphere) of 385 live ticks, spirochaetes were observed. Spirochaetes were successfully cultivated from three ticks collected on the Faeroe Islands, one tick from Iceland, and two ticks from Sweden.

To increase the sensitivity of the screening of ticks and to further characterise the spirochaetes, total DNA from one half of each tick was extracted and PCR-amplified with flagellin- and ospA-gene specific primers. Amplification with the flagellin primers was successful for 113 ticks. Using the ospA specific primers, 115 ticks gave a PCR product of the expected size. The results are summarised in Table 1, Paper 3.

Smears of ticks from which DNA amplification was successful were examined by IFA using the Borrelia genus- and B. burgdorferi s.l.-specific monoclonal antibodies, H9724 (Barbour et al. 1986) and 84C (Shoberg et al. 1994), respectively. In one of 18 live ticks from Campbell Island (south of New Zealand), spirochaetes were seen after staining with H9724. The spirochaetes from the ticks collected from Alaska, the Faeroe Islands, Iceland and Sweden reacted with both the anti-flagellin monoclonal antibody H9724 and the anti OspB monoclonal antibody 84C (Shoberg et al. 1994), indicating that they belong to B. burgdorferi s.l. We could not detect any spirochaetes by this method in any of the dead ticks.

To further characterise the spirochaetes, an amplified, species variable part of the flagellin gene from all PCR positive ticks from
Campbell Island, Crozet Island (located between the Antarctic and South Africa) and of randomly selected samples from other localities were subjected to DNA sequencing. The *fla* fragments showed a high degree of mutual sequence homology regardless of the source.

Altogether, six different sequences were found (Figure 8) all more closely related to the *fla* gene of *B. garinii* than to that of any other Lyme disease *Borrelia* species.

**Figure 8.** Nucleotide sequence of 156 bp internal *fla* gene fragment from *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and from *I. uriae* ticks. Sequence 1 is representative of three ticks at Campbell Island, three ticks from Egg Island and eleven ticks from Crozet Island, sequence 2 of six ticks from Crozet Island, sequence 3 of four ticks from Crozet Island and two ticks from Nolsøy Island, sequence 4 of five ticks from Flatey Island, three ticks from Nolsøy Island and two ticks from Crozet Island, sequence 5 of eight ticks from Egg Island and sequence 6 of five ticks from Bonden.
When sequencing the *fla*-PCR products from the 23 positive ticks from Crozet Island, four different sequence patterns were observed. Three of these sequences (sequence 1, 3 and 4, Figure 8) were also found in ticks collected from different localities in the Northern hemisphere, i.e. Iceland, the Faroe Islands and Alaska. This result is compatible with an exchange of *B. garinii* strains between regions at the extreme ends of the northern and the southern hemispheres. There are several species of seabirds; albatrosses, shearwaters, stormpetrels, terns and auks, that can theoretically serve as vehicles of spirochaetes or infected ticks within or between the hemispheres.

When we later analysed the different isolates of *B. garinii* from the Faroe Islands and Iceland, it was shown that they represented the same ribotype according to the 16S rRNA gene pattern (see Paper 4).

We found *B. garinii* DNA in ticks from two localities in the southern hemisphere, Crozet Island and Campbell Island. Although isolation attempts were unsuccessful, the PCR and IFA results strongly suggest the presence of *B. garinii* in the southern hemisphere. In these regions the prerequisites are apparently lacking for an exchange between the marine enzootic cycle of *B. garinii* and a putative terrestrial cycle. In general, the seabirds and *I. uriae* in the southern oceans are restricted to offshore islands and peninsulas. They arrive only accidentally, by extreme winds, on the inner mainland. In accordance with the separation of the marine and the theoretical terrestrial enzootic cycle of Lyme disease *Borrelia* is the apparent absence of *B. burgdorferi s.l.* in the Australian terrestrial tick, *I. holocyclus* (Russel *et al.* 1994).
These results suggest that *B. garinii* is the prevalent infecting species of *I. uriae* ticks in both the northern and southern hemispheres. The *fla* gene sequences of the different *B. garinii* infecting *I. uriae* show a noticeable heterogeneity. The occurrence of identical *fla* sequences from localities in the northern and southern hemispheres as well as within the same hemisphere is compatible with an interchange of *B. garinii* by seabirds.

2.4. Molecular polymorphism of *B. garinii* in the Baltic Sea is influenced by different Lyme disease enzootic foci. (Paper 4).

The results presented in Papers 1 and 2 indicated that there is a close spatial relationship between terrestrial and marine enzootic foci of *B. garinii* in the Baltic Sea. This relationship may have generated a heterogeneous population of *B. garinii*. Could different enzootic foci from various ecological niches contribute to this heterogeneity? To approach these questions, *Borrelia* were isolated from *I. ricinus* and *I. uriae* ticks from the northern Atlantic and Baltic Sea and characterised both at a phenotypic and genotypic level (Table 1, Paper 4).

All strains studied were identified as *B. garinii* using species-specific PCR amplification primers complementary to the 16S-rDNA (Marconi and Garon 1992c). Three patterns of variable signature nucleotide positions were found in the partial 16S-rDNA sequence of the isolates. Before this study, only a single set of variable and signature nucleotides had been delineated in the *B. garinii* 16S-rRNA gene (Marconi and Garon 1992c). This previously defined ribotype
corresponded to RT1 and included isolates from humans and ticks from Germany, Switzerland, southern Sweden and the Asian part of Russia.

The RT1 (Table 1) strains NBS23A and Lab originated from Norrbyskär (see Paper 1) and Lithuania, respectively, suggesting a wide distribution of this *B. garinii* ribotype (Table 1, Paper 4). The wide geographic occurrence of RT1 might be due to the occurrence of two competent vectors, *I. ricinus* and *I. persulcatus* which cover wide geographic areas. The spread of RT1 borreliae may rely on the involvement of birds, either as vehicles of infected ticks or as reservoirs of borreliae (Paper 5). Some passerine bird species breeding in northern Scandinavia find their winter quarters in east and south east Asia (Alerstam 1982).

The partial 16S-rRNA sequence of RT2 is distinct from RT1 by a single nucleotide (Table 1). These two RT's share a common signature nucleotide, which distinguishes them from the RT3 isolates, as well as from other Lyme disease *Borrelia* species. In contrast to the wide distribution of RT1 borreliae, RT2 strains were limited to neighbouring islands in the Bothnian Gulf (Table 1, Paper 4). RT2 as well as RT3 strains were recovered from both *I. uriae* and *I. ricinus*, two tick species which occupy different ecological niches. Thus, *B. garinii* is associated with more than one tick species. It is possible that this ecological plasticity of *B. garinii* ensures their survival in different ecological systems and allows them to expand their host range.

The occurrence of RT3 borreliae (Table 1) on two islands in the North Atlantic and the Baltic Sea (Table 1, Paper 4), suggests a marine spread of certain *B. garinii* variants between these two regions. The recently recognised exchange of different populations of seabirds
(Guillemots) from the North Atlantic to the Baltic Sea or vice versa (Staav 1995) may favour the dispersal of this ribotype of *B. garinii*.

**TABLE 1.** Analysis of variable and signature nucleotide positions of the partial 16S-rDNA in the studied borreliae strains

<table>
<thead>
<tr>
<th><em>Borrelia</em> isolate</th>
<th>Nucleotide at the following 16S-rDNA position</th>
<th>Ribotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>126</td>
<td>170</td>
</tr>
<tr>
<td>Ip90, Lab, NBS23a</td>
<td>C</td>
<td>A</td>
</tr>
<tr>
<td>NBS16, IUB18</td>
<td>T</td>
<td>A</td>
</tr>
<tr>
<td>IUB19, Mal01, Mal02, Far01, Far02, Fis01</td>
<td>C</td>
<td>G</td>
</tr>
<tr>
<td>B31</td>
<td>T</td>
<td>G</td>
</tr>
<tr>
<td>R-IP3</td>
<td>T</td>
<td>G</td>
</tr>
</tbody>
</table>

Respective sequences of *B. burgdorferi* B31 and *B. afzelii* R-IP3 are included for comparison.

All RT3 strains in the Atlantic were isolated from the seabird tick *I. uriae*. Due to the association of *B. garinii* with this vector species in the Atlantic region, a transmission of *B. garinii* between Europe and North America may be expected to occur. Such a transmission has not yet been demonstrated. It should be noted, however, that seabirds ringed in Iceland and on the Faeroe Islands are recovered along the eastern coast of North America (Petersen 1982).

The ribotyping correlated well with phenotypic analysis based on reactivity against Osp-specific Mabs. RT2 and RT3 isolates had discrete OspA serotypes (Table 3, Paper 4). Moreover, RT2 strains had one single OspC serotype, whereas two geographically discrete groups of RT3 strains branched into two OspC types (Table 4, Paper 4). Unlike RT2 and RT3, all RT1 isolates showed individual anti-OspA
reactivities (Table 3, Paper 4). Notably, one RT1 isolate shared OspA serotype 6 with RT3 strains, although the serotype identities of these strains could be differentiated using an additional Mab. Recently, Osp serotyping was shown to be Lyme disease *Borrelia* species-specific (Wilske *et al.* 1993, Wilske *et al.* 1995). Results of this study also validate the specificity of Osp serotyping at the subspecies level. Furthermore, delineation of new ribotypes and anti-Osp reactivity patterns of *B. garinii* strengthen the view of considerable genetic and phenotypic heterogeneity.

Strains of OspA serotype 3 and 6 have been isolated from *I. uriae* (this study) and human CSF (Wilske *et al.* 1993), indicating that *I. uriae* might serve as a vector of human pathogenic borreliae strains. It should be emphasised, however, that the pathogenicity to humans of *I. uriae* associated *B. garinii* has not yet been proven.

In conclusion, the heterogeneity of the *B. garinii* population in the Baltic Sea might be influenced by two geographically opposite enzootic foci, one from the North Atlantic (RT3) and the other (RT1) from Euroasia. Although isolated from *I. ricinus*, spirochaetes representing RT3 appear to have a predilection for *I. uriae* and seabirds. These spirochaetes may represent a distinct *Borrelia* species. If this can be corroborated by species delineation, an appropriate name would be *Borrelia atlantica.*
2.5. The role of migrating passerine birds in the epidemiology of Lyme disease in Europe. (Paper 5).

Our previous results demonstrate the importance of seabirds in the global distribution of the Lyme disease spirochaete *B. garinii*. Are terrestrial birds also important as tick carriers and involved in the spread of Lyme disease *Borrelia*?

In Japan and the United States, avian reservoirs of the Lyme disease *Borrelia* have been suggested since *B. burgdorferi s.l.* has been isolated from passerine birds and from tick larvae carried by birds (Anderson *et al.* 1986, Anderson *et al.* 1990, Magnarelli *et al.* 1992, McLean *et al.* 1993, Miyamoto *et al.* 1993).

To investigate the importance of passerine birds in the epidemiology of Lyme disease in Europe a study involving seven bird observatories in Sweden and one in Denmark was designed (Figure 1, Paper 5). A total of 22,998 birds were caught and surveyed for ticks. For practical reasons we limited the collection of ticks to the head of the birds. Nine hundred and sixty seven ticks of five different tick species were found. *I. ricinus* was the most common species and constituted 98.2% of all tick specimens. A total of 283 larvae, 664 nymphs, three adult females and one adult male of *I. ricinus* were found. This is in agreement with previous observations that nymphs are more common than larvae on birds, and that adult *I. ricinus* rarely feed on birds (Humair *et al.* 1990).

The number of more exotic tick species was low. Interestingly, one *I. persulactus* was collected on a spring migrating Willow Warbler (*Phylloscopus trochilus*). This is the first record of *I. persulcatus* in Sweden, indicating an influx of ticks from eastern Europe and Asia. Only 10 *Hyalomma marginatum*, and one *Haemaphysalis punctata*
were collected from spring migrating birds heading north. This is lower than observed in previous studies (Hoogstraal et al. 1961).

In the Western Palearctic, it has been shown that the prevalence of tick infestation is low on birds migrating from their wintering areas in Africa. These studies, performed in Egypt, showed that the highest prevalence of ticks on migrating birds is observed during the autumn migration (Hoogstraal et al. 1961, Hoogstraal et al. 1963). In contrast to this and other studies (Weisbrod and Johnson 1989), we found a higher prevalence of tick infestation in the spring than in autumn. This difference in seasonal occurrence of ticks together with the low prevalence of tropical ticks probably reflects the geographic location of Sweden. The lower prevalence of ticks on migrating birds in the autumn is probably due to the northern location of the breeding grounds where the tick density is low, and the fact that the ticks are less active at this time of the year. Thus, the prevalence of tick infestation on migrating birds partly reflects the season and the location of the study area. The relatively few tick-infested Lyme borreliosis enzootic areas in this region are concentrated on the coast of the Baltic sea (Paper 1., Jaenson et al. 1994).

After identification of the species and age of the ticks, all *I. ricinus* larvae were analysed by an immunofluorescence assay using the anti-flagellum monoclonal antibody H9724 (Barbour et al. 1986). By this method, spirochaete-like organisms were detected in 8.8% (25 of 283) of the larvae. This is higher than found in tick larvae (4.7%) collected from passerine birds in USA (Magnarelli et al. 1992).

All ticks were subjected to total DNA extraction (Ischizawa et al. 1991). By PCR, using *ospA* and *fla* specific primers, we found that 15%
(44 of 283) of the larval *I. ricinus* from 15 of the 37 bird species examined were infected with *B. burgdorferi* s.l. This is indirect evidence that there are indeed avian reservoirs of *B. burgdorferi* s.l. in Europe.

**Figure 9.** The main migratory routes of Swedish birds. A) Birds migrating from south-west during spring, B) Birds migrating from south-east during spring and C) Fall migrators take either a south-west or south-east route. The distribution of the various genomic species of Lyme disease *Borrelia* spirochetes detected in tick larvae are shown in the boxes beside the respective arrows.
The prevalence of infected larvae was higher for ticks removed from thrushes than other species such as pipits and warblers (Table 1 and 2, Paper 5). This is similar to results from the USA (Magnarelli et al. 1992) and suggests that certain species act as reservoirs for \textit{B. burgdorferi s.l.} The frequency of infected tick larvae collected from birds was higher than would be expected by a transovarial transmission of spirochaetes to ticks (Doby et al. 1990, Mejlon and Jaenson 1993).

In Europe the distribution of the different Lyme disease \textit{Borrelia} species appear to be scattered (Nohlmans et al. 1995). When analysing the DNA from \textit{I. ricinus} larvae with the 16S rRNA gene primer sets (Marconi and Garon 1992c) we found that all three Lyme disease \textit{Borrelia} species occurring in Europe were present. There was a different prevalence of \textit{B. burgdorferi s.l.} species in tick larvae attached to birds migrating from the south-east compared to south-west migrants (Figure 9). Interestingly, \textit{B. burgdorferi s.s.} sequences were found in tick larvae collected on birds arriving from south west Europe (Figure 9). This is an indication that, even though no isolates have been obtained in Sweden, \textit{B. burgdorferi s.s.} may be brought into Sweden by migratory birds.

Our results indicate two coexisting mechanisms for the spread of \textit{B. burgdorferi s.l.} by birds. i) Migratory birds are carrying \textit{B. burgdorferi s.l.} infected ticks over great distances. The results of Paper 5, together with previous studies by others (Anderson and Magnarelli 1984, Anderson \textit{et al.} 1986, Magnarelli \textit{et al.} 1992), show that birds are involved in the dispersal of infected ticks to new sites. The dissemination of infected ticks by birds into areas where mammalian reservoir hosts and mammalian blood hosts prevail can contribute to the
establishment of new foci for Lyme borreliosis. ii) The relatively high prevalence of *Borrelia*-infected *I. ricinus* larvae collected from birds is a strong indication that several species of birds play a role, not just as tick carriers, but also as spirochetal reservoirs infective to ticks. It is possible that birds are a significant epizootological factor in the occurrence of Lyme borreliosis. They may also be, in part, responsible for the heterogeneous distribution of *B. burgdorferi* s.l. species in Europe (Nohlmans *et al.* 1995). It may also be possible that the dispersal and introduction of spirochaetes into new areas is more efficient via a spirochaemtic reservoir (bird) than by the infected vector. This hypothesis requires further investigation.
CONCLUSIONS

*B. burgdorferi s.l.* and Lyme disease are present in Northern Sweden.

The seabird tick *I. uriae* is a competent vector of *B. garinii* in both the northern and southern hemispheres.

Certain species of seabirds seem to be reservoirs of *B. garinii*. Together with *I. uriae* they may maintain a marine enzootic cycle of *B. garinii*.

The molecular polymorphism of *B. garinii* in the northern Baltic Sea suggests involvement of different Lyme borreliosis enzootic foci.

Migratory birds are important for the spread of *B. burgdorferi s.l.* in Europe.
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