TICK-BORNE DISEASES: OPENING PANDORA'S BOX

Seta Jahfari

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SETA JAHFARI

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Tick-borne Diseases: Opening Pandora's Box

Teken-overdraagbare ziekten: het openen van de doos van Pandora

Proefschrift

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Dedicated to
my caring mother,
and my loving Shahin

CONTENT

The vermin only teaze and pinch
Their foes superior by an inch.
So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed ad infinitum.

Jonathan Swift from "On Poetry: a Rhapsody" (1733)

CHAPTER 1

General Introduction
Ticking on Pandora's Box

Partially based on
'Emerging tick-borne pathogens: Ticking on Pandora's Box',
S. Jahfari and H. Sprong
'Ecology and the prevention of Lyme borreliosis',
4th volume of Ecology and Control of Vector Borne diseases,
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1. INTRODUCTION

Since the discovery of *Borrelia burgdorferi* as the causative agent of Lyme borreliosis in 1982, tick-borne pathogens have been the focus of extensive attention in scientific research, in the fields of veterinary and human medicine. The list of new or re-emerging tick-borne pathogens transmitted by the *Ixodes* species is growing and constantly evolving [1]. These developments may be attributed to various factors: i) novel approaches in the field of molecular biology have led to discovery of new tick-borne pathogens, ii) new evidence for human disease by microbes previously not considered as such, or thought to be restricted to animals [2], and iii) the geographical spread of tick vectors, hosts and their pathogens into new areas in Europe [3]. Furthermore, there are other factors that have resulted in an increase in the number of humans who are bitten by ticks in Europe, namely: iv) changes in land use that enabled the resurgence of large hosts, contributing to a sharp increase in tick densities [4, 5]; v) habitat encroachment and alterations due to recreational activities and tourism in areas with high tick densities [6]; and vi) demographic changes such as an aging population and a higher number of chronically ill people [7, 8]. The increase in the number of reported tick bites demonstrates that the risk of acquiring a tick-borne infection is growing [9]. Hence, these tick-borne diseases constitute a novel public health burden of unknown proportions.

Lyme borreliosis is the most prevalent vector-borne disease in North America and Europe [10, 11]. Furthermore, in the past decades, human exposure to tick bites has risen in most European countries. Therefore, there have been marked increases in the incidence of Lyme borreliosis and tick-borne encephalitis over the past ten to twenty years [12-14]. Lyme borreliosis can present with a wide range of clinical manifestations. The most common and earliest manifestation is an expanding rash at the site of the tick bite (*erythema migrans*), which -when left untreated- can progress towards disseminated disease. Occasionally, the infection disseminates and affects a patient's nervous system, joints, skin, and in rare cases the heart or eyes [10]. The causative agents of Lyme borreliosis are spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s. l.) complex. They are generally transmitted by ticks of the *Ixodes ricinus* complex [15] and are maintained in enzootic cycles by different vertebrate hosts [16-18]. At least five genospecies of *B. burgdorferi* s. l. complex have been shown to be pathogenic to humans, namely *Borrelia burgdorferi* sensu stricto (s. s.), *Borrelia afzelii, Borrelia garinii*, *Borrelia spielmanii* and *Borrelia bavariensis* [10, 19]. Other *Borrelia* genospecies, such as *Borrelia lusitaniae,* have occasionally been found to cause disease as well [20-23]. In the Netherlands, a retrospective study among general practitioners has shown a continuing increase in consultations for tick bites and *erythema migrans* between 1994 and 2014 [9, 24, 25]. The increasing number of tick bites, adding up to 1.1 million tick bites in 2009 [9], poses a growing risk of disseminated Lyme borreliosis and of other tick-

borne diseases. Despite the high exposure through tick bites, tick-borne diseases other than Lyme borreliosis are rarely diagnosed.

Besides the *Borrelia burgdorferi* s. l. genospecies which, the generalist tick species *Ixodes ricinus* can transmit other established tick-borne pathogens, namely *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *Rickettsia monacensis*, several *Babesia* species, Louping ill virus, and tick-borne encephalitis virus. Besides these established pathogens, there are other bacteria, such as *Coxiella* and *Francisella*, and possibly *Bartonella* that can be transmitted by *I. ricinus*. Ticks may play a role in the enzootic cycles of these pathogens; however, other transmission routes have a more prevalent role in the transmission to humans [26-28]. Furthermore, there are new potential pathogens currently emerging [29, 30]. The human infection incidence of these microbes caused by a tick bite is still a matter of debate; none of these debatable pathogens will be covered in this thesis. This thesis focuses on tick-borne pathogens transmitted by *I. ricinus* that pose an emerging public health concern in Europe, with an emphasis on the Netherlands.

2. THE VECTOR

Ticks are classified into two superfamilies: the *Ixodidae* (hard ticks) and the *Argasidae* (soft ticks). In Europe, *Ixodes ricinus* species complex is by far the most important and widespread tick while *I. persulcatus* is found in Russia and Asia and *I. pacificus* and *I. scapularis* are found in western North America and eastern North America, respectively [10, 31] (Figure 1). Typically, *I. ricinus* have a three-host life cycle that usually takes two to four years to complete where each stage (except for the egg) of the tick feeds only once, such that it can enter the next stage of its lifecycle [32] (Figure 2). In contrast to many other tick species *I. ricinus* is known to have a very large host range, it feeds on over 300 vertebrate species [31]. The larvae, nymphs and adults tend to feed on animals of different sizes, but all life stages can be found feeding on humans. The larvae are mostly found on smaller animals, mostly rodents. Nymphs feed mostly on rodents and birds [18], whereas the adult typically feed on larger animals, such as red and roe deer and wild boar but also livestock, cattle and domestic animals [33]. Mating takes place in the vegetation and on the host during blood feeding. The fertilized female lays between 1,000-5,000 eggs in the shrub layer and subsequently dies [31].

After attachment to its host, the tick pierces the host's skin and inserts a hypostome [34]. Various substances are produced and injected in the host by the tick salivary gland including; a cement to anchor the mouthparts to the skin, enzymes, vasodilators, anti-inflammatory, immunosuppressive and anti-hemostatic substances but also pathogens [34]. When it is not seeking a host, *I. ricinus* can be found at the base of vegetation, where the relative humidity is higher [33].

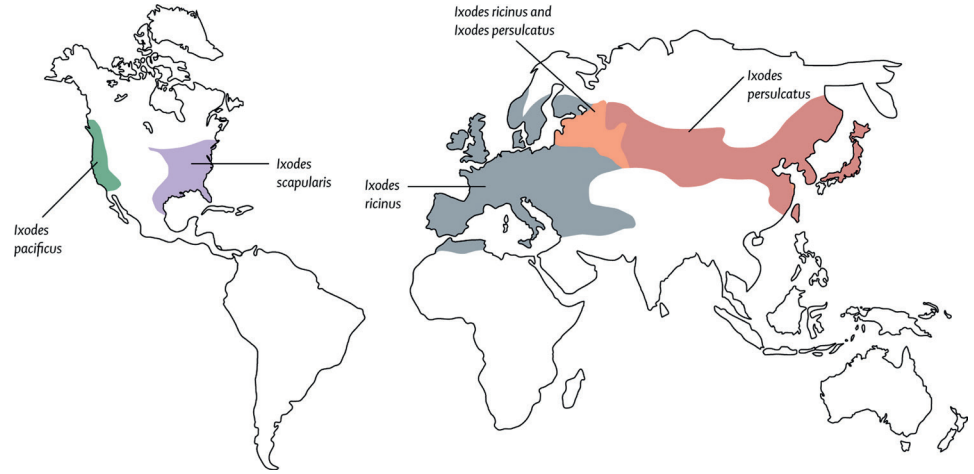


Figure 1: Global distribution of the vectors *Ixodes ricinus* species complex, map from European Union Concerted Action on Lyme Borreliosis [10] (figure used with approval of EUCALB).

Transmission dynamics of tick-borne pathogens in ticks and vertebrate hosts

An understanding of the relative risks of (re-)emergence of a tick-borne pathogen requires insight into the transmission dynamics of that pathogen in ticks and vertebrate hosts. As humans are considered accidental hosts of all tick-borne pathogens, these pathogens require one or more vertebrate host species for maintenance in enzootic cycles in nature [35]. A greater understanding of the biology of the vector, the host and the vector-borne pathogens therefore provides the best basis for risk assessment in the field of public health and public health policy-making. An interdisciplinary approach offers benefits because it addresses questions regarding tick-borne pathogens in the context of public health.

Since *I. ricinus* is a three-host tick, the feeding behavior of *I. ricinus* in each life stage has an impact on the risk of tick-borne infection for humans (Figure 2). The transmission dynamics are multi-faceted and different for each pathogen. Overall, several conditions must be met for transmission cycles to be sustained. Firstly, a vertebrate host must be present that is susceptible to infection with the pathogen. In addition, the host must experience a sufficient level of infection in the blood or skin tissue to enable the pathogen to be transmitted to other ticks during blood feeding. Thirdly, it must be possible to maintain a pathogen in the tick for extended periods, including molting into the next life stage. After molting, the tick must be able to transmit the infection to another vertebrate host. Finally, sufficient numbers of susceptible vertebrate hosts must be present in an area to maintain both the ticks and the pathogens in enzootic cycles.

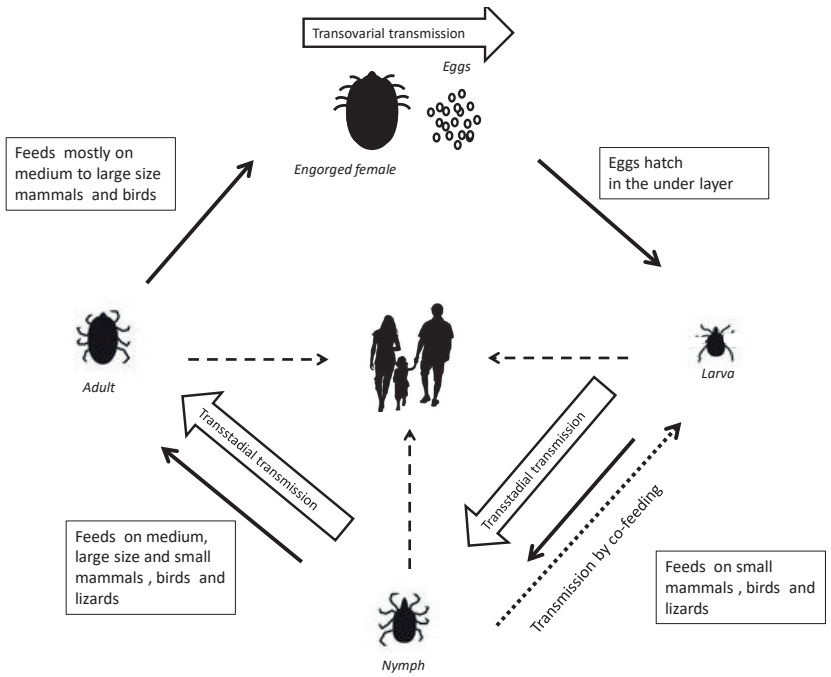


Figure 2: Life cycle of *Ixodes ricinus*. A blood meal is required to develop from one stage into the next, and for the adult female to mate and lay eggs. The arrows depict transovarial and transstadial transmission routes of pathogens and transmission by co-feeding. Humans are incidental hosts for the ticks.

Transmission cycles of pathogens among ticks and vertebrate hosts can only be preserved when ticks transfer pathogens between susceptible hosts (horizontal transmission), and will not be sustained when transmission is directed toward dead-end hosts. These dead-end hosts are incapable of experiencing high levels of the pathogen in blood or tissue (tangential transmission), or are occasionally bitten by ticks, such as humans. Depending on the micro-organism, the reservoir or amplification host responds differently to infection with a tick-borne pathogen. This has a direct impact on transmission dynamics. For example, *Babesia* species are parasites of red blood cells and are often associated with relatively mild - or asymptomatic-, but chronic infections of the reservoir host (e.g. cattle). These long-term infected animals therefore offer many opportunities for feeding ticks to become infected. On the other hand, most viral and bacterial infections are either potentially fatal or can induce an immune response in the vertebrate host. This limits the period during which the pathogen circulates in high numbers. In these situations, where the time window for other feeding ticks to become infected is limited, the tick itself becomes the crucial link in maintaining the enzootic cycle in nature. When the perpetual route of survival of the pathogen is the tick, ticks can serve as the reservoir for the pathogens, mostly due to the ability of that pathogen to be transmitted transstadially and transovarially. But also because

ticks have a longer lifespan than some of their host (e.g. small mammals like rodents). In that case, the vertebrate host acts as a so-called amplifier of the pathogen. Pathogens can be transmitted between the different life stages of ticks in different ways: i) through transstadial transmission, i.e. by transmission between different stages of tick development, from larva to nymph or from nymph to adult; ii) through transovarial transmission, i.e. by transmission of the pathogen between generations, or more precisely, from an adult female to her eggs; or iii) by means of a mechanism called co-feeding, where the micro-organism is transmitted from one tick to another during feeding in close proximity on the same host.

3. TICK-BORNE PATHOGENS ENDEMIC TO THE NETHERLANDS

Anaplasma phagocytophilum

Anaplasma phagocytophilum is a gram-negative obligate intracellular pathogen of the family *Anaplasmatacae* in the order *Rickettsiales*. It causes disease in humans and animals by infecting neutrophils, granulocytes and monocytes [36]. In 2001, the taxonomical description of the family *Anaplasmatacae* underwent extensive reorganization, supported by detailed phylogenetic analyses. Bacteria that had been known under various designations were all renamed *A. phagocytophilum*, including the organisms formerly known as *Ehrlichia phagocytophila*, *E. equi* and *Cytoecetes phagocytophila* [37]. *Anaplasma phagocytophilum* have been detected in questing nymphal and adult *I. ricinus* ticks in studies across Europe, with infection rates ranging from 0.4% to 34% [36] and with lower prevalence rates in nymphs in comparison to adult ticks. In the Netherlands, infection rates in questing nymphs and adults vary between 0% and 11% in questing *I. ricinus* ticks [38, 39]. Transovarial transmission of *A. phagocytophilum* has not been proven experimentally to occur in *Ixodes* species. However, there are some indications that transovarial transmission does occur in low frequencies. Since, low rates of *A. phagocytophilum* in questing larvae have been reported [39], and *A. phagocytophilum* has even been detected in larvae of a breeding colony after two to four generations of molting [40]. In the field, however, the bacteria are thought to be primarily maintained through enzootic cycles between vector and vertebrate hosts.

Various reservoir hosts are implicated to play a role in the maintenance of the endemic life cycle of *A. phagocytophilum* in nature. The animals identified as reservoir hosts range from domestic and wild ungulates, to small mammals like rodents and insectivores, to birds and lizards [1, 36].

In domestic ruminants and horses, clinical manifestations of anaplasmosis have been described since the 1950s [41] and 1960s [42], respectively. However, the first cases of Human Granulocytic Anaplasmosis (HGA) were not reported until 1994 in the US [43]. Cases of human infection were later reported worldwide [44]. Symptoms are generally mild, virus-like and aspecific.

In the Netherlands, the first and strangely enough the only reported human case dates from 1999 [45], despite the widespread presence of *A. phagocytophilum* in questing *I. ricinus* ticks. Furthermore, seropositivity against *A. phagocytophilum* has been observed

in the Netherlands in risk groups such as forestry workers (1.3%), and in febrile patients (3.7%), and potential Lyme borreliosis patients (4%), but not in healthy controls [46]. Since several *A. phagocytophilum* strains are reported in nature, some could be more virulent to humans than others. This may well explain the discrepancy in infection rates reported in questing ticks and the exposure levels measured using serological methods in comparison to reported symptomatic human cases. Antibodies to *A. phagocytophilum* antigens appear to have extensive serological cross-reactivity [47]. Serological tests are generally group-specific and cannot be used to distinguish antibodies resulting from exposure to, or infection with individual strains. Human granulocytic anaplasmosis (HGA) is currently thought to be the third most common tick-transmitted disease in Europe [48], and therefore poses a growing public health concern. Since HGA is not a nationally reportable disease in most European countries, an accurate estimate of HGA incidence remains a challenge.

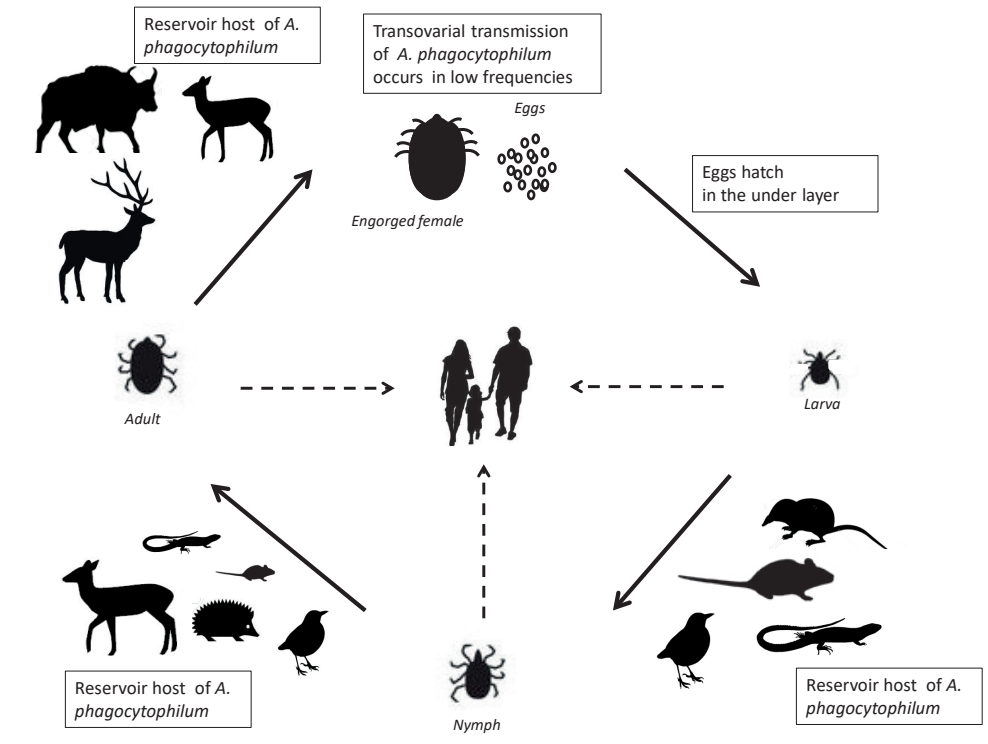


Figure 3: Life cycle of *Ixodes ricinus* and the most relevant reservoir host of *Anaplasma phagocytophilum* for each life stage of the tick.

Candidatus Neoehrlichia mikurensis

Candidatus Neoehrlichia mikurensis is a relatively novel clade in the family of *Anaplasmataceae* and was first described in wild rats and found in *Ixodes ovatus* ticks in Japan [49]. This bacterium has retained its '*Candidatus*' status, since it has not been cultivated *in vitro* thus far [50]. This obligatory intracellular bacterium was initially detected in several

variously designated species of ticks and rodents from Europe and Asia [51-57]. The Asian strains showed a 99% similarity, based on the 16S rRNA component, to the 'Schotti variant' found in *I. ricinus* that was first described in the Netherlands [51]. Since then, *Candidatus* Neoehrlichia mikurensis has been found in *I. ricinus* all over mainland Europe, with infection rates varying from 0.1% to 22% [58, 59].

In the Netherlands, infection rates of *Candidatus* Neoehrlichia mikurensis in questing *I. ricinus* nymphs range from 6% to 8%, rising to 11% for questing adult *I. ricinus* [60]. Transovarial transmission in ticks is thought not to occur [61]. So far, *Candidatus* Neoehrlichia mikurensis is thought to depend entirely on vertebrate hosts to maintain its endemic life cycle in nature. However, one study found *Candidatus* Neoehrlichia mikurensis in questing larvae [62].

Rodents are the assumed reservoir hosts of this bacterium. Experimentally, infected wild rodents have proven to be competent hosts that transmit *Candidatus* Neoehrlichia mikurensis to laboratory ticks [61]. In Europe, six rodent species (*Apodemus agrarius*, *A. flavicollis*, *A. sylvaticus*, *Myodes glareolus*, *Microtus agrestis* and *Mi. arvalis*) have been shown to be infected with *Candidatus* Neoehrlichia mikurensis [58]. Prevalence rates in rodents and ticks follow a seasonal pattern [38, 63, 64]. Furthermore, infection rates may vary considerably between rodent species.

In studies where ticks from humans were tested for *Candidatus* Neoehrlichia mikurensis in Italy, Germany and the Netherlands, infection rates varied from 4% to 10% [52, 65-67]. These figures indicate that human exposure to *Candidatus* Neoehrlichia mikurensis is significant and real. The relatively low number of human neoehrlichiosis cases reported is possibly due to the virtual absence of routine diagnostic tools, and a lack of awareness. Since, *Candidatus* Neoehrlichia mikurensis has not yet been cultivated in the laboratory, serological assays such as whole-cell IFA or specific ELISA tests are not yet available.

In 2010, the first case of human neoehrlichiosis was reported in a patient from Sweden [68]. In the same year, five other human infections were described in Germany, Switzerland and the Czech Republic [69-71], and curiously a report of neoehrlichiosis in an immunocompromised dog [72]. The symptoms described in all the human cases (febrile illnesses with fever, headache, myalgia and malaise fever) were generally non-specific and are usually seen in any other ordinary inflammatory reaction and many other infectious diseases. These reports of human infection suggested that re-evaluation was needed regarding the human disease risk with this pathogen. Most of the reported neoehrlichiosis cases occurred in immunocompromised patients who often showed severe clinical manifestations and prolonged disease [58, 59, 73]. Other neoehrlichiosis cases have been reported more recently in immunocompetent patients who had relatively mild symptoms or were even asymptomatic in Poland, China, Sweden and the Netherlands [74-78]. Two of these studies also showed that the DNA of *Candidatus* Neoehrlichia mikurensis can be detected for several months in the blood of these patients, who nevertheless did not display major clinical manifestations or complications [74, 76]. These findings indicate that the true infection rates have been underestimated.

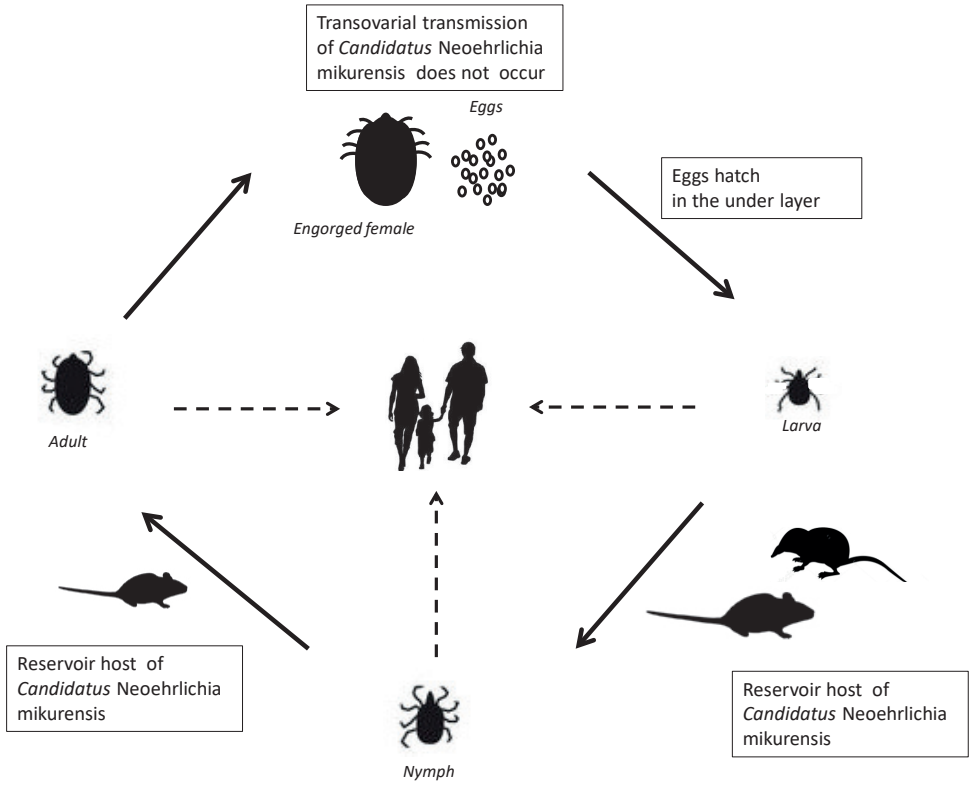


Figure 4: Life cycle of *Ixodes ricinus* and the most relevant reservoir host of *Candidatus* Neoehrlichia mikurensis for each life stage of the tick.

Borrelia miyamotoi

Borrelia miyamotoi belongs to the relapsing fever group of the *Borrelia* genus and was discovered in Japan in 1995 [79, 80]. This relapsing fever bacterium is more distantly related to the group of spirochetes that are the causative agents of Lyme borreliosis [81, 82]. It is the only member of the relapsing fever family that is transmitted by the hard tick *Ixodes* species. In Europe, *B. miyamotoi* infection rates vary between 0.5% and 4% in *I. ricinus* tick vectors [83]. In the Netherlands, these figures are the same for questing *I. ricinus* ticks, and the bacterium is found in all three life stages [84, 85]. *Borrelia miyamotoi* is thought to be transmitted transovarially and transstadially by ticks, and coexists with *B. burgdorferi* s. l. [81, 82, 86]. Experimentally, transovarial transmission has only been shown in *I. scapularis* [81], but since *B. miyamotoi* is widely found in questing *I. ricinus* larvae, it is likely that transovarial transmission does occurs [87], although probably inefficiently with an infection rate of 0,5% of questing larvae [83]. Nonetheless, since the total abundance of larvae is substantial, transovarial transmission plays an essential role in maintaining the enzootic cycle [87].

Interestingly, *B. miyamotoi* results in a short-term systemic infection in rodents, therefore making rodents excellent but transitory amplifying hosts of this bacterium [61]. *Borrelia miyamotoi* was detected in blood or tissue of various rodent species, namely *Myodes glareolus, M. arvalis, Apodemus flavicollis* and *A. sylvaticus* [61, 88, 89]. Whether vertebrates other than rodents may also become infected is not clear.

It took more than 15 years after the first discovery of *Borrelia miyamotoi* before the first human cases were recognized [90]. Mainly due to the non-specific nature of the illness [91], cases may have been confused with viral infections or other tick-borne diseases such as anaplasmosis [91]. Relapsing fever *Borrelia* infections are characterized by febrile-like illness and one or more relapse episodes of bacteremia and fever. In 2011 a series of cases was reported in Russia [90]. After this report, the pathogenicity of this bacterium was reconsidered and in 2013, several case reports from the US and the Netherlands followed in rapid succession [92-95]. Cases were later also reported from Japan [96] and Germany [97]. Additionally, another study revealed that people in the Netherlands are indeed exposed on a wide scale to ticks that carry this pathogen [85]. In this study, 3.6% of the ticks that fed on humans tested positive for *B. miyamotoi.* How widespread the levels of *B. miyamotoi* exposure, infection and disease are remains to be examined.

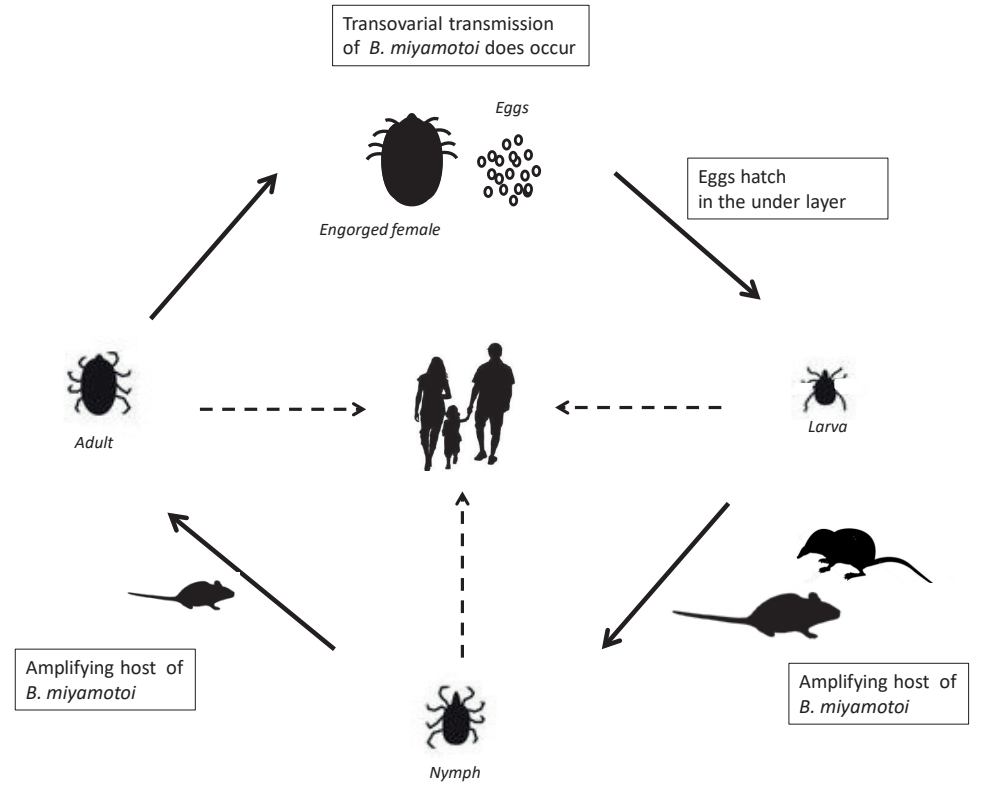


Figure 5: Life cycle of *Ixodes ricinus* and the most relevant amplifying host of *Borrelia miyamotoi* for each life stage of the tick.

Spotted fever group *Rickettsiae: Rickettsia helvetica* and *Rickettsia monacensis*

In Europe, *Rickettsia helvetica* and *Rickettsia monacensis* are the two main *Rickettsia* bacteria found in *I. ricinus*. These obligate intracellular bacteria belong to the spotted fever group of the *Rickettsiae*. *Rickettsia helvetica* was first described in 1979 from a questing *I. ricinus* tick in Switzerland [98], but it was not until 1993 that it was officially recognized as a spotted fever group *Rickettsia* [99]. *Rickettsia monacensis,* on the other hand, is a relatively novel bacterium in this same group. It was first described in 2000 in ticks from Slovakia [100], and later also in Germany [101]. Since their discovery, *R. helvetica* and *R. monacensis* have both been reported in questing *I. ricinus* ticks all over Europe. The infection rates vary from 0.5% to 66% and from 0.5% to 57% for *R. helvetica* and *R. monacensis*, respectively [102]. In the Netherlands, *R. helvetica* is widespread in the questing tick population, with infection rates ranging from 6% to 66% [38, 103, 104]. In contrast, *R. monacensis* is reported in only about 0.8% of questing ticks [38]. Like all spotted fever *Rickettsiae*, transmission occurs transstadially and transovarially. Therefore, ticks in nature are usually thought to be the main reservoir and vectors of this group of *Rickettsiae* [105]. Although transovarial transmission has not been studied in *R. monacensis,* it is not thought to differ from the process in other *Rickettsia* species in the spotted fever group. Still, despite the transovarial transmission, vertebrate hosts act as an amplifier of *Rickettsiae*, playing a vital role in transmission cycles. A study investigating transmission competence found that rodents were not able to transmit *R. helvetica* or *R. monacensis* to *I. ricinus* larvae [61]. However, various reports of bird-feeding ticks that tested positive for *R. helvetica* suggest that birds may play a role in the transmission cycle [102]. In a recent study, it was shown that birds are indeed important amplifying host for *R. helvetica* [106].

The amplification hosts of *R. monacensis* seem to be more elusive. A study where *R. monacensis* was detected in lizard tissue (7%) and fed *I. ricinus* ticks (41%) on Madeira Island, Portugal [107] suggest that lizards may be a potential reservoir or amplification host for *R. monacensis*.

Both *Rickettsiae* have been reported in a small number of human cases. Infections with *Rickettsia helvetica* were reported in different cases in Sweden [108-111]. Furthermore, *R. helvetica* infections were reported in France, Italy and Slovakia [112-114]. *Rickettsia monacensis* infections have been reported in patients with Mediterranean spotted fever-like illness in Spain and Italy [115, 116]. In the Netherlands, *R. helvetica* or *R. monacensis* were identified using serological and molecular methods in skin biopsies of *erythema migrans* patients. Interestingly, co-infections with both bacteria were also found in Lyme neuroborreliosis patients [117, 118]. However, the clinical relevance of these findings are difficult to interpret from that study, it is not yet clear whether co-infection affected the clinical manifestations and the severity of the disease, especially in Lyme borreliosis patients. However, further evaluation and isolation of the bacterium from clinical samples and further studies are required to determine the pathogenicity of *R. helvetica*.

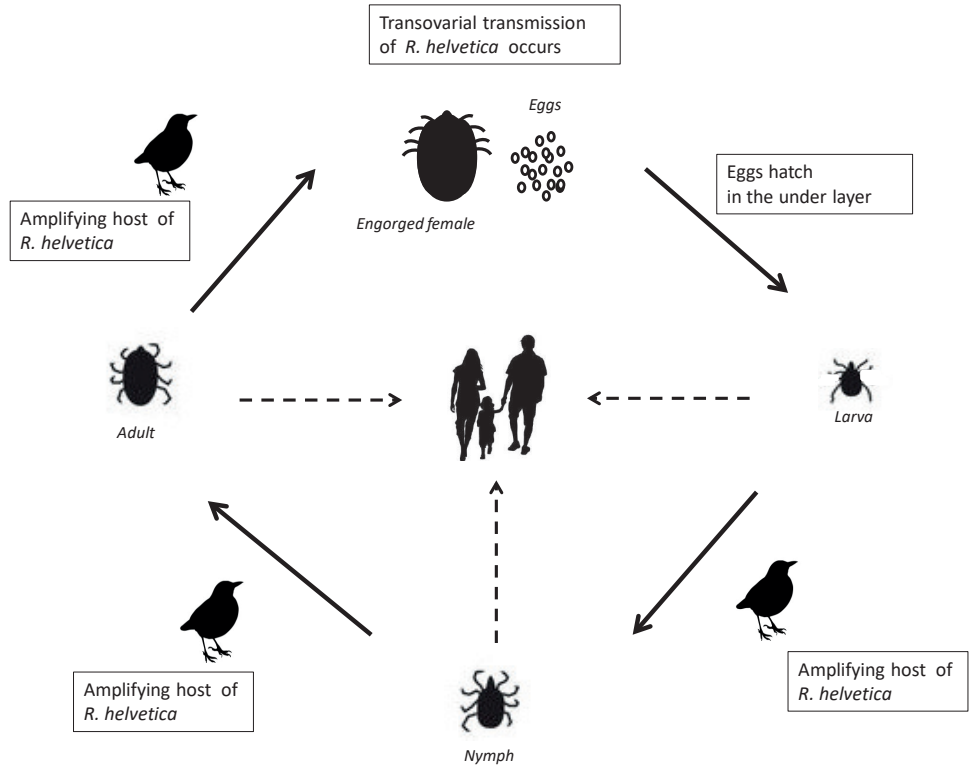


Figure 6: Life cycle of *Ixodes ricinus* and the most relevant amplifying host of *Rickettsia helvetica* for each life stage of the tick.

***Babesia* species**

In Europe, three intra-erythrocytic protozoan parasites are known to have *I. ricinus* as their vector: *Babesia divergens*, *Babesia venatorum* and *Babesia microti.* Like Malaria (*Plasmodium*), they are classified as unicellular and obligate endoparasites (apicomplexan) of the Piroplasmida suborder and the *Babesiidae* family. The infection rate of *Babesia spp.* in questing ticks ranges from 0.9% to 20% [119]. The infection rate of *Babesia* species in questing *I. ricinus* ticks in the Netherlands is around 2% [38, 120], and all three *Babesia* genospecies have been found to occur. The maintenance and persistence of *Babesia*'s within the tick vector is ensured through transstadial and transovarial transmission, with the exception of *B. microti* where transovarial transmission does not appear to occur in *I. ricinus* [121]. Cattle have been identified as the main reservoir host of *B. divergens*. Other ungulates like roe deer, fallow deer, red deer, mouflon and sheep can also be infected with this protozoan parasite [121]. The main reservoir of *B. venatorum* seems to be roe deer, and small rodents and shrews appear to be the main reservoir of *B. microti* [1]. Although babesiosis is mainly known to cause disease in animals, it is a zoonotic disease that can also infect humans. However, the main impact of babesiosis is still in the veterinary field, where

it causes disease in livestock and in companion animals [122]. In humans, *B. divergens* has been reported as the causative agent in most of the cases of babesiosis in Europe [1, 122]. Only four clinical cases caused by *B. venatorum* have been described in Europe, namely in Austria, Italy and Germany [123-125]. Only one case of *B. microti* infection has been reported in Germany [119]. All these babesiosis reports concerned immunocompromised patients, mainly patients without a (functional) spleen [122]. This is largely due to the fact that disease manifestations are more severe and often life-threatening in such heavily immunocompromised patients. In immunocompetent patients, *Babesia* infection is often mild with viral-like symptoms or even asymptomatic. The reported seroprevalence rates in Germany (5.4% to 8% for *B. microti* and 3.6% for *B. divergens*) suggest that exposure to *Babesia* is more widespread and that the number of patients is underestimated, probably due to the relatively mild symptoms or asymptomatic infections. Only two cases of human babesiosis have been reported in immunocompetent patients from France, of which one was caused by *B. divergens* [126].

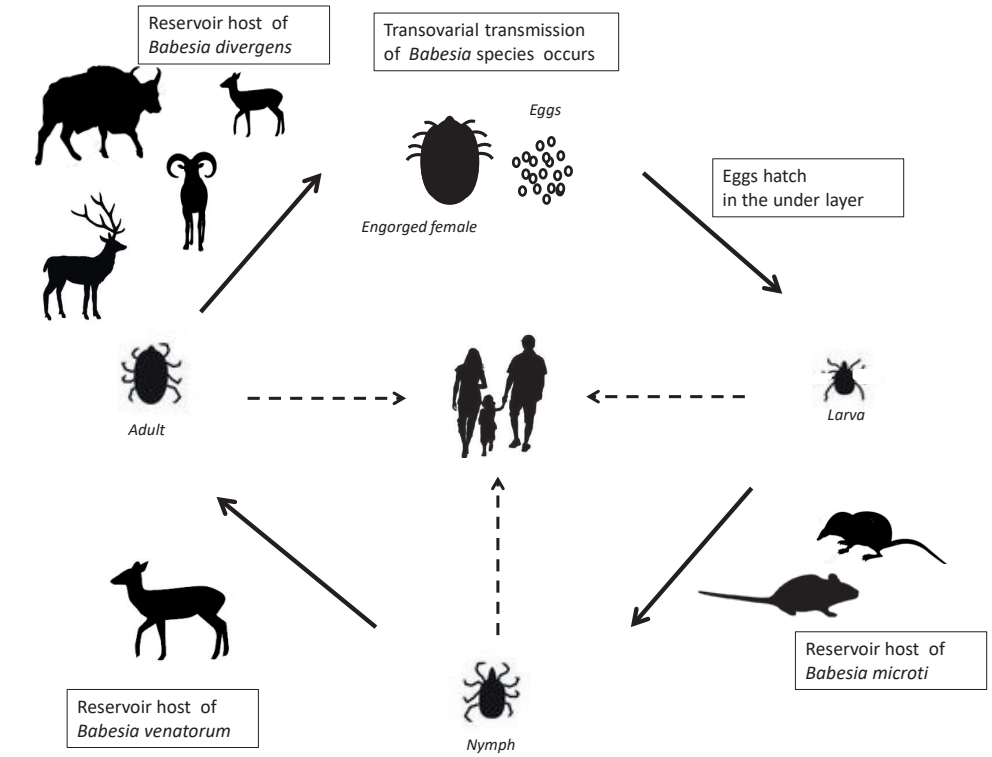


Figure 7: Life cycle of *Ixodes ricinus* and the most relevant reservoir host of *Babesia* species for each life stage of the tick.

Tick-borne encephalitis viruses

In Europe, the first reported case of Tick-borne encephalitis (TBE) occurred in 1931 in Austria, when an outbreak of meningitis was reported [127]. The causative agent – tick-borne encephalitis virus (TBEV) – was not isolated until 1937 in the former Soviet Union [128]. Tick-borne encephalitis virus is a member of the genus *Flavivirus* and the *Flaviviridae* family. Most members of this virus genus are arthropod-borne. This group of tick-borne flaviviruses (the TBEV serocomplex) comprises ten other viruses, including Omsk hemorrhagic fever virus, Powassan virus, and Louping ill virus [129, 130]. The virus has a linear positive-stranded RNA genome that consists of a single open reading frame [131]. TBEV can be divided into three subtypes: Siberian (TBEV-Sib), Far Eastern (TBEV-Fe) and European (TBEV-Eu) [132]. Only the TBEV-Eu subtype has *I. ricinus* as its main vector; the other two subtypes are associated with *I. persulcatus*.

In endemic areas in Europe, TBEV infection rates vary between 0.1 and 5% in ticks. Infection rates increase during the tick's development from stage to stage. Reported infection rates are usually less than 1% [133], with fairly high infection rates (up to 27%) in microfoci [134]. Transmission in tick populations occur transstadially, with co-feeding identified as the most efficient route of infection in naive larvae. Transovarial transmission also occurs, and even sexual transmission has even been suggested in ticks [133, 135].

Various rodent species serve as the main reservoir or amplifying host. They are able to transmit the virus via viremia to feeding ticks and by co-feeding. The rodent species *A. flavicollis*, *A. sylvaticus, M. glareolus* and *M. arvalis* are important reservoir hosts for TBEV-Eu [133]. These species can even maintain the virus in nature through latent persistent infection [136-138]. Migratory birds have been described as playing an essential role in the geographical distribution of TBEV-infected ticks, thus contributing to new foci. Still, little is currently known about the possible role of birds as TBEV reservoirs [139]. However, the prevalence of TBEV-infected bird-feeding *I. ricinus* is relatively low [1]. Ungulates that roam freely – such as goats, sheep, deer and wild boar – are thought not to contribute to the amplification of the virus. These animals are only viremic during a very short period and do not display any clinical symptoms, although they can serve as sentinels for the identification of TBEV foci in serological studies [133, 140]. In the Netherlands some sero-reactivity has been observed in wild animals and horses, although none of these cases could be confirmed through Hemagglutination Inhibition (HI) or Serum Neutralization Testing (SNT) for neutralizing antibodies [141, 142]. Although the *I. ricinus* vector of the TBEV-Eu subtype as well as the rodent reservoir are widely present in the Netherlands, it is unclear why TBEV does not thrive in an enzootic cycle. This is particularly notable considering recent reports from Belgium, where wild cervids and 2% to 4% of cattle have tested positive for TBEV with neutralizing antibodies [143-145]. These findings indicate that TBEV foci are present.

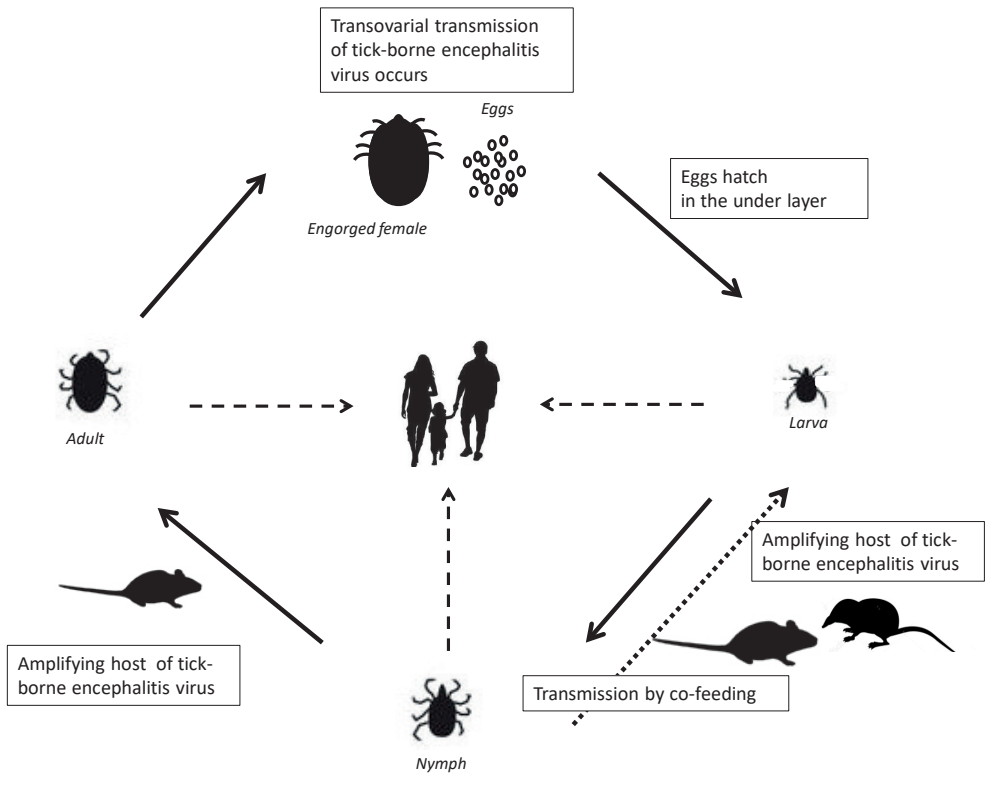


Figure 8: Life cycle of *Ixodes ricinus* and the relevant amplifying host of tick-borne encephalitis virus species for each life stage of the tick.

According to the European Centre for Disease Prevention and Control (ECDC), TBEV is currently endemic in 27 European counties [133, 146]. Furthermore, expansion northwards and to higher altitudes has been reported in recent years [147, 148]. Still, no known autochthonous cases have been reported from Spain, Portugal, the United Kingdom, Ireland or Belgium [133]. Until 2015, this was also the case for the Netherlands. The western European TBE subtype often has a biphasic course. The first phase is associated with non-specific flu-like symptoms (e.g. fever, fatigue, myalgia, nausea, or headache). This initial phase is followed by an afebrile asymptomatic interval that could precede the second phase, when the central nervous system is affected (such as meningoencephalitis, myelitis or paralysis) [146, 149]. Considering that two-thirds of human TBEV infections are believed to be asymptomatic [150] and that the TBEV-Eu subtype is associated with milder disease [149], it is possible that human cases are underreported in Europe. This underreporting could also be due to notification policies of different countries, since notification of the disease is not mandatory in all countries, but only notifiable in 17 European countries [151]. However since 2012, TBE is included in the list of notifiable diseases and under surveillance in the European Union [152].

The incidence of TBE appears to be increasing in some European countries such as Poland, Germany, Czech Republic, Slovakia, and Switzerland but also Scandinavian countries, have seen an increasing trend in TBE cases [133, 151]. The number of human TBE cases increased during the last 2 decades, with a mean of 892 annual cases reported to ECDC from 1990 to 1999 and a mean of 1382 annual cases from 2000 to 2009 [133]. Besides tick-bite prevention, vaccination is the most effective protective measure against TBE according to the World Health Organization (WHO) [153]. The WHO and the ECDC recommend TBE vaccination for people who live in TBE risk areas or people who frequently visit grasslands and forests in TBE risk areas [146, 153].

In several European countries, TBE vaccination currently is included in official government vaccination programs, under certain conditions [151]. In Austria, the vaccination program has been implemented, a coverage rate of 88% of the total population received at least one dose of the vaccine and more than half the population (58%) being within the officially recommended vaccination schedule (a shot every couple of years) [154]. In contrast, other highly endemic counties have much lower vaccination coverage for instance: Latvia 39% (2008), Estonia 20% (2008), Lithuania 19% (2008), Switzerland 17% (2007), the Czech Republic 16% (2009), Slovenia 13% (2009) and Sweden 13% (2008) [8].

4. CO-INFECTION

Since all the previously described agents can coexist in *I. ricinus* ticks, co-infections in ticks are frequently reported. However, only a small number of systematic and large-scale studies have been conducted to investigate the composition of mixed tick-borne pathogen infection rates [155]. Some studies have attempted to determine the infection rates of the entire range of pathogens among *I. ricinus* through reverse line blot analysis and other PCR amplification methods. However, the findings of these studies are difficult to compare due to differences in methodology. The outcomes of such studies are strongly affected by the methods used for tick collection, the sample size, the selection of tested tick life-stages, the selection of tested tick-borne pathogens, the DNA extraction methods, and the selection of primers and probes. Furthermore, without sequence analysis there is little discrimination amongst strain variants that are not associated with human disease. Consequently, in most European countries there is little accurate information about the co-infection rates for all tick-borne pathogens among *I. ricinus*.

According to two studies, co-infection of two or more tick-borne pathogens occurs relatively frequently in questing *I. ricinus* ticks. For instance, about 6% to 7% of questing ticks is infected with more than one pathogen [38, 156]. In a more recent study that used a novel molecular platform to test an entire range of tick-borne microbes in adult ticks, 45% of the ticks was found to be co-infected with at least two microorganisms [30].

These findings suggest that people bitten by ticks run the risk of being exposed to multiple pathogens at once or concomitantly. In other words, human co-infection with tick-borne

pathogens can occur following a single bite from a tick infected with multiple pathogens, or following several simultaneous bites from ticks that carry single pathogens. Both these scenarios can potentially result in co-infection of different tick-borne pathogens in humans [155]. Co-infections may affect the severity of disease and influence clinical outcomes. However, to what extend co-infection occurs, and influences disease outcome in a clinical setting is not known yet.

5. OTHER FACTORS THAT INFLUENCE HUMAN EXPOSURE TO TICKS AND TICK-BORNE PATHOGENS

The transmission cycles of tick-borne pathogens have multiple drivers. Many factors influence vector distribution and pathogen dynamics, such as land use, habitat destruction, degradation and fragmentation. These factors influence host density and host composition in specific areas. In addition, weather factors are important since they affect the intensity and temporal patterns of vector activity throughout the year, leading particularly to increased biting rates in humans. Climate also influences habitat suitability and therefore the survival and reproduction rates of (new) vectors and hosts. In the Netherlands, climate change is one of the many factors that influence vector habitats. Changes in landscape management (e.g. the conversion of agricultural land into habitats suitable for the maintenance of large populations of deer and other ungulates) contribute to a sharp increase in tick densities [4-6, 157]. In addition, socio-economic factors such as recreational activities in rural areas with high tick densities have resulted in increased human exposure to ticks. Demographic changes are another important factor, with elderly people making up a substantial portion of the population and improvements in healthcare for chronically ill or immunocompromised patients. In 2013, for instance, 16% of the Dutch population was older than 65 and almost one-third of the entire Dutch population suffered from one or more chronic diseases [7]. These groups can suffer more complications and severe disease when infected with a tick-borne pathogen. Furthermore, public awareness of ticks and the pathogens carried by them also plays a role in the increased reports of tick bites.

Table 1: Tick-borne pathogens transmitted by *Ixodes ricinus* in the Netherlands. With the pathogen; reservoir or amplifying host, mode of transmission in the vector, human or veterinary disease, cell tropism, and characteristics

Pathogen	Reservoir or amplifying host	Transmission in vector	Human or veterinary disease	Cell tropism	Characteristics
Anaplasma phagocytophilum	ungulates, rodents, insectivores, birds and lizards	transstadial and transovarial	Anaplasmosis, human granulocytic anaplasmosis (HGA)	neutrophils, granulocytes and monocytes	obligatory intracellular bacterium
Babesia divergens	cattle, ungulates	transstadial and transovarial	Babesiosis	erythrocytes	protozoan parasites
Babesia microti	rodents, shrews	transstadial	Babesiosis	erythrocytes	protozoan parasites
Babesia venatorum	roe deer	transstadial and transovarial	Babesiosis	erythrocytes	protozoan parasites
Borrelia afzelii	Rodents	transstadial	Lyme borreliosis	extra-cellular pathogen	spirochete bacterium
Borrelia bavariensis	rodents, hedgehogs	transstadial	Lyme borreliosis	extra-cellular pathogen	spirochete bacterium
Borrelia burgdorferi sensu stricto	Rodents	transstadial	Lyme borreliosis	extra-cellular pathogen	spirochete bacterium
Borrelia garinii	Birds	transstadial	Lyme borreliosis	extra-cellular pathogen	spirochete bacterium
Borrelia miyamotoi	Rodents	transstadial and transovarial	hard tick-borne relapsing fever, *Borrelia miyamotoi* disease	extra-cellular blood pathogen	spirochete bacterium
Borrelia spielmanii	rodents hedgehogs	transstadial	Lyme borreliosis	extra-cellular pathogen	spirochete bacterium

Table 1: Tick-borne pathogens transmitted by *Ixodes ricinus* in the Netherlands. With the pathogen; reservoir or amplifying host, mode of transmission in the vector, human or veterinary disease, cell tropism, and characteristics *(continued)*

Pathogen	Reservoir or amplifying host	Transmission in vector	Human or veterinary disease	Cell tropism	Characteristics
Candidatus Neoehrlichia mikurensis	rodents	transstadial	Neoehlrichiosis	leukocytes and endothelium	obligatory intracellular bacterium
Rickettsia helvetica	Birds	transstadial and transovarial	Rickettsiosis	endothelium	obligatory intracellular bacterium
Rickettsia monacensis	-	-	Rickettsiosis	-	obligatory intracellular bacterium
Tick-borne encephalitis virus (TBEV-Eu)	Rodents	transstadial, transovarial, sexual transmission, co-feeding	tick-borne encephalitis	neural tissue	*Flavivirus*

6. THESIS AIM AND OUTLINE

The overall aim of this dissertation is to determine to what extent endemic tick-borne pathogens other than Lyme spirochetes cause disease and/or aggravate Lyme borreliosis in humans. The disease incidence and disease burden of tick-borne diseases other than Lyme borreliosis is unknown, mostly because of poor medical awareness and absence of (good) supportive laboratory diagnostic tools. Raising awareness and improvement of the diagnosis for tick-borne diseases other than Lyme borreliosis is only warranted when there is more knowledge about the substantial risk of acquiring these diseases. To this end, we used a multidisciplinary approach to assess prevalence, enzootic cycles, epidemiology and human exposure, infection and disease of a range of pathogens transmitted by *Ixodes ricinus* ticks, as the dominant tick species present in the Netherlands.

Therefore, the objectives of the thesis are:

1. Identifying the enzootic cycles and main reservoir hosts for the various tick-borne pathogens in the Netherlands transmitted by *Ixodes ricinus*
2. Determining the human exposure, and possibly infection and disease of tick-borne pathogens other than Lyme spirochetes

This thesis has been divided in two parts, in part I, the different tick-borne pathogens (as described in the introduction and table 1 of the introduction) found in the Netherlands are studied in the field and their enzootic cycles are unraveled. To be more precise, in **Chapter 2**, we assessed the acarological risk of exposure to several tick-borne pathogens (*Borrelia burgdorferi* s. l. genospecies, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *Rickettsia monacensis*, several *Babesia* species), in the Netherlands. While testing whether these pathogens might share similar enzootic cycle, this by determining patterns of co-infection and spatial and seasonal dynamics of infection in questing *Ixodes ricinus* nymphs.

In the following chapters, we focus on the individual tick-borne pathogens by identifying their main reservoir hosts. *Candidatus* Neoehrlichia mikurensis is an emerging and vector-borne zoonosis. The first human disease cases were reported in 2010. Limited information is available about the prevalence, distribution, its natural life cycle and reservoir hosts. In **Chapter 3**, we aim to investigate the distribution and prevalence of *Candidatus* Neoehrlichia mikurensis in questing ticks the Netherlands, Belgium and the UK. To understand the enzootic cycle and main vector transmitting this newly emerging *Candidatus* Neoehrlichia mikurensis, we assess the infection rate in different tick species and possible transmission routes of *Candidatus* Neoehrlichia mikurensis in non-experimental settings and its putative mammalian hosts.

Anaplasma phagocytophilum is the etiological agent of Human Granulocytic Anaplasmosis (HGA) and anaplasmosis in animals. Knowledge on the distribution of *A. phagocytophilum* in ticks and wildlife in the Netherlands and Belgium is scarce. Wild animals and ticks play key roles in the enzootic cycles of this pathogen. The host range, zoonotic potential and transmission dynamics has only incompletely been resolved for *A. phagocytophilum.* The aim of **Chapter 4** is to investigate the distribution of *A. phagocytophilum* in different stages of endemic tick species and in wildlife hosts and free ranging domestic animals. The potential vectors and animal samples are tested by qPCR and conventional PCR, to determine whether they are infected with *A. phagocytophilum*. We investigated whether genetic delineation, based on *groEL* gene correlates with host distribution/species and zoonotic potential. To assess whether the differential distribution of the genetic variants is due to geographic variation, all available *groEL* sequences of European *A. phagocytophilum* isolates are collected and subjected to similar analyses. Population genetic analyses are used to determine which of the ecotypes is expanding.

Tick-borne encephalitis virus (TBEV) is an important public health concern and endemic in 27 European countries. The number of recognized human tick-borne encephalitis (TBE) cases in endemic regions of Europe has increased in the last decades, and expansion TBEV subtypes northwards and to higher altitudes is reported in recent years. The Netherlands and Belgium are not considered endemic for TBEV. However, recent reports from Belgium, where wildlife and ~3% of cattle have tested positive for TBEV with neutralizing antibodies, prompted us to reinvestigate the presence of TBEV in the Netherlands, in **Chapter 5**. In this

chapter, we investigate whether there is an endemic cycle tick-borne encephalitis virus in the Netherlands. By using roe deer as sentinels, we screen their sera for TBEV neutralizing antibodies. In addition, molecular screening for TBEV of questing *I. ricinus* ticks.

European hedgehogs (*Erinaceus europaeus*) are urban dwellers and host both *I. ricinus* and *Ixodes hexagonus*. These ticks transmit several zoonotic pathogens like *B. burgdorferi* s. l., *A. phagocytophilum*, *R. helvetica*, *B. miyamotoi* and *Candidatus* Neoehrlichia mikurensis. It is unclear to what extent hedgehogs in (sub)urban areas contribute to the presence of infected ticks in these areas, which subsequently pose a risk for acquiring a tick-borne disease for humans. In **Chapter 6**, we aim to investigate to what extent hedgehogs contribute to the enzootic cycle of these tick-borne pathogens, and to shed more light at the mechanisms of the transmission cycles involving hedgehogs and both ixodid tick species. By determining the prevalence of *B. burgdorferi* s. l. genospecies, *B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis and *R. helvetica* in the different stages of the *I. hexagonus* and *I. ricinus* tick species sampled from European hedgehogs from Belgium. Furthermore, assessing the role of these tick species and that of the hedgehog in the enzootic cycle of the different pathogens. By using epidemiological analysis and comparing the infection prevalences of the different pathogens from engorged ticks collected from hedgehogs with questing nymphs from the vegetation, we determine the reservoir status of the European hedgehog for *B. burgdorferi* s. l. genospecies, *B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis and *R. helvetica.* We also investigate the vector competence of *I. hexagonus* for tick-borne pathogens in non-experimental settings.

In part II of this thesis and following chapters, I translate these environmental findings to human exposure, infection and disease, in relation to public health.

Both early localized and late disseminated forms of Lyme borreliosis are caused by *B. burgdorferi* s. l. Differentiating between the spirochetes that only cause localized skin infection from those that cause disseminated infection, and tracing the group of medically-important spirochetes to a specific vertebrate host species, are two critical issues in disease risk assessment and management. It has been postulated that this genetic diversity is at the base of the multiple clinical manifestations that infection with these bacteria can display. At least five genospecies of *B. burgdorferi* s. l. are commonly associated with Lyme borreliosis in Europe: *B. afzelii*, *B. garinii*, *B. burgdorferi* s. s., *B. spielmanii*, and *B. bavariensis.* In **Chapter 7,** we want to understand the transmission and/or amplification host for each *Borrelia burgdorferi* s. l. that can cause disease and link that to a clinical manifestation of Lyme borreliosis. Therefore, we aim to directly link a transmission and/or amplification host for *B. burgdorferi* s. l. to a clinical manifestation of Lyme borreliosis. We hypothesize that a transmission cycle for a genotype is one factor that determines the clinical manifestation of Lyme borreliosis. We test this hypothesis by a quantitative molecular epidemiologic approach. Our sample collection covers both clinical sources (Lyme borreliosis patients having *erythema migrans*, Lyme neuroborreliosis, acrodermatitis chronica atrophicans, or

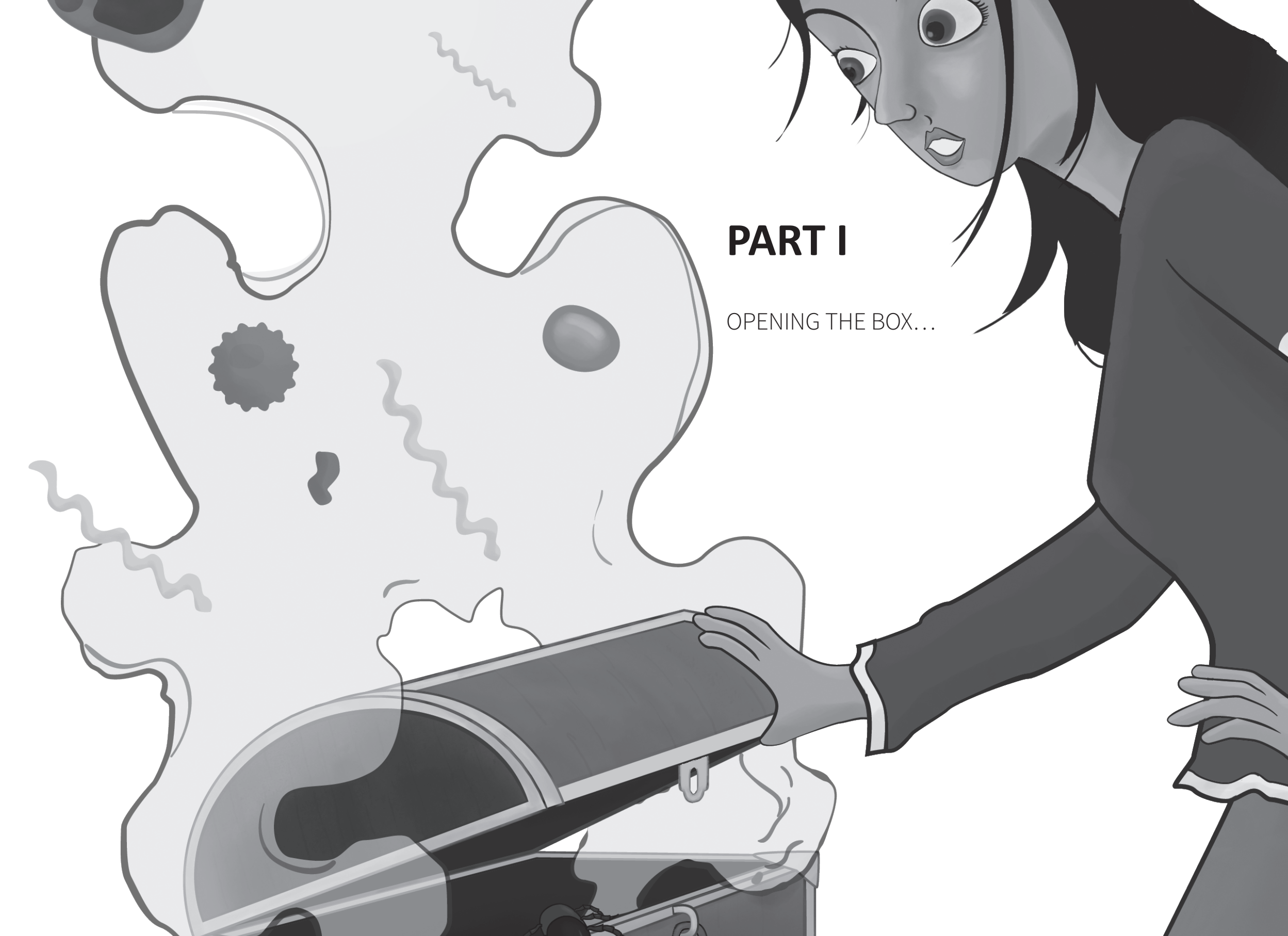
Lyme arthritis) and field sources (ticks feeding on birds, rodents and hedgehogs). Plasmid genes, regulatory, housekeeping and neutral genetic loci from a number of samples are input to the statistical test of the hypothesis.

Tick-borne diseases are the most prevalent vector-borne diseases in Europe. Still, knowledge on the incidence and clinical presentation of other tick-borne diseases than Lyme borreliosis and tick-borne encephalitis is minimal, despite the high human exposure to these pathogens through tick bites. Using molecular detection techniques, the frequency of tick-borne infections after exposure through tick bites was estimated. In **Chapter 8**, we aim to investigate whether infection with tick-borne pathogens other than *B. burgdorferi* s. l. can be shown in patients with early localized Lyme borreliosis and in people exposed to tick bites in the Netherlands, by using molecular detection techniques, the frequency of tick-borne infections after exposure through tick bites will also be estimated. In addition, we aim to determine the clinical picture of patients with DNA of tick-borne pathogens in their blood.

Ixodes ricinus ticks transmit *B. burgdorferi* s. l., the causative agent of Lyme borreliosis and transmit *B. miyamotoi,* which was recently found to cause infections and disease in humans. In **Chapter 9**, we aim to determine the prevalence of *B. miyamotoi* infection in ticks and natural amplifying hosts in the Netherlands and to what extent ticks are co-infected with *B. burgdorferi* s. l.. In addition, *erythema migrans* has been sporadically described in *B. miyamotoi* infected patients, but these skin lesions might as well represent co-infections with *B. burgdorferi* s. l.. However, thus far only one PCR-confirmed patient has been described in the Netherlands, suggesting under-diagnosis due to a lack of awareness, lack of severe symptoms, lack of widely available diagnostic tools and/or misdiagnosis. Because no routine diagnostics are currently performed for *B. miyamotoi* in the AMC (tertiary) multidisciplinary Lyme disease center, we aimed to investigate if (co-) infections with *B. miyamotoi* were missed over the past years. By using *B. miyamotoi*-specific real-time PCR on ticks and in spleen samples from potential reservoir hosts in nature and in Lyme borreliosis-suspected human skin biopsies, which were previously tested for *B. burgdorferi* s. l. by PCR.

Substantial exposure to *B. miyamotoi* occurs through bites from *I. ricinus* ticks in the Netherlands, this tick species transmits besides *B. burgdorferi* s. l. also *A. phagocytophilum* and other pathogens. Direct evidence for *B. miyamotoi* infection in European populations is scarce. A viral-like illness with high fever, resembling Human Granulocytic Anaplasmosis (HGA), has been attributed to *B. miyamotoi*-infections in relatively small groups. *Borrelia miyamotoi*-infections associated with chronic meningoencephalitis have also been described in occasional case reports. In **Chapter 10**, the objective is to gain more insight in the public health risk of *B. miyamotoi.* As a first attempt to describe the exposure of *B. miyamotoi* in the Netherlands, using a newly in-house developed serological assay based on the Glycerophosphodiester phosphodiesterase (GlpQ) antigen, which appears to be highly conserved among all members of the relapsing fever *Borreliae*, including *B. miyamotoi,* but distinct for the spirochetes causing Lyme borreliosis and their near relatives. In addition, we

determined here the seroprevalence of anti-*B. miyamotoi* antibodies in different risk groups within the general population. Apart from important epidemiologic insights, our findings will facilitate the future identification of the clinical symptoms of *B. miyamotoi* infections and might serve as a starting point for further development of serological assays.
In the synthesis (**Chapter 11**), I summaries the findings in the different chapters and discuss the implications of the data presented in this thesis.



PART I

OPENING THE BOX…

CHAPTER 2

Spatiotemporal dynamics of emerging pathogens in questing *Ixodes ricinus*

E. C. Coipan, S. Jahfari, M. Fonville,
C. B. Maassen, J. van der Giessen, W. Takken,
K. Takumi and H. Sprong (2013).
Frontiers in Cellular and Infection Microbiology

ABSTRACT

Ixodes ricinus transmits *Borrelia burgdorferi* sensu lato, the etiological agent of Lyme disease. Previous studies have also detected *Rickettsia helvetica*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, and several *Babesia* species in questing ticks in the Netherlands. In this study, we assessed the acarological risk of exposure to several tick-borne pathogens (TBPs), in the Netherlands. Questing ticks were collected monthly between 2006 and 2010 at 21 sites and between 2000 and 2009 at one other site. Nymphs and adults were analyzed individually for the presence of TBPs using an array-approach. Collated data of this and previous studies were used to generate, for each pathogen, a presence/absence map and to further analyze their spatiotemporal variation. *Rickettsia helvetica* (31.1%) and *B. burgdorferi* sensu lato (11.8%) had the highest overall prevalence and were detected in all areas. *Candidatus* Neoehrlichia mikurensis (5.6%), *A. phagocytophilum* (0.8%), and *Babesia spp.* (1.7%) were detected in most, but not all areas. The prevalences of pathogens varied among the study areas from 0 to 64%, while the density of questing ticks varied from 1 to 179/100 m^2. Overall, 37% of the ticks were infected with at least one pathogen and 6.3% with more than one pathogen. One-third of the *Borrelia*-positive ticks were infected with at least one other pathogen. Coinfection of *B. afzelii* with *Candidatus* Neoehrlichia mikurensis and with *Babesia spp.* occurred significantly more often than single infections, indicating the existence of mutual reservoir hosts. Alternatively, coinfection of *R. helvetica* with either *B. afzelii* or *Candidatus* Neoehrlichia mikurensis occurred significantly less frequent. The diversity of TBPs detected in *I. ricinus* in this study and the frequency of their coinfections with *B. burgdorferi* s. l., underline the need to consider them when evaluating the risks of infection and subsequently the risk of disease following a tick bite.

INTRODUCTION

In the Netherlands, the hard tick *Ixodes ricinus* is the main vector of a variety of human pathogens. The most prevalent tick-borne disease is Lyme borreliosis [10]. This multi-systemic disorder is caused by several members of the *Borrelia burgdorferi* sensu lato complex. Of the 18 genospecies of this complex [158], *B. afzelii, B. garinii, B. spielmanii, B. bavariensis*, and *B. burgdorferi* sensu stricto have already been detected in the Netherlands, in both patients and questing ticks. *Borrelia lusitaniae*, and *B. valaisiana* were detected in questing *I. ricinus*, but their public health significance is less clear [21, 159-161]. Over the last decades, the incidence of Lyme borreliosis has increased significantly in Europe [13]. A long-term retrospective study among general practitioners in the Netherlands has shown a continuing increase in consultations for tick bites and *erythema migrans* in the last decade [162]. The incidence of *erythema migrans* patients increased from 39 per 100,000 inhabitants in 1994 to 134 per 100,000 inhabitants in 2009. Previous studies in the Netherlands have identified the presence of other pathogens in questing *I. ricinus* as well. Human babesiosis is caused by the intra-erythrocytic protozoa *Babesia divergens, B. venatorum (EU1)*, and *B. microti* [163]. A recent study reported these three *Babesia* species in approximately 1% of questing *I. ricinus* [120]. The spotted fever syndrome is caused by at least 15 different *Rickettsia* species, some of which are transmitted by *I. ricinus* [164]. *Rickettsia conorii* and *R. monacensis* are probably the most common tick-borne *Rickettsiae* to cause disease in Europe [164], whereas the pathogenicity of *R. helvetica* is still questionable [165]. All three *rickettsia*l species have been previously found in the Netherlands [103] with local prevalences varying from <1% (*R. conorii*) to as high as 66% (*R. helvetica*). *Anaplasma phagocytophilum*, the etiologic agent of human granulocytotropic anaplasmosis [103], has been detected in ticks, from the Netherlands in several studies [66, 104]. *Candidatus* Neoehrlichia mikurensis can be considered an emerging zoonosis, as more than eight human cases have been described in Europe since 2010, while previously it was considered non-pathogenic. Despite an overall prevalence of *Candidatus* Neoehrlichia mikurensis in questing ticks of approximately 7% [60], no human cases have been reported in the Netherlands.

Autochthonous tick-borne diseases other than Lyme disease have not been reported, except for a single case of granulocytotropic anaplasmosis in 1999 [45]. This may be caused by lower pathogenicity, lack of overt symptoms, or lack of awareness of public and health professionals. Multiple studies have reported coinfection with some of the tick-borne pathogens (TBPs) [156, 166-170]. It is known that the severity of Lyme disease may be affected by simultaneous infections with other TBPs [155, 166]. Some of them, such as *A. phagocytophilum*, modulate host immunity and increase susceptibility to various second pathogens, including *B. burgdorferi* [171, 172]. Thus, coinfection might be partly responsible for the variability in clinical manifestations that are usually associated with Lyme borreliosis. The acquirement of a tick-borne disease depends on many environmental, societal, and immunological factors, but it is always preceded by the bite of a tick infected with the causal agent. Previous studies have shown that the risk of infection of humans by TBPs depends

mainly on the density of questing infected ticks—the acarological risk [173-176]. The study of mixed infections in questing ticks might therefore, reveal patterns of coinfection of *B. burgdorferi* s. l. with two or more other pathogens, allowing us to generate hypotheses on the transmission cycle of some more obscure pathogens from the dynamics of better-known ones. The aim of this study was to assess the acarological risk of exposure to TBPs in the Netherlands by comparing the abundances of questing ticks infected with *B. burgdorferi* s. l. and with other TBPs.

METHODS

Collection of ticks and tick data

All ticks were collected on a monthly basis between 2006 and 2010 in 21 sites. In Duin & Kruidberg field sampling was conducted between 2000 and 2009. The sites were spread all over the Netherlands and they have been selected based on Lyme borreliosis incidence in humans, and the availability of volunteers. The same sites were described in some previous studies regarding ticks and TBPs in the Netherlands [103, 177-179]. Sampling of ticks was done by blanket dragging, using a 1 m^2 cloth on a 100 m long transect. Based on morphological criteria, ticks were identified to species level, with stage and sex recorded. The density of ticks was estimated as the number of questing ticks *per* 100 m^2. Additionally, data on the presence of ticks and TBPs in other 39 areas were collected from some previous studies that have used the same sampling and analysis methodology [51, 60, 66, 104].

DNA Extraction of ticks

All the collected ticks were immersed in 70% alcohol and stored at −20◦C until the DNA extraction. DNA from questing ticks was extracted by alkaline lysis [177]. Questing larvae were not taken into account as humans are generally bitten by either nymphs or adult *I. ricinus* [66, 180].

PCR detection and identification of pathogens

The presence of the DNA of different TBPs (*Rickettsia spp., B. burgdorferi* s. l., *Ehrlichia/ Anaplasma spp.,* and *Babesia spp.*) was determined by polymerase chain reaction (PCR) followed by reverse line blotting (RLB) as described before [104, 177]. To minimize cross contamination and false-positive results, positive and negative controls were included in each batch tested by PCR and RLB assays. Furthermore, DNA extraction, PCR mix preparation, sample addition, and PCR analysis were performed in assigned separate labs. PCR products of some samples were sequenced by dideoxy-dye termination sequencing of both strands, and compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/), using BLAST [181]. The sequences were aligned and analyzed using BioNumerics 6.6 (Applied Maths, Kortrijk, Belgium). The prevalence of infection was calculated as the percentage of ticks infected with a certain microorganism.

Acarological risk

To calculate the densities of questing ticks infected with each of the five pathogens' genera, we multiplied the prevalence of infection with the density of questing ticks in each of the investigated sites.

Correlation between prevalence and tick density

For some pathogens, we noticed that the prevalence might correlate with the density of questing ticks at the sampling locations. To test this possibility we fitted a binomial model to our data, by defining the prevalence of infection as an exponential function of the tick density (d) at each sampling location. Knowing that the number of infected ticks (k) out of the total number of ticks tested (n), is binomially distributed with a probability (p), we used the function $p = aExp[bd]$, $0 < a < 1$, to test an alternative model ($b < 0$) against a null model ($b = 0$) by a likelihood-ratio test. The alternative (decreasing exponential) model fitted significantly better to our data with p-value $P \leq 0.05$.

Seasonal dynamics

To test for the seasonal dynamics of the prevalence against a constant prevalence of infection, a binomial model for the prevalence of infection (p) was fitted to our tick abundance data, in combination with the sampling days (d). The prevalence of infection was thus a cosine function of the sampling days. We tested an alternative model ($f = \frac{\text{PDF}[\text{BinomialDistribution}[n,p],x]}{\partial p} \to \frac{e^{a+a1\text{Cos}[t]+b1\text{Sin}[t]}}{1+e^{a+a1\text{Cos}[t]+b1\text{Sin}[t]}}$) against a null model ($f = \frac{\text{PDF}[\text{BinomialDistribution}[n,p],x]}{\partial p} \to \frac{e^{a}}{1+e^{a}}$). We calculated the test statistic (difference) for the likelihood-ratio and we determined whether the alternative (seasonal) model fitted better our data by deriving the probability of the difference (where a difference with a *p-value* ≤ 0.05 was considered significant). Based on the binomial likelihood, a significant seasonal dependence of the prevalence was assessed for each pathogen. All the statistical analyses were performed with Wolfram Mathematica 9.

RESULTS

The mean density of questing nymphs and adult ticks varied greatly between sites, from as low as 1 (at Houtvesterijen Heide) up to 179/100 m^2 (at Duin & Kruidberg; **Table 2**), results that are consistent with previous Dutch studies [177].

Pathogen detection and identification

A total of 5570 questing nymphs and adult *I. ricinus* from 22 different study areas were tested for the presence of TBPs by PCR amplification followed by RLB (Table 1). The recently identified *B. bavariensis* reacted consistently with our *B. garinii* probe [182], and therefore we grouped these two *Borrelia* genospecies. Five *Borrelia* genospecies were found in this study in all twenty-two study areas (Table 1), with the overall prevalence (11.8%) inscribed

in the interval of average European prevalence [183], and comparable with previous studies in the Netherlands [177, 178]. *Borrelia afzelii* was predominant (6.7%), followed by *B. garinii*/*B. bavariensis* (1.5%), *B. valaisiana* (1.2%), and *B. burgdorferi* sensu stricto (0.2%). The remaining fraction of the *Borrelia* positive samples could not be further identified to the species level by RLB. Sequencing several of these samples revealed the presence of *B. spielmanii*, corroborating previous findings of this genospecies in the Netherlands [184]. *Borrelia lusitaniae*, which was recently found in the Netherlands [104], was not detected in this study.

Rickettsia helvetica was most frequently detected in tick lysates, its 31.1% average prevalence (Table 1) being among one of the highest in Europe [range 1.5 to more than 40.6% [185, 186]]. A previous study from our laboratory found *R. helvetica* not only in vertebrate hosts, but also in tick larvae at comparable prevalences as for the other tick stages, indicating a high efficiency of transovarial transmission [103]. Thirty-three *Rickettsia* isolates could not be identified up to the species level by RLB. Sequencing of these samples revealed the presence of *R. monacensis*, which was reported in the Netherlands before [103]. *Rickettsia conorii* was detected in only three questing ticks from one study area (Veldhoven). *Anaplasma phagocytophilum*-infected ticks were recorded with an overall prevalence of only 0.8% (Table 1). *Candidatus* Neoehrlichia mikurensis DNA was found with a global prevalence of 5.6% (Table 1). *Ehrlichia canis* DNA was detected in only 5 tick lysates from four different study areas, which resulted in an overall prevalence of 0.1% (5/5343). Ninety-nine *Ehrlichia* isolates could not be identified to the species level neither by RLB nor by sequencing. *Babesia venatorum*, formerly also known as *B.EU1* [187], was present with a global prevalence of 1.0% (41/4238). The prevalence of *B. microti* in questing ticks was 0.4% (17/4238), and the protozoan was detected in 6 from 19 sites. Only one tick from the Duin & Kruidberg area contained the DNA of previously reported *B. divergens* [120]. Twelve *Babesia sp.* could not be further identified by neither RLB, or sequencing. The average prevalence of *Babesia*-positive ticks in the study areas was 1.6% (Table 1).

Spatial distribution and variation

Collated data were used to generate presence/absence maps of the five major TBPs in the Netherlands (Figure 1). The presence/absence of *Borrelia spp.*, *R. helvetica*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, and *Babesia spp.* was assessed for 61, 24, 39, 39, and 25 locations, respectively. The presence of these pathogens was observed in 58, 24, 33, 20 and 18 areas, respectively, heterogeneously distributed across the Netherlands. The few absence points were scattered over the Netherlands as well, and did not cluster in any geographic region (Figure 1).

Table 1: Observed and expected coinfections. Chi-square tests were used to calculate the associations of several combinations of pathogens *Significant positive associations and ** and significant negative associations (P<0.05) are shown in bold.

Observed (%)	***R. helvetica***	***A. phagocytophilum***	***N. mikurensis***	***Babesia* (all)**
Borrelia (all)	3.3%	0.1%	1.6%	0.4%
B. afzelii	1.8%	0.0%	1.3%	0.3%
R. helvetica		0.3%	2.2%	0.5%
A. phagocytophilum			0.0%	0.0%
N. mikurensis				0,1%
Expected (%)	*R. helvetica*	*A. phagocytophilum*	*N. mikurensis*	*Babesia (all)*
Borrelia (all)	3.9%	0.1%	0.7%	0.2%
B. afzelii	2.2%	0.1%	0.4%	0.1%
R. helvetica		0.3%	1.9%	0.5%
A. phagocytophilum			0.0%	0.0%
N. mikurensis				0.1%
Chi-test (p-value)	*R. helvetica*	*A. phagocytophilum*	*N. mikurensis*	*Babesia (all)*
Borrelia (all)	0.03**	0.30	0.00*	0.01*
B. afzelii	0.03**	0.24	0.00*	0.00*
R. helvetica		0.80	0.05**	0.77
A. phagocytophilum			0.10	0.42
N. mikurensis				0.66

Borrelia prevalence was between 5% (Houtvesterijen Heide) and 50% (Bellingwedde; where only six ticks were tested), while for *R. helvetica* it varied even more, from 3% in some sites (Apeldoorn), to 64% in others (Duin & Kruidberg) (Table 2). Lower variations in prevalences were observed for *Candidatus* Neoehrlichia mikurensis, *A. phagocytophilum* and *Babesia spp.* (Table 2). For *Candidatus* Neoehrlichia mikurensis, the prevalence was on average of 5%, but some areas displayed values of over 10% (Table 2). *Babesia spp.* showed an over- all prevalence of 1.7%, similarly to Germany and Luxembourg [169, 188]. *Anaplasma phagocytophilum* was the least prevalent pathogen in our study, with a mean prevalence of 0.8%—comparable with the 0.5–1% prevalence found in different European countries [186, 189, 190]. However, one of the sites displayed a 10-fold higher prevalence than average (Bilthoven 8%, Table 2).

Table 2: Prevalence (%) of the five major pathogens found in the 22 study areas. Tick density/activity of each study area was expressed as the average density/activity of the questing ticks ticks collected from April to September from at least three consecutive years. Average prevalences of the study areas (n=22) were calculated. +: positive samples; T: tested; ND: Not determined.

Geographic location	*B. burgdorferi sl.*			*R. helvetica*			*N. mikurensis*			*A. phagocytophylum*			*Babesia spp.*			*Density (/100m2)*	
	+	T	%	+	T	%	+	T	%	+	T	%	+	T	%	Nymphs	Adults
Apeldoorn	15	38	39	1	38	3	3	38	8	0	38	0	5	38	13	17	5
Appelscha	10	79	13	11	76	14	3	79	4	0	79	0	4	79	5	19	5
Bellingwedde	3	6	50	2	6	33	0	6	0	0	6	0	0	6	0	7	0.3
Bijlmerweide	34	330	10			ND	1	330	0,3	0	330	0			ND	12	1
Bilthoven	4	40	10	6	40	15	0	40	0	3	40	8	1	40	3	9	3
D&Kruidberg	123	1640	8	848	1327	64	113	1676	7	11	1676	1	12	1499	1	160	19
Ede	48	354	14	23	354	6	36	353	10	3	353	1	2	353	1	54	7
Eijsden	28	232	12	23	232	10	0	232	0	1	232	0,4	10	232	4	34	1
Gieten	10	136	7	31	136	23	6	136	4	2	136	1	2	136	1	59	5
Haaksbergen	9	105	9	11	105	10	1	105	1	4	105	4	2	105	2	77	2
Hoge Veluwe	2	8	25	2	8	25	0	8	0	0	8	0	0	8	0	25	1
Hoog Baarlo	28	311	9	24	311	8	2	311	1	4	311	1	9	311	3	34	2
Hoogeveen	47	163	29	48	163	29	11	163	7	0	163	0	5	163	3	63	3
Houtvest_Bos	49	510	10			ND	35	510	7	4	510	1			ND	32	2
Houtvest_Hei	4	88	5			ND	5	88	6	1	88	1			ND	1	0.2
Kwade Hoek	43	162	27	13	162	8	23	162	14	0	162	0	3	162	2	9	4
Montferland	18	1470	12	12	147	8	11	147	7	0	147	0	3	147	2	40	3
Nijverdal	24	127	19	13	127	10	18	127	14	1	127	1	8	127	6	34	2
Ruinen	25	94	27	30	94	32	2	94	2	2	94	2	1	94	1	18	1
Twiske	46	292	16	62	292	21	13	292	4	1	292	0,3	0	292	0	36	2
Veldhoven	25	242	10	14	239	6	13	242	5	6	242	2	1	242	0,4	47	17
Wassenaar	33	204	15	91	204	45	4	204	2	1	204	0	3	204	1	46	3
Total/Average	628	5308	11.8	1265	4061	31.1	300	5343	5.6	44	5343	0.8	71	4238	1.7	38	4

Identification of high risk-areas depends on both pathogen prevalence and density of questing ticks (nymphs and adults). The density of questing ticks varied between 1/100 m2 (Houtvesterijen Heide) and 179/100 m2 (Duin & Kruidberg; Table 2). The density of questing *Borrelia*-infected ticks varied between 0 and 19 ticks per 100 m2 (Figure 2), whereas the maximum densities of *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis and *Babesia spp.* infected ticks were 3.0, 13, and 2.9 ticks per 100 m2, respectively. The density of questing *R. helvetica*-infected ticks varied between 0 and 22 ticks per 100 m2, with one notable exception: Duin & Kruidberg area had both a high tick density and an exceptionally high *R. helvetica* prevalence, which resulted in a density of questing *R. helvetica*- infected ticks of up to 119 ticks per 100 m2. Considering that these are calculated as average values for an entire season, it is therefore inevitable that the densities of infected questing ticks are actually higher for peak months of tick activity [i.e., May-June [178]]. Based on a likelihood ratio test, performed for a decreasing model and a constant one, we detected a significant negative correlation between the density of questing ticks and the infection prevalence with *B. burgdorferi* s. l. ($p = 3.6 \times 10{-}10$) and *Babesia spp.* ($p = 4.9 \times 10{-}5$) (Figure 3). On the other hand, there was no correlation found between these variables for *R. helvetica* (p = 1.0), *Candidatus* Neoehrlichia mikurensis (p = 1.0) and *A. phagocytophilum* (p = 0.69) (Figure 3). Graphs for the density of infected questing ticks against the density of questing ticks revealed that the former is linearly increasing with the latter for *R. helvetica*, *Candidatus* Neoehrlichia mikurensis and *A. phagocytophilum* (Figure 4). For the other two pathogens— *Babesia spp.* and *B. burgdorferi* s. l., the density of infected questing ticks reached the maximum values at densities of questing ticks of 119 and 268, respectively (Figure 4).

Temporal variation

To gain insight into long-term dynamics of ticks and their pathogens, we analyzed the data obtained from Duin & Kruidberg, where a 10-year (2000–2009) tick-surveillance was performed. This area was selected at that time because of its unusual high tick density/activity. The prevalences of all pathogens were relatively stable over the past decade (*B. burgdorferi* s. l. 7.0%, *B. afzelii* 4.6%, *A. phagocytophilum* 0.7%, *R. helvetica* 65%, *Babesia spp.* 1.1%), except for *Candidatus* Neoehrlichia mikurensis, whose prevalence increased from 3.5% (2000–2007) to 12% in the last 2-year interval (2008–2009). The average density/activity of adult ticks remained relatively low with 7–34 ticks per 100 m2. The average density/activity of nymphal ticks was more pronounced (102–410 ticks per 100 m2) and peaked in 2004–2005 (Figure 5). The likelihood ratio test detected similar decreasing trends in the temporal relation between the prevalence and the tick density as for the spatial variation analysis (not shown). Despite the inverse relationship between the prevalence and the tick density, the peaks of density/activity of infected ticks coincided with the peak of high densities of questing ticks in 2004–2005 (Figure 5).

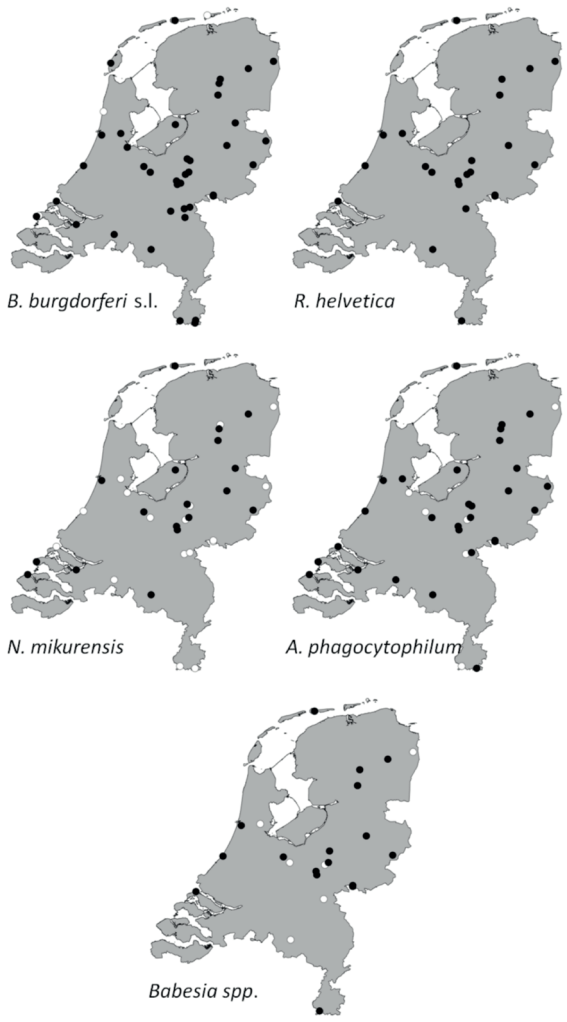


Figure 1: Aggregated presence/absence map of questing *I. ricinus* nymphs/adults infected with *B. burgdorferi s.l.* (A), *R. helvetica* (B), *N. mikurensis* (C), *A. phagocytophylum* (D), *Babesia species* (E). Presence/absence points from previous studies [205-212] were also incorporated.

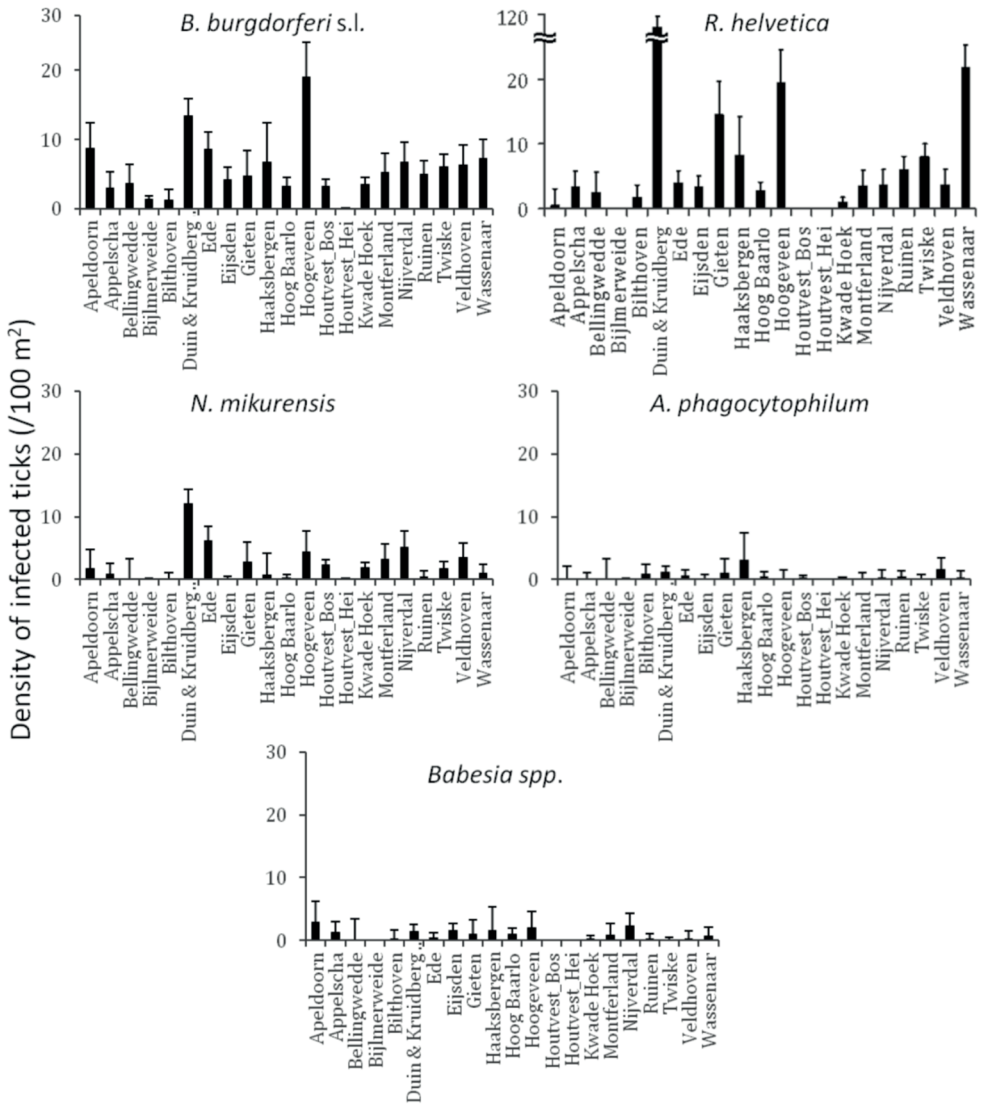


Figure 2: Identification of high risk-areas depends on both prevalence and tick density/activity. Their calculated product defines the density/activity of infected ticks (nymphs and adults/100 m2). The error bars depict the upper limit of the 95% confidence interval. Duin en Kruidberg's density of *R. helvetica* infected ticks reaches to 119/100 m2.

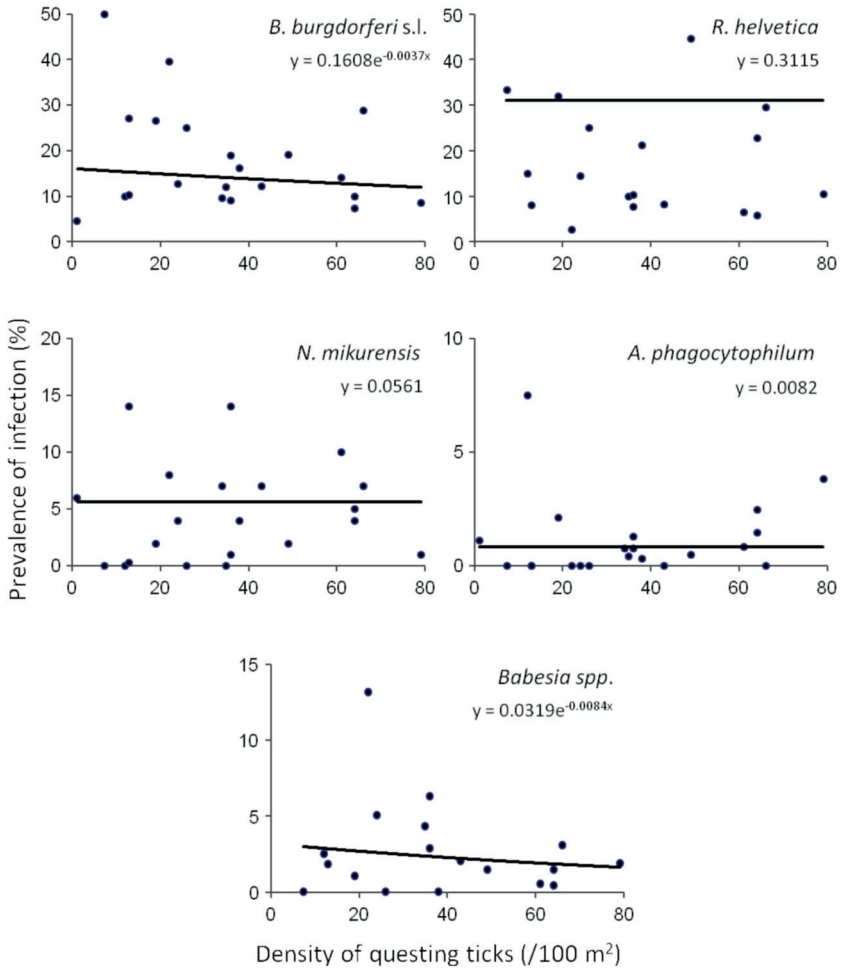


Figure 3: Density and prevalence relations. Significant negative correlations between the density of questing ticks and the infection prevalence were found for *B. burgdorferi* s.l. (p = 3.6 × 10−10) and *Babesia spp.* (p = 4.9 × 10−5). On the other hand, there was no correlation found between these variables for *R. helvetica* (p = 1.0), N. mikurensis (p = 1.0), and *A. phagocytophilum* (p = 0.69). Note that due to the very small exponents, the curves look approximately linear, although they are in fact exponential, as explained in the text. The data set included all of the areas except for Duin&Kruidberg.

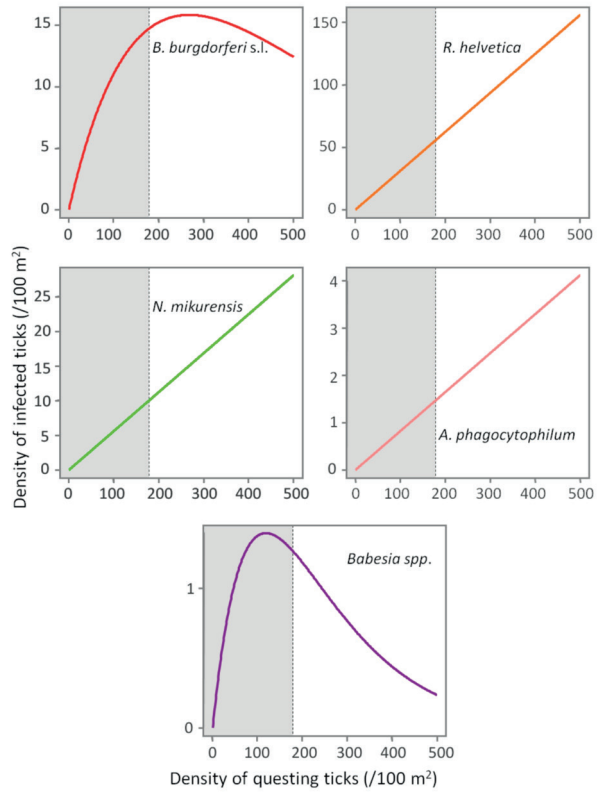


Figure 4: Evolution of the density of infected ticks (y-axis) with the density of questing ticks (x-axis). The density of infected ticks is calculated as the product of the infection prevalence (from the highest likelihood model) with the density of questing ticks. The numbers are expressed as ticks/100 m2. The grey area marks the normal questing ticks densities (0-179/100 m2) in The Netherlands.

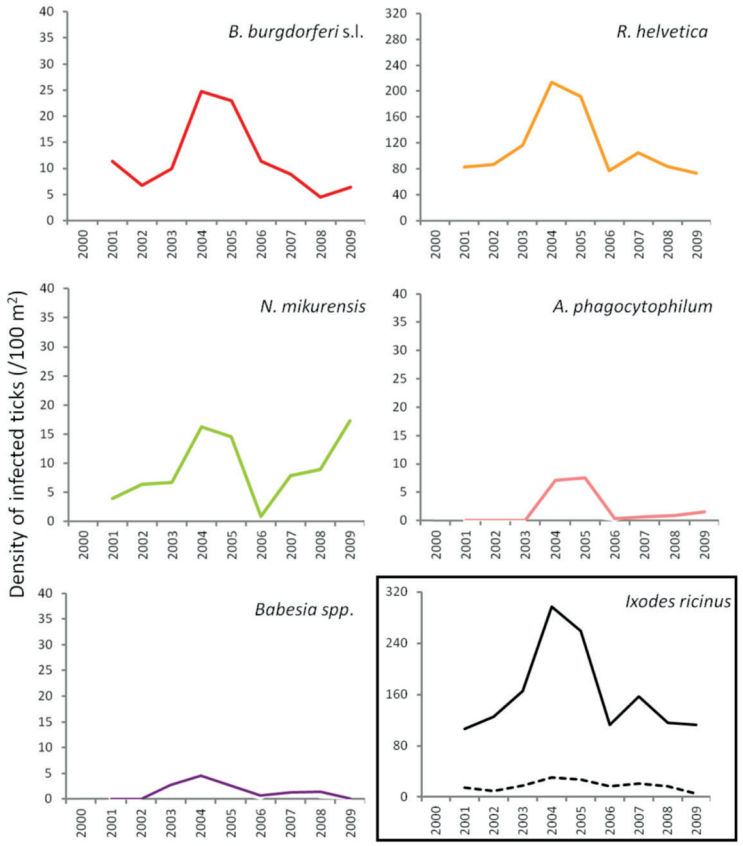


Figure 5: Changing average of density of infected ticks and tick density/activity in Duin & Kruidberg area. Density/activity of nymphs and adults are depicted in the bottom right graph as continuous and dotted line, respectively.

Coinfection

Overall, 37% (2064/5570) of the ticks was infected with one or more pathogens and 6.3% (350/5570) with more than one pathogen of different genera. Furthermore, 37% (234/628) of the *Borrelia*-positive ticks were infected with at least one other pathogen of a different genus. Almost 5% (29/628) of the *Borrelia*-positive ticks were also positive for three or more other pathogens. One tick carried the DNA of *B. afzelii*, *R. helvetica*, *Candidatus* Neoehrlichia mikurensis, and *B. microti*. Mixed infections, involving two or three *Borrelia* genospecies, occurred in only 0.3% (15/5308) of the tick lysates. Coinfection of *B. afzelii* with *Candidatus* Neoehrlichia mikurensis or with *Babesia spp.* occurred significantly more than random, whereas infection of *R. helvetica* with either *B. afzelii* or *Candidatus* Neoehrlichia mikurensis occurred significantly less frequent (Table 3).

Seasonal dynamics

Seasonality modeling of the prevalence indicated a different periodicity of the analyzed pathogens (Figure 6). Thus, *B. afzelii*, *Candidatus* Neoehrlichia mikurensis and *Babesia spp.* showed highest prevalences in ticks at time periods corresponding to October, while non- *afzelii B. burgdorferi* and *R. helvetica* had the highest prevalence around June. Annual prevalence of *A. phagocytophilum* was not seasonal.

Table 3: Observed and expected coinfections. Chi-square tests were used to calculate the associations of several combinations of pathogens *Significant positive associations and **significant negative associations ($p < 0.05$) are shown in bold.

Observed (%)	***R. helvetica***	***A. phagocytophilum***	***N. mikurensis***	***Babesia spp.***
Borrelia (all)	3.3	0.1	1.6	0.4
B. afzelii	1.8	0.0	1.3	0.3
R. helvetica		0.3	2.2	0.5
A. phagocytophilum			0.0	0.0
N. mikurensis				0.1
Expected (%)	*R. helvetica*	*A. phagocytophilum*	*N. mikurensis*	*Babesia spp.*
Borrelia (all)	3.9	0.1	0.7	0.2
B. afzelii	2.2	0.1	0.4	0.1
R. helvetica		0.3	1.9	0.5
A. phagocytophilum			0.0	0.0
N. mikurensis				0.1
Chi²-test (p-value)	*R. helvetica*	*A. phagocytophilum*	*N. mikurensis*	*Babesia spp.*
Borrelia (all)	**0.03****	0.30	**0.00***	**0.01***
B. afzelii	**0.03****	0.24	**0.00***	**0.00***
R. helvetica		0.80	**0.05****	0.77
A. phagocytophilum			0.10	0.42
N. mikurensis				0.66

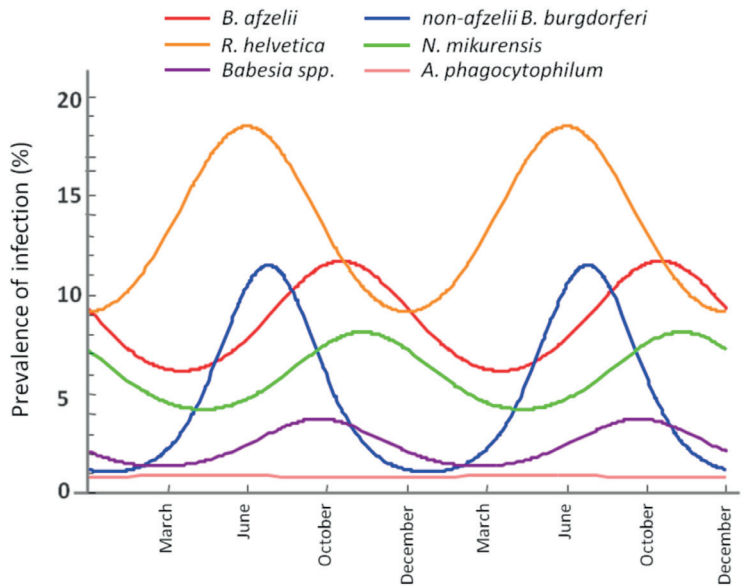


Figure 6: Seasonal variation of the infection rate in ticks. The maximum infection rates of non-*afzelii B. burgdorferi*, and *R. helvetica* are in June, while the amplitudes of *B. afzelii*, N. mikurensis, and *Babesia spp.* overlap in October.

DISCUSSION

In order to assess the acarological risk of acquiring a tick-borne infection in the Netherlands, the abundance of questing ticks infected with *B. burgdorferi* s. l. and four other genera of TBPs were compared. Our study revealed the nationwide circulation of TBPs in enzootic cycles. Although the most common tick-borne infection is acknowledged to be Lyme borreliosis, our results showed that there are other pathogens present in questing ticks at prevalences comparable with *B. burgdorferi* (i.e., *R. helvetica*, Table 2). Due to the fact that our investigations only detected the DNA of the microorganisms under discussion, and not the viable cells, we cannot asseverate their infectiousness for other vertebrate hosts. However, previous studies implicate *Ixodes ricinus* ticks as vectors for these microorganisms [32, 60, 103, 164, 191-197], and therefore the risk for public health should not be neglected. Although no human disease with the organisms other than *B. burgdorferi* s. l. was reported so far in the Netherlands, it is known that infection with some of them (e.g., *Ehrlichia*) is generally either asymptomatic or mild, self-limiting diseases [198].

Spatial distribution and variation

All the pathogens were observed in most of the areas in which investigations were conducted, regardless of the geographical position. The absence in certain areas might be explained by the relatively low number of ticks collected/tested (Table 2). The prevalences

of infection in the ticks varied significantly between the areas investigated. The lack of a full perspective on the host community at each of the sites does not allow us to make a definite statement on why we see such a variation of the prevalence of infection. We propose, however, that the extremely high local variability of the pathogens may be associated with the differences in host assemblages in the investigated habitats. As ticks can feed on many different animals and every host species has a unique reservoir competence [e.g., rodents being the most competent reservoirs of *B. afzelii* [199]], the presence of different hosts in different communities affects the prevalence of infection with various microorganisms.

In terms of the risk for public health, neither the density of questing ticks, nor the prevalence of infection alone, has any significance. Instead, it is their product—the density of infected questing ticks—that defines high or low risk areas [173-176]. We noticed that in some areas, where tick densities were highest, the mean prevalence of *Borrelia* infection had very low values (8% for Duin & Kruidberg; Table 2). Using a log-likelihood ratio statistics, we tested the hypothesis of a constant prevalence over the range of questing ticks density. The test confirmed the independence of the two variables but only for *R. helvetica*, *Candidatus* Neoehrlichia mikurensis, and *A. phagocytophilum*, while for *B. burgdorferi* and *Babesia spp.* it indicated a slight negative correlation of the prevalence with the tick density (Figure 3). Thus, we would expect that the density of ticks infected with *B. burgdorferi* and *Babesia spp.* would decrease as the density of questing ticks increases. Plotting the density of infected questing ticks as an exponential function of the questing ticks' densities, however, revealed that over the usual range of questing ticks densities the density of infected ticks is also increasing, and the downward trend might be observed only for questing ticks densities of over 100 (for *Babesia spp.*) or 200/100 m2 (for *B. burgdorferi*) (Figure 4). This observation is consistent with the finding made by Randolph [200] that, in Europe the density of *Borrelia* infected ticks depends much more on the density of all ticks than on the infection prevalence, and that only in areas where the tick density is unusually high (100–450/100 m2) is the infection prevalence consistently low.

Temporal variation

In terms of temporal variation, the longest series of data we had was for 10 successive years (Duin & Kruidberg, Figure 5). At this site, the density of questing ticks was highest in 2004–2005, and it was due to a steep increase in the number of questing nymphs. The variations in tick density might indicate yearly fluctuations in the composition and availability of reservoir hosts. For example, a mast year might have been responsible for the increment in small mammals' population size (i.e., rodents), with the upsurge of nymphs at a consequential rate. The trend line indicated the maintenance of relatively constant prevalences for *B. burgdorferi*, *A. phagocytophilum*, and *R. helvetica*. *Babesia* prevalence showed a slight decrease over time while, on the contrary, *Candidatus* Neoehrlichia mikurensis showed a steep increase (almost 3-fold). The maintenance of relatively constant prevalences of infection in time implies that the acarological risk is predominantly dependent on the density/activity of ticks (Figure 5).

Coinfection

One-third of the ticks infected with *Borrelia* were also infected with at least one other TBP. Recent studies in other European countries have shown that mixed infections of the TBPs do not represent an exception but more likely the rule. A negative significant association was found between all *Borrelia* (and *B. afzelii* alone) and *R. helvetica*, as well as between *Candidatus* Neoehrlichia mikurensis and *R. helvetica* (Table 3). On the other hand, significant positive associations were found between *Borrelia* (and particularly *B. afzelii*) and *Candidatus* Neoehrlichia mikurensis and between *Borrelia* and *Babesia spp.* (Table 3). These findings lead us to the hypothesis that *B. afzelii*, *Candidatus* Neoehrlichia mikurensis, and *Babesia* might share the same reservoir hosts, while *R. helvetica* is maintained in other enzootic cycles.

Seasonal dynamics

Further evidence for our hypothesis came from the seasonality modeling of the infection prevalence. This indicated a variation in the same phase for *B. afzelii*, *Candidatus* Neoehrlichia mikurensis and *Babesia spp.* on the one side and for non-*afzelii B. burgdorferi* and *R. helvetica* on another (Figure 6). That means that the infection peak in questing ticks is different for different pathogens, further suggesting that they were acquired from the distinct vertebrate hosts. Scientific literature confirms this. Rodents are known to be competent transmission hosts for *B. afzelii* [199, 201] and *B. microti* [196], and they have been designated as potential reservoirs for *Candidatus* Neoehrlichia mikurensis [167, 202]. On the other hand, non-*afzelii Borrelia*, like *B. garinii* and *B. valaisiana* have been shown to be associated with birds [199, 203], while a study of de la Fuente and co-workers [204] found that *A. phagocytophilum* infections occurred in deer, cattle and various bird species, meaning that birds might serve as reservoirs for both these bacteria.

Rikettsia helvetica was previously found at high rates in both rodents (29%) and roe deer (19%) [103]. The fact that *R. helvetica* was negatively associated with *B. afzelii*, although they might share the same hosts, is possibly due to that the former is transovarially transmitted in ticks which act thus as both vectors and reservoirs of the *rickettsiae* [103]; therefore, they alone can be responsible for the maintenance of the bacteria, without the intervention of a rodent host in the cycle. Hence, our findings are not coincidental, and indicate that certain coinfections are more likely to occur than the others, given particular combinations of vertebrate hosts. Although previous meta-analyses indicate that coinfection and co-exposure for some of the TBPs appear to occur somewhat unpredictably across different areas and different hosts [168], it is anticipated that future wildlife studies will help define geographical risks of coinfection and provide insight into the dynamics of infection within reservoir hosts.

CONCLUSION

We have shown that ticks and the five genera of TBPs have a ubiquitous distribution in the Netherlands, with the few absence point presumably determined by the small number of collected ticks. The pathogens were found in sites all over the Netherlands, encompassing a variety of habitats, from open areas such as dune and heather to deciduous or coniferous forests.

This study brings valuable information on the prevalence, geographic distribution and temporal variation of *B. burgdorferi* s. l., *R. helvetica*, *Candidatus* Neoehrlichia mikurensis, *A. phagocytophilum* and *Babesia spp.* in questing *I. ricinus*. Due to their omnipresence, we underline the need to consider all of these pathogens when evaluating the risks of infection and subsequently of disease following a tick bite. Whereas the incidence of Lyme disease is on the rise, other tick-borne diseases remain heavily unreported, and even knowledge on the human exposure to them is scarce. Our study suggests that there are pathogens positively associated with *Borrelia* (i.e., *Candidatus* Neoehrlichia mikurensis and *Babesia spp.*) in questing ticks. This strengthens the idea of established enzootic cycles (common reservoir hosts) in which these microorganisms are maintained, and it is consequently possible that they might follow the same upward trend as the Lyme spirochetes. In the case of *Candidatus* Neoehrlichia mikurensis we have in fact witnessed the beginning of what might be a following upward trend.

Human activity in any natural habitat is accordingly accompanied by an imminent risk of exposure to any of the pathogens. Although the risk, as measured by the density of infected ticks, may vary in time and space, its driving factor appears to be the tick density/activity. It is therefore possible that the risk of exposure to TBPs would be minimized by developing effective and sustainable methods for the control of *Ixodes ricinus* populations.

CHAPTER 3

Prevalence of *Candidatus* Neoehrlichia mikurensis in ticks and rodents from North-west Europe

S. Jahfari, M. Fonville, P. Hengeveld, C. Reusken,
E. J. Scholte, W. Takken, P. Heyman,J. M. Medlock,
D. Heylen, J. Kleve and H. Sprong

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ABSTRACT

Background: *Candidatus* Neoehrlichia mikurensis is an emerging and vector-borne zoonosis. The first human disease cases were reported in 2010. Limited information is available about the prevalence, distribution, its natural life cycle and reservoir hosts. An *Ehrlichia*-like "schotti" variant has been described in questing *Ixodes ricinus* ticks, which could be identical to *Candidatus* Neoehrlichia mikurensis.

Methods: Three genetic markers, *16S rDNA*, *gltA* and *groEL* of *Ehrlichia* schotti-positive tick lysates were amplified, sequenced and compared to sequences from *Candidatus* Neoehrlichia mikurensis. Based on these DNA sequences, a multiplex real-time PCR was developed to specifically detect of *Candidatus* Neoehrlichia mikurensis in combination with *Anaplasma phagocytophilum* in tick lysates. Various tick species from different life-stages, particularly *Ixodes ricinus* nymphs, were collected from the vegetation or wildlife. Tick lysates and DNA derived from organs of wild rodents were tested by PCR-based methods for the presence of *Candidatus* Neoehrlichia mikurensis. Prevalence of *Candidatus* Neoehrlichia mikurensis was calculated together with confidence intervals using Fisher's exact test.

Results: The three genetic markers of *Ehrlichia* schotti-positive field isolates were similar or identical to *Candidatus* Neoehrlichia mikurensis. *Candidatus* Neoehrlichia mikurensis was found to be ubiquitously spread in the Netherlands and Belgium, but was not detected in the 401 tick samples from the UK. *Candidatus* Neoehrlichia mikurensis was found in nymphs and adult *Ixodes ricinus* ticks, but neither in their larvae, nor in any other tick species tested. *Candidatus* Neoehrlichia mikurensis was detected in diverse organs of some rodent species. Engorging ticks from red deer, European mouflon, wild boar and sheep were found positive for this bacterium.

Conclusions: *Ehrlichia* schotti is similar, if not identical, to *Candidatus* Neoehrlichia mikurensis. *Candidatus* Neoehrlichia mikurensis is present in questing *Ixodes ricinus* ticks throughout the Netherlands and Belgium. We propose that *Ixodes ricinus* can transstadially, but not transovarially, transmit this microorganism, and that different rodent species may act as reservoir hosts. These data further imply that wildlife and humans are frequently exposed to *Candidatus* Neoehrlichia mikurensis-infected ticks through tick bites. Future studies should aim to investigate to what extent *Candidatus* Neoehrlichia mikurensis poses a risk to public health.

BACKGROUND

The most prevalent tick-borne infection of humans in the Northern hemisphere is Lyme borreliosis [12]. The same tick species transmitting the etiologic agents of Lyme borreliosis also serve as the vector of pathogens causing tick-borne encephalitis and several forms of rickettsiosis, anaplasmosis and ehrlichiosis [162]. Members of the family *Anaplasmataceae* are obligatory intracellular bacteria that reside within membrane-enclosed vacuoles. Human ehrlichiosis and anaplasmosis are two closely related diseases caused by various members of the genera *Ehrlichia* and *Anaplasma*. A major difference between these two members is their cellular tropism. *Ehrlichia chaffeensis*, the etiologic agent of human monocytotropic ehrlichiosis (HME), is an emerging zoonosis that causes clinical manifestations ranging from a mild febrile illness to a fulminant disease characterized by multi-organ system failure [164]. *Anaplasma phagocytophilum* causes human granulocytotropic anaplasmosis (HGA), previously known as human granulocytotropic ehrlichiosis [198]. Despite the presence of *Anaplasma phagocytophilum* in questing *Ixodes ricinus* ticks in the Netherlands [177], only one human case has been reported [45]. Seropositivity against anaplasmosis was observed in risk groups, such as forestry workers and suspected Lyme borreliosis patients, but not in control groups [46]. Still, the incidence of these tick-borne diseases and the associated public health risks remain largely unknown.

A novel candidate species in the family of *Anaplasmataceae*, called *Candidatus* Neoehrlichia mikurensis, was first isolated from wild rats and was also found in *I. ovatus* in Japan [49]. *Candidatus* Neoehrlichia mikurensis can be distinguished from other genera based on sequence analysis of *16S rDNA*, citrate synthase (*gltA*) and heat shock protein *groEL* genes [49]. This recently identified bacterium is detected in several tick species and rodents in different parts of the world under different names [49, 51-53, 213]. *Candidatus* Neoehrlichia mikurensis found in *I. ricinus* ticks in Italy has been referred to as *Candidatus Ehrlichia* walkerii [52, 54] and the *Ehrlichia* species isolated from a rat in China was called "Rattus strain" [53]. Furthermore, a *Candidatus* Neoehrlichia mikurensis has been described in *I. persulcatus* in Russia [56, 57] and *I. ovatus* from China and Japan [49, 53, 214] In the US, an *Ehrlichia*-like organism, closely related to *Candidatus* Neoehrlichia mikurensis, was previously detected in raccoons. This variant is called *Candidatus* Neo*ehrlichia* lotoris [215, 216]. The Asian *Candidatus* Neoehrlichia mikurensis strains showed a 99% similarity based on the *16S rDNA* to the *Ehrlichia* schotti. *Ehrlichia* schotti was first described in 1999 in *I. ricinus* in the Netherlands by Leo Schouls and was named after his technician [51]. Later this species was reported in *I. ricinus* in Russia [217], and subsequently in Germany and Slovakia [218, 219]. These findings raised the question whether *Ehrlichia* schotti is the same as *Candidatus* Neoehrlichia mikurensis.

It is unclear whether *Candidatus* Neoehrlichia mikurensis poses a risk to public health. Until recently, there were no human infections reported. In 2010, the first case of human *Candidatus* Neoehrlichia mikurensis infection was reported in a patient from Sweden [68]. In the same year, five other human infections were described in Germany, Switzerland and

the Czech Republic [69-71]. More recently, a canine infection was reported in Germany [72]. The symptoms described in all of these cases were generally non-specific and usually seen in any other ordinary inflammatory reaction (Table 1). These reported cases of human infections imply that re-evaluation is needed regarding the pathogenesis of this species. All but one case that have been described so far have occurred in patients who were immuno-compromised. The non-specificity of the reported symptoms, poor diagnostic tools and the lack of awareness of public health professionals could explain the absence of (reported) patients.

In this study we aim to investigate (i) whether *Ehrlichia* schotti is similar to the described *Candidatus* Neoehrlichia mikurensis family, (ii) the distribution and prevalence of *Candidatus* Neoehrlichia mikurensis in the Netherlands, Belgium and the UK, (iii) possible transmission routes of *Candidatus* Neoehrlichia mikurensis in non-experimental settings and (vi) its putative mammalian hosts.

METHODS

Collection, identification and DNA extraction of ticks questing *I. ricinus* from all stages and *Dermacentor reticulatus* adults were collected in 2009 and 2010 by flagging the vegetation at geographically different locations in the Netherlands and Belgium. Ticks collected in the UK and Vrouwenpolder (NL) have been described before [179]. Questing *I. arboricola* were collected from bird nests in two different areas in Belgium. *Ixodes hexagonus* feeding on hedgehogs were collected in a hedgehog-shelter in 2010. *Ixodes ricinus* feeding on red deer (*Cervus elaphus*), European mouflon *(Ovis orientalis musimon*), wild boar (*Sus scrofa*) and sheep (*Ovis aries*) were collected. All the collected ticks were immersed in 70% alcohol and stored at −20°C until the DNA extraction. Based on morphological criteria, tick species and stages were identified to species level, with stage and sex recorded [224]. In doubtful cases, sequencing of tick mitochondrial 16S rDNA confirmed the tick-species [225]. DNA of questing ticks was extracted by alkaline lysis [162]. DNA of engorged ticks was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's manual (Qiagen, 2006, Hilden; Germany) following the manufacturer's protocol for the purification of total DNA from ticks.

Preparation of DNA lysates from wild rodents

Longworth traps (Bolton Inc., UK), baited with hay, apple, carrot, oatmeal and mealworm were used to capture different species of rodents and insectivores at 7 different locations in the Netherlands between 2007 and 2010. Animals were anaesthetized with isoflurane and euthanized by cardiac puncture. Serum was collected and stored at – 20°C. Spleen, liver, kidney, brain and other organs were collected and frozen at −80°C. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer's manual (Qiagen, 2006, Hilden; Germany). All animals were handled in compliance with Dutch laws on animal handling and welfare (RIVM/DEC permits).

Table 1: Reported human cases of *Candidatus* Neoehrlichia mikurensis (until October 2011)

Location	Case	Symptoms and clinical signs	Ref.
Germany	Male, 69yr Immunosuppressive therapy	Episodes of fever, nonproductive cough, left thoracic pain, vein thrombosis, hypochromic anemia, reduced numbers of leukocytes, decreased percentage of lymphocytes, increased proportion of monocytes and elevated levels of CRP, microbiological analysis were negative.	[220]
Germany	Male, 57yr Previously healthy	Headaches, fever, intracerebral and subarachnoid hemorrhage, aneurysm, elevated CRP, pulmonary infiltration, microbiological analyses were negative, elevated infection parameters. Patient died from septic multi-organ failure.	[220]
Sweden	Male, 77yr Chronic lymphocytic leukemia	Transitory ischemic attack, hemolytic anemia, fever, erysipelas-like rash, transitory weakness of the left side of face and arm, hemolytic anemia, thrombocytopenia, thrombosis, pulmonary infiltration, increased proportion of monocytes and elevated levels of CRP, blood and other cultures were negative	[221]
Switzerland	Male, 61yr CABG surgery	Malaise, fever, moderate dyspnea, elevated leukocytes/neutrophils, elevated CRP, microbiological analysis were negative	[222]
Czech Republic	Female 55 yr Mantle Cell Lymphoma	Spiking fever, myalgias, arthralgias, erthema nodosum, elevated CRP, blood-, urine culture and pharyngeal swabs were negative. Antinuclear-, antinucleolar antigens and rheumatoid factor screens were negative.	[223]
Czech Republic	Male, 58yr Liver transplantation and splenectomy	Spiking fever, extreme fatigue, joint pain, skin erythema, painful and stiffened subcutaneous veins, mild leukocytosis and elevated CPR, blood and urine cultures and pharyngeal swab were negative.	[223]

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2006, Hilden; Germany). All animals were handled in compliance with Dutch laws on animal handling and welfare (RIVM/DEC permits).

Polymerase chain reactions

Polymerase chain reaction (PCR) amplifications were performed in a *P*x2 Thermal Cycler (Thermo Electron Corporation, Waltham, Massachusetts, USA). The presence of *Ehrlichia* schotti in questing *I. ricinus* was studied by Reverse Line Blotting as described [177]. Fragments of the *16S rDNA*, citrate synthase gene *gltA,* and the chaperonin *groEL* of ehrlicial species were amplified from tick lysates and rodent tissue samples using novel primers and primers that were previously described (Table 2). Amplification of *gltA* and *groEL* were both done in 50 µl reaction volumes containing 5 µl template DNA. *gltA* DNA was amplified using a final concentration of 800 nM of each primer, NMik fo-gltA and NMik re-gltA with the following PCR program, 15 min at 95°C, 40 cycles each consisting of 30 sec at 94°C, 25 sec at 53°C, and 10 min at 72°C. *groEL* DNA was amplified using, 500 nM of each primer NMik fo-groEL and NMik re-groEL. The PCR program used is as followed: 15 min at 95°C, 40 cycles each consisting of 30 sec at 94°C, 75 sec at 49°C, and 10 min at 72°C. The nested reaction was carried out at the same temperature as the first reactions; only 25 cycles were carried out with 1 µL of the first amplification product. The HotStarTaq Polymerase Kit (Qiagen) was used for all PCR experiments. PCR products were detected by electrophoresis in a 1.5% agarose gel stained with SYBR gold (Invitrogen).

Multiplex real-time PCR

Oligonucleotide primer and probe sequences were designed to be specific for the *Candidatus* Neoehrlichia mikurensis *groEL* gene using Visual OMP DNA (Software, Inc., Ann Arbor, USA). Primer sequences for the *Candidatus* Neoehrlichia mikurensis *groEL* gene were NMikGroEL-F2a and NMikGroEL-R2b and generated a 99-bp fragment which was detected with the NMikGroEL-P2a TaqMan probe (Table 2). Sequences were evaluated on the basis of the following criteria: predicted cross-reactivity with closely related organisms, internal primer binding properties for hairpin and primer-dimer potential, length of the desired amplicon, G-C content, and melting temperatures (*Tm*s) of probes and primers. The specificity of the *groEL* primers for *Candidatus* Neoehrlichia mikurensis in the multiplex real-time PCR assay was tested with DNA extracted from the following microorganisms: *R. rickettsii*, *A. phagocytophilum*, *R. helvetica*, *Bartonella henselae*, *Ehrlichia canis*, *B. afzelii*, *B. garinii, B. burgdorferi* sensu stricto*, Babesia microti, Candidatus* Midichloria mitochondrii and tick lysates containing *Wolbachia species* [177, 226]. None were amplified. Random samples of tick lysates which were *Candidatus* Neoehrlichia mikurensis *groEL* positive in the q-PCR were routinely confirmed by conventional PCR using NMik fo-gltA and NMik re-gltA primers, followed by DNA sequencing.

Optimized conditions for multiplex PCR

PCR was performed in a multiplex format with a reaction volume of 20 µl, using the iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, USA), in the LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche, Basel, Switzerland). Final PCR reaction concentrations were 1x iQ Powermix, primers ApMSP2F and ApMSP2R at 250 nM each, probe ApMSP2P-FAM at 125 nM, primers NMikGroEL-F2a and NMikGroEL-R2b at 250 nM each, probe NMikGroEL-P2a-RED at 250 nM, and 3 µl of template DNA. Cycling conditions were: 95°C for 5 min, followed by 60 cycles of a 5 sec denaturation at 95°C followed by a 35 sec annealing-extension step at 60°C.

Ticks lysates were considered positive if the Ct-value of a proper sigmoid curve was maximally three cycles more than the highest dilution of the positive control sample. For each PCR and real-time multiplex PCR, positive, negative controls and blank samples were included. A 10-3 to 10-5 dilution of a mixture of sequencing-confirmed *Candidatus* Neoehrlichia mikurensis-positive tick lysates were used as positive controls. In order to minimize contamination, the reagent setup, the extraction and sample addition, and the real-time PCR as well as sample analysis were performed in three separate rooms, of which the first two rooms were kept at positive pressure and had airlocks.

Table 2: Primers used for amplification and sequencing of *gltA* and *groEL* genes of *Candidatus* Neoehrlichia mikurensis, and the amplification of the *msp2* gene of *A. phagocytophilum*. Primers were either identical to or slightly modified from the primers described in the reference papers.

Gene	Name	Type	Sequence	Reference
gltA	NMik fo-gltA	Primer (forward)	5'-aagtgcatgctttgctacatt-'3	This study
gltA	NMik re-gltA	Primer (reverse)	5'-tcatgatctgcatgtaaaataaat-'3	This study
GroEL	NMikGroEL-F2a	Primer (forward)	5'-ccttgaaaatatagcaagatcaggtag-'3	This study
GroEL	NMikGroEL-R2b	Primer (reverse)	5'-ccaccacgtaacttatttagtactaaag -'3	This study
GroEL	NMikGroEL-P2a	Probe (RED)	5'-RED-cctctactaattattgctgaagatgtagaag gtgaagc-BHQ2-'3	This study
GroEL	NMik fo-groEL	Primer (forward)	5'-gaagyatagtytagtatttttgtc-'3	[220]
GroEL	NMik re-groEL	Primer (reverse)	5'-ttaacttctacttcacttgaacc-'3	[220]
GroEL	NMik seq1groEL	Primer (reverse)	5'-acatcacgcttcatagaaag-'3	[220]
GroEL	NMik seq2groEL	Primer (forward)	5'-aaaggaattagtattagaatcttt-'3	[220]
GroEL	NMik seq3groEL	Primer (forward)	5'-aatatagcaagatcaggtagac-'3	[220]
GroEL	NMik seq4groEL	Primer (reverse)	5'-cttccattttaactgctaattc-'3	[220]
Msp2	ApMSP2F	Primer (forward)	5'-atggaaggtagtgttggttatggtatt-'3	[227]
Msp2	ApMSP2R	Primer (reverse)	5'-ttggtcttgaagcgctcgta-'3	[227]
Msp2	ApMSP2P	Probe (FAM)	5'-FAM-tggtgccagggttgagcttgagattg-BHQ1-'3	[227]

DNA sequencing and genetic analysis

PCR amplicons were sequenced using the described primers (Table 2) and the BigDye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystems). All sequences were confirmed by sequencing both strands. Sequences were compared with sequences in Genbank, using BLAST after subtraction of the primer sequences (http://www.ncbi.nlm.nih.gov/genbank/). The collected sequences were assembled, edited, and analyzed with BioNumerics version 6.5 (Applied Maths NV, Sint-Martens-Latem, Belgium). Resulting sequences were aligned with those from related organisms in Genbank. Phylogenetic analyses of the sequences and related organisms were conducted using the BioNumerics program using the neighbour-joining algorithm with Kimura's two-parameter model. Bootstrap proportions were calculated by the analysis of 1000 replicates for neighbour-joining trees. DNA sequences are available upon request.

RESULTS

Comparison of *Ehrlichia* schotti with *Candidatus* Neoehrlichia mikurensis

Twenty-three tick lysates, which were previously tested positive for the presence of *Ehrlichia* Schotti-variant by PCR and Reverse Line Blotting were amplified by PCR on the three loci *16S rDNA* gene, *gltA* and *groEL* using primers specific for *Candidatus* Neoehrlichia mikurensis (Table 2). Almost all *Ehrlichia* schotti-variant-positive tick lysates were also PCR-positive on these three markers. None of these three loci were successfully amplified in fifteen *Ehrlichia* schotti-variant-negative ticks. The PCR products of parts of the *16S rDNA*, *gltA* and *groEL* were sequenced and compared with *Candidatus* Neoehrlichia mikurensis sequences available in Genbank. The 1740 base pairs of the *16S rDNA* sequences from the *Ehrlichia* schotti-variant were 99.6% to 100% similar to the *Candidatus* Neoehrlichia mikurensis sequences and the *Candidatus Ehrlichia* walkerii sequence in Genbank (Table 3). The 233 base-pair fragment of the *gltA* sequences from the *Ehrlichia* schotti were identical to the *Candidatus Ehrlichia* walkerii *gltA* sequence (Table 3). The 1238 base- pairs of the *groEL* isolates amplified from the tick lysates showed a 94.3% and 95.5%, 98.7% and 100% (AB084583 and AB074461, EF633745 and FJ966365) match with the *Candidatus* Neoehrlichia mikurensis *groEL* sequences in Genbank, respectively. Phylogenetic analyses of the *gltA* and *groEL* sequences showed that the *Ehrlichia* schotti clustered with *Candidatus* Neoehrlichia mikurensis isolates, but not with *A. phagocytophilum* or any of the *Ehrlichia* species present in Genbank (Figure 1).

Table 3: Members of the *Candidatus* Neoehrlichia mikurensis group are distinguished from other genera based on sequence analysis of *16SrDNA*, citrate synthase (*gltA*) and heat shock protein *groEL* genes. This strain has been reported in different parts of the world under diverse nominations. The similarity of these isolates with *Candidatus* Neoehrlichia mikurensis isolates present in tick isolates from the Netherlands, were calculated.

Country (Ref.)	Species	Named	Gene	AccessionN	Similarity
Netherlands [210]	*I. ricinus*	*Ehrlichia*-like 'schotti variant'	*16S*	AF104680	100%
Russia [228]	*I. ricinus* *I. persulcatus*	*Ehrlichia*-like 'schotti variant'	*16S*	AF104680	100%
Germany [229]	*I. ricinus*	*Ehrlichia*-like 'schotti variant'	*16S* *groEL*	AF104680 EU810407	100% 100%
Italy [230]	*I. ricinus*	C. *Ehrlichia* walkerii	*16S* *gltA*	AY098730 AY098729	100% 100%
Italy [231]	*I. ricinus*	C. *Ehrlichia* walkerii	*16S* *gltA*	AY098730 AY098729	100% 100%
China [232]	*Rattus norvegicus*	*Ehrlichia*-like 'Rattus variant'	*16S*	AY135531	98.9%
Japan [233]	*Rattus norvegicus* *I. ovatus*	C. N. mikurensis (TK4456 and IS58)	*16S* *groEL*	AB084582 AB074460 AB084583 AB074461	99.1% 99.4% 94.3% 95.5%
USA [234]	*Procyon lotor*	*Ehrlichia*-like organism	*16S*	AY781777	99.8%
Japan [235]	*A. argenteus* *A. speciosus and* *Eothenomys.smithii*	C. N. mikurensis (FIN686 and Nagano21)	*16S*	AB196304 AB196305	99.5% 99.6%
Russia [236]	*I. persulcatus*	*Ehrlichia*-like 'schotti variant'	*16S*	AF104680	100%
Italy [55]	*C. glareolus*	C. N. mikurensis	*16S*	AB213021	99.6%
Russia [237]	*M. rossiaemeridionalis* *I. persulcatus*	C. N. mikurensis	*16S*	EF445398	100%
USA [238]	*Procyon lotor*	C. N. lotoris (RAC413)	*16S* *groEL* *gltA*	EF633744 EF633745 EF633746	97.8% 98.7% 79.5%
Slovakia [239]	*I. ricinus*	C. N. mikurensis	*16S*	AB196305	99.7%
Russia [237]	*I. persulcatus* *A. peninsulae*	C. N. mikurensis	*16S* *groEL*	FJ966364 FJ966363 FJ966366 FJ966365	99.6% 100% 98.7% 98.7%
Germany [220]	Human	C. N. mikurensis	*16S,* *groEL*	EU810404 EU810406	99.9% 100%
Switzerland [222]	Human	C. N. mikurensis	*16S* *groEL*	GQ501089 HM045824	100% 98.9%
Germany [240]	Dog	C. N. mikurensis	*groEL*	EU432375	100%

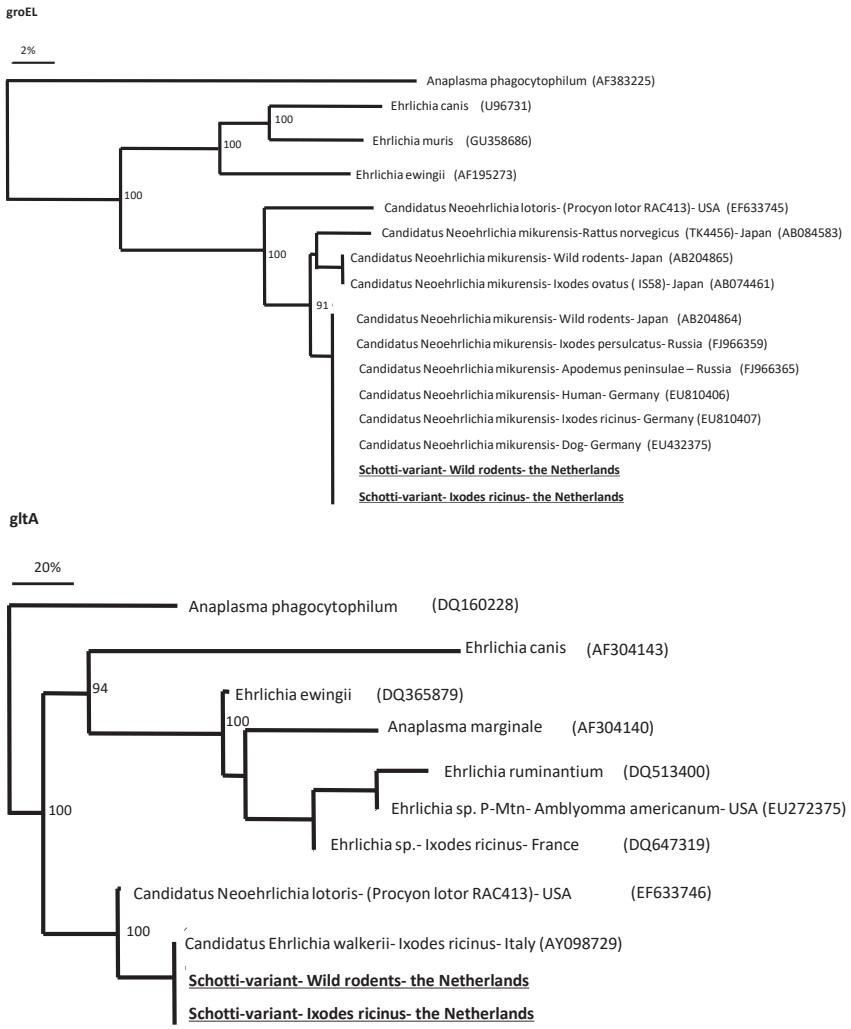

Figure 1: Phylogenetic tree of the *groEL* (top) and *gltA* (bottom) of different *Anplasma* and *Ehrlichia* species and their relation with *Candidatus* Neoehrlichia mikurensis and related species found around the world. Different *groEL* and *gltA* sequences were taken from Genbank. Their accession numbers are shown between brackets. The evolutionary distance values were determined by the method of Kimura, and the tree was constructed according to the neighbor-joining method. Bootstrap values higher than 90% are indicated at the nodes.

Prevalence and distribution of *Candidatus* Neoehrlichia mikurensis

In order to estimate the prevalence and distribution of *Candidatus* Neoehrlichia mikurensis in North-West Europe, questing *I. ricinus* nymphs (~88%) and adults (~12%) were tested using a q-PCR for the simultaneous detection of *Candidatus* Neoehrlichia mikurensis and *A. phagocytophilum.* In all 12 study-areas in the Netherlands, *Candidatus* Neoehrlichia

mikurensis was detected with a prevalence varying from 1% to 16% (Table 5). Ticks from one study area (Duin en Kruidberg) were tested in two consecutive years. In 2009, 16% of the questing nymphs and adults *I. ricinus* were infected with *Candidatus* Neoehrlichia mikurensis. The prevalence of *Candidatus* Neoehrlichia mikurensis in questing ticks decreased to 8% in 2010. *Candidatus* Neoehrlichia mikurensis positive *I. ricinus* ticks were found in two out of three regions in Belgium. A fraction of the ticks from the *Candidatus* Neoehrlichia mikurensis negative area (Brussels) were positive for *A. phagocytophilum,* which was comparable to other regions (data not shown), indicating that the processing and testing of ticks from this area was not affecting the outcome of the results. The results for the *A. phagocytophilum* will be published elsewhere. To determine whether *Candidatus* Neoehrlichia mikurensis is present in the UK, 338 *I. ricinus* and 63 *D. reticularis* ticks from a previous study were tested [241]. These ticks were collected at 7 dispersed study areas in the UK and were partially caught by blanket dragging and removed from wildlife, pets and humans. *Anaplasma phagocytophilum,* but not *Candidatus* Neoehrlichia mikurensis, was detected in these tick lysates.

Table 4: The prevalence and distribution of *Candidatus* Neoehrlichia mikurensis in questing *I. ricinus* in the Netherlands and Belgium. Confidence intervals (95%), which were calculated using Fisher's exact test, are between brackets.

Location	Tested (n)	Positive (n)	Prevalence (%)
Boswachterij Hardenberg	90	7	8% (3-15%)
Dintelse Gorzen	122	9	7% (3-14%)
Drents-Friese Wold	29	1	3% (0-18%)
Duin en Kruidberg (2009)	320	52	16% (12-21%)
Duin en Kruidberg (2010)	137	11	8% (4-14%)
Hoog Soeren	217	3	1% (0-4%)
Kop van Schouwen	238	23	10% (6-14%)
Denekamp	104	4	4% (1-10%)
Pyramide van Austerlitz	270	32	12% (8-16%)
Rijk van Nijmegen	53	1	2% (0-10%)
Ulvenhoutse bos	8	1	13% (0-53%)
Vijlenerbos	328	10	3% (2-5%)
Vrouwenpolder	86	6	7% (3-15%)
Brussel-area (Belgium)	153	0	0% (<2%)
Vlaanderen-area (Belgium)	114	3	3% (1-8%)
Wallonië-area (Belgium)	106	3	3% (1-8%)
Total of all ticks	***2375***	***166***	***7% (6-8%)***

Role of ticks in the transmission of *Candidatus* Neoehrlichia mikurensis

Transovarial (vertical) transmission has been implicated for *Rickettsia* [103] and *Anaplasma* [242], but not for *Ehrlichia* species [243]. Whether *Candidatus* Neoehrlichia mikurensis is transmitted transovarially in *I. ricinus* has not been investigated so far. The prevalence of *Candidatus* Neoehrlichia mikurensis was determined in 55 pools of 5 questing *I. ricinus* larvae from Vrouwenpolder, where nymphal and adult ticks were found to be positive for *Candidatus* Neoehrlichia mikurensis (Table 4). None of the 55 pools were *Candidatus* Neoehrlichia mikurensis positive (Table 5). Some of the pools were positive for *A. phagocytophilum*, approving the used methodology. The prevalence of *Candidatus* Neoehrlichia mikurensis in questing *I. ricinus* nymphs was ~7%, whereas the prevalence in adult ticks was ~11% (Table 5). No significant differences were observed in the prevalence between questing male and female *I. ricinus* ticks. To investigate the role of other tick species in the transmission of *Candidatus* Neoehrlichia mikurensis: *Dermacentor reticulatus*, *I. hexagonus* and *I. arboricola* were analyzed for the presence of *Candidatus* Neoehrlichia mikurensis (in the multiplex real-time PCR). None were found positive (Table 6). Again, some were found positive for the *A. phagocytophilum msp2* gene (data not shown), indicating that there is no significant inhibition within these samples.

Table 5: Prevalence of *Candidatus* Neoehrlichia mikurensis in questing *I. ricinus*, divided by lifecycle stage. *Pools of 5 larvae. 95% Confidence intervals of the prevalence are between brackets.

Stage	Tested (n)	Positive (n)	Prevalence (%)
Larvae	55*	0	0% (<1%)
Nymph	2003	137	7% (6-8%)
Female	92	10	11% (5-20%)
Male	173	19	11% (7-17%)

Table 6: *D. reticularis, I. hexagonus* and *I. arboricola* tested in the multiplex real-time PCR for *Candidatus* Neoehrlichia mikurensis. 95% Confidence intervals of the prevalence are between brackets.

Tick species	Tested (n)	Positive (n)	Prevalence (%)
I. arboricola	79	0	0% (<5%)
I. hexagonus	169	0	0% (<2%)
Dermacentor reticulatus	177	0	0% (<2%)

Potential reservoir hosts of *Candidatus* Neoehrlichia mikurensis

To investigate the possible mammalian hosts for *Candidatus* Neoehrlichia mikurensis, 79 spleen samples of different wild small mammals were tested by (nested)-PCR for the presence of *gltA* and *groEL* (Table 7). PCR-positive samples were sequenced to confirm the presence of *Candidatus* Neoehrlichia mikurensis. Both the *groEL* and *gltA* sequences isolated from spleen were identical to the *Candidatus* Neoehrlichia mikurensis sequences

found in the questing ticks in the Netherlands (Figure 1). Spleen samples from *Apodemus sylvaticus*, *Microtus arvalis* and *Myodes glareolus* were *Candidatus* Neoehrlichia mikurensis positive. After the spleen was found positive, other organs (kidney, liver and brain) were also tested for *Candidatus* Neoehrlichia mikurensis. All the tested organs were positive.

Table 7: Spleens of wild rodent and insectivore species were tested by PCR and sequencing using *Candidatus* Neoehrlichia mikurensis specific primers.

Rodent species	Tested (n)	Positive (n)
Apodemus flavicollis	2	0
Apodemus sylvaticus	23	5
Crocidura russula	5	0
Microtus arvalis	8	2
Myodes glareolus	35	4
Sorex araneus	6	0
Total	***79***	***11***

Whether other mammals in the Netherlands are reservoir hosts is difficult to address, due to the protective status of these animals. An animal can be considered a potential reservoir host when the prevalence of *Candidatus* Neoehrlichia mikurensis in ticks feeding on this animal is significantly higher than the prevalence in questing ticks. This is for example the case for *A. phagocytophilum* [244-248]. *Ixodes ricinus* feeding on red deer (*Cervus elaphus*), European mouflon (*Ovis orientalis musimon*), wild boar (*Sus scrofa*) and sheep (*Ovis aries*) were tested by multiplex real-time PCR. The prevalence of *Candidatus* Neoehrlichia mikurensis in feeding ticks was comparable to the prevalence in questing ticks (Table 8).

Table 8: *Ixodes ricinus* ticks feeding on animals living in nature reserve areas in the Netherlands were tested by multiplex real-time PCR for the presence of *Candidatus* Neoehrlichia mikurensis.

Ticks from	Ticks tested (n)	Ticks Positive (n)	Prevalence in ticks (%)	Animals tested (n)	Animals with positive ticks (n)
Cervus elaphus	409	26	6% (4-9%)	17	10
Sus scrofa	48	4	8% (2-20%)	8	2
Ovis aries	264	33	13% (9-17%)	24	13
Ovis orientalis musimon	233	10	4% (2%-8%)	18	4

DISCUSSION

Recently, six human and one canine case of *Candidatus* Neoehrlichia mikurensis infection were reported in different locations in Europe. These reports advocate a re-assessment of the occurrence of this microorganism in questing ticks. Schouls and colleagues described an *Ehrlichia*-like organism (*Ehrlichia* schotti) in Dutch ticks [51]. In our study, the three genetic markers *16S rDNA*, *gltA* and *groEL* of *Ehrlichia* schotti-positive field isolates turned out to be similar and identical to DNA sequences available from *Candidatus* Neoehrlichia mikurensis. Thus, *Ehrlichia* schotti and *Candidatus* Neoehrlichia mikurensis are most likely one and the same species. Previous findings on *Ehrlichia* schotti can be interpreted as findings on *Candidatus* Neoehrlichia mikurensis. Thus, *Candidatus* Neoehrlichia mikurensis has already been present in the Netherlands in 1999 [104, 177]. Furthermore, 11% of 289 engorged *I. ricinus* removed from humans were *Candidatus* Neoehrlichia mikurensis positive, indicating that the Dutch population is being exposed to ticks infected with *Candidatus* Neoehrlichia mikurensis [66]. Remarkably, human and animal cases of *Candidatus* Neoehrlichia mikurensis infection in the Netherlands have not yet been described.

The development of a real time-PCR specific for *Candidatus* Neoehrlichia mikurensis allowed us to test significant numbers of ticks without having to perform the labor-intensive Reverse-Line blotting. These analyses showed that the *Candidatus* Neoehrlichia mikurensis is present in vegetation ticks throughout the Netherlands and Belgium. No *Candidatus* Neoehrlichia mikurensis -positive ticks were found in one location in Belgium. One possible explanation is that this location in the Brussels-area is exceptional due to its reduced fauna and flora caused by human interference. This forest in the Brussels-area is also highly fragmented because of a railroad and several major motorways that run through the forest. Several parts of it can be ecologically considered 'islands,' which could -through isolation of mammal and tick populations- explain the absence of the pathogen in this forest. More ticks of this unique area need to be tested in order to address this hypothesis. *Candidatus* Neoehrlichia mikurensis was also not detected in ticks from the UK. This could indicate that these species have not (yet) been established on this island.

The overall prevalence of *Candidatus* Neoehrlichia mikurensis in questing nymphs and adults is approximately 7%. From the public health point of view, it indicates that a significant proportion of people contracting a tick bite are exposed to *Candidatus* Neoehrlichia mikurensis. Transmission of the *Candidatus* Neoehrlichia mikurensis in ticks appears to occur horizontally rather than vertically. None of the tested larvae were found positive, even though the prevalence of nymphs is approximately 7% and 11% for adults. Other tick species, with more restricted host preference than *I. ricinus*, were also tested for the presence of *Candidatus* Neoehrlichia mikurensis. *Dermacentor reticulatus, I. hexagonus* and questing *I. arboricola* were found negative. The data indicate that these tick species probably play insignificant roles in the transmission of *Candidatus* Neoehrlichia mikurensis. In contrast, *I. ricinus* can be considered as its main vector in the Netherlands and Belgium.

A potential group of reservoir hosts for *Candidatus* Neoehrlichia mikurensis are wild rodents. Indeed, spleen samples and other organs (kidney, liver and brain) of some rodent species turned out to be *Candidatus* Neoehrlichia mikurensis positive, which indicates a systemic infection of these rodents with *Candidatus* Neoehrlichia mikurensis. The *Candidatus* Neoehrlichia mikurensis isolates from ticks and wild rodents (Table 7) were genetically identical, indicating that rodents are potential reservoir hosts [202]. However, the reservoir potential of rodents can only be by xeno-diagnosis or experimental infection. The prevalence of *Candidatus* Neoehrlichia mikurensis in *I. ricinus* ticks feeding on red deer, European mouflon, wild boar and sheep were comparable to the prevalence in questing ticks. From these prevalence data, it was not possible to infer the role of these animals in the transmission of *Candidatus* Neoehrlichia mikurensis. However, it is clear that these animals are being exposed to the *Candidatus* Neoehrlichia mikurensis through tick bites. Further experiments are necessary to determine whether there are other mammalian reservoirs than wild rodents.

CONCLUSION

Although human infection of the *Candidatus* Neoehrlichia mikurensis has not been reported in the Netherlands, it is unclear to what extent *Candidatus* Neoehrlichia mikurensis poses risks to public health. The symptoms described in all of the *Candidatus* Neoehrlichia mikurensis infection cases were generally non-specific and usually seen in any other ordinary inflammatory reaction. What's more, most of the *Ehrlichia* infections are known to be either asymptomatic or mild, self-limiting diseases [198]. In other words, infection can occur without causing disease. So far, diagnosis has relied only on PCR amplification of the *Candidatus* Neoehrlichia mikurensis. The lack of serological tests makes diagnosis particularly difficult. Against these backdrops, the actual incidence of human infection with *Candidatus* Neoehrlichia mikurensis is likely to be much higher than currently reported in Europe. Thorough surveillance and improvement of diagnostic tools will probably increase the number of identified human cases, and consequently provide more insight in the public health relevance of *Candidatus* Neoehrlichia mikurensis.

CHAPTER 4

Circulation of four *Anaplasma phagocytophilum* ecotypes in Europe

S. Jahfari, E. C. Coipan, M. Fonville, A. D. van Leeuwen,
P. Hengeveld, D. Heylen, P. Heyman, C. van Maanen,
C. M. Butler, G. Foldvari, S. Szekeres, G. van Duijvendijk,
W. Tack, J. M. Rijks, J. van der Giessen, W. Takken,
S. E. van Wieren, K. Takumi and H. Sprong

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ABSTRACT

Background

Anaplasma phagocytophilum is the etiological agent of granulocytic anaplasmosis in humans and animals. Wild animals and ticks play key roles in the enzootic cycles of the pathogen. Potential ecotypes of *A. phagocytophilum* have been characterized genetically, but their host range, zoonotic potential and transmission dynamics has only incompletely been resolved.

Methods

The presence of *A. phagocytophilum* DNA was determined in more than 6000 ixodid ticks collected from the vegetation and wildlife, in 289 tissue samples from wild and domestic animals, and 69 keds collected from deer, originating from various geographic locations in the Netherlands and Belgium. From the qPCR-positive lysates, a fragment of the *groEL*-gene was amplified and sequenced. Additional *groEL* sequences from ticks and animals from Europe were obtained from GenBank, and sequences from human cases were obtained through literature searches. Statistical analyses were performed to identify *A. phagocytophilum* ecotypes, to assess their host range and their zoonotic potential. The population dynamics of *A. phagocytophilum* ecotypes was investigated using population genetic analyses.

Results

DNA of *A. phagocytophilum* was present in all stages of questing and feeding *Ixodes ricinus,* feeding *I. hexagonus, I. frontalis*, *I. trianguliceps,* and deer keds, but was absent in questing *I. arboricola* and *Dermacentor reticulatus.* DNA of *A. phagocytophilum* was present in feeding ticks and tissues from many vertebrates, including roe deer, mouflon, red foxes, wild boar, sheep and hedgehogs but was rarely found in rodents and birds and was absent in badgers and lizards. Four geographically dispersed *A. phagocytophilum* ecotypes were identified, that had significantly different host ranges. All sequences from human cases belonged to only one of these ecotypes. Based on population genetic parameters, the potentially zoonotic ecotype showed significant expansion.

Conclusion

Four ecotypes of *A. phagocytophilum* with differential enzootic cycles were identified. So far, all human cases clustered in only one of these ecotypes. The zoonotic ecotype has the broadest range of wildlife hosts. The expansion of the zoonotic *A. phagocytophilum* ecotype indicates a recent increase of the acarological risk of exposure of humans and animals.

INTRODUCTION

Anaplasma phagocytophilum is an obligate intracellular bacterium of the family *Anaplasmataceae* in the order *Rickettsiales* that causes disease in humans and animals [47]. It infects neutrophils, resulting in influenza-like symptoms clinically and on rare occasions is even a fatal condition in humans [249]. The first cases of Human Granulocytic Anaplasmosis (HGA) were reported in the USA in 1994 [43]. This incidence has increased gradually to 6.1 cases per million persons in 2010. The first European case was reported in Slovenia in 1995. Since, HGA cases have been occasionally reported throughout Europe [36]. It is unclear to what extent HGA poses a risk to public health in Europe: epidemiological data on the disease incidence and disease burden is either incomplete or lacking from most European countries [250]. The non-specificity of the reported symptoms, poor diagnostic tools and lack of awareness of public health professionals further complicate these estimations [36, 164, 251].

Anaplasma phagocytophilum is transmitted to humans by the bite of an infected tick [252]. The main vector in Europe is *I. ricinus,* which also transmits *Borrelia burgdorferi* sensu lato, the causative agent of Lyme borreliosis. In the Netherlands, Lyme borreliosis is on the rise: there has been a threefold increase in consultations of general practitioners for tick bites and Lyme borreliosis since 1994. This rise can be partially explained by spatiotemporal increases in the abundance and activity of questing ticks [161, 253, 254]. It is to be expected that growth in tick abundance and activity will also increase the risk of human exposure to other tick-borne pathogens such as *A. phagocytophilum*, but evidence for this extrapolation is lacking [38, 255].

Anaplasma phagocytophilum is maintained in nature through enzootic cycles between ticks and wild animals [252]. The pathogen has been detected in ticks in most European countries and the infection rates range from 0.4% to 67% [36]. In the Netherlands, infection rates in questing nymphs and adults vary between 0% and 8% [38]. *Anaplasma phagocytophilum* has been detected in a wide range of wildlife species, including ruminants, rodents, insectivores, carnivores, birds and even reptiles [36]. The relative roles of each tick stage and wildlife species in the enzootic life cycles of *A. phagocytophilum* have not been fully elucidated yet [36].

Anaplasma phagocytophilum is currently considered as a single bacterial species. Cross-infection experiments, where isolates from distinct host origins were not uniformly infectious for heterologous hosts, indicate that host specialization of *A. phagocytophilum* may occur [256, 257]. Furthermore, *Anaplasma phagocytophilum* can be genetically divided into either a few or many subclusters, depending on the genetic markers used. As sequence-based clusters in the bacterial world appear to correspond to an “ecotype”, defined as a population of cells in the same ecological niche [258], subclustering may be related to variation in space, host preference, and pathogenicity. Initially, sequences from the *16S rRNA* gene have been

used for subclustering, but this gene was shown to not be informative enough to delineate distinct ecotypes of *A. phagocytophilum* [259, 260]. Highly variable gene fragments encoding for major surface proteins [261, 262], and *ankA*, a secretory protein [263-265], have been used as well. The *groEL* heat shock operon has an intermediate genetic variability and is expected to act as a marker for demographic analyses [266-269]. Sequences from the *groEL* operon have been shown to more clearly delineate ecotypes of *A. phagocytophilum* than do sequences of the *16S rRNA* gene [266, 270].

Knowledge on the distribution of *A. phagocytophilum* in ticks and wildlife in the Netherlands and Belgium is scarce. The aim of this study was to investigate the distribution of *A. phagocytophilum* in different stages of endemic tick species and in wildlife hosts and free ranging domestic animals. The potential vectors and animal samples were tested by qPCR and conventional PCR, to determine whether they were infected with *A. phagocytophilum*. We investigated whether genetic delineation, based on *groEL*, correlates with host distribution/species and zoonotic potential. To assess whether the differential distribution of the genetic variants was due to geographic variation, all available *groEL* sequences of European *A. phagocytophilum* isolates were collected and subjected to similar analyses. Population genetic analyses were used to determine which of the ecotypes is expanding.

METHODS

Collection of samples and DNA extraction

Questing *I. ricinus* and *Dermacentor reticulatus* were collected by blanket dragging at 17 different sites in the Netherlands and Belgium [205, 271]. *Ixodes arboricola* and *I. frontalis* (nymphs and adults and fed larvae) were collected from bird nest boxes and from birds that were captured with mistnets and nest traps in forested areas around the city of Antwerp (Belgium) [271]. *Ixodes hexagonus* feeding on European hedgehogs (*Erinaceus europaeus*) were collected in a hedgehog-shelter [205]. *Ixodes trianguliceps and I. ricinus* feeding on bank voles (*Myodes glareolus)* and wood mice (*Apodemus sylvaticus*) were collected at several different sites in the Netherlands and Belgium. *Ixodes ricinus* feeding on red deer (*Cervus elaphus*), European mouflon (*Ovis orientalis musimon*), wild boar (*Sus scrofa*), sheep (*Ovis aries*), wood mouse (*A. sylvaticus*), and sand lizard (*Lacerta agilis*) has been described in previous studies [205, 207, 272]. Volunteers collected *I. ricinus* feeding on roe deer (*Capreolus capreolus*) at various localities from deer-shelters. Hunters collected deer keds (*Lipoptena cervi*) from culled roe deer. Spleen samples were obtained from 19 animal species. These included samples from roe deer, several bird species and badgers (*Meles meles*), which were found dead or were euthanized and sent to the Dutch Wildlife Health Centre for postmortem examination. The spleen samples obtained from foxes (*Vulpes vulpes*) as well as the capture of wild rodents have been described elsewhere [205, 273]. EDTA-blood from clinically and laboratory confirmed anaplasmosis from horses were collected in a veterinary hospital [274]. DNA from questing ticks was extracted by alkaline lysis [207].

Blood and spleen samples were kept frozen (-80 °C) until testing. DNA from engorged ticks, deer keds, and tissue samples was extracted using the Qiagen DNeasy Blood & Tissue Kit [205].

Polymerase chain reactions and sequencing

All samples were screened for the presence of *A. phagocytophilum* DNA with a real-time polymerase chain reaction (qPCR) targeting a 77-bp portion of the *msp2* gene. The primers used were ApMSP2F (5'-atggaaggtagtgttggttatggtatt-'3) and ApMSP2R (5'-ttggtcttgaagcgctcgta-'3), and the probe was ApMSP2P (5'-tggtgccagggttgagcttgagattg-'3) labeled with FAM6 [227]. This qPCR was performed in a multiplex format with *Candidatus* Neoehrlichia mikurensis [205]. qPCR-positive samples were analyzed further with primers targeting a fragment of the *groEL* gene of *A. phagocytophilum* [275]. All sequences were confirmed by sequencing both strands. The sequences were stored and analyzed in Bionumerics (Version 7.1, Applied Math, Belgium), after subtraction of the primer sequences.

Molecular epidemiological database

Anaplasma phagocytophilum groEL DNA sequences with the geographical origin (country) and the host species from which the isolate originated were also downloaded from the Entrez Nucleotide Database (GenBank, NCBI). *Anaplasma phagocytophilum* sequences originating from Northern white-breasted hedgehogs (*Erinaceus roumanicus*) were from a previous study [276]. Sequences that did not originate from natural isolates were excluded. Sequences that were too short to cover regions of variation were also excluded from further analysis. A literature search was performed to specifically extract *A. phagocytophilum groEL* DNA sequences from human patients in Europe [277-282]. DNA sequences and epidemiological data used for this study are given in the supplementary Table 1.

Phylogenetic and population genetic analysis

We delineated four *A. phagocytophilum* clusters (called ecotypes) by visually inspecting a phylogenetic tree (Supplementary Figure 1). A best-scoring maximum likelihood tree was obtained using RAxML 7.5.5 [283] with the option rapid bootstraps (n = 100). Each codon-position was separately analyzed using a general-time-reversible model of base substitutions, gamma-distributed rates and invariant proportions. These models of DNA evolution were determined using PartitionFinder 1.0.1 [284]. The sequences were aligned using MAFFT [285] with default options. Alignment was trimmed (position 642 to 1084) to exclude short sequences to visualize genealogy of *A. phagocytophilum* haplotypes using Haploviewer (http://www.cibiv.at/~greg/haploviewer). Population genetics measures (Ewens-Watterson test, Tajima's D, Fu's Fs) were calculated using Arlequin [286] using untrimmed alignment.

Host distributions between and within ecotypes

One *A. phagocytophilum* ecotype might be over-represented among lysates from a particular host species. We tested this possibility using a multinomial model in which a lysate from a particular host species is evenly associated across all four ecotypes, i.e. with the probability of 1/4 per ecotype. We then estimated by the Monte Carlo method the probability that the number of most numerous ecotypes in a random realization from the multinomial is equal to or greater than the observed maximum among our lysates. The probability (i.e. P-values) less than 0.05 were considered significant support for selective distribution. Counting distinct host species is an alternative measure of host diversity per ecotype. However, observed number of distinct host species is best avoided because the sample availability varied by ecotype and a straightforward comparison in this case is invalid. Therefore, we applied the rarefaction analysis to our datasets and calculated whether the differences in observed number of distinct host species per ecotype were statistically significant and not a random variation due to the sampling bias. For this purpose, we computed p-values using EstimateS (Version 9, R. K. Colwell, http://purl.oclc.org/estimates).

RESULTS

A total of 3493 questing nymphs and adult *I. ricinus* from various geographical areas in the Netherlands and Belgium were tested for the presence of *A. phagocytophilum* by PCR. DNA of *A. phagocytophilum* was found in 2.6% of the tested ticks (90/3493), and in 13 of the 17 investigated areas (Table 1). *Anaplasma phagocytophilum* DNA was also detected in 1.3% (5/386) questing *I. ricinus* larvae (Table 2). The infection rate of adult *I. ricinus* was significantly higher than that of larvae or nymphs. No significant difference was observed between the infection rates of larvae and nymphs (Table 2). *Anaplasma phagocytophilum* DNA was not detected in *I. arboricola* (n=79) and *I. frontalis* (n=13) collected from nest boxes, nor in questing *Dermacentor reticulatus* (n=59), but was found in 42% of the deer keds (29/69) feeding on 10 roe deer.

Table 1: Infection rates of *A. phagocytophilum* in questing I. ricinus nymphs and adults.
Ticks were collected by blanket dragging on various locations in The Netherlands and Belgium (three locations). The 95%-confidence intervals, which were calculated using Fisher's exact test, are between brackets. The five locations with infection rates significantly lower than 3% are indicated in **bold**. The four locations with infection rates significantly higher than 3% are indicated in ***italic bold.***

Location	Tested (n)	Positive (n)	Infection rate (CI)	
Denekamp	104	0	0.0%	(<2.8%)
Vlaanderen-area (Belgium)	114	0	0.0%	(<2.6%)
Pyramide van Austerlitz	270	1	0.3%	(<1.8%)
Vijlenerbos	328	1	0.3%	(0-1.5%)
Kop van Schouwen	238	2	0.8%	(0.1-2.7%)
Rijk van Nijmegen	53	0	0.0%	(<5.5%)
Ulvenhoutse bos	61	0	0.0%	(<36%)
Wallonië-area (Belgium)	106	1	0.9%	(0-5%)
Dintelse Gorzen	122	2	1.6%	(0.2-5.8%)
Duin en Kruidberg	457	8	1.8%	(0.8-3.4%)
Boswachterij Hardenberg	90	2	2.2%	(0.3-7.8%)
Dwingeloo-area	1071	35	3.3%	(2.3-4.5%)
Drents-Friese Wold	29	2	6.9%	(0.8-22%)
Hoog Soeren	217	14	6.5%	(3.7-10.3%)
Brussels-area (Belgium)	153	10	6.5%	(3.1-11.7%)
Vrouwenpolder	86	7	8.0%	(3.3-16%)
Hoge Veluwe	47	5	10.6%	(4.0-22%)
Total of all ticks	**3493**	**90**	2.5%	(2.0-3.1%)

Table 2: Infection rates of *A. phagocytophilum* in questing *I. ricinus*, divided by life stage.
The 95%-confidence intervals, which were calculated using Fisher's exact test, are between brackets. The infection rate of adults is significantly higher than larvae or nymphs ($p<0.05$).

Stage		Tested (n)	Positive (n)	Infection rate (CI)	
Larvae		386	5	1.3%	(0.4-3.0%)
Nymph		3090	68	2.2%	(1.7-2.8%)
Adult		306	18	5.9%	(3.5-9.1%)
	Female	113	5	4.4%	(1.5-10.2%)
	Male	193	13	6.7%	(3.6-11.2%)

To investigate the possible vertebrate host species for *A. phagocytophilum*, tissue samples of many different animals were tested (Table 3). Spleen samples from roe deer (26/38), red foxes (8/81), one wood mouse (1/23) and one common black bird (1/11) were positive (Table 3). Other organs, except brain, of the wood mouse and common black bird were also tested positive in the *A. phagocytophilum* qPCR (data not shown). *Anaplasma phagocytophilum* DNA was also amplified from 14 clinically- and laboratory confirmed horses. No *A. phagocytophilum* DNA was detected in the spleen samples of other rodents (n=45), insectivores (n=11), songbirds (n=26), and badgers (n=40).

Table 3: Presence of *A. phagocytophilum* in vertebrate tissue samples.
DNA extracts from spleen and EDTA-blood of wildlife and horses were tested by qPCR. The presence of *A. phagocytophilum* was confirmed in most cases by conventional PCR using *groEL* specific primer pairs, followed by sequencing. Positive animal species are shown in bold.

Species	Common name	Tested (n)	Positive (n)
Apodemus flavicollis	Yellow-necked mouse	2	0
Apodemus sylvaticus	Wood mouse	23	1
Crocidura russula	White-toothed shrew	5	0
Microtus arvalis	Common vole	8	0
Myodes glareolus	Bank vole	35	0
Sorex araneus	Common shrew	6	0
Carduelis chloris	Greenfinch	4	0
Coccothraustes coccothraustes	Hawfinch	2	0
Fringilla coelebs	Common chaffinch	3	0
Parus major	Great tit	4	0
Phylloscopus trochilus	Willow warbler	1	0
Pyrrhula pyrrhula	Bullfinch	1	0
Turdus iliacus	Redwing	5	0
Turdus merula	Common blackbird	11	1
Turdus philomelos	Song thrush	6	0
Capreolus capreolus	Roe deer	38	26
Equus ferus caballus	Domestic horse	14	14
Vulpes vulpes	Red fox	81	8
Meles meles	Badger	40	0
Total		289	50

Due to their protected status in the Netherlands and Belgium, it is very difficult to address the infection rate of *A. phagocytophilum* in wildlife. As a proxy for their infection rates, ticks feeding on wildlife were collected and tested. The infection rates of *I. ricinus* feeding on roe deer, red deer, hedgehog, sheep, and mouflon (Table 4) were significantly higher than the infection rate of questing adult *I. ricinus* (Table 2). Ticks from wild boar were also positive

(5/48), but not significantly more than *I. ricinus* from the vegetation (Table 1). Only one of the 109 *I. ricinus* larvae feeding on wood mice were positive for *A. phagocytophilum*. This same wood mouse carried 18 *A. phagocytophilum*-negative larvae (data not shown). Only nine *I. trianguliceps* feeding on four wood mice (n=4) and three bank voles (n=5) were collected. All eight larvae were negative, whereas one female *I. trianguliceps* feeding on a wood mouse was *A. phagocytophilum*-positive (Table 4). Both *I. ricinus* (11/117) and *I. frontalis* (4/7) feeding on common black birds were *A. phagocytophilum*-positive (Table 4). One *I. frontalis* (1/194) and none of the *I. arboricola* feeding on great/blue tit were *A. phagocytophilum*-positive. *Ixodes ricinus* ticks feeding on sand lizards were all negative for *A. phagocytophilum* (Table 4).

Table 4: Infection rates of *A. phagocytophilum* in different Ixodid species feeding on wildlife. Larval (L), nymphal (N) and adult (A) stages of *Ixodes ricinus* (IR)*, I. trianguliceps* (IT), *I. frontalis* (IF), *I. arboricola* (IA) *and I. hexagonus* (IH) feeding on different vertebrate species were tested for the presence of *A. phagocytophilum* DNA. The infection rates of ticks from animal species in **bold** are significantly higher than those of ticks from the vegetation (Table 1). The 95%-confidence intervals of these infection rates, which were calculated using Fisher's exact test, are between brackets. Data from sand lizards are derived from a previous study [207].

Ticks from	Ticks species tested (n)		Tick stage	Ticks positive (n)	Infection rate ticks (%)	Animals tested (n)	Animals with positive ticks (n)
Apodemus sylvaticus	IR	109	L	1	0.9% (0-5%)	26	1
Apodemus sylvaticus	IT	4	A/L	1	25% (1-81%)	4	1
Myodes glareolus	IT	5	L	0	0% (<52%)	5	0
Turdus merula	IR	117	N/L	11	9% (5-16%)	42	6
Turdus merula	IF	7	N	4	57% (18-90%)	6	3
Parus major/caeruleus	IF	194	A/N/L	1	1% (<3%)	120	3
Parus major/caeruleus	IA	13	A/N/L	0	0% (<25%)	13	0
Lacerta agilis	IR	165	A/N/L	0	0% (<2%)	93	0
Sus scrofa	IR	48	N	5	10% (3.5-23%)	8	4
Erinaceus europaeus	IH	193	A/N	44	23% (17-29%)	ND	ND
Ovis orientalis musimon	IR	233	A	120	52% (45-58%)	18	18
Ovis aries	IR	264	A	173	66% (59-71%)	24	24
Capreolus capreolus	IR	301	A/N/L	245	81% (77-86%)	38	35
Cervus elaphus	IR	409	A/N	351	86% (82-89%)	16	16
Total		2062		956		413	111

In total, 162 *groEL* sequences were obtained from the qPCR-positive samples. Together with the *groEL* sequences from Genbank a phylogenetic tree was reconstructed (Supplementary Figure 1). Four major *A. phagocytophilum* clusters, called ecotypes, could clearly be delineated from this tree. Bootstrap support values were: 98% (ecotype I), 100% (ecotype III) and 100% (ecotype IV). Bootstrap support values for ecotype II was not explicitly computed, but the high support values for the ecotypes I, III and IV imply that the ecotype II is similarly well supported. These four ecotypes were also visually distinguishable in haplotype genealogies in samples both from all over Europe (Figure 1) and from the Netherlands/Belgium (not shown). Based on these four ecotypes, all the available sequences were subdivided further based on their vertebrate host (Table 5). The majority of *A. phagocytophilum* samples belonged to ecotype I or II (Table 5). Ecotype I was isolated significantly more often from cattle, dogs, hedgehogs, horses, mouflons, red deer, sheep, and humans, while ecotype II was isolated significantly more often from roe deer. *Anaplasma phagocytophilum* from wood mouse was identical to the *groEL* sequence found in the engorged *I. trianguliceps.* Both these samples belonged to ecotype III. Likewise, 18 European isolates from rodents, and two isolates originating from *I. persulcatus* belonged to ecotype III. Four *I. frontalis*, one *I. ricinus* feeding on common blackbirds, and one spleen from a common blackbird contained *A. phagocytophilum* isolates belonging to ecotype IV. Samples from birds were significantly more often associated with ecotype IV than with other ecotypes (Table 5).

Ecotype I contained the largest number of distinct hosts, whereas the observed host range of the other three ecotypes was significantly smaller than expected (Table 6), indicating a broad host range for ecotype I and much smaller host ranges for the others. The most abundant host species in ecotype II, III and IV were roe deer, rodents and birds, respectively (Table 5). Visual inspection of the haplotype genealogies within ecotype I indicates a mixture of *A. phagocytophilum* samples of all kind of vertebrate species and *I. ricinus*, indicating transmission of *A. phagocytophilum* between these host species via *I. ricinus*. The presence of ecotypes in European countries were plotted to test whether the clustering could be explained by differences in geographic distribution. All four ecotypes were spread over Europe, and no geographic clustering of the ecotypes was observed (Figure 2).

Considerable sequence variation was found between and within ecotypes I, II and III. This prompted us to investigate whether this genetic marker could be used to detect changes in the population dynamics of *A. phagocytophilum* in the wild. The Ewens-Watterson test was performed separately on ecotypes I, II and III to infer the neutrality of the *groEL* marker [287-289]. It was not possible to apply the neutrality test for ecotype IV because only one haplotype was identified in this ecotype (Figure 1). The probability that two randomly chosen samples share the same haplotype (the F-values) agreed to the expectation of neutrality for ecotype III, but not ecotype I and II indicating that a particular haplotype was over-represented within each ecotype. Population expansion was tested using estimates of

Fu's Fs and Tajima's D. Estimates of Fu's Fs were significantly negative for ecotype I from the Netherlands and ecotype I from Europe (Table 7). These results demonstrate a large excess of rare genetic variants, over the expected genetic variants under the hypothesis of neutral selection and constant population size.

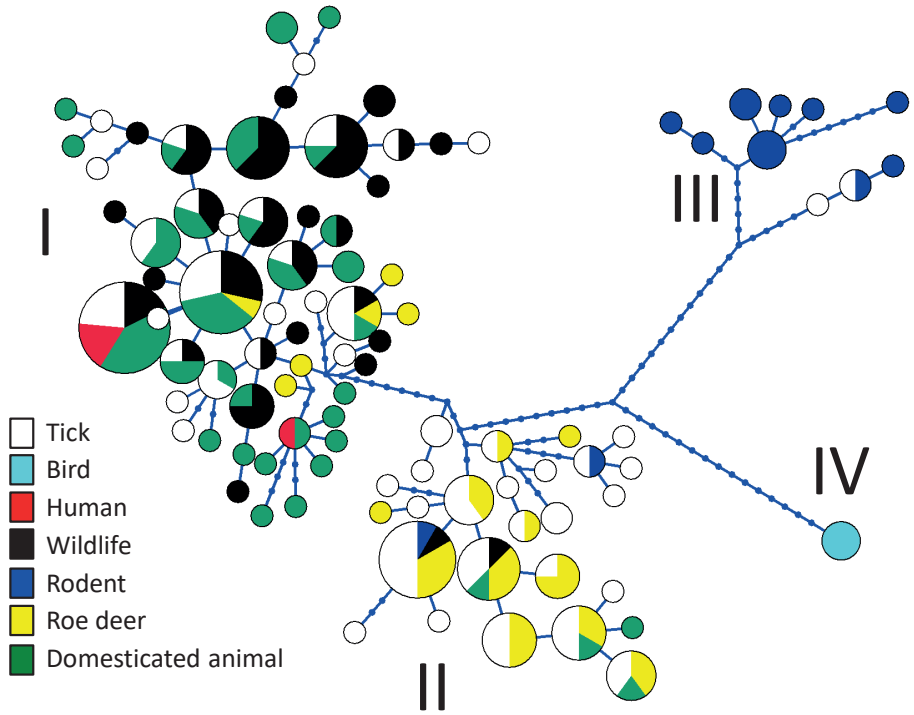


Figure 1: Genealogy of *A. phagocytophilum* haplotypes
Only 228 isolates of out of 548, representing all 97 haplotypes are shown. Of each haplotype, only one isolate per host from each country is used. An open circle is a haplotype. It is colored by the isolation origin (host species) and drawn in proportion to the sample size. A small blue dot is a missing haplotype. A blue edge is a mutation. The haplotype genealogies were made using Haploviewer software [300]. Roman numerals label the four ecotypes, which were inferred from a phylogenetic tree (Supplementary Figure 1).

Table 5: Host distributions between ecotypes.
European samples (All) and Dutch and Belgian samples (Part) are divided in the four ecotypes, which are derived from Figure 1. European samples included the Dutch and Belgian samples. Asterisks* indicate that the *A. phagocytophilum* samples were (partially) derived from ticks feeding on these hosts (Table 3). The most numerous ecotype in bold numerals indicate significant deviations from the hypotheses that ecotypes are evenly represented in that host species (P< 0.05).

	Ecotype I		Ecotype II		Ecotype III		Ecotype IV		
Animal	***All***	***Part***	***All***	***Part***	***All***	***Part***	***All***	***Part***	***Total***
Bird	0	0	0	0	0	0	8*	6*	8
Rodent	0	0	3	0	27*	3*	0	0	30
Hedgehog	59*	7*	0	0	0	0	0	0	59
Cattle	5	0	0	0	0	0	0	0	5
Dog	9	0	0	0	0	0	0	0	9
Red fox	3	3	0	0	0	0	0	0	3
Goat & sheep	24*	11*	5	0	0	0	0	0	29
Horse	36	14	0	0	0	0	0	0	36
Moose	1	0	1	0	0	0	0	0	2
Mouflon	18*	14*	0	0	0	0	0	0	18
Red deer	45*	26*	2	0	0	0	0	0	47
Wild boar	3	2*	0	0	0	0	0	0	3
Roe deer	6	3	66	39	0	0	0	0	72
Human	34	0	0	0	0	0	0	0	34
I. persulcatus	0	0	**12**	0	3	0	0	0	15
I. ricinus	**101**	**23**	68	7	0	0	0	0	169
Deer ked	3	0	**6**	**6**	0	0	0	0	9
Total	347	103	163	52	30	3	8	6	548

Phylogenetic analyses of *groEL* sequences from all *A. phagocytophilum* samples (Table 5) were performed as described in the Methods section. Roman numerals label the four ecotypes.

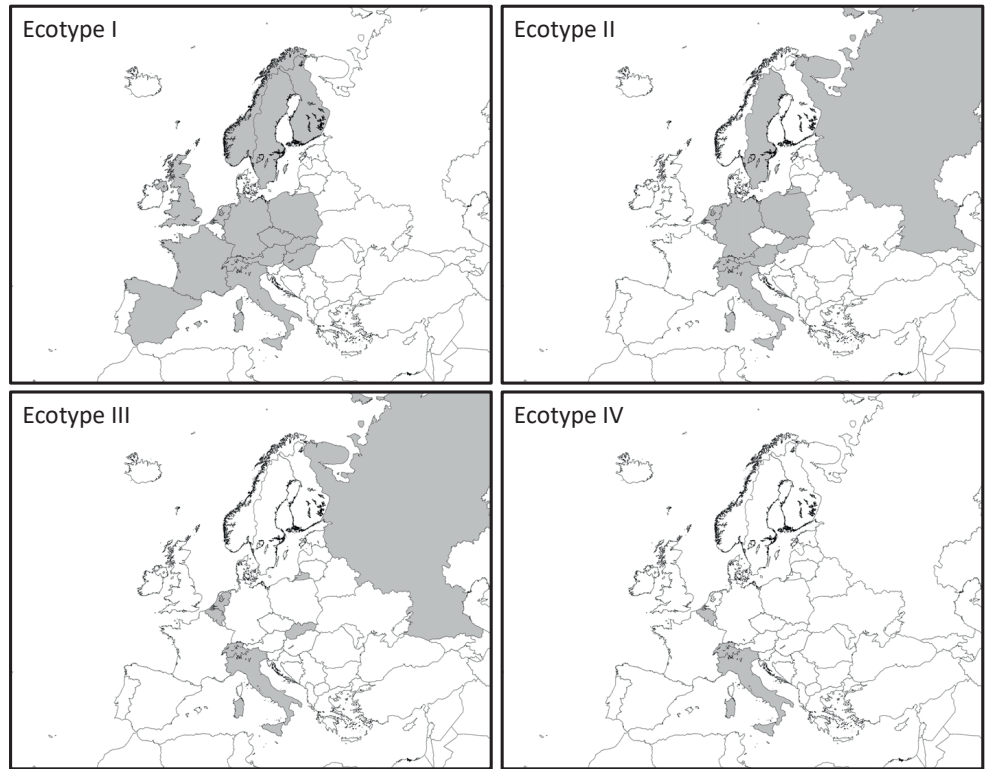


Figure 2: Geographic distributions of *A. phagocytophilum* ecotypes in Europe.
Countries in which one or more isolates from an ecotype are found are filled with grey. A country in which an ecotype was not detected or which was not sampled is depicted in white. Data are based on isolates from Table 5. Number of isolates per country can be found in Supplementary Table II.

Table 6: Host distributions within ecotypes.
The expected and observed host range of the four ecotypes were calculated for the European samples (All) and Dutch and Belgium samples (Part). Observed: observed number of distinct host species. Expected: expected number of distinct host species given the sample size. Bold italic numerals indicate p-value < 0.025, hence observed host-species richness is significantly less than expectation from the ecotype I. Expected species richness and its p-value were computed using EstimateS (Version 9, R. K. Colwell, http://purl.oclc.org/estimates).

Hosts (17)	**Ecotype I**		**Ecotype II**		**Ecotype III**		**Ecotype IV**	
Sample	***All***	***Part***	***All***	***Part***	***All***	***Part***	***All***	***Part***
Size	347	103	163	52	30	3	8	6
Observed	14	9	***8***	***3***	***2***	1	***1***	***1***
Expected	14	9	12	8	8	2	5	4

Table 7: Summary of the population genetic test for *A. phagocytophilum*
Ewens and Watterson is a test of neutrality. Bold numerals indicate p-values less than 0.05 indicating that a particular haplotype was identified in the ecotype more frequently than the expectation. This test returned Not applicable (NA) when only one haplotype is identified in the sample. Fu's Fs statistic is a measure of a population expansion based on population genetics. Bold numerals indicate p-values less than 0.05, hence a significant evidence for a population expansion.

Cluster	**Ecotype I**		**Ecotype II**		**Ecotype III**		**Ecotype IV**	
Sample	***All***	***Part***	***All***	***Part***	***All***	***Part***	***All***	***Part***
Ewens Watterson	**0.19**	0.10	0.18	0.48	0.18	NA	NA	NA
Tajima's D	**-1.8**	-0.18	-0.56	-1.45	-0.01	0.00	0.00	0.00
Fu's Fs	**-25,47**	**-8,71**	-1,48	-1,57	1.16	0.00	0.00	0.00

DISCUSSION

This study addressed the circulation of *A. phagocytophilum* in ticks and vertebrates. Our investigations only detected the DNA of this microorganism and not their viability or infectivity. However, previous studies implicate *I. ricinus* ticks as vectors and all investigated animals as potential hosts for *A. phagocytophilum* [36]. Therefore, the inability of a DNA-based detection method to asseverate infectiousness in host species is expected to be a minor issue in this study. In any case, we revealed the widespread circulation of *A. phagocytophilum* in enzootic cycles in Belgium and the Netherlands. In some cases, the infection status of hosts was inferred from the infection rate of infected tick feeding. Vertebrates were considered positive when the infection rate of engorged ticks was significantly higher than that of questing *I. ricinus* (Table 4).

The absence of *A. phagocytophilum* in questing ticks in four out of 17 areas might be attributable to the relatively low number of ticks collected and tested (Table 1). Still, infection rates of *A. phagocytophilum* in questing ticks varied significantly between some geographic locations (Table 1), corroborating results from previous studies from other locations in Europe [36]. Knowledge on the ecological factors driving these differences is of relevance to public and animal health [290], but was not in the scope of this study. Significant differences between the infection rates of *I. ricinus* nymphs and adults were observed as well (Table 2). This may reflect that *A. phagocytophilum* ecotypes I and II cycle mainly between (infected) adults and nymphal *I. ricinus,* which become infected when feeding on larger vertebrates.

Also, in another study a small but significant proportion of *I. ricinus* larvae were found *A. phagocytophilum*-positive [40]. These larvae may have become positive due to transovarial transmission or due to drop-off from *A. phagocytophilum*-positive hosts after partial feeding and continued to quest as larvae. Transovarial transmission and whether *A. phagocytophilum*-positive larvae can transmit the microorganism to vertebrate hosts needs

to be investigated. Together, these findings indicate that all three tick stages should be taken into account when calculating the acarological risk of a given area [38].

In terms of the risk for public health, not only the product of the density of questing ticks and their infection rate defines high or low risk areas, but also the zoonotic potential of the microorganism should be taken into account [38]. The identification of four different *A. phagocytophilum* ecotypes (Figure 1 and 2) with significantly different host ranges and zoonotic potential supports this. A significant correlation between the genetic clustering of *groEL* sequences and different host ranges was found (Table 5 and 6). Further genetic subclustering within ecotypes I, II and IV was also observed (Supplementary Figure 1). These subclusters could not be statistically linked to a further restriction in host ranges or to limitations in geographic distributions (not shown), probably due to lack of resolution in the *groEL locus,* and due to the limitations in the number and origin of the used samples, particularly of rodents and birds.

Clustering of *A. phagocytophilum* isolates can also be achieved using other genetic loci, such as the *ankA* gene, which is presumably involved in host-specific adaptation [263, 291]. Combining several genetic loci, such as *groEL* and *AnkA*, in future analyses could reveal more refined host ranges, especially within ecotype I. Recently, a multilocus sequence typing scheme for *A. phagocytophilum* was presented, which was shown to be informative concerning host species, geographic distribution, and zoonotic potential [291]. The advent of this standardized multilocus sequence typing scheme and a freely available molecular epidemiological database (http://pubmlst.org/aphagocytophilum/) will facilitate more elaborate analyses in the future.

Ecotype I had the broadest host range, but lacked birds and rodents, indicating that the latter two do not contribute directly to the transmission cycle. The generalist feeding behavior of *I. ricinus* nymphs and adults probably facilitates the continuous exchange of ecotype I between the different vertebrate species. All human isolates on the *groEL*-gene from Genbank and the literature [277-281] belong to ecotype I, demonstrating that members of this ecotype are zoonotic. Hence, ecotype I is the most plausible cause of infection regarding the one case of Human Granulocytic Anaplasmosis (HGA) in the Netherlands reported in 1999 [292]. Whether all or only a subset of the members of ecotype I are zoonotic remains to be examined [293].

In this study, ecotype II was found in roe deer, *I. ricinus*, and deer keds (Table 5). Therefore, ecotype II may circulate between roe deer via *I. ricinus*, or deer keds, or both. Whether deer keds may act as a host specific vector for ecotype II remains to be investigated [294, 295]. When the generalist tick *I. ricinus* would transmit ecotype II, then the observed host specificity might be attributed to *A. phagocytophilum* and the possibility that the vectors play a role in host specificity could be excluded. Only three isolates belonging to ecotype III were

found in this study. One isolate was found in the spleen of a wood mouse. The kidney, liver and ear of this rodent were all *A. phagocytophilum*-positive, indicating a systemic infection of this wood mouse with *A. phagocytophilum.* Two isolates were found in two different tick species, *I. trianguliceps* and *I. ricinus,* feeding on one wood mouse. Ecotype III was not found in questing *I. ricinus* or in any other wildlife, except rodents (Table 5). Our finding supports the notion that ecotype III might be adapted to a life cycle involving exclusively some rodent species and a rodent specific vector, such as *I. trianguliceps* [260, 265, 296, 297]. Ecotype IV is most likely associated with one or more bird species, but not with other vertebrates. Ecotype IV was not found or in any other animal species. As ecotype IV was not found in questing *I. ricinus* either, it might be adapted to a life cycle involving exclusively birds and a bird-specific vector, such as *I. frontalis.* Although *A. phagocytophilum* was not detected in questing *D. reticulatus* or *I. arboricola* ticks, their role in the transmission of one or more *A. phagocytophilum* ecotypes cannot be excluded due to the relatively low numbers of ticks tested [298]. Although many ticks and animal samples have been included in this study, some animal species, particularly birds, rodents and carnivores, and some geographical locations (Figure 2) are underrepresented. Future studies should include broader and randomized sampling strategies.

Before the considerable sequence variation between and within ecotype I and II in the *groEL* gene (Figure 1) could be used to address their population dynamics, several statistical tests were performed to address the neutrality of this genetic marker. The Ewens-Watterson test detected significant departure from neutrality for ecotype I. This outcome indicated that a particular haplotype was identified in ecotype I more frequently than the neutral expectation, indicating that this haplotype is under positive selection. Fu's Fs statistic detected genetic traces of demographic changes for ecotype I in the Netherlands and Belgium. Fu's Fs is more sensitive than Tajima's D to an excess of rare genetic variants in the samples [299], and this has proved to be true for our datasets (Table 7). The increase in ecotype I population sizes might have occurred through an increase in either the population of ixodid ticks, or in the vertebrate host species, or in both [161, 253].

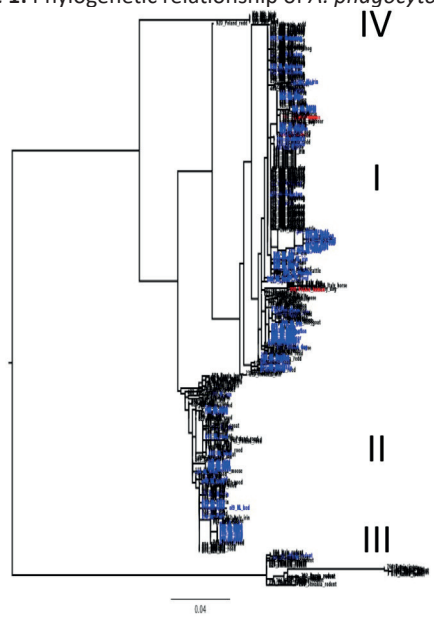
CONCLUSION

In conclusion, we identify the *groEL* gene as a suitable marker to discriminate between *A. phagocytophilum* ecotypes. These ecotypes can be linked to distinct host ranges. Furthermore, all three ecotypes have enzootic cycles in the Netherlands and Belgium. In these countries, ecotype I is expanding. This is probably caused by the increase in abundance (and activity) of their vertebrate hosts and vectors. Based on the analyses of the *groEL* marker, we infer that: 1. Ecotype I has the highest zoonotic potential, and 2. the acarological risk of exposure to *A. phagocytophilum* ecotype I has been increasing in time. However, future studies concerning the evolution, population dynamics, and ecology of naturally occurring *A. phagocytophilum* will shed light on identifying risks for public health.

Supplementary Table I: Geographic distributions of *A. phagocytophilum* ecotypes in Europe. Number of isolates per country. Data are based on isolates from Table 3.

Cluster	Ecotype I	Ecotype II	Ecotype III	Ecotype IV
Austria	16	11		
Belgium	1		1	6
Czech Republic	2	1		
Finland	1			
France	5			
Germany	42	23		
Hungary	93			
Italy	9	15	3	1
Luxembourg	2			
Netherlands	103	24	2	
Norway	2	52		
Poland	3			
Russia		9	13	
Slovakia	4	13	10	
Slovenia	44	1		
Spain	11	8		
Sweden	3			
Switzerland	4	1	1	1
United Kingdom	2	5		

Supplementary Figure 1: Phylogenetic relationship of *A. phagocytophilum*



CHAPTER 5

Tick-borne encephalitis virus in ticks and roe deer, the Netherlands

S. Jahfari, A. de Vries, J. M. Rijks, S. Van Gucht,
H. Vennema, H. Sprong, B. Rockx

Emerging Infectious
diseases (2017)

ABSTRACT

Here we report the first evidence of tick-borne encephalitis virus (TBEV) in the Netherlands. A serological screening found TBEV-neutralizing antibodies in six roe deer, five of whom were from one national park. In addition, TBEV RNA was detected in two questing ticks from the same location. Enhanced surveillance and increased awareness among medical professionals should clarify the significance of this finding.

INTRODUCTION

Tick-borne encephalitis virus (TBEV) can infect humans causing febrile illness, with neurological complications including encephalitis in humans (TBE) [301]. TBEV is transmitted through a bite of infected ticks and small rodents are considered the main amplifying hosts, while larger animals including deer, serve as feeding hosts for the ticks [302, 303]. TBEV can be divided into three subtypes, the European (TBEV-Eu), Siberian (TBEV-Si) and Far Eastern (TBEV-Fe), and the clinical course of disease and prognosis depend on the subtype [304]. TBEV is endemic in 27 European countries, and therefore is considered an important public health concern [305]. The number of recognized human tick-borne TBE cases in endemic regions of Europe has increased in the last decades, and expansion TBEV subtypes northwards and to higher altitudes is reported in recent years, this expansion is thought to be related to climate change [306, 307]. No autochthonous human cases had been reported from the Netherlands or the neighboring country, Belgium [308]. However, recent reports from Belgium, where wildlife and ~ 3% of cattle have tested positive for TBEV with neutralizing antibodies, prompted us to reinvestigate the presence of TBEV in the Netherlands [309, 310].

METHODS

Collection of roe deer sera, antibody detection

Between January and September 2010, hunters collected 297 blood samples from roe deer (*Capreolus capreolus*) according to a sampling scheme designed to obtain a representative sample of the roe deer population from locations across the Netherlands. Serum samples were stored at -80ºC until testing. TBEV reactive IgG was detected in roe sera using a commercial ELISA based on inactivated TBEV for IgG detection in a range of hosts (All species ELISA- Progen, Heidelberg, Germany) according to the manufacturer's recommendations. TBEV neutralizing antibody titration in the roe deer sera was performed at the Belgian Scientific Institute of Public Health, with the TBEV Neudörfl reference strain NCPV#848, which is considered a gold standard for TBE diagnosis as previously described [309].

Collection of tick samples, molecular detection and analyses

Ticks were collected by blanket dragging on seven locations in or close to the national park Sallandse Heuvelrug in September 2015. 1160 *Ixodes ricinus* nymphs and 300 adult ticks were collected.

Pools of 5 nymphs or two adults were lysed and RNA was extracted as described with some modifications [311]. Collected ticks were ground up with Lysis matrix I (MPbio, USA) in a FastPrep FP120 homogenizer (Thermo Fisher, Waltham, MA, USA) for 40 sec at speed 6.0. RT-qPCR reactions were done in a final volume of 20 µl with TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher scientific, USA), 5 µl of sample and 0.4 µM for all primers (TBpanFlavi-1 5'-TAYAACATGATGGGCAAGAGAGAGAA-3', TBpanFlavi-2

5'-TATAACATGATGGGCAAAAGAGAGAA-3', TBpanFlavi-3 5'-GTGTCCCAGCCAGCTGTGTCATC-3', TBpanFlavi-4 5'-GTGTCCCATCCGGCTGTGTCGTC-3') and 0.4 µM probe (TBpanFlavi-P 5'-FAM-TGGTACATGTGGCTGGGGAG-BH1-3'). With 20 min reverse transcription step at 50°C, denaturation at 95°C for 30 s and 55 cycles of 95°C for 10 s and 60°C for 30 s. Amplification was performed on a Roche LightCycler 480 instrument. To obtain genomic sequences, primers and protocols were used as described previously [312]. Distance-based analyses were conducted using Kimura 2- parameters distance estimates and trees were constructed using the Neighbour-Joining (NJ) algorithm, implemented in the in Bionumerics 7.1. (Applied Math, Belgium). Bootstrap proportions were calculated by the analysis of 1000 replicates for NJ trees.

RESULTS

TBEV reactive antibodies were detected in roe deer sera using a commercial ELISA based on inactivated TBEV (All species ELISA; Progen, Heidelberg, Germany). Serologic screening of roe deer sera by ELISA yielded six positive (VIEU/ml > 125) and eight borderline (VIEU/ml 64-98) sera. To confirm these serological findings, all positive, and three negative sera were tested in a serum neutralization test (SNT) with the TBEV Neudörfl reference strain NCPV#848 [309]. Five out of six ELISA positive samples were confirmed positive by SNT. Interestingly, four out of five SNT-confirmed roe deer were shot at or near a popular recreation area, the national park Sallandse Heuvelrug (Figure 1).
In response to the serological findings, ticks were collected by blanket dragging on seven locations at the national park in September 2015.

1160 *Ixodes ricinus* nymphs and 300 adult ticks were collected. Pools of five nymphs or two adults were lysed and RNA was extracted as described [311]. The extracted RNA were tested for the presence of flavivirus RNA using a RT-qPCR. RNA of a flavivirus was detected in two samples, one nymph pool and one pool of adult female ticks. To obtain sequences of the two qPCR-positive samples, primers and protocols were used as described previously [312]. Both sequences obtained from the tick lysates were identical. The sequences obtained in this study clustered within the TBEV-Eu subtype complex (Figure 2), but is different from the currently known TBEV-Eu sequences

Figure 1: Spatial distribution of TBEV-positive in roe deer sera. The spatial distribution of TBEV-ELISA test results are shown in the Netherlands (black dot, positive; grey dot, borderline; white dot, negative). The enlargement of the National Park Sallandse Heuvelrug area indicates the locations the TBEV-ELISA positive roe deer (black dots) in relation to the site with PCR positive ticks (black star) from 2015.

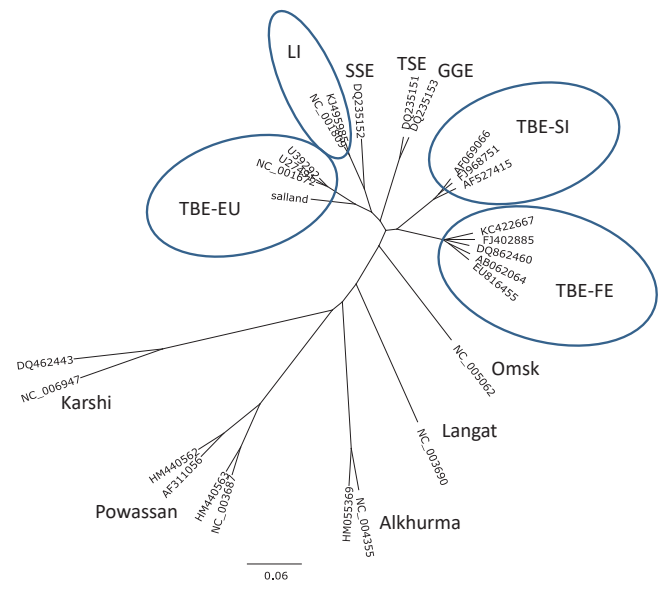


Figure 2: Genetic cluster analysis of Dutch TBEV sequences. Genetic cluster analysis of Dutch TBEV sequences with tick-borne viruses. The description 'salland' being the obtained sequences from the questing ticks in the Netherlands. The sequence consists of one large fragment (3756 base pairs) of the first part of the genome, which includes the envelope gene. This fragment clusters with the TBEV-Eu. Distance-based analyses were conducted using Kimura 2- parameters distance estimates and trees were constructed using the Neighbour-Joining (NJ) algorithm, implemented in the in Bionumerics 7.1. (Applied Math, Belgium). Bootstrap proportions were calculated by the analysis of 1000 replicates for NJ trees.

DISCUSSION

The presence of TBEV RNA in two questing ticks collected through surveillance in one national park confirms the presence of TBEV in the Netherlands. Serological evidence that roe deer from the same location had been infected with a flavivirus, most probably TBEV, five years prior to the detection of TBEV in ticks, suggests that TBEV has been endemic in the Netherlands for some time. In addition, the finding of at least one serologically positive roe deer south of the national park, suggests that TBEV is distributed more widely within the Netherlands. Disseminating information about the occurrence of TBEV in ticks and wildlife is important for both medical professionals and the general at large. In response to the current finding, the National Institute for Public Health and the Environment provided this type of information to medical professionals and the public at large. Within a week, this resulted in the report of an autochthonous TBE infection in the Netherlands (9). Further studies have been initiated to collect data on the circulation of TBEV in ticks and animals in other geographical areas in the Netherlands, while the prevalence of human infections will be assessed through increased surveillance of clinical cases with TBEV-like symptoms and serosurveillance of, for example, risk groups.

CHAPTER 6

Melting pot of tick-borne zoonoses: the European hedgehog contributes to the maintenance of various tick-borne diseases in natural cycles urban and suburban areas

S. Jahfari and S.C. Ruyts, E. Frazer-Mendelewska,
R. Jaarsma, K. Verheyen, H. Sprong

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ABSTRACT

Background

European hedgehogs (*Erinaceus europaeus*) are urban dwellers and host both *Ixodes ricinus* and *Ixodes hexagonus*. These ticks transmit several zoonotic pathogens like *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia miyamotoi* and *Candidatus* Neoehrlichia mikurensis. It is unclear to what extent hedgehogs in (sub) urban areas contribute to the presence of infected ticks in these areas, which subsequently pose a risk for acquiring a tick-borne disease. Therefore, it is important to investigate to what extent hedgehogs contribute to the enzootic cycle of these tick-borne pathogens, and to shed more light at the mechanisms of the transmission cycles involving hedgehogs and both ixodid tick species.

Methods

Engorged ticks from hedgehogs were collected from (sub)urban areas via rehabilitating centers in Belgium. Ticks were screened individually for presence of *Borrelia burgdorferi* sensu lato, *Borrelia miyamotoi*, *Anaplasma phagocytophilum, Rickettsia helvetica* and *Candidatus* Neoehrlichia mikurensis using PCR-based methods. Infection rates of the different pathogens in ticks were calculated and compared to infection rates in questing ticks.

Results

Both *Ixodes hexagonus* (n = 1132) and *Ixodes ricinus* (n = 73) of all life stages were found on the 54 investigated hedgehogs. Only a few hedgehogs carried most of the ticks, with 6 of the 54 hedgehogs carrying more than half of all ticks (624/1205). *Borrelia miyamotoi*, *Anaplasma phagocytophilum, Rickettsia helvetica* and *Borrelia burgdorferi* genospecies (*Borrelia afzelii*, *Borrelia bavariensis*, and *Borrelia spielmanii*) were detected in both *Ixodes hexagonus* and *Ixodes ricinus*. *Anaplasma phagocytophilum, Rickettsia helvetica*, *Borrelia afzelii*, *Borrelia bavariensis* and *Borrelia spielmanii* were found significantly more in engorged ticks in comparison to questing *Ixodes ricinus*.

Conclusions

European hedgehogs seem to contribute to the spread and transmission of tick-borne pathogens in urban areas. The relatively high prevalence of *B. bavariensis*, *B. spielmanii*, *B. afzelii*, *A. phagocytophilum* and *R. helvetica* in engorged ticks suggests that hedgehogs contribute to their enzootic cycles in (sub)urban areas. The extent to which hedgehogs can independently maintain these agents in natural cycles, and the role of other hosts (rodents and birds) remain to be investigated.

BACKGROUND

The incidence of tick-borne diseases has increased the last decades and poses important economic and medical consequences [313]. Lyme borreliosis is the most prevalent tick-borne disease in Europe and presents itself under a wide range of clinical manifestations. The most common and earliest manifestation is an expanding rash at the site of the tick bite (*erythema migrans*), and left untreated it can progress towards more severe disease manifestations. The disseminated infection can affect a patient's nervous system, joints, lymph nodes, skin, and in rare cases the heart or eyes [10]. The causative agents of Lyme borreliosis are spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s. l.) complex. They are generally transmitted by ticks of the *Ixodes ricinus* complex [15] and are maintained in enzootic cycles by different vertebrate hosts [16-18]. At least five genospecies of *B. burgdorferi* s. l. complex have been shown to be pathogenic to humans, namely *B. burgdorferi* sensu stricto (s. s.), *B. afzelii, B. garinii*, *B. spielmanii* and *B. bavariensis* [10, 19]. Each of these genospecies is maintained in nature through a distinct enzootic cycle involving ixodid ticks and vertebrates acting as reservoirs [33, 314]. The different *Borrelia* genospecies are generally associated with different clinical manifestations [19]. Current knowledge is that *B. afzelii* is predominantly involved in dermal infections (*erythema migrans* and acrodermatitis chronica atrophicans) [10] and is adapted to rodents and other small mammals [314, 315]. *Borrelia garinii* is associated with neurological manifestations and birds maintain most of the *B. garinii* strains [203, 271, 314]. *Borrelia bavariensis* is frequently found in rodents [316, 317] and hedgehogs [318] and is also associated with neuroborreliosis in humans [19]. *Borrelia spielmanii* infection in humans is rare, and only found in patients with *erythema migrans*. Its reservoir hosts are of the family Gliridae [319], but this *Borrelia* genospecies has also been detected in hedgehogs and their ticks [318, 320]. Besides the *B. burgdorferi* s. l. genospecies, other established pathogens circulate in enzootic cycles including the same ixodid ticks and vertebrate reservoirs, for example *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia miyamotoi* and *Candidatus* Neoehrlichia mikurensis. These pathogens can cause non-characteristic, viral-like symptoms in humans, and often confused with Lyme borreliosis [321]. Their infections are often self-limiting, but in immunocompromised patients, they can cause severe clinical manifestations [58, 68, 83, 95]. In the framework of human health, therefore, it is important to identify the different components of the enzootic cycle of these tick-borne diseases, and to shed more light at the mechanisms of the transmission cycles.

The generalist tick species *Ixodes ricinus* actively quests in the vegetation for hosts to feed on and may readily bite humans, thereby possibly transmitting pathogens. *Ixodes hexagonus* is a host specialist, feeding primarily on hedgehogs. It shows an endophilic behavior preferring dark, humid places, and is usually found in the nests of its host species [322]. Besides hedgehogs, this tick species has been found to infest other host species as well [323]. Despite their nest dwelling behavior, it is known to occasionally bite humans and companion animals, even though less frequently than *I. ricinus* does [323, 324]. Both

ixodid species can be found on hedgehogs and are competent vectors for transmitting *B. burgdorferi* s. l. [325-327].

The European hedgehog (*Erinaceus europaeus* Linnaeus, 1758) is a nocturnal insectivorous mammal commonly found throughout Western Europe [328]. They seem to have adjusted to a wide variety of habitats and occur in rural, suburban, and urban areas but generally prefer grassland with sufficient edge habitats. Hedgehogs can reach up to nine times higher densities in urban areas with parks and garden, than in rural areas, with lowest densities in forests and open grassland fields and agricultural land without cover such as shrubs or dead wood [329-331]. Since they are one of the most successful urban adapters, hedgehogs and *I. hexagonus* could contribute to the spread and persistence of pathogens in a (sub)urban habitat via secondary enzootic cycles, even when the contact between *I. hexagonus* and humans is low [318, 332].

Only a few studies have been performed on the reservoir competence of the European hedgehog. These studies have shown that these mammals can be infected with different *B. burgdorferi* s. l. genospecies [318, 320, 322] as well as other tick-borne pathogens, such as *A. phagocytophilum* [333, 334], tick-borne encephalitis virus (TBEV) [327] and *R. helvetica* [335]. The role of the European hedgehog and both ixodid tick species feeding on it in the transmission cycle of many tick-borne pathogens like *B. miyamotoi* and *Candidatus* Neoehrlichia mikurensis is not completely illuminated, yet [336].

In this study we aim to investigate the prevalence of *B. burgdorferi* s. l. genospecies, *B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis and *R. helvetica* in the different stages of the *I. hexagonus* and *I. ricinus* tick species sampled from European hedgehogs (*E. europaeus*) from Belgium. Furthermore, we aim to investigate the role of these tick species and that of the hedgehog in the enzootic cycle of the different disease pathogens. By using epidemiological analysis and comparing the infection prevalences of the different pathogens from engorged ticks collected from hedgehogs with questing nymphs from the vegetation, we aim to i) determine the reservoir status of the European hedgehog for *B. burgdorferi* s. l. genospecies, *B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis and *R. helvetica,* and ii) find indications for the vector competence of *I. hexagonus* for tick-borne pathogens.

METHODS

Hedgehog and tick sampling

Since European hedgehogs are legally protected in Belgium, the current investigation was carried out on ticks sampled from hedgehogs that were brought to the rehabilitation centers of Herenthout and Heusen-Zolder in the Campine region, Belgium. In general, these hedgehogs were captured in gardens and urban areas by civilians. To grant the hedgehogs an easy and full recovery, removal of all ectoparasites upon arrival at the rehabilitation center is a standard procedure. For this study, attached ticks of all life stages were collected by the centers' volunteers in 2014 (both centers) and 2015 (only Herenthout) between the

end of April and the end of October. Tick specimens were stored in 70% ethanol at room temperature until further investigation. Ticks were identified to species and life stage [337]. The number of attached ticks (tick burden) was recorded for each hedgehog. Since only hedgehogs that harbored ticks were used in this study, there is no data on the percentage of hedgehogs that were infested by ticks. Age (adult or juvenile) was determined based on weight [318] for all hedgehogs, except two. The questing *I. ricinus* ticks, that were caught by drag-sampling the vegetation in the same region as where the hedgehogs were collected, are part of a previously published study [106].

Sample preparation and molecular detection of tick-borne pathogens

All ticks were processed individually. Nucleic acids were extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted DNA was stored at -20 degrees Celsius until further use. Ticks were tested individually for presence of *B. burgdorferi* s. l., *B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis and *R. helvetica* DNA using two separate multiplex real-time PCR assays as described before [60, 106, 338, 339], followed by sequencing for species identification. For the identification of *B. burgdorferi* s. l. genospecies a conventional PCR assay targeting the 5S-23S intergenic region (IGS) was performed. *Borrelia burgdorferi* s. l. genospecies identification was determined by comparison of sequences to isolate in-house molecular databases [161]. As genospecies identification was successful for only 43.4% of the *B. burgdorferi* s. l. positive sequences, we proportionally assigned these unidentifiable sequences in each life stage per tick species to the different *Borrelia burgdorferi* s. l. genospecies present in stage, using the proportion of each genospecies detected in that stage as a weighting factor (Hofmeester *et al*., submitted). We assumed that the probability to successfully identify a particular genospecies is equal for all *Borrelia burgdorferi* s. l. genospecies. For confirmation of *B. miyamotoi* conventional PCR targeting *glpQ* gene was done [95]. The *groEL* gene of *A. phagocytophilum* was amplified and sequenced [39]. For all conventional PCR's, both strands of PCR products were sequenced by BaseClear (Leiden, the Netherlands).

Statistical tests

All statistical tests were performed using R version 3.2.0 (R Core Team, 2016 [340]) and all graphs were made with the package *ggplot2* [341]. To test the differences in distribution of tick species, tick burden and infection prevalence of the different pathogens in ticks on hedgehogs from different age classes, Kruskal-Wallis tests were employed. The number of mixed tick species infestations (both tick species on the same hedgehog) was compared to the number of single species infestations (only *I. ricinus* or *I. hexagonus*) with Pearson's Chi-squared test. With the *prop.test* function, we tested if the pathogens in the ticks occurred more frequently alone or co-existing with a different pathogen in the same tick. Afterwards we compared the infection prevalence of the pathogens in *I. hexagonus* with

the prevalence in *I. ricinus.* Finally, to assess the transmission capabilities of the hedgehog for each pathogen, we compared the infection prevalence in the engorged ticks collected from hedgehogs with the infection prevalence in questing *I. ricinus* from the same region [106], and used this as a proxy to evaluate the reservoir status of the hedgehog. In order to evaluate the reservoir status of a host species, it is best to compare engorged *I. ricinus* larvae with questing *I. ricinus* larvae and nymphs. As the amount of engorged *I. ricinus* larvae is too low to perform these analyses (n = 7), we decided to compare the infection prevalence of each pathogen in engorged *I. ricinus* larvae and nymphs collected from hedgehogs with the infection prevalence in host-seeking *I. ricinus* nymphs and adults. This way, we compare ticks that fed once (engorged larvae and questing nymphs) or twice (engorged nymphs and questing adults), and omit ticks that had the chance to feed three times (engorged adults). Engorged adults have a higher chance to be infected than a questing tick anyway, that has never fed more than twice. The difference between the pathogen communities in engorged and questing ticks is thus that the engorged ticks will have certainly fed, at least once, on hedgehogs, while the chance that the questing ticks will have fed on hedgehogs is rather low. Differences between the infection prevalence of the pathogens in engorged and questing ticks may then reflect the importance of the hedgehog in the transmission of pathogens. A higher infection prevalence of a certain pathogen in engorged larvae and nymphs will then suggest that the hedgehog has an important role in the transmission of that pathogen.

RESULTS

Of the 54 hedgehogs investigated, 24 were adults and 28 were juveniles. For two hedgehogs, age was not determined. Both *I. hexagonus* and *I. ricinus* ticks of all life stages were found on the hedgehogs. The number of ticks per hedgehog ranged from one to 167. Most hedgehogs in our study carried only few ticks, while only few individuals harbored the majority of the ticks. Six of the 54 hedgehogs carried more than half of all ticks (624/1205) and only 15 hedgehogs carried 25 or more ticks. Tick burden did not significantly differ between hedgehog age classes (p = 0.97). In total, we collected 1205 ticks and found significantly more *I. hexagonus* (n = 1132) than *I. ricinus* (n = 73) (p < 0.05). The most common life stage of *I. hexagonus* retrieved from the hedgehogs were nymphs (n = 586) (p = 0.03). Of *I. ricinus*, all life stages were equally common (p = 0.07, Figure 1). Some hedgehogs were found to harbor both species of ticks (n = 10), but infestations with only one tick species were more common (n = 44, p < 0.05).

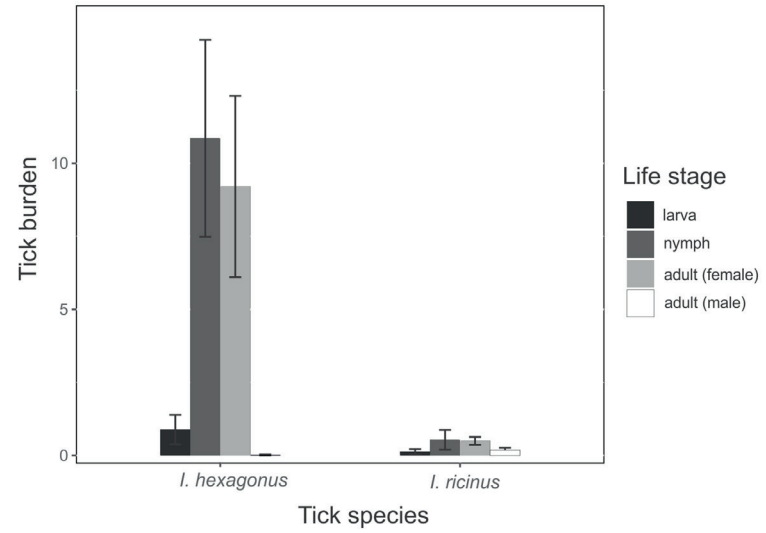


Figure 1: The distribution of the different life stages of *Ixodes ricinus* (IR) and *Ixodes hexagonus* (IH) collected from 54 hedgehogs in the Campine region, Belgium (mean ± S.E.)

Of the 1205 collected ticks, two got lost during sample preparation, hence the molecular analyses were performed on 1203 ticks. A total of 859 (71.4%) ticks was infected with at least one of the tested pathogens. Of these infected ticks, 524 (61%) had a single infection, and 335 ticks (39%) were infected with more than one pathogen of another genus. The number and percentage of infected *I. ricinus* and *I. hexagonus* ticks per life stage can be found in Table 1. A more detailed overview, including the different *Borrelia burgdorferi* s. l. genospecies, is provided in Additional file 1. *Anaplasma phagocytophilum* and *R. helvetica* were the two most common pathogens and occurred in 466 ticks (38.7% of all analyzed ticks or 54% of all infected ticks) coming from 34 hedgehogs and in 481 ticks (40% of all analyzed ticks or 56% of all infected ticks) coming from 37 hedgehogs, respectively. An infection with *Borrelia burgdorferi* s. l. occurred in 297 ticks (24.7% of all analyzed ticks or 34.6% of all infected ticks) from 28 hedgehogs. We were able to identify the *B. burgdorferi* s. l. genospecies in 129 (43.4%) of these infected ticks, of which *B. afzelii* (n = 80), *B. spielmanii* (n = 28) and *B. bavariensis* (n = 17) were the most common. *Borrelia turdi* occurred once in both tick species and *Borrelia garinii* and *B. valaisiana* each in one *I. ricinus* tick. An infection with *B. miyamotoi* occurred in 20 ticks from five hedgehogs. Only three *Ixodes ricinus* ticks from two hedgehogs were infected with *Candidatus* Neoehrlichia mikurensis. The pathogen prevalence per tick species is depicted in Figure 2. *Ixodes ricinus* seems to be more likely infected with at least one pathogen (59/72, 81.9%) than *I. hexagonus* (800/1131, 70.7%) but the difference between the two tick species was only marginally significant (p = 0.06). More specifically, the infection prevalence of *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *B. afzelii*, *B. garinii*, *B. valaisiana* and *B. turdi* was highest in *I. ricinus* while

infection with *R. helvetica* was highest in *I. hexagonus* ($p < 0.05$). For the infection prevalence of *B. miyamotoi*, *B. spielmanii* and *B. bavariensis*, no difference between the tick species could be observed. There was no difference in infection prevalence between adult and juvenile hedgehogs for any of the detected pathogens ($p > 0.05$).

Table 1: The number (#) of *Ixodes ricinus* and *Ixodes hexagonus* ticks infected with a certain pathogen, for all life stages together or for larvae (L), nymphs (N) or adults (A) separately, and the percentage (%) of infected ticks of the two species on all analyzed ticks from that species per life stage.

			B. burgdorferi s. l.	*B. miyamotoi*	*R. helvetica*	*A. phagocytophilum*	*Candidatus N. mikurensis*
I. hexagonus	**L**	**#**	3	2	10	2	0
		%	6.3	4.2	20.8	4.2	0
	N	#	166	8	192	279	1
		%	28.4	1.4	32.8	47.7	0.2
	A	#	91	7	267	137	0
		%	18.3	1.4	53.6	27.5	0
	all	#	260	17	469	418	1
		%	23	1.5	41.5	37	0.09
I. ricinus	**L**	**#**	1	0	0	3	0
		%	14.3	0	0	42.9	0
	N	#	20	0	3	23	2
		%	71.4	0	10.7	82.1	7.1
	A	#	16	3	9	22	0
		%	43.2	8.1	24.3	59.5	0
	all	#	37	3	12	48	2
		%	51.4	4.2	16.7	66.7	2.8

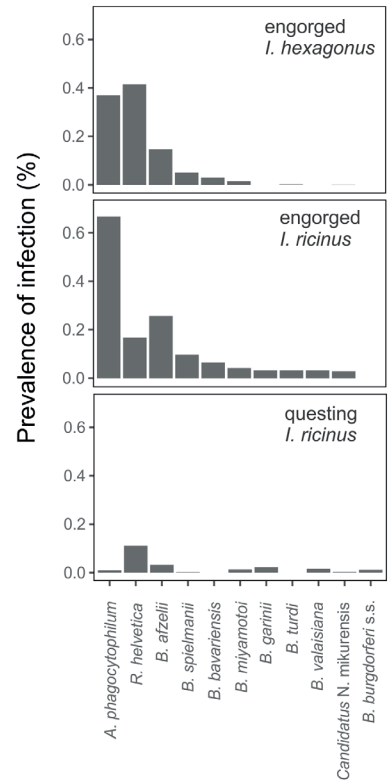


Figure 2: The prevalence of the distinct pathogens in *Ixodes ricinus* and *Ixodes hexagonus* ticks collected from hedgehogs (mean ± S.E.).

Co-infections of other pathogens with *B. burgdorferi* s. l. were investigated. For *I. ricinus*, 37 of the 59 infected ticks (62.7%) carried two (n=31) or three (n=6) pathogens. The most common co-infection in *I. ricinus* (24/37) was with *B. burgdorferi* s. l. and *A. phagocytophilum*. Of the 800 infected *I. hexagonus* ticks, 298 (37.3%) had a co-infection composed of two (n=232) or three (n=65) pathogens. Co-infections of *A. phagocytophilum* and *R. helvetica* (102/298), *A. phagocytophilum* and *B. burgdorferi* s. l. (86/298) and *A. phagocytophilum*, *R. helvetica* and *B. burgdorferi* s. l. (64/298) occurred most often. One *I. hexagonus* tick was infected with four pathogens: *A. phagocytophilum*, *R. helvetica*, *B. burgdorferi* s. l. and *B. miyamotoi*. All pathogens were found more often co-existing with another pathogen in a tick, than as the single pathogen infecting the tick ($p < 0.05$).

Ixodes ricinus larvae and nymphs from hedgehogs were infected more often (28/35) than questing *I. ricinus* nymphs and adults (367/1874) ($p < 0.05$). We could not detect any difference in prevalence of *R. helvetica*, *B. miyamotoi*, *B. garinii* and *B. valaisiana*. For all other pathogens, infection prevalence was significantly higher in the engorged ticks from the hedgehogs ($p < 0.05$).

Afterwards we repeated these analyses for *I. hexagonus* collected from hedgehogs and compared the larvae and nymphs of this tick species with the questing *I. ricinus* nymphs and adults collected from the vegetation. This enables us to interpret more comprehensively the reservoir role of the hedgehog for the different pathogens, and the vector competence of *I. hexagonus*. We observed that *B. garinii* and *B. valaisiana* were more prevalent in the questing *I. ricinus* ticks. No significant difference in infection prevalence between questing or engorged ticks could be detected for *Borrelia turdi*, *B. miyamotoi* and *Candidatus* Neoehrlichia mikurensis. The prevalence of all other pathogens, including *R. helvetica,* is higher in the engorged than the questing ticks. Furthermore, when comparing just the ticks collected from hedgehogs that carried 25 or more ticks, we obtain the same outcome.

For *A. phagocytophilum*, *R. helvetica*, *B. bavariensis* and *B. miyamotoi*, the distribution of the infections was clustered in some hedgehogs, with most hedgehogs harboring no, or only few infected ticks, while only few hedgehogs were responsible for the majority of the infected ticks. This is visualized in Figure 3. Twelve of the 17 ticks infected with *Borrelia bavariensis* and 16 of the 20 ticks infected with *B. miyamotoi* came from one individual hedgehog (hedgehog #18). Hedgehog #33 harbored a total of 125 ticks of which 118 were infected with *A. phagocytophilum* (25.3% of all *A. phagocytophilum* infections). Still, there are hedgehogs that harbor many ticks, while no or few or these ticks are infected with one of these pathogens (Figure 3).

Of the *A. phagocytophilum* positive ticks, 43 were sequenced of which 33 *I. hexagonus* and 10 *I. ricinus* from 18 different individual hedgehogs. All the *groEL* sequences of the *A. phagocytophilum* isolates clustered with the zoonotic ecotype, ecotype I (not shown [39]).

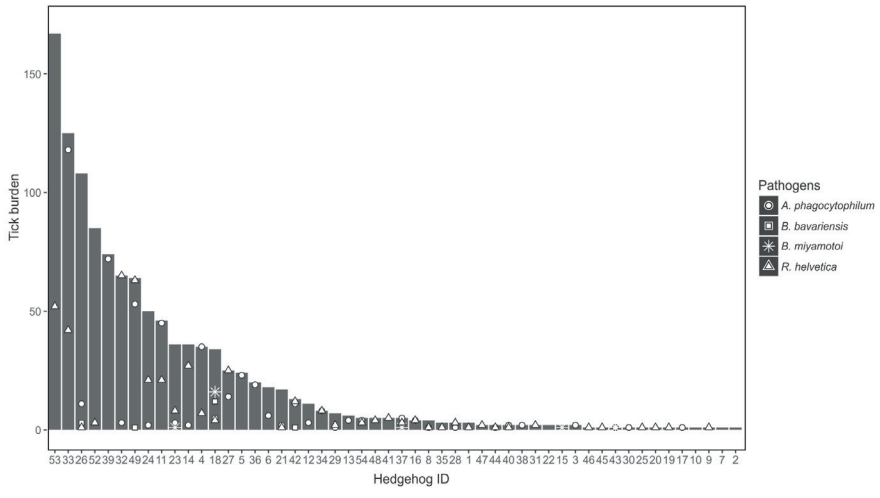


Figure 3: The tick burden per hedgehog with the number of ticks per hedgehog harboring infection with *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Borrelia bavariensis* and *Borrelia miyamotoi*.

DISCUSSION

Our results confirm that hedgehogs are a host of all three stages of *I. hexagonus* and *I. ricinus*. Still, more *I. hexagonus* were found feeding on hedgehogs than *I. ricinus* ticks (Figure 1). The aggregation of ticks on hedgehogs varied vastly between the individual hedgehogs, as only a few hedgehogs were recorded to carry most of the ticks (Figure 3). This means that just a few hedgehogs contribute to tick maintenance, similar to what is seen on rodents [342]. This seems to be especially the case for *I. hexagonus*, and to a lesser extend for *I. ricinus*, since the burdens of *I. ricinus* on hedgehogs appears to be relatively low. Moreover, it is less likely that hedgehogs can maintain the *I. ricinus* life cycle as the sole host species because, even though it can feed all life stages of this generalist tick species, hedgehog densities in forested areas, the preferred habitat of *I. ricinus*, are too low [329, 330]. Namely, if all *I. ricinus* stages should rely only on the hedgehog to feed on, many ticks would starve and perish since the amount of encounters with this host would be low. We believe, rather, that a host community without large mammals but composed only of small or medium sized hosts such as rodents, birds and hedgehogs (like in (sub)urban area's and parks), can already be sufficient to complete the life cycle of *I. ricinus*. This because, as we show, large mammals are not the only hosts adult *I. ricinus* ticks feed on. More research is needed, however, to elucidate the role of hedgehogs in the life cycle of this generalist tick species.

Since 71.4% of the ticks retrieved from hedgehogs were infected by at least one pathogen, hedgehogs can be considered as amplifying hosts and epidemiologically important wildlife species. Moreover, 39% of all infected ticks carried more than one pathogen of another genus. High prevalence of tick-borne pathogens *B. bavariensis*, *B. spielmanii*, *B. afzelii*, *A. phagocytophilum* and *R. helvetica* in engorged *I. hexagonus* and *I. ricinus* ticks obtained from *E. europaeus*, indicates that hedgehogs contribute to pathogen maintenance in natural cycles in urban and suburban areas. For *B. bavariensis*, *B. spielmanii*, *B. afzelii*, *A. phagocytophilum* and *R. helvetica*, the infection prevalence was higher in the engorged ticks of both species, in comparison to the infection rates in questing ticks from the same region (Figure 2). This indicates that the hedgehog is a possible reservoir host of these pathogens and contributes to their enzootic cycle. On the other hand, *Candidatus* Neoehrlichia mikurensis infection rate was not significantly higher in questing *I. ricinus* ticks than in engorged hedgehog ticks, indicating that hedgehogs do not play a main role in the maintenance of the enzootic cycle of this pathogen.

Engorged *I. ricinus* ticks tend to be more infected with any pathogen in comparison to engorged *I. hexagonus*, except for *R. helvetica* which was significantly more prevalent in *I. hexagonus* ticks. Perhaps this observation can be subscribed to transmission pathway of *R. helvetica*, which occurs transovarially as well as transstadially. Therefore, ticks in nature are usually thought to be the main reservoir and vectors of *R. helvetica* [105]. However, since transovarial transmission rates are less than 100%, vertebrate hosts like the hedgehog can act as an amplifier of this pathogen, playing a vital role in transmission cycles. The pathogens that are present in engorged *I. ricinus* ticks can originate from a previous blood meal from

another host species, while the pathogens *I. hexagonus* carries are most probably coming from the hedgehog, since hedgehogs are their preferred host species. This way infection prevalence in engorged *I. ricinus* can be higher than engorged *I. hexagonus*, when they fed in a previous stage on a host species that functions as an efficient reservoir species for some of the investigated pathogens, such as small rodents or birds.

Remarkably, the infection of some pathogens such as *B. bavariensis*, *B. miyamotoi*, *R. helvetica* and *A. phagocytophilum* seem to be clustered per individual hedgehog, meaning that only a few hedgehogs contribute to the gross of the infected ticks. *Borrelia miyamotoi* is known to give short-term systemic infection in rodents, therefore making rodents excellent but transitory amplifying hosts of this bacterium [61]. Vertebrates other than rodents may also become infected: *B. miyamotoi* DNA was also found in the tissue of an European greenfinch and a great tit [89]. The clustering of infected fed ticks on only one hedgehog in this study indicate that *B. miyamotoi* might result in a short-term systemic infection in hedgehogs as well. The role of these animals in the transmission cycle is not clear; they could be transitory hosts. Another possible explanation for the fact that many ticks were infected with the same pathogen on the same hedgehog could be co-feeding transmission [343, 344]. With this route of transmission, no systemic infection of the vertebrate host is necessary. The host is only a transient bridge, bringing together infected and uninfected ticks in both space and time, thereby facilitating pathogen exchange. The host does not necessarily have to be infected himself [343, 344]. The bird associated *Borrelia*e, *B. garinii* and *B. valaisiana*, were each detected in one *I. ricinus* adult tick, and *B. turdi* occurred in one *I. hexagonus* female and one *I. ricinus* nymph. We can thus confirm the indication that hedgehogs are no reservoir hosts for the bird associated, only for the rodent-associated, *B. burgdorferi* s. l. genospecies [318].

Hedgehogs and their host-specific parasite *I. hexagonus* seem to play a role in maintaining some pathogens, like *B. bavariensis*, *B. spielmanii*, and *A. phagocytophilum* in cryptic cycles. The generalist feeding behavior of *I. ricinus* and the low prevalence of these pathogens in questing *I. ricinus* suggest that they do not play a main role in the maintenance of the enzootic cycle of these pathogens. However, when feeding on hedgehogs *I. ricinus* may still be infected by *I. hexagonus*-associated pathogens and transmit them to humans. *Borrelia bavariensis* can cause neurological disease in humans [19], and *B. spielmanii* has been linked to EM in humans. Both pathogens have already been linked to hedgehogs [318, 320]. Moreover, co-infection of *R. helvetica* and *B. burgdorferi* s. l. has been shown in neuroborreliosis patients [118]. Also, co-infections are thought to affect the severity of disease and influence clinical outcomes in some cases [155]. Since hedgehogs seem to be large contributors to co-infection rates in ticks, this poses an increased health risk. The variant of *A. phagocytophilum* detected in these samples were all linked to human cases of anaplasmosis (ecotype I) [39].

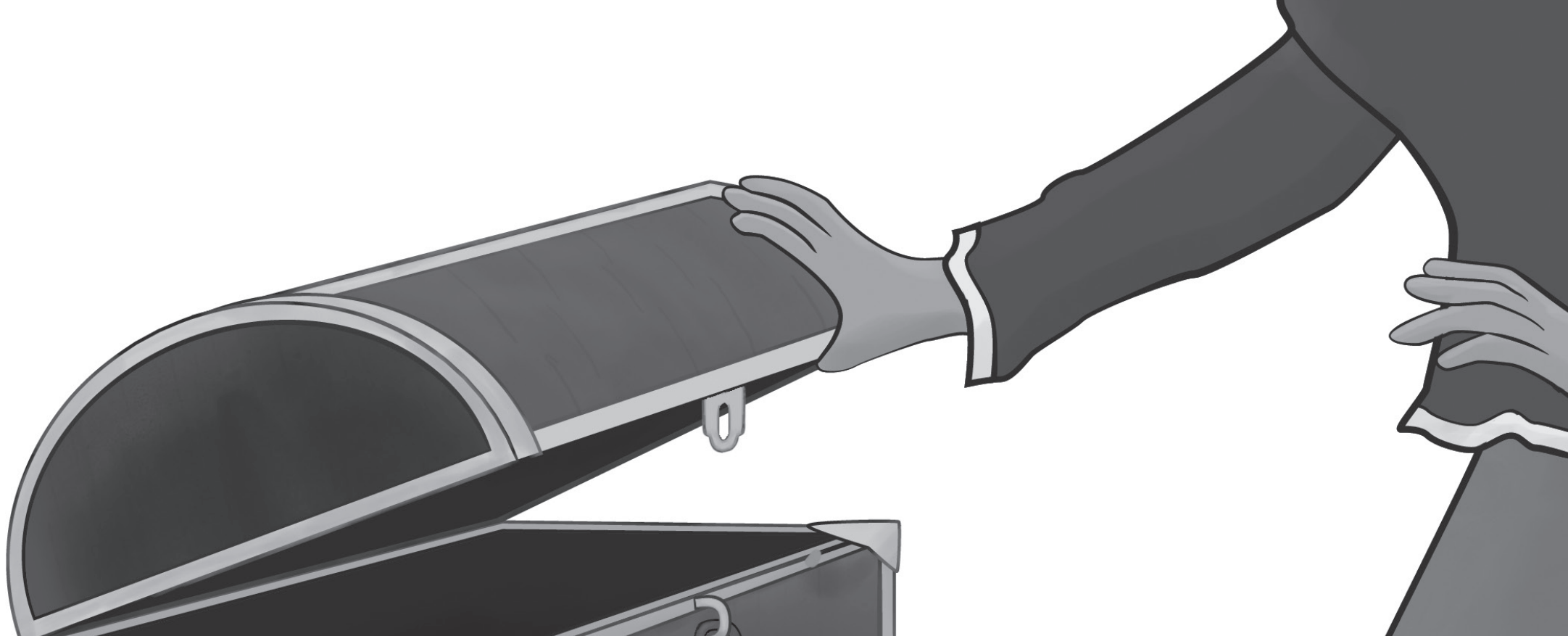
CONCLUSIONS

From these findings we conclude that hedgehogs are important components in the enzootic cycle of a diverse set of human pathogens, thereby contributing to the maintenance of various tick-borne diseases in (sub)urban areas. Humans are likely to come into contact with ticks infected with one or several of these pathogens while gardening or recreating in parks [345]. This poses a potential human health risk. Most hedgehogs, however, carry only few ticks and hedgehog densities are relatively low, thus hedgehogs will probably infect only few ticks with a certain pathogen.

Further research is necessary to elucidate the interaction between hedgehog densities, tick burden and tick infection prevalence and to assess the precise impact of hedgehogs on the enzootic cycle of the various tick borne human pathogens, and the associated human health risk.

PART II

...LEAVING ONLY
HOPE INSIDE



CHAPTER 7

Enzootic origins for clinical manifestations of Lyme borreliosis

S. Jahfari, A. Krawczyk, E. C. Coipan, M. Fonville,
J. Hovius, H. Sprong, K. Takumi

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ABSTRACT

Both early localized and late disseminated forms of Lyme borreliosis are caused by *Borrelia burgdorferi* senso lato. Differentiating between the spirochetes that only cause localized skin infection from those that cause disseminated infection, and tracing the group of medically-important spirochetes to a specific vertebrate host species, are two critical issues in disease risk assessment and management. *Borrelia burgdorferi* senso lato isolates from Lyme borreliosis cases with distinct clinical manifestations (*erythema migrans*, neuroborreliosis, acrodermatitis chronica atrophicans, and Lyme arthritis) and isolates from *Ixodes ricinus* ticks feeding on rodents, birds and hedgehogs were typed to the genospecies level by sequencing part of the intergenic spacer region. In-depth molecular typing was performed by sequencing eight additional loci with different characteristics (plasmid-bound, regulatory, and housekeeping genes). The most abundant genospecies and genotypes in the clinical isolates were identified by using odds ratio as a measure of dominance. *Borrelia afzelii* was the most common genospecies in acrodermatitis patients and engorged ticks from rodents. *Borrelia burgdorferi* senso stricto was widespread in *erythema migrans* patients. *Borrelia bavariensis* was widespread in neuroborreliosis patients and in ticks from hedgehogs, but rare in *erythema migrans* patients. *Borrelia garinii* was the dominant genospecies in ticks feeding on birds. Spirochetes in ticks feeding on hedgehogs were overrepresented in genotypes of the plasmid gene *ospC* from spirochetes in *erythema migrans* patients. Spirochetes in ticks feeding on hedgehogs were overrepresented in genotypes of *ospA* from spirochetes in acrodermatitis patients. Spirochetes from ticks feeding on birds were overrepresented in genotypes of the plasmid and regulatory genes *dbpA*, *rpoN* and *rpoS* from spirochetes in neuroborreliosis patients. Overall, the analyses of our datasets support the existence of at least three transmission pathways from an enzootic cycle to a clinical manifestation of Lyme borreliosis. Based on the observations with these nine loci, it seems to be justified to consider the population structure of *B. burgdorferi* senso lato as being predominantly clonal.

INTRODUCTION

Lyme borreliosis is one of the vector-borne disease with the highest incidence in Europe [14]. The most common manifestation of Lyme borreliosis is *erythema migrans*, an expanding skin lesion occurring after several days or weeks at the site of the tick bite. Medical guidelines advise antibiotic treatment of *erythema migrans*, as it commonly prevents the development of late and more severe disease stages [10]. The disseminated and more severe manifestations of Lyme borreliosis can involve a patient's nervous system (neuroborreliosis), joints (Lyme arthritis), skin (acrodermatitis chronica atrophicans), and in rare cases heart (Lyme carditis), ocular and lymph nodes (*borrelia*l lymphocytoma). These disseminated manifestations seldom occur simultaneously in individual patients. Epidemiological surveys from Germany and the Netherlands found that 89-95% of the reported Lyme borreliosis cases had *erythema migrans*, 2-5% Lyme arthritis, 2% (early) neuroborreliosis, 0.4-2% *borrelia*l lymphocytoma, 0.9-1% acrodermatitis chronica atrophicans, 0.1% Lyme carditis, and 0.1% ocular manifestations [25, 346].

Lyme borreliosis is caused by spirochetes belonging to the *B. burgdorferi* sensu lato (s. l.) complex. These bacteria have a high genetic diversity, at intergenospecies and intragenospecies level. It has been postulated that this genetic diversity is at the base of the multiple clinical manifestations that infection with these bacteria can display [347-349]. At least five of the more than 19 known genospecies of *B. burgdorferi* s. l. are commonly associated with Lyme borreliosis in Europe: *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto (s. s.), *B. spielmanii*, and *B. bavariensis* [350]. Other *Borrelia* genospecies, such as *B. lusitaniae,* have occasionally been found to cause disease as well [20-23]. All the five pathogenic genospecies are able to cause *erythema migrans* [351]. Although there is not a clear-cut differentiation between genospecies and disseminated disease manifestations, current knowledge is that *B. afzelii* is predominantly associated with acrodermatitis, *B. garinii* and *B. bavariensis* with neurological manifestations, and *B. burgdorferi* s. s. with Lyme arthritis [10, 19]. Remarkably, not all the *Borrelia* genotypes within the pathogenic genospecies can cause Lyme borreliosis [117]. Genotype is a subset of spirochetes belonging to the same genospecies which share an identical sequence. From the 251 multilocus sequence types (MLST) that were identified in a study investigating 775 *B. burgdorferi* s. l. isolates from various sources, only six sequence types significantly associated to clinical manifestations in humans [19]. In Northern America, *B. burgdorferi* s. s. has some major sequence types of the outer surface protein C (*ospC*) and certain sequence types of *16S-23S rRNA* intergenic spacer that are more frequently found in disseminated cases of Lyme borreliosis [347-349]. Furthermore, significant associations between clusters of multilocus sequence types (clonal complexes) of *B. burgdorferi* s. s. and localized or disseminated forms of Lyme borreliosis have been found [352]. What genetic information determines the differential invasiveness of *Borrelia* genospecies, but also of genotypes within a genospecies is not known.

Ixodes ricinus is the main vector of *B. burgdorferi* s. l. in Western Europe [353], although other tick species, such as *I. hexagonus, I. uriae* and *I. frontalis* might be able to transmit *B. burgdorferi* s. l. as well [322, 354, 355]. Ticks become infected during a blood meal on an infected vertebrate host or when co-feeding near an infected nymph [356, 357]. Many small mammals, birds and lizards act as transmission and/or amplification hosts for *B. burgdorferi* s. l. [358]. Interestingly, ungulates are among the few vertebrates known as incompetent for transmission of *B. burgdorferi* s. l. [359, 360]. The full range of transmission and/or amplification hosts and their relative contribution to the generation of infected ticks has not been well understood for all *Borrelia* genospecies. It has been hypothesized that the genetic diversity of *B. burgdorferi* s. l. is maintained by a multiple-niche polymorphism balancing selection [361]. This seems to be the case at the genospecies level: Small mammals mainly transmit *B. afzelii*, while *B. garinii* is mainly transmitted by birds [201, 203, 361]. There is evidence that this is also the case within the genospecies: In the United States, four important host species of *B. burgdorferi* s. s. were infected with different sets of *ospC* major groups [362]. Furthermore, different host species differ in their ability to transmit *B. burgdorferi* s. s. [363]. Whether this is also the case for two major *Borrelia* genospecies in Europe, *B. afzelii* and *B. garinii*, needs further investigation [364].

This study aims to directly link a transmission and/or amplification host for *B. burgdorferi* s. l. to a clinical manifestation of Lyme borreliosis. We hypothesize that a transmission cycle for a genotype is one factor that determines the clinical manifestation of Lyme borreliosis. We test this hypothesis by a quantitative molecular epidemiologic approach. Our sample collection covers both clinical sources (Lyme borreliosis patients having *erythema migrans*, neuroborreliosis, acrodermatitis, or Lyme arthritis) and field sources (ticks feeding on birds, rodents and hedgehogs). Plasmid, regulatory, house-keeping and neutral genetic loci from a number of samples are input to the statistical test of the hypothesis.

MATERIAL AND METHODS

Borrelia samples

For this study, 183 previously described isolates [19] of *B. burgdorferi* s. l. which were derived from Lyme borreliosis cases across Europe (Austria, Switzerland, Denmark, Spain, Finland, France, Germany, Hungary, Italy, Netherlands, Portugal, Slovenia, Sweden), with distinct clinical manifestations were used. In addition, 26 isolates of *B. burgdorferi* s. l. from questing *I. ricinus* and 184 *B. burgdorferi* s. l. positive lysates of questing *I. ricinus* from the Netherlands were used. *Ixodes ricinus* ticks that had fed on rodents (n=217), birds (n=153) and hedgehogs (n=28) from the Netherlands were used. Ticks of all stages were included in this study.

DNA extraction, screening of samples, target gene amplification

Upon arrival in the laboratory, the ticks were identified by an experienced technician using morphological keys [224, 365]. Only *Ixodes ricinus* tick were used for further analysis. DNA extraction from the individual questing ticks was done by alkaline lysis in ammonium hydroxide, as described previously [51], while the DNA extraction from the bacterial isolates and fed ticks was performed using DNeasy® Blood & Tissue Kit (QIAGEN N.V., Venlo, the Netherlands). Screening of the questing and fed ticks for *B. burgdorferi* s. l. was done by qPCR, as described [271].

For all the *Borrelia* positive samples, we performed PCR targeting the variable 5S–23S rDNA intergenic spacer region (IGS), to determine and type the genospecies of the *Borrelia*. The PCR was performed according to the protocol described before [161]. All *B. burgdorferi* s. l. samples were sequenced using the 5 regulatory virulence -associated genes (*bosR*, *rrp2*, *rpoS* and *rpoN*) and 3 plasmid genes (*ospC*, *ospA* and *dbpA*). The gene rpoN is involved in the regulation of plasmid genes [366-368]. The genes bosR, rpoS, and rrp2 are located downstream of rpoN [369, 370]. Furthermore, these genes are thought to be involved in the uptake of essential metal and ions from the surroundings, which is important for survival of the spirochetes in the host [371]. PCR procedure and primers as described in Table A of the supplementary data. All 8 genes were amplified and subsequently sequenced in forward and reverse directions. Trimming and manual cleaning of sequences was performed in Bionumerics 7.1. (Applied Math, Belgium). Genes *ospC*, *ospA* and *dbpA* available in Genbank were downloaded and used for the analyses.

Molecular epidemiological database

Borrelia burgdorferi s. l. *ospC*, *ospA* and *dbpA* DNA sequences with the geographical origin (country) and the host species from which the isolate originated were also downloaded from the Entrez Nucleotide Database (GenBank, NCBI). A sequence was excluded if its host species was unknown or it did not originate from Europe. Sequences that were too short to cover regions of variation were also excluded from further analysis. DNA sequences and epidemiological data used for this study are given in Supplementary material.

Genetic analyses

DNA sequences for each gene were aligned using MAFFT version 7.271 [372] with the option localpair turned on and the option maxiterate set to 1000. A phylogenetic tree was estimated using raxml version 8.1.17 [373]. We set an option for a general time reversible model of DNA evolution and CAT approximation for a gamma model of rate heterogeneity (-m GTRCAT). We also set an option for rapid bootstrap analysis and maximum likelihood tree search (-f a) to compute confidence values for each internal node. We collapsed the internal nodes in the estimated maximum likelihood tree for which a confidence value was less than 80% using a program Python (2.7.10 Release) and importing a module Phylo [374].

Statistical analyses

We estimated association between a particular source and a genotype or a genospecies as odds ratio, using Fishers Hypergeometric distribution [375] by means of the maximum likelihood method. Three outcomes are possible: (a) a particular source is overrepresented in a genospecies (odds ratio > 1), (b) present evenly in each genospecies (odds ratio = 1), and (c) underrepresented in a particular genospecies (odds ratio < 1). P-value for odds ratio was calculated based on the likelihood ratio test with 1 degree of freedom. When P-value is greater than 0.05, we interpret that the estimated odds ratio is not equal to 1.
Plasmid, regulatory, and house-keeping genes are analyzed for identification of a possible virulent cluster. Each internal node in a gene tree was examined for virulence by depth-first search, by estimating Bayes factor [376] at each internal node. This calculation was based upon Dirichlet compound multinomial distribution [377] with a flat prior. Null hypothesis is that the observed numbers of eight sources (Lyme borreliosis patients having *erythema migrans*, neuroborreliosis, acrodermatitis, or Lyme arthritis, questing ticks and engorged ticks from birds, rodents and hedgehogs) within a cluster are congruent with the proportions of eight sources in the whole gene tree. Alternative hypothesis is that observed numbers within a cluster are better explained by assigning cluster-specific proportions. Estimated Bayes factors are translated into decision categories following a classification scheme [376]. Category 'Decisive' supports that some source is overrepresented within the cluster. All other decision categories are deemed insufficient support and we discard them for subsequent steps in the analyses.

A 'Decisive' clusters could indicate significant excess in any of the eight sources of our sample collection. Our primary interest is one of four clinical sources. To find out which, we calculate a distance from a cluster to each of four clinical sources. Mathematically, we assemble observed numbers of eight sources into a data matrix, where 'Decisive' clusters are rows and eight distinct sources are columns (Supplementary material Table B). Then we identify the top two principal components by applying singular value decomposition [378] to the data matrix. Cosine similarity defines the distance between a cluster and a clinical source. After identifying the shortest distance, we label a cluster by the matching clinical source. We checked the result by examining the number of sequences with regard to the matching clinical source (Supplementary material Table B). In case of failure to check, the cluster remains unlabeled. Failure is often due to the absence of any clinical source, or unusual amount of excess or deficit in one of field sources.

A test on nine genetic markers is performed to divide the markers into two subgroups based on odds ratio. Genetic markers are ordered by the estimated odds ratio values, then they are split into a high odds ratio group and a low odds ratio group. Each arrangement is tested based on the likelihood ratio test against the null hypothesis: all genes are equal in odds ratio. We choose the arrangement for which the P-value is: (1) the lowest among all eight

splits, and (2) less than the threshold value (p = 0.05). A test on genospecies and the genetic markers is performed similarly.

Depth-first tree-traversal and Dirichlet compound multinomial distribution are implemented using Python (2.7.10 Release) and importing two modules Phylo [374] and Scipy [379]. Odds ratio calculation and singular value decomposition are implemented using Mathematica 10.4.1.0 (Wolfram Research, Inc., Campaign, IL).

RESULTS

Genospecies frequencies based on IGS

A set of 467 samples from seven sources (four clinical manifestations and three field sources) were typed to the genospecies level using the intergenic spacer region (IGS, Table 1,[161]). Clinical sources were Lyme borreliosis patients having *erythema migrans* (n=136), acrodermatitis (n = 36), neuroborreliosis (n=9) and Lyme arthritis (n = 1). Six patients developed both *erythema migrans* and neuroborreliosis, and their *Borrelia* sequences were added once to both clinical manifestations for further analyses. Field sources were engorged ticks from birds (n = 51), engorged ticks from hedgehogs (*Erinaceus europaeus;* n = 182), and engorged ticks from rodents (*Apodemus sylvaticus* and *Myodes glareolus;* n = 56). The most common genospecies was *B. afzelii* (n = 329: 133 clinical and 205 field isolates), followed by *B. garinii* (n = 59: 23 clinical and 35 field), *B. spielmanii* (n = 40: 3 clinical and 37 field), *B. bavariensis* (n = 27: 7 clinical and 20 field), and *B. burgdorferi s. s.* (n = 12: 10 clinical and 2 field). *Borrelia valaisiana* was identified only once in a tick feeding on a hedgehog, and ignored in further analyses. Since it is the feeding tick nymph, it is possible that the tick was infected in a previous developmental stage.

Table 1: Summary of intergenic spacer region sequences by isolate origin and genotype. Each row lists the numbers of successful sequencing products using the specified isolate. Columns are organized according to *Borrelia* genotypes.

	Afzelii	*Bavariensis*	Senso stricto	*Garinii*	*Spielmanii*	Valaisiana	Total
Acrodermatitis chronica atrophicans	35	0	1	0	0	0	36
Lyme arthritis	1	0	0	0	0	0	1
Erythema migrans	97	1	9	20	3	0	130
Neuroborreliosis	0	3	0	0	0	0	3
Erythema migrans & neuroborreliosis	0	3	0	3	0	0	6
Engorged bird tick	17	0	0	34	0	0	51
Engorged hedgehog tick	122	20	2	1	37	1	182
Engorged rodent tick	56	0	0	0	0	0	56
Unknown/Others	1	0	0	1	0	0	2
Total	329	27	12	59	40	1	467

Genospecies association with clinical manifestations

Based on IGS genetic typing, *B. afzelii* was the dominant genospecies identified from acrodermatitis patients as measured by odds ratio (OR = 17, Table 2). *Borrelia burgdorferi* s. s. (OR = 8) was overrepresented in *erythema migrans* patients. *B. bavariensis* was the dominant genospecies in patients with neuroborreliosis (OR = 35), and it was underrepresented in *erythema migrans* patients (OR = 0.4). *Borrelia afzelii* was absent and therefore underrepresented in neuroborreliosis patients (OR = 0).

Genospecies association with enzootic cycles

Borrelia afzelii was the dominant genospecies in engorged ticks from rodents (OR = 29, Table 2), whereas *B. garinii* was the dominant genospecies in engorged ticks from birds (OR = 29). *B. bavariensis* was overrepresented in engorged ticks from hedgehog (OR = 3).

Frequencies of nine other genetic markers

Next to IGS, one or more loci (Table 3) were obtained from a larger subset of the sample collection (n = 966). These loci complement IGS as additional genetic markers for this study because of their potent roles in a sequelae following an acute *Borrelia* infection [370, 380-383]. Between 500 and 700 sequences were located on plasmids (*dbpA*, *ospA* and *ospC*, Table 3). Between 200 and 300 sequences were loci from regulatory genes (*bosR*, *rpoN*, *rpoS*, and *rrp2*, Table 3), and approximately 700 sequences were located on house-keeping genes (*clpA* and *clpX*, Table 3).

Table 2: Estimates for odds ratio values by genotype. Numerals are bold when P-value is less than 0.05: Odds ratio is not equal to 1.

	Afzelii	*Bavariensis*	Senso stricto	*Garinii*	*Spielmanii*	Valaisiana
Acrodermatitis chronica atrophicans	**17**	**0**	1	**0**	**0**	0
Lyme arthritis	0.4	0	0	0	0	0
Erythema migrans	1	**0.4**	**8**	2	**0.2**	0
Neuroborreliosis	**0**	**35**	0	4	0	0
Engorged bird tick	**0.2**	**0**	0	**29**	**0**	0
Engorged hedgehog tick	0.7	**3**	0.3	**0**	**24**	2
Engorged rodent tick	**29**	**0**	0	**0**	**0**	0

Table 3: Summary of isolates and sequences in this study. Each row lists the numbers of successful sequencing products using the specified isolate. Columns are organized according to plasmid, regulatory and MLST genes.

	Plasmid			Regulatory				MLST	
	dbpA	ospA	ospC	bosR	rpoN	rpoS	rrp2	clpX	clpA
Acrodermatitis	33	35	36	36	36	36	35	38	38
Lyme arthritis	2	3	3	1	1	1	1	2	2
Erythema migrans (EM)	125	152	127	126	129	128	128	162	163
Neuroborreliosis (NB)	28	42	44	3	3	3	3	19	22
NB and EM	6	6	6	6	6	6	6		
Vegetation tick	98	335	217	27	31	61	29	514	494
Engorged bird tick	46		40		14	49			
Engorged hedgehog tick		15	9			3			
Engorged rodent tick	101	40	99			56			
Unknown/Others	233	27	8	1	2	1	1		
Total	672	655	581	199	220	344	203	735	719

Association of genetic markers with clinical manifestations

Sequences of each of the nine genetic markers grouped into clusters by genetic similarity (Supplementary material Table B). The number of genetic clusters was the lowest for the regulatory gene (9 clusters, *bosR*) and the highest for the plasmid gene (108 clusters, *ospC*). One or more clusters were identified in each genetic marker, which contain an excess of isolates from neuroborreliosis patients (Supplementary material Table B), as supported by OR estimates, all exceeding 1 (Table 4). A likelihood test on the set of nine genetic markers supported that eight markers were statistically equal in OR and *ospA* was significantly lower

than the rest (Table 4). Distinct clinical clusters were identified for acrodermatitis in all nine genetic markers as well (Supplementary material Table B). Excess of acrodermatitis in the identified clusters were quantified in OR estimates (Table 4). Seven genetic markers were statistically equal in OR and *dbpA* and *ospC* were lower than the other seven markers (Table 5). *Erythema migrans* clusters were only identified in *ospC* (Supplementary material Table B). OR estimate for the *erythema migrans* clusters (Table 6) was a factor ten lower than the estimates for neuroborreliosis and acrodermatitis (Table 4 and 5).

Association of genetic markers with enzootic cycles

We investigated whether spirochetes from ticks feeding on a particular host species appear exceedingly often in the genotypes of the nine markers from spirochetes in neuroborreliosis patients (Supplementary material Table B), or that their occurrence follows the expectation (2.5 Statistical analyses). Spirochetes from ticks feeding on birds were overrepresented in the genotypes of the three genetic markers from spirochetes in neuroborreliosis patients, as supported by the estimated OR values significantly exceeding the value one at these loci (*dbpA*, *rpoN*, *rpoS* in Table 4). No evidence was found for the association of the spirochetes in ticks feeding on hedgehog with the neuroborreliosis, i.e. estimated OR values for *ospC* and *rpoS* in Table 4 are not significantly different from one.

Similar analysis was performed regarding the genotypes of the nine markers of spirochetes in acrodermatitis patients (Supplementary material Table B). Spirochetes from ticks feeding on rodents were overrepresented in the acrodermatitis patients: the estimated OR significantly exceeded the value one at three genetic markers (*dbpA*, *ospA*, and *rpoS* in Table 5). Spirochetes from ticks feeding on hedgehog were overrepresented in acrodermatitis: estimated OR using *ospA* significantly exceeded one (Table 5).

Spirochetes from ticks feeding on hedgehogs (OR = 4.6) and on rodents (OR = 2.7) were overrepresented in the genotypes of spirochetes from *erythema migrans* patients (Table 6). No evidence was found for the association of spirochetes from ticks feeding on birds with *erythema migrans* patients (Table 6).

OR estimates were calculated so far separately for association of genospecies and genetic markers with clinical manifestations and enzootic cycles. We applied the likelihood ratio test to the separate cases with the aim to identify main contributing factors. Genospecies *B. afzelii* and the seven genetic markers equally predicted the excess of acrodermatitis. Genospecies *B. bavariensis* and the eight genetic markers equally predicted the excess of neuroborreliosis. Genospecies *B. burgdorferi s. s.* was better predictor for the excess of *erythema migrans* than the specific genotype of the plasmid gene *ospC*.

Table 4: Estimates for odds ratio support neuroborreliosis clusters in three plasmid four regulatory and two MLST genes. Rows list plasmid, regulatory and house-keeping genes. A cell contains estimated odds ratio (see Method for detail). Numerals are bold when P-value is less than 0.05. A subgroup of genes having higher estimated odds ratio values is identified by applying the likelihood ratio test. The members are indicated by the + symbol in the class column.

	Gene	Class	Neuroborreliosis	Engorged bird tick	Engorged hedgehog tick	Engorged rodent tick
Plasmid	*dbpA*	+	92	7		0.3
	ospA		9			
	ospC	+	**30**	**0.3**	0.6	0.04
Regulatory	*bosR*	+	36			
	rpoN	+	35	55		
	rpoS	+	**28**	**7**	1.4	**0.04**
	rrp2	+	53			
MLST	*clpA*	+	58			
	clpX	+	53			

Table 5: Estimates for odds ratio support acrodermatitis clusters in in three plasmid four regulatory and two MLST genes. See Table 4 for detail.

	Gene	Class	Acrodermatitis	Engorged bird tick	Engorged hedgehog tick	Engorged rodent tick
Plasmid	*dbpA*		11	0.1		40
	ospA	+	37		15	4
	ospC		**5**	0.6	1.5	0.8
Regulatory	*bosR*	+	13			
	rpoN	+	18	0.03		
	rpoS	+	**14**	**0.1**	0.7	**25**
	rrp2	+	13			
MLST	*clpA*	+	39			
	clpX	+	42			

Table 6: Estimates for odds ratio support *erythema migrans* clusters in only one plasmid gene. See Table 4 for detail.

	Gene	*Erythema migrans*	Engorged bird tick	Engorged hedgehog tick	Engorged rodent tick
Plasmid	*dbpA*				
	ospA				
	ospC	2	1.4	4.6	2.7
Regulatory	*bosR*				
	rpoN				
	rpoS				
	rrp2				
MLST	*clpA*				
	clpX				

DISCUSSION

From a public health perspective, it is important to be able to differentiate between the pathogenic and non-pathogenic *Borrelia* spirochetes and to differentiate between the genotypes that only cause localized infections and the ones that are able to cause more disseminated, and consequently more severe disease manifestations. Discriminating between these types and linking them to specific vertebrate hosts could also be useful for disease risk assessment and management. From the medical perspective, the development of more accurate diagnostic tools, and effective treatment regimens require knowledge on the diversity and etiology of the different *Borrelia* genospecies and genotypes, which can be identified based on IGS [161].

Our quantitative molecular epidemiological approach based on genospecies level testifies that hedgehogs are a host of *B. bavariensis* and that this genospecies is widespread in neuroborreliosis patients but rare in patients with *erythema migrans*. Birds are a host of *B. garinii*. While rodents are a host of *B. afzelii* and this genospecies is strongly associated with acrodermatitis. *Borrelia burgdorferi* s. s. is widespread in *erythema migrans*, and its host could not be identified in our sample collection. Statistical support (odds ratio) for these links are substantial. The results in this study express the currently prevailing hypotheses in a more precise and quantitative manner.

The same quantitative approach based on the set of nine genetic markers revealed extra links on top of those identified by the genospecies-based analyses (Figure 1). Based on the significant associations found in these analyses, tentative transmission pathways of *B. burgdorferi* s. l. were identified in a number of situations: (1) from hedgehogs to *erythema migrans* patients based on the genetic similarity of the plasmid gene *ospC*, (2)

from hedgehogs to patients with acrodermatitis using the plasmid gene *ospA*, (3) from birds to neuroborreliosis patients using the plasmid gene *dbpA* and two virulence-associated regulatory genes *rpoN* and *rpoS*, and (4) from rodents to patients with *erythema migrans* using *ospC* as a marker. Overall, the analyses of our datasets support the existence of at least three transmission pathways from an enzootic cycle to a clinical manifestation of Lyme borreliosis (Figure 1). Nevertheless, these pathways are not mutually exclusive and overlap to some extent. All three clinical manifestations could be traced by one of those pathways to hedgehogs. We are aware that more transmission pathways exist, and that not all of them were identified in this study. Two primarily limiting factors are; the lack of a significant statistical support and, the lack of samples from more vertebrate hosts species from which engorged tick were analyzed (limited dataset). For example, the lack of *B. burgdorferi* s. s. from vertebrate hosts (Table 3) might be masking a medically important transmission pathway of this genospecies from an unknown enzootic cycle to *erythema migrans*. Due to these factors, the understanding of *Borrelia* transmission pathways at this stage (illustrated by Figure 1) is incomplete. We contemplate that a more comprehensive picture would emerge when further *B. burgdorferi* s. l. sequences become available from increasing number of a variety of amplification hosts and human Lyme borreliosis patients.

Borrelia bavariensis was identified both in neuroborreliosis and in *erythema migrans* patients. Interestingly, this genospecies seems to be abundant in neuroborreliosis patients, but rare in *erythema migrans* patients (Table 2), and rare in questing *Ixodes ricinus* ticks [38, 384, 385]. All sequences of the nine genetic markers from neuroborreliosis patients genetically cluster together with at least one sequence from *Borrelia* isolates from *erythema migrans* patients (Supplementary material Table B). If we maintain the evidence solely on the basis of genetic identity, the hypothesis that *erythema migrans* always precedes neuroborreliosis is supported by our data. An alternative argument can be made based on the number of sequences in combination with the genetic identity. A high propensity of this genospecies to manifest neuroborreliosis in a patient, and to a significantly lesser extent *erythema migrans*, is consistent with the high odds ratio in neuroborreliosis and the low odds ratio in *erythema migrans*, while both clinical manifestations co-exist within the same genetic cluster. Medical guidelines advise antibiotic treatment of *erythema migrans*, as it commonly prevents the development of late and more severe disease stages [10]. Based on our observations, this strategy does not seem to work well for the prevention of neuroborreliosis caused by *B. bavariensis,* because of the scarcity of *B. bavariensis* infections causing *erythema migrans*.

Our analysis attests that all the nine genetic markers except *ospA* are equally suitable candidate marker for neuroborreliosis. Equality among the majority of genetic markers applies to the other severe sequela, acrodermatitis. Odds ratio estimates exceeding the baseline (= 1) by a wide margin (Table 4 and 5) can be explained by high disequilibrium among the plasmid and chromosomal loci, whose phenotypes are associated to regulatory and

house-keeping tasks. These results are less compatible with the possibilities that multiple *Borrelia* genotypes exchange a virulent plasmid gene by horizontal gene transfer. For the purpose of disease risk assessment and management, it might be justified to consider the population structure of *B. burgdorferi* s. l. as being predominantly clonal.

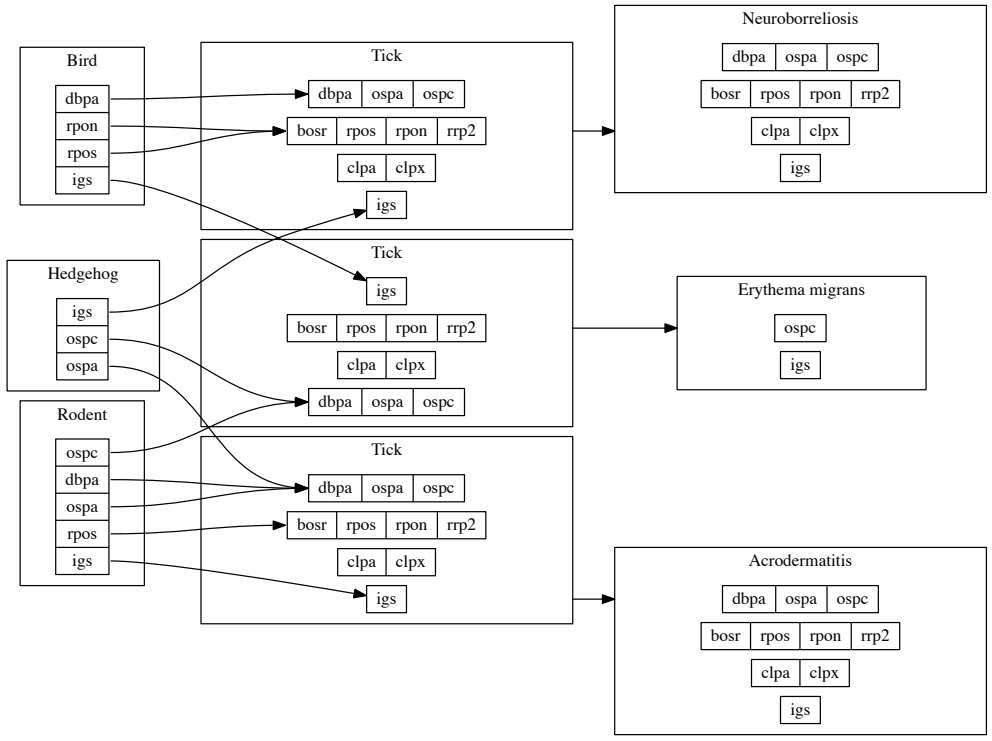

Figure 1: Schematic drawing illustrating the genetic linkages from engorged *Ixodes ricinus* ticks on distinct wild animal species to different clinical outcomes of Lyme borreliosis. Square boxes represent clinical and field sources of the sample collection in this study. A *Borrelia* gene overrepresented in a source is listed in the box. Directed edges between two boxes represents the routes identified in this study, by which a *Borrelia* gene transmits from a certain wild animal species to a specific sequela of Lyme borreliosis.

CHAPTER 8

Molecular detection of tick-borne pathogens in humans with tick bites and erythema migrans in the Netherlands

S. Jahfari, A. Hofhuis, M. Fonville,
J. van der Giessen, W. Van Pelt, H. Sprong

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ABSTRACT

Background

Tick-borne diseases are the most prevalent vector-borne diseases in Europe. Knowledge on the incidence and clinical presentation of other tick-borne diseases than Lyme borreliosis and tick-borne encephalitis is minimal, despite the high human exposure to these pathogens through tick bites. Using molecular detection techniques, the frequency of tick-borne infections after exposure through tick bites was estimated.

Methods

Ticks, blood samples and questionnaires on health status were collected from patients that visited their general practitioner with a tick bite or *erythema migrans* in 2007 and 2008. The presence of several tick-borne pathogens in 314 ticks and 626 blood samples of this cohort were analyzed using PCR-based methods. Using multivariate logistic regression, associations were explored between pathogens detected in blood and self-reported symptoms at enrolment and during a three-month follow-up period.

Results

Half of the ticks removed from humans tested positive for *Borrelia burgdorferi* sensu lato, *Anaplasma phagocytophilum, Candidatus* Neoehrlichia mikurensis*, Rickettsia helvetica, Rickettsia monacensis, Borrelia miyamotoi* and several *Babesia* species*.* Among 92 *Borrelia burgdorferi* s. l. positive ticks, 33% carried another pathogen from a different genus. In blood of sixteen out of 626 persons with tick bites or *erythema migrans*, DNA was detected from *Candidatus* Neoehrlichia mikurensis (n=7), *Anaplasma phagocytophilum* (n=5)*, Babesia divergens* (n=3)*, Borrelia miyamotoi* (n=1) and *Borrelia burgdorferi* s. l. (n=1). None of these sixteen individuals reported any overt symptoms that would indicate a corresponding illness during the three-month follow-up period. No associations were found between the presence of pathogen DNA in blood and; self-reported symptoms, with pathogen DNA in the corresponding ticks (n=8), reported tick attachment duration, tick engorgement, or antibiotic treatment at enrolment.

Conclusions

Based on molecular detection techniques, the probability of infection with a tick-borne pathogen other than Lyme spirochetes after a tick bite is roughly 2.4%, in the Netherlands. Similarly, among patients with *erythema migrans*, the probability of a co-infection with another tick-borne pathogen is approximately 2.7%. How often these infections cause disease symptoms or to what extend co-infections affect the course of Lyme borreliosis needs further investigations.

AUTHORS SUMMARY

Two most common tick-borne diseases in Europe are Lyme borreliosis and tick-borne encephalitis. Ticks transmit many more pathogens, causing neglected diseases such as anaplasmosis, babesiosis, rickettsiosis and neoehrlichiosis. These diseases are seldom diagnosed, due to their mild and non-characteristic symptoms, but also due to lack of awareness and availability of diagnostic tests. Using molecular detection techniques (polymerase chain reaction or PCR), we estimated the frequency of tick-borne infections in humans after a tick bite and in patients with the first symptoms of Lyme borreliosis, an *erythema migrans*. About half of the ticks that fed on humans carried one or more tick-borne pathogens, and approximately 2.5% of people that were bitten by ticks were infected with a tick-borne pathogen other than Lyme borreliosis or tick-borne encephalitis. Co-infections of a tick-borne pathogen in patients with an *erythema migrans* was also approximately 2.5%. Based on these findings, we estimated the incidence of tick-borne infections other than Lyme borreliosis in the Netherlands. How often these infections cause disease or to what extend co-infections affect the course of Lyme borreliosis needs further investigations.

INTRODUCTION

Lyme borreliosis is the most prevalent tick-borne disease in humans, and is caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex [10, 350, 386]. The most common clinical manifestation of early localized Lyme borreliosis is *erythema migrans* (EM), an expanding skin lesion occurring after several days or weeks at the site of the tick bite. Other sporadically reported symptoms in this early stage of disease are malaise and viral-like symptoms. Disseminated Lyme borreliosis displays more severe manifestations that can involve a patient's nervous system, joints, skin, and in rare cases the heart [10, 350, 386]. Tick-borne encephalitis (TBE) is the most common tick-borne central nervous system infection caused by the tick-borne encephalitis virus (TBEV). Its clinical spectrum ranges from fever to mild meningitis and severe meningoencephalitis with or without paralysis [387].

In several European countries, there have been marked increases in the incidence of Lyme borreliosis and TBE over the past ten to twenty years [12-14]. In the Netherlands, a retrospective study among general practitioners has shown a continuing increase in consultations for tick bites and EM between 1994 and 2009 [9, 24]. The increasing number of tick bites, adding up to 1.1 million tick bites in 2009 [9], poses a growing risk of disseminated Lyme borreliosis and perhaps also of other tick-borne diseases. In the Netherlands, *Ixodes ricinus* ticks transmit several *Borrelia burgdorferi* s. l. genospecies, but are also infected with a variety of established or potentially pathogenic microorganisms, such as *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, several *Babesia* species, *Rickettsia helvetica*, *R. monacensis* and TBEV [38, 60, 103, 120, 177, 388]. These ticks often carry multiple pathogens; at least one-third of the *I. ricinus* ticks carrying

B. burgdorferi s. l. are co-infected with one or more pathogens from a different genus [38], implying frequent exposure and possibly subsequent infection with several pathogens when humans are bitten by ticks.

Remarkably, little is known about the incidences and clinical presentations of other tick-borne diseases than Lyme borreliosis and TBE. In general, disease caused by these other tick-borne pathogens, are associated with febrile illnesses with fever, headache, myalgia and malaise [36, 73, 83, 95, 122, 389]. However, in immunocompromised patients chronic infections with severe clinical manifestations and even mortality have been described [73, 95, 122, 390]. In the Netherlands, one single case of anaplasmosis has been reported in 1999 [45], and one case of *B. miyamotoi* disease in an immunocompromised patient in 2012 [95]. It has been suggested that the severity of disease in Lyme borreliosis is affected by co-infections with other tick-borne pathogens [11, 155, 166, 391, 392]. Therefore, co-infections of *B. burgdorferi* s. l. with different tick-borne pathogens may possibly contribute to the high variety of clinical manifestations that are associated with Lyme borreliosis.

Several reasons can be appointed for the absence in reporting of tick-borne diseases other than Lyme borreliosis and TBE, and the diagnosis of co-infections with other pathogens in Lyme borreliosis patients. Firstly, most of these infections might be self-limiting without overt or characteristic symptoms, often a clear-cut case definition of patients infected with one of these pathogens has not been established yet. Secondly, a poor performance or non-existence of supportive laboratory tests in routine medical microbiological settings. Thirdly, the lack of awareness among health professionals.

Here, we aim to investigate i) whether infection with tick-borne pathogens other than *B. burgdorferi* s. l. can be shown in patients with early localized Lyme borreliosis and in people exposed to tick bites in the Netherlands, and to determine ii) the clinical picture of patients with DNA of tick-borne pathogens in their blood.

Our approach is to test for the presence of nucleic acid (DNA/RNA) of the specific pathogens in human blood through amplification with PCR, especially since currently; there is no other specific laboratory diagnostic to detect infection with most of these tick-borne pathogens. Compared to DNA amplification with PCR, available serological tests generally have a low specificity and or sensitivity, particularly during the early phase of infection. In addition, although culturing is considered the most reliable method in proving the presence of microorganisms, it is time consuming, costly and often not possible for all pathogens.

MATERIAL AND METHODS

Study design, ticks, human samples and questionnaires

Ticks, EDTA-blood and questionnaire data were available from a nationwide prospective observational study among patients who consulted one of 307 enrolling general practitioners for a tick bite or EM between May 2007 and December 2008 in the Netherlands, as described in detail [254]. All participants gave written informed consent, all minors who participated in the study had consent given from a parent/guardian, and the study protocol (number 07-032/K) was approved by the medical ethics committee of the University Medical Centre in Utrecht, the Netherlands. Patients were not eligible for participation when they were younger than six years of age, or when the tick bite had occurred outside the Netherlands. At enrolment, participants received the first set of study materials, containing a brochure about the study, an enrolment questionnaire, and materials for collection and mailing of first blood samples and removed ticks. Ticks removed from the skin were submitted in a small tube with 70% ethanol. In total, 314 ticks were obtained from 293 participants, of which 278 patients consulted their physician for a tick bite, and fifteen patients consulted their physician with an EM. Four ticks (1%) were larvae, 167 (53%) nymphs, 135 (43%) adult ticks, and for eight ticks, the developmental stage could not be determined, as they had been damaged too much during removal from the patient's skin. No other tick species than *I. ricinus* were identified. At enrolment, two tubes of blood were collected, 7 ml in a serum tube and 5 ml in an EDTA tube. Three months after enrolment, follow-up questionnaires and a consecutive 7 ml serum sample was collected from the tick bitten patients and from the EM patients after standard antibiotic treatment [254, 393]. Seven patients who consulted their physician for a tick bite and in whom EM developed within the three month follow-up duration of the prospective study, were categorized in EM patient-group of the current study. EDTA-blood samples were available for molecular testing from 335 tick bitten patients and 291 EM patients.

Tick analyses for detection of tick-borne pathogens

After arrival at the laboratory, ticks were stored at –20°C in ethanol. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions extraction for ticks. After total DNA extraction from ticks and amplification by PCR, reverse line blotting (RLB) was performed for *Borrelia*-, *Ehrlichia*-, *Anaplasma*-, *Rickettsia*- and *Babesia*-species. Further identification by DNA sequencing was performed as described [104, 177]. PCR products that specifically reacted to the generic ("catch all") probes, but that could not be further specified to the (geno)species level were designated as "untypeable". Furthermore, our RLB analysis could not distinguish between *B. garinii* and *B. bavariensis* [38]. The presence of *B. miyamotoi* in ticks was tested by a real-time PCR amplification in 302 ticks, and *Candidatus* Neoehrlichia mikurensis in 312 ticks. The presence of TBEV RNA could not be screened in the tick samples, since only DNA had been

extracted from these samples. Individual test results of the tick analyses were not reported to the participants or their physicians, in accordance with the informed consent form.

Molecular analyses for detection of tick-borne pathogens in EDTA-blood

Extraction of whole nucleic acid of the EDTA-blood samples were performed using robot-extraction (MagNA Pure Compact Extraction Robot; Roche, Basel, Switzerland) from 400 µL of EDTA-plasma (Nucleic Acid Isolation Kit I; Roche) according to the manufacturer's instructions in a diagnostic laboratory setting. All 626 samples were analyzed with different real-time PCRs based on various genes specific for the microorganism of interest namely; *B. burgdorferi* s. l., *B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, spotted fever *Rickettsia*'s carried out on a LightCycler 480 (Roche Diagnostics Nederland B.V, Almere, the Netherlands). For primers and probes, see supplementary data, Table 1. Reactions were done in a final volume of 20 µl with iQ multiplex Powermix, 3 µl of sample and 0.2 µM for all primers and different concentrations for probes. Positive plasmid controls and negative water controls were used on every plate tested. For detection of TBEV, multiplex a reverse transcription real-time PCR was performed as described before [394]. In brief, reactions were done in a final volume of 20 µl with TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher scientific, USA), 5 µl of sample and 0.2 µM for all primers and 0.2 µM probes (Table 1 of the supplementary data) were added to the master mix and internal control was added to all the samples. With 20 min reverse transcription step at 50°C, denaturation at 95°C for 30 s and 50 cycles of 95°C for 10 s and 60°C for 30 s. The amplification was performed on a Roche LightCycler 480 instrument. For *Babesia* genospecies, we performed a conventional PCR targeting the *18S rRNA* gene on all the blood samples [177], followed by sequencing. To minimize cross contamination and false-positive results, negative controls were included in each batch tested by PCR. In addition, DNA/RNA extraction, PCR mix preparation, sample addition, and PCR analyses were performed in separated air locked dedicated labs. On all samples that were found positive in the real-time PCR, conventional PCRs were performed for confirmation on one or more targets followed by Tris-Borate-EDTA-agarose gel-electrophoresis. PCR products were sequenced, and these were compared with reference sequences from Genbank using Unweighted Pair Group Method with Arithmetic Mean-based (UPGMA) hierarchical clustering. Individual test results of these molecular analyses on EDTA-blood were not reported to the participants or their physicians, in accordance with the informed consent form.

Statistical analyses

The prevalence of microorganism DNA detection in ticks and in EDTA-blood was calculated with 95% confidence intervals (95%CI) based on mid-P exact. Characteristics of persons with or without DNA detected in blood by PCR were compared in Chi-square or Fisher's exact test. We looked for associations between DNA detected in EDTA-blood by PCR and DNA detected in available ticks from the participants, tick engorgement, patient-reported tick

attachment duration, antibiotic treatment at enrolment, and patient-reported symptoms at enrolment and after three months. Using multivariate logistic regression, we explored for associations between DNA detected in blood by PCR and self-reported symptoms at enrolment and follow-up. All reported clinical symptoms (at enrolment and follow-up) were included as predictive variables in the multivariate logistic regression models, after which the models were optimized using backwards elimination, until all predictive variables that were maintained in the model were statistically significant contributors ($p<0.05$). Statistical analyses were performed with SAS 9.4 (SAS Inc.)

RESULTS

Tick-borne pathogens in ticks removed from humans

Table 1 shows the number of DNA sequences of the pathogens detected in 314 ticks obtained from 293 participants. *Borrelia burgdorferi* s. l. DNA was detected in 92 (29%) ticks, as published earlier [254]. The ticks contained DNA of *Candidatus* Neoehrlichia mikurensis (5.4%), *A. phagocytophilum* (1.0%), *Rickettsia* species (22%), *Babesia* species (3.5%). and *B. miyamotoi* (2.3%). DNA of microorganisms of two or more genera were detected in 34 ticks (11%). Among the 92 *B. burgdorferi* s. l. positive ticks, 30 (33%) also carried a pathogen of a different genus. About half of the ticks (149/314, 47%) tested negative for all genera.

Table 1: Detected DNA sequences in 314 ticks obtained from 293 participants. The results on *B. burgdorferi* s. l. have been published by Hofhuis et al. 2013 [254].

Detected DNA sequences	n/ *N*	*% (95%CI)*	Estimated human exposure with 1.1 million tick bites
Borrelia burgdorferi sensu lato [254]	92/ *314*	29.3% *(24.5%-34.5%)*	322293
B. afzelii [254]	36/ *314*	11.5% *(8.3%-15.4%)*	126115
B. garinii [254]	11/ *314*	3.5% *(1.9%-6.0%)*	38535
B. burgdorferi senso stricto [254]	7/ *314*	2.2% *(1.0%-4.4%)*	24522
B. valaisiana [254]	4/ *314*	1.3% *(0.4%-3.0%)*	14013
Untypeable* *Borrelia burgdorferi* [254]	36/ *314*	11.5%	
Borrelia miyamotoi	7/ *302*	2.3% *(1.0%-4.5%)*	*25497*
Babesia spp	11/ *314*	3.5% *(1.8%-6.0%)*	
B. microti	6/ *314*	1.9% *(0.8%-3.9%)*	21019
B. venatorum (B. EU1)	4/ *314*	1.3% *(0.4%-3.0%)*	14013
B. divergens	1/ *314*	0.3% *(0.0%-1.6%)*	3503
Ehrlichia spp / *Anaplasma* spp	8/ *314*	2.5% *(1.2%-4.8%)*	
A. phagocytophilum	3/ *314*	1.0% *(0.2%-2.6%)*	10510
Untypeable* *Ehrlichia* / *Anaplasma* spp	5/ *314*	1.6%	
Candidatus Neoehrlichia mikurensis	17/ *312*	5.4% *(3.3%-8.4%)*	59936
Spotted fever *rickettsia*'s	70/ *314*	22.3% *(18.0%-27.2%)*	
R. helvetica	59/ *314*	18.8% *(14.8%-23.4%)*	206688
R. monacensis	1/ *314*	0.3% *(0.0%-1.6%)*	3503
Untypeable* *Rickettsia* spp	10/ *314*	3.2%	
Co-infections with *B. burgdorferi* sensu lato**	30		105096
Babesia spp	3/ *314*	1.0% *(0.2%-2.6%)*	
Ehrlichia / *Anaplasma* spp *Candidatus* Neoehrlichia mikurensis	10/ *314*	3.2% *(1.6%-5.6%)*	
Spotted fever *rickettsia*'s	21/ *314*	6.7% *(4.3%-9.9%)*	
Borrelia miyamotoi	1/ *302*	0.3% *(0.0%-1.6%)*	

Using the observed prevalence of tick-borne pathogens in 314 ticks, national annual numbers of human exposure were estimated among 1.1 million tick bites in the Netherlands [9].

* PCR products that specifically reacted to the generic ("catch all") probes, but that could not be further specified to the (geno)species level were designated as Untypeable. Within *B. burgdorferi* s. l., RLB analysis could not distinguish between *B. garinii* and *B. bavariensis* [254].

** these categories of co-infections with *B. burgdorferi* s. l. are not mutually exclusive.

Tick-borne pathogens in human EDTA-blood

Table 2 shows the prevalence of DNA detection of tick-borne pathogens in EDTA-blood samples of 335 tick bitten patients and 291 EM patients, using various (real-time) PCRs. Only one (0.2%) of 626 blood samples tested positive for *B. burgdorferi* s. l. and one (0.2%) for *B. miyamotoi* in the real-time PCRs multiplex, both with high Ct values. In another multiplex, five blood samples (0.8%) were positive for *A. phagocytophilum* and seven (1.1%) for *Candidatus* Neoehrlichia mikurensis. Three (0.5%) blood samples for *Babesia* genospecies yielded a sequence in conventional PCR, in which genetic analyses showed to be *B. divergens*. None of the samples were found positive for spotted fever *Rickettsia*'s or TBEV.

All seven of the *Candidatus* Neoehrlichia mikurensis sequence yielded a partial *groEL* sequence and five out of seven could also be confirmed on a separate gene, namely *gltA*. The seven *groEL* are 100% identical to each other as were the five *gltA* sequences (Figure 1). Four out of five *A. phagocytophilum* positives yielded a partial *groEL* sequence after nested PCR. The four *groEL* are almost identical to each other, with just one or two mismatches. Nevertheless, all four sequences are part of zoonotic variant of the *A. phagocytophilum*, ecotype I [39]. Three of the tested blood samples for *Babesia* genospecies yielded a sequence in conventional PCR for the ribosomal *18S rRNA* gene, and showed to be identical to *B. divergens* sequences. Extensive efforts to generate a *B. miyamotoi* sequence failed. Accession numbers of the obtained sequences are: LC167302, LC167303, LC167304, LC167305.

Table 2: Prevalence of DNA detection of tick-borne pathogens in blood of persons with tick bites or *erythema migrans* (EM), as determined by PCRs.

	EM patients (n=291)		Tick bitten patients (n=335)		Total (n=626)		Estimated number of infection among 1.1 million tick bites
	n	*%*	n	*%*	n	*% (95%CI)*	n
Borrelia burgdorferi s. l.	1	*0.3%*	0	-	1	*0.2% (0.0% - 0.8%)*	1757
Borrelia miyamotoi	1	*0.3%*	0	-	1	*0.2% (0.0% - 0.8%)*	1757
Anaplasma phagocytophilum	2	*0.7%*	3	*0.9%*	5	*0.8% (0.3% - 1.8%)*	8786
Candidatus Neoehrlichia mikurensis	4	*1.4%*	3	*0.9%*	7	*1.1% (0.5% - 2.2%)*	12300
Babesia divergens	1	*0.3%*	2	*0.6%*	3	*0.5% (0.1% - 1.3%)*	5272
Spotted fever *Rickettsia* species	0	-	0	-	0	*0.0% (0.0% - 0.5%)*	-
Tick-borne encephalitis virus	0	-	0	-	0	*0.0% (0.0% - 0.5%)*	-
Total (excluding *B. burgdorferi* s. l.)	8	*2.7%*	8	*2.4%*	16	*2.6% (1.5% - 4.0%)*	28115

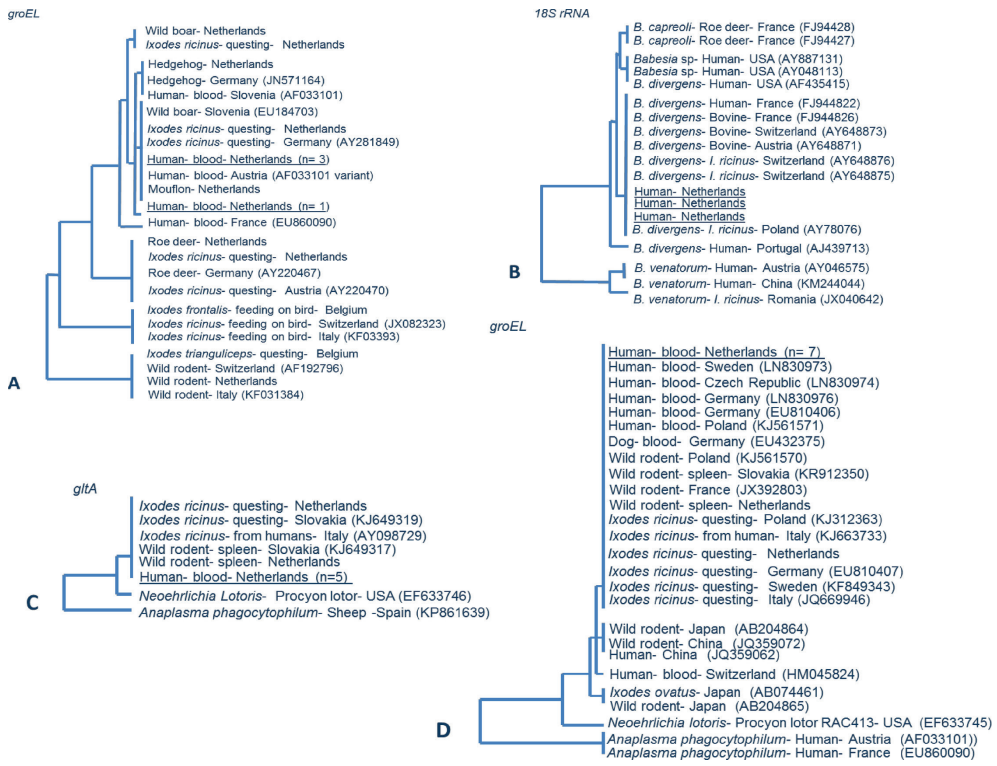

Figure 1: Phylogenetic tree of the sequences obtained from human blood samples.
PCR and sequencing was performed on the real-time PCR-positive blood samples. Sequences were clustered with (reference) sequences from Genbank. The evolutionary distance values were determined by Kimura method, and the tree was constructed according to the neighbor-joining method. A.) *Anaplasma phagocytophilum*: Phylogenetic tree of partial heat shock protein gene *groEL* of *Anaplasma phagocytophilum* of the four, one sequences is slightly different by couple of mismatches. All four are part of zoonotic variant of *Anaplasma phagocytophilum*. B.) *Babesia* genospecies: Three of the tested blood samples for *Babesia* genospecies yielded a sequence for the ribosomal *18S rRNA* gene, and showed to be identical to *B. divergens* sequences. C.) *Candidatus* Neoehrlichia mikurensis: Five out of seven *Candidatus* Neoehrlichia mikurensis yielded a partial sequence of the citrate synthase gene *gltA*. D.) *Candidatus* Neoehrlichia mikurensis: All seven *Candidatus* Neoehrlichia mikurensis yielded a partial sequence of the heat shock protein gene *groEL*.

EDTA blood samples testing were tested in various (real-time) PCRs for the presence of tick-borne pathogens. Using the observed prevalence of infection with tick-borne pathogens, national numbers of infections per year were estimated among 1.1 million tick bites in the Netherlands [9]. Note that the prevalence of DNA confirmed *Borrelia burgdorferi* s. l. detection in blood is a small fraction of the number of manifest borreliosis cases. For explanation, see results section. 95%CI = 95% confidence intervals.

Characteristics of 16 participants with DNA of tick-borne pathogens detected in blood

The prevalence of DNA of a tick-borne pathogen other than *B. burgdorferi* s. l. detected in blood from persons after a tick bite was 2.4% (Table 2), this number was similar to EM patients (2.7%). In the blood of one person DNA of both *A. phagocytophilum* and *B. divergens* were detected. Altogether, DNA of tick-borne pathogens was detected in the blood of sixteen persons. The characteristics of these sixteen participants are summarized in Table 3, with regard to age, gender, self-reported clinical symptoms, antibiotic treatment and tick exposure at enrolment and during the three month follow-up period. Eight of the sixteen cases had submitted ticks at enrolment. Among these eight ticks, six ticks tested negative in PCR, and in two ticks DNA was detected of a different genus than the tick-borne pathogens that had been detected in the EDTA-blood of the corresponding participants (Table 3). We did not observe associations between detection of tick-borne pathogen DNA in EDTA-blood and; patient-reported tick attachment duration, with tick engorgement, with antibiotic treatment at enrolment. Seven out of sixteen cases reported clinical symptoms at enrolment or during the three month follow-up period, such as myalgia (3 cases), skin rash (2 cases), tingling sensations in limbs (2 cases), fatigue, arthralgia, headache, pain in limbs, and gastrointestinal symptoms/vomiting. Using multivariate logistic regression, we compared the prevalence of self-reported symptoms, tick attachment duration and tick engorgement among cases with and without DNA of tick-borne pathogens detected in blood. Compared to the cases that tested negative by PCR, the cases with DNA of tick-borne pathogens detected in their blood sample were not more likely to report any of the named clinical symptoms at enrolment or at follow-up.

DISCUSSION

In this study, DNA of tick-borne microorganisms was detected and identified in ticks and human blood samples (Table 1 and 2). The limitations of this methodology are well known; hence, the interpretation of these results should be done with caution [2]. In order to unequivocally prove the presence of the corresponding infectious agents in ticks or blood, their viability should be tested by *in vitro* culture or infection experiments of laboratory animals. Also, the absence of DNA of a pathogen cannot be interpreted as the absence of the infectious agent. Besides the technical detection limits of PCR-based methods, the timing of sample collection after a tick bite and start of an antibiotic treatment, as well as the tissue tropism of the pathogen strongly affect the ability of pathogen detection [395, 396]. The latter is corroborated in this study: Only in one out of the 291 patients with an *erythema migrans* (EM) -a skin infection caused by *B. burgdorferi* s. l. - the DNA of this pathogen was detected in blood (Table 2). This finding confirms that the chance of detecting *B. burgdorferi* s. l. DNA in blood samples of confirmed Lyme borreliosis patients is very low [395]. *Rickettsia helvetica* and *R. monacensis* were both not detected in the 626 blood samples whereas, recently molecular evidence for their presence in cerebrospinal fluid of neuroborreliosis patients and in a skin sample of an EM patient was found [117, 118]. The absence of TBEV in

blood samples can further be explained by its extremely low infection rates in ticks and focal geographic distribution in the Netherlands [388]. *Candidatus* Neoehrlichia mikurensis, *A. phagocytophilum, B. miyamotoi* and *Babesia* species are all pathogens that can be expected in blood because of their biology and tissue tropism [36, 58, 83, 119].

The tick samples were screened by a different method (RLB) than the blood samples (real-time PCR). In 314 ticks removed from humans a wide variety of tick-borne pathogens were detected namely, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia burgdorferi* sensu stricto, *Borrelia valaisiana*, *Babesia microti, Babesia venatorum, Babesia divergens, Anaplasma phagocytophilum, Candidatus* Neoehrlichia mikurensis*, Rickettsia helvetica, Rickettsia monacensis* and *Borrelia miyamotoi*. All these pathogens have been found in questing ticks from field studies in the Netherlands before [84, 161, 397]. The infection rate of tick-borne pathogens other than *B. burgdorferi* s. l. varied from 0.3% (*B. divergens* and *R. monacensis*) up to 18.8*%* (*R. helvetica*)*.* With an estimated incidence of 1.1 million tick bites per year, human exposure to a tick-borne pathogen other than *B. burgdorferi* s. l. and TBEV varies from roughly 3500 for *B. divergens,* and 3500 for *R. monacensis* to 207,000 persons for *R. helvetica.* Among the 322,000 persons exposed to *B. burgdorferi* s. l. through a tick bite, roughly 105,000 are simultaneously exposed to another pathogen. In addition, exposure to more than one tick-borne pathogen can occur when people have more than one tick bite at once or several consecutive tick bites.

Clearly, not all exposure to tick-borne pathogens results in human infection. Based on the development of an EM or seroconversion, the risk of infection with *B. burgdorferi* s. l. after tick bites was estimated to be 5.1% [254]. In this study, evidence for infection comes from the detection of *Candidatus* Neoehrlichia mikurensis, *A. phagocytophilum, B. divergens, B. miyamotoi* and *B. burgdorferi* s. l. DNA in the blood of sixteen individuals after exposure to a tick bite. None of these cases reported to be immunocompromised, and all the EM patients were treated with antibiotics according to the guidelines for treatment of Lyme borreliosis [393]. Mild clinical symptoms were reported by seven out of sixteen PCR-positive cases. However, using multivariate logistic regression, we did not detect associations between DNA detected in blood and self-reported symptoms at enrolment and follow-up. Furthermore, we did not find associations between detection of DNA of tick-borne pathogens in blood and; PCR positive ticks, patient-reported tick attachment duration, tick engorgement, and antibiotic treatment at enrolment. The lack of statistically significant associations may be due to the mildness of symptoms amongst immune-competent patients, and to a lesser degree due to insufficient numbers of PCR-positive cases per pathogen genus in our analyses.

In this study, *Candidatus* Neoehrlichia mikurensis infection was observed in 1.1% (95%CI 0.5% - 2.2%). *Candidatus* Neoehrlichia mikurensis infections have been described in immunocompromised patients [398], and more recently in immune-competent patients

with relatively mild symptoms in China, Poland, and Sweden [74, 76, 77, 399]. *Anaplasma phagocytophilum* infection was found in 0.9% (95%CI 0.3% - 2.0%) of the persons exposed to tick bites in the Netherlands (Table 2). Genetic analyses of the DNA sequences showed the highest similarity to the zoonotic *A. phagocytophilum* ecotype I [39]. Evidence for *A. phagocytophilum* infection is primarily based on its molecular, microscopic or serological detection most disease cases [1, 36]. There is serological evidence that *A. phagocytophilum* infection occurs in the absence of disease symptoms [400]. *Babesia divergens* infection was observed in 0.5% (95%CI 0.1% - 1.3%) of the persons exposed to tick bites. In Europe, only two cases of human babesiosis have been reported in immune-competent patients, one due to *B. divergens* [126]. Only one patient with EM was possibly infected with *B. miyamotoi* 0.2% (95%CI 0.0% - 0.8%). The presence of *B. miyamotoi* DNA could only be determined by real-time PCR, and several attempts to confirm this finding by conventional PCR was unsuccessful. This patient had received antibiotic treatment at enrolment for his EM, so a low bacterial load in blood due to the treatment could be an explanation for the high Ct value. Evidence for infection with *B. miyamotoi* in Europe comes from one immunocompromised case [95], and a seroprevalence study in people exposed to tick bites [401].

Altogether, the probability of infection with a tick-borne pathogen other than Lyme spirochetes after tick bites in the Netherlands is roughly 2.4% (95%CI 1.1% – 4.5%). This number is similar to the probability of a co-infection with another tick-borne pathogen in patients with EM (2.7%, 95%CI 1.3% – 5.2%). Interestingly, one patient in this study had a co-infection with *A. phagocytophilum* and *B. divergens*. The severity of self-reported symptoms of the seven EM patients with a co-infection was indistinguishable from patients only having EM. No indications were found that infection with a tick-borne pathogen other than *B. burgdorferi* s. l. caused overt symptoms that would indicate a corresponding illness. The low number of persons with a tick bite or EM that were identified with an tick-borne pathogen infection other than *B. burgdorferi* s. l., in combination with the limited medical assessments, and the used method of pathogen detection are not sufficient to infer how often tick-borne pathogens other than *B. burgdorferi* s. l. (and TBEV) cause disease. Also, to what extend they affect the diagnoses and the etiology of Lyme borreliosis. Furthermore, the ability for a pathogen to cause disease depends also on extrinsic factors for example the immune status of its host.

The high exposure to tick-borne pathogens other than *B. burgdorferi* s. l. and TBEV, and their ability to cause infection in the general population, warrants increased awareness, knowledge, improvement of diagnostic tests and a clear-cut clinical case definitions in an European setting. Only when better laboratory tests are available for these tick-borne diseases, their impact as a co-infection with Lyme borreliosis can be assessed.

Table 3 : Characteristics of participants with DNA of tick-borne pathogens detected in blood

Patient	**Case 1** **Male, age 60**	**Case 2** **Male, age 42**	**Case 3** **Female, age 58**	**Case 4** **Female, age 56**	**Case 5** **Female, age 63**
EM and self-reported symptoms	t=0: EM	t=0: EM	t=0: skin rash	No	t=0: myalgia, tingling in limbs, fatigue. t=12: vomiting, gastrointestinal symptoms.
Antibiotic treatment	t=0: doxycycline 100 mg b.i.d. 10 days	t=0: doxycycline 100 mg b.i.d. 10 days	No	t=12: doxycycline 1 wk treatment of bronchitis	No
Microorganism DNA detected in EDTA-blood	*B. burgdorferi* s.l.	*B. miyamotoi*	*A. phagocytophilum*	*A. phagocytophilum*	*A. phagocytophilum* *Babesia divergens*
Tick (bite) characteristics: reported tick attachment duration, engorgement, detected DNA of microorganisms in tick	Reported attachment duration of tick bite before the EM at enrollment: 24 hours.	Reported attachment duration of tick bite before the EM at enrollment: 72 hours.	Submitted tick: unengorged adult female *Ixodes ricinus*. Tick PCR positive for *B. burgdorferi* sensu stricto, Untypeable* *Ehrlichia* / Anaplasma spp, Ca. Neoehrlichia mikurensis, and *R. helvetica*. Reported tick attachment duration: 17 hours.	Submitted tick: unengorged nymph *Ixodes ricinus*. Tick PCR negative. Reported tick attachment duration: 14 hours.	Submitted tick: unengorged nymph *Ixodes ricinus*. Tick PCR negative. Reported tick attachment duration: 2 hours.
Reported tick exposure history	1 tick bite in past 7 days. No other tick bites in past 5 years.	1 tick bite in past 7 days. 3 other tick bites in past 5 years, >6 weeks ago.	1 tick bite at t=0. 30 other tick bites in past 5 years, 6 in past 6 weeks, 2 in past 7 days.	2 tick bites at t=0. >15 other tick bites in past 5 years, 1 in past 6 weeks.	1 tick bite at t=0. No other tick bites in past 5 years.

Table 3 : Characteristics of participants with DNA of tick-borne pathogens detected in blood *(continued)*

Patient	**Case 6** **Female, age 38**	**Case 7** **Female, age 46**	**Case 8** **Female, age 15**	**Case 9** **Male, age 55**	**Case 10** **Female, age 63**	**Case 11** **Male, age 79**
EM and self-reported symptoms	t=0: EM t=12: myalgia	t=0: EM	No No information on t=12.	t=0: EM	t=12: arthralgia	No
Antibiotic treatment	t=0: amoxicillin 500 mg q.i.d. 14 days	t=0: doxycycline 100 mg b.i.d. 14 days	No	t=0: doxycycline 100 mg b.i.d. 14 days	No	No
Microorganism DNA detected in EDTA-blood	*A. phagocytophilum*	*A. phagocytophilum*	*Babesia divergens*	*Babesia divergens*	*Ca*. Neoehrlichia mikurensis	*Ca*. Neoehrlichia mikurensis
Tick (bite) characteristics: reported tick attachment duration, engorgement, detected DNA of microorganisms in tick	Submitted tick: partially engorged nymph *Ixodes ricinus*. Tick PCR negative. Reported tick attachment duration: 36 hours.	Reported attachment duration of tick bite before the EM at enrollment: 25 hours.	Submitted tick: partially engorged female adult *Ixodes ricinus*. Tick PCR negative. Reported tick attachment duration: 3 hours.	Reported attachment duration of tick bite before the EM at enrollment: 96 hours.	Submitted tick: unengorged nymph *Ixodes ricinus*. Tick PCR positive for *R. helvetica*. Reported tick attachment duration: 8 hours.	Submitted tick: partially engorged female adult *Ixodes ricinus*. Tick PCR negative. Reported tick attachment duration: 12 hours.
Reported tick exposure history	1 tick bite in past 10 days 1 other tick bite in past 5 years, >6 weeks ago.	2 tick bites in past 7 days. 2 other tick bites in past 5 years.	1 tick bite at t=0. No other tick bites in past 5 years.	2 tick bites in past 7 days. No other tick bites in past 5 years.	1 tick bite at t=0. 25 other tick bites in past 5 years, 4 in past 6 weeks.	1 tick bite at t=0. No other tick bites in past 5 years.

Table 3 : Characteristics of participants with DNA of tick-borne pathogens detected in blood *(continued)*

Patient	Case 12 Male, age 40	Case 13 Female, age 60	Case 14 Female, age 61	Case 15 Male, age 48	Case 16 Male, age 71
EM and self-reported symptoms	t=0: skin rash t=12: EM	t=0: EM	t=0: faded EM (not inspected by physician), headache, myalgia, pain in limbs. t=12: myalgia.	t=0: EM, tingling in limbs. No information on t=12.	No
Antibiotic treatment	t=12: doxycycline 100 mg b.i.d. 10 days	t=0: doxycycline 100 mg b.i.d. 10 days	t=0: doxycycline 100 mg b.i.d. 14 days	t=0: doxycycline 100 mg b.i.d. 10 days	No
Microorganism DNA detected in EDTA-blood	*Ca*. Neoehrlichia mikurensis	*Ca*. Neoehrlichia mikurensis	*Ca*. Neoehrlichia mikurensis	*Ca*. Neoehrlichia mikurensis	*Ca*. Neoehrlichia mikurensis
Tick (bite) characteristics: reported tick attachment duration, engorgement, detected DNA of microorganisms in tick	Reported attachment duration of tick bite before the EM at enrollment: 8 hours.	Unknown attachment duration of tick bite before the EM at enrollment.	Reported attachment duration of tick bite before the EM at enrollment: 16 hours.	Reported attachment duration of tick bite before the EM at enrollment: 10 hours.	Submitted tick: partially engorged female adult *Ixodes ricinus*. Tick PCR negative. Unknown tick attachment duration.
Reported tick exposure history	3 tick bites in past 3 weeks. Other tick bites (number unknown) in past 5 years, >6 weeks ago.	1 tick bite in past 7 days. 10 other tick bites in past 5 years, 3 in past 6 weeks.	1 tick bite in past weeks. No other tick bites in past 5 years.	5 in past 7 days. >20 other tick bites in past 5 years, >6 weeks ago.	1 tick bite at t=0. No other tick bites in past 5 years.

Legend for Table 3

EM: *erythema migrans*.

t=0: time of enrolment, at the time of blood sample collection for PCR-testing.

t=12: twelve weeks after enrolment.

b.i.d.: bis in die.

q.i.d.: quarter in die.

* PCR products from tick analyses that specifically reacted to the generic ("catch all") probes, but that could not be further specified to the (geno)species level were designated as 'Untypeable'.

Supplementary Table 1.

Microorganism	Target gene	Name	Sequence (sense)	Reference
Anaplasma phagocytophilum	msp2	ApMSP2F	5'-ATGGAAGGTAGTGTTGGTTATGGTATT-3'	[402]
		ApMSP2R	5'-TTGGTCTTGAAGCGCTCGTA-3'	
		ApMSP2P	5'-FAM530-TGGTGCCAGGGTTGAGCTTGAGATTG-BHQ1-3'	
Candidatus Neoehrlichia mikurensis	groEL	GroEL-F2a	5'-CCTTGAAAATATAGCAAGATCAGGTAG-3'	[60]
		GroEL-R2a	5'-CCACCACGTAACTTATTTAGCACTAAAG-3'	
		GroEL-P2a	5'-X-CCTCTACTAATTATTGCtGAAGAT GTAGAAGGTGAAGC-BHQ2-3' X= CALFluorRed590nm	
Borrelia burgdorferi s. l.	ospA	B-OspA_modF	5'-AAT ATT TAT TGG GAA TAG GTC TAA-3'	[403]
		B-OspA_borAS	5'-CTTTGTCTTTTTCTTTRCTTACA-3'	
		B-OspAmod-Patto	5'-Atto520-AAG CAA AATGTTAGC AGC CTT GA-BHQ1-3'	
	flaB	B-FlaB-F	5'-CAGAIAGAGGTTCTATACAIATTGAIATAGA-3'	
		B-FlaB-Rc	5'-GTGCATTTGGTTAIATTGCGC-3'	
		B-FlaB-Rt	5'-GTGCATTTGGTTAIATTGTGC-3'	
		B-FlaB-Patto	5'-Atto425-CAACTIACAGAIGAAAXTAAIAGAAT TGCTGAI CA-Pho-3' X = BHQ-1-dT	

Supplementary Table 1. *(continued)*

Microorganism	Target gene	Name	Sequence (sense)	Reference
Borrelia miyamotoi	flaB	FlabBm.motoiF2	5’-AGAAGGTGCTCAAGCAG-3’	[95]
		FlabB.m.motoiR3	5’-TCGATCTTTGAAAGTGACATA T-3’	
		FlabBm.motoiPro	5’-ATTO647N-AGCACAACAGGAGGGAGTTCAAGC-BHQ2-3’	
Rickettsia genospecies	gltA	RickgltA-F-Stenos	5’- TCGCAAATGTTCACGGTACTTT -3’	[339]
		RickgltA-R-Stenos	5’- TCGTGCATTTCTTTCCATTGTG -3’	
		Rickglt-probe-stenos	5’- Atto520-TGCAATAGCAAGAAC CGTAGG CTGGATG-BHQ1 -3’	
Rickettsia helvetica	gltA	Rick_HelvgltA_F2	5’- ATGATCCGTTTAGGTTAATAGGCTTCGGTC -3’	[106]
		Rick_HelvgltA_R2	5’- TTGTAAGAGCGGATTGTTTTCTAGCTGTC -3’	
		Rick_HelvgltA_pr3	5’-Atto425-CGATC+C+ACG+TG+CCGCAGT-BHQ1-3’ X = BHQ-1-dT	
Tick-borne encephalitis virus	3’ non-coding region	F-TBE	5’-GGGCGGTTCTTGTTCTCC-3’	[404]
		R-TBE	5’-ACACATCACCTCCTTGTCAGACT-3’	
		TBE-Probe-WT	5’-FAM-TGAGCCACCATCACCCAGACACA-BHQ1-3’	
	E gene	TBEE-F6	5’-GGCTTGTGAGGCAAAAAAGAA-3’	[405]
			5’-TCCCGTGTGTGGTTCGACTT-3’	
			5’-JOE-AAGCCACAGGACATGTGTACGACGCC-BHQ2-3’	

Supplementary Table 2.

Microorganism	Target gene	Name	Sequence (sense)
Anaplasma phagocytophilum	*groEL*	Ap-groEL-For	5’-ATGGTATGCAGTTTGATCGC-3’
		Ap-groEL-Rev	5’-TTGAGTACAGCAACACCACCGGAA-3’
		Ap-groEL_nested_For	5’-GTGGAATTTGAAAATCCATAC-3’
		Ap-groEL_nested_Rev	5’-GTCCTGCTAGCTATGCTTTC-3’
Candidatus Neoehrlichia mikurensis	*groEL*	Neoehrl-groEL-For	5’-GAAGCATAGTCTAGTATTTTTGTC-3’
		Neoehrl-groEL-Rev	5’-TTAACTTCTACTTCACTTG-3’
		Neoehrl-groEL_nested-1	5’-ACATCACGCTTCATAGAA-3’
		Neoehrl-groEL_nesetd-2	5’-AAAGGAATTAGTATTAGAATCTTT-3’
		Neoehrl-groEL_nested-3	5’-AATATAGCAAGATCAGGTAGAC-3’
		Neoehrl-groEL_nested-4	5’-CTTCCATTTTAACTGCTAA-3’
Candidatus Neoehrlichia mikurensis	*gltA*	Neoerhl-gltA-For	5’-AAGTGCATGCTTTGCTACATT-3’
		Neoerhl-gltA-Rev	5’-TCATGATCTGCATGAAAATAA AT-3’
Borrelia miyamotoi	*glpQ*	glpQ-BM-F1	5’-CTCATAATTTCATGCTTTAAA-CAAGAAATG-3’
		glpQ-BM-F2	5’-ATGGGTTCAAACAAAAAGTCACC-3’
		glpQ-BM-F3	5’-GCTCACAGGGGTGCTAGTGGG-3’
		glpQ-BM-R1	5’-CCAGGGTCCAATTCCATCAGAATATTGT-GCAAC-3’
		glpQ-BM-R2	5’-CATTACTGTGTCAGTAAAATCTGTA-AATATACCATCTAC-3’
Babesia species	*18S rRNA*	Bath-F	5’-TAAGAATTTCACCTCTGACAGTTA-3’
		Bath-R	5’-ACACAGGGAGGTAGTGACAAG-3’

CHAPTER 9

Borrelia miyamotoi in vectors and hosts in the Netherlands

A. Wagemakers and S. Jahfari, B. de Wever,
L. Spanjaard, M. Starink, H. de Vries, H. Sprong and J. W. Hovius

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ABSTRACT

Introduction

Ixodes ticks transmit *Borrelia burgdorferi* sensu lato (s. l.), the causative agent of Lyme borreliosis (LB). These tick species also transmit *Borrelia miyamotoi,* which was recently found to cause infections in humans. We were interested in the prevalence of *B. miyamotoi* infection in ticks and natural hosts in the Netherlands, and to what extent ticks are co-infected with *B. burgdorferi.* In addition, *erythema migrans* has been sporadically described in *Borrelia miyamotoi* infected patients, but these skin lesions might as well represent co-infections with *B. burgdorferi* s.l. We therefore investigated whether *B. miyamotoi* was present in LB-suspected skin lesions of patients referred to our tertiary Lyme disease clinic.

Methods

3360 questing *Ixodes ricinus* nymphs as well as spleen tissue of 74 rodents, 26 birds and 10 deer were tested by PCR for the presence of *B. miyamotoi*. Tick lysates were also tested for the presence of *B. burgdorferi* s. l.. Next, we performed a PCR for *B. miyamotoi* in 31 biopsies from LB-suspected skin lesions in patients visiting our tertiary Lyme center. These biopsies had been initially tested for *B. burgdorferi* s. l. by PCR, and the skin lesions had been investigated by specialized dermatologists.

Results

Out of 3360 unfed (or questing) nymphs, 313 (9.3%) were infected with *B. burgdorferi* s. l., 70 (2.1%) were infected with *B. miyamotoi*, and 14 (0.4%) were co-infected with *B. burgdorferi* s. l. and *B. miyamotoi*. Co-infection of *B. burgdorferi* s. l. with *B. miyamotoi* occurred more often than expected from single infection prevalences (p=0.03). Both rodents (9%) and birds (8%) were found positive for *B. miyamotoi* by PCR, whereas the roe deer samples were negative. Out of 31 LB-suspected skin biopsies, 10 (32%) were positive for *B. burgdorferi* s. l. while none were positive for *B. miyamotoi*.

Conclusion

The significant association of *B. burgdorferi* s. l. with *B. miyamotoi* in nymphs implies the existence of mutual reservoir hosts. Indeed, the presence of *B. miyamotoi* DNA indicates systemic infections in birds as well as rodents. However, their relative contributions to the enzootic cycle of *B. miyamotoi* requires further investigation. We could not retrospectively diagnose *Borrelia miyamotoi* infection using biopsies of LB-suspected skin lesions, supporting the hypothesis that *B. miyamotoi* is not associated with LB-associated skin manifestations. However, this warrants further studies in larger sets of skin biopsies. A prospective study focused on acute febrile illness after a tick bite could provide insight into the incidence and clinical manifestations of *Borrelia miyamotoi* infection in the Netherlands.

INTRODUCTION

Borrelia miyamotoi is a tick-borne relapsing fever (TBRF) spirochete in *Ixodes* ticks, the same vector that transmits *Borrelia burgdorferi* s. l. and a range of other tick-borne pathogens. The prevalence of *B. miyamotoi* in reservoir hosts, as well as the reservoir host range in nature, is yet scarcely described, but points towards small rodents and certain bird species being infected [406-411]. Interestingly, *B. miyamotoi* can cause short-term systemic infection in rodents, and it is experimentally shown that ticks can acquire this pathogen from rodents [61]. While *B. burgdorferi* s. l. causes Lyme borreliosis (LB), *B. miyamotoi* was recently found to cause infections in humans [412]. The incidence of *Borrelia miyamotoi* infection in humans is yet unknown. Two studies have described the prevalence of *Borrelia miyamotoi* in febrile patients suspected of a tick-borne infection, which found 97/11,515 (0.84%) of patients in north-eastern United States and 51/302 (16.9%) of hospital-admitted Russian patients to be PCR positive for *B. miyamotoi* on blood [412, 413]. *Borrelia miyamotoi* infection presents around two weeks after a tick bite with a high fever and viral-like symptoms such as headache, myalgia, arthralgia and malaise [412-414]. In two patients with severe immunodeficiency, including one case in our tertiary Lyme clinic (AMLC, Amsterdam, The Netherlands), *B. miyamotoi* infection was found to cause a chronic meningoencephalitis [415, 416]. Several studies have described *Borrelia miyamotoi* infected patients presenting with *erythema migrans* (EM), a classical symptom of early LB. In one study, 9% of Russian patients with *Borrelia miyamotoi* infection presented with EM, and it was hypothesized that EM was a sign of co-infection with *B. burgdorferi* s. l. rather than a true manifestation of *Borrelia miyamotoi* infection [412]. A study in North America revealed several *Borrelia miyamotoi* infected patients to be co-infected with *B. burgdorferi* s. l., one of which presenting with an EM [413]. Furthermore, a study in Japan described two febrile *Borrelia miyamotoi* infected patients that presented with EM and were shown to have antibodies against *B. burgdorferi* s. l. antigens, suggesting co-infections with both *B. burgdorferi* s. l. and *B. miyamotoi* [417]. In the Netherlands, exposure levels to *B. miyamotoi* as measured by serology showed that risk groups such as forestry workers (10%) had higher seroprevalence levels than the control group. In the same study, *B. miyamotoi* antibodies were also found in patients suspected of human granulocytic anaplasmosis (HGA) (14.6%), suggesting that *B. miyamotoi* infections occur in Dutch high-risk populations and that they could be misdiagnosed [418]. However, thus far only one PCR-confirmed patient has been described in the Netherlands, suggesting under-diagnosis due to a lack of awareness, lack of severe symptoms, lack of widely available diagnostic tools and/or misdiagnosis. A Russian study recently revealed a relatively high transmission efficiency of *B. miyamotoi* by adult *I. persulcatus* ticks to humans (~8%), and *B. miyamotoi* was recently demonstrated to be transmitted by *I. ricinus* larvae, bites of which would likely go unnoticed in humans [419, 420]. Indeed, a Russian study described *B. miyamotoi* to be under-diagnosed, leading to relapses in the absence of adequate antibiotic treatment [421]. Because no routine diagnostics are currently performed for *B. miyamotoi* in our (tertiary) multidisciplinary Lyme disease center, we aimed to investigate

if (co-) infections with *B. miyamotoi* were missed over the past years. Thus, in the current study, we performed *B. miyamotoi*-specific real-time PCR on ticks and in spleen samples from potential reservoir hosts in nature and in LB-suspected human skin biopsies, which were previously tested for *B. burgdorferi* s. l. by PCR.

METHODS

Collection of ticks and vertebrate hosts

Questing ticks were collected by blanket dragging at 11 different forested areas throughout the Netherlands in 2014-2015. Spleen tissues were collected from different species of rodents, roe deer and birds (table 2) at several sites in the Netherlands. The obtained spleen samples of roe deer, birds as well as the capturing of wild rodents have been described elsewhere [422, 423]. Spleen samples were kept frozen (-80 °C) until testing. DNA from tissue samples was extracted using the Qiagen DNeasy Blood & Tissue Kit according to manufacturer's protocol. Based on morphological criteria, tick species and stages were identified, and DNA from *I. ricinus* nymphs was extracted by alkaline lysis [162].

Polymerase chain reactions and sequencing of DNA from ticks and vertebrate hosts

At the beginning of the extraction process, an internal amplification and extraction control (IAC) consisting of a random DNA sequence in the Invitrogen pCR(tm)2.1-TOPO® vector was added to the samples. All samples were screened for the presence of *B. miyamotoi* DNA with a real-time polymerase chain reaction (RT-PCR) targeting portion of the *flagellin* gene. We used 200 nM forward primer (5'-AGA AGG TGC TCA AGC AG-3') and 200 nM reverse (5'-TCG ATC TTT GAA AGT GAC ATA T-3') primer, 200 nM probe (5'-Atto647N-AGC ACA ACA GGA GGG AGT TCA AGC-BHQ2-3'), and 3 to 8 µl of template DNA [95]. Samples with doubtful qPCR-results were re-tested with 3 µl sample volume, to reduce the contribution of inhibitory factors, and with 8 µl sample volume, to (slightly) increase the detection limit of the test.
RT-PCR -positive samples were analyzed further with primers targeting a fragment of a 700bp fragment of the glycerophosphodiester phosphodiesterase (*glpQ*) gene. The PCR was performed with the HotStarTaq master mix (Qiagen, Venlo, the Netherlands) using forward 5'-ATG GGT TCA AAC AAA AAG TCA CC-3' and reverse primers 5'-CCA GGG TCC AAT TCC ATC AGA ATA TTG TGC AAC-3' under the following conditions: 15 min 94°C, then 40 cycles of 30 s 94°C, 30 s 53°C, 90 s 72°C and finishing with 10 minutes at 72°C.The sequences were stored and analyzed in Bionumerics (Version 7.1, Applied Math, Belgium), after subtraction of the primer sequences. Of all the *B. miyamotoi* positive tick samples (n=70), 30 were sequenced for the glpQ and/or p66 gene. A total of 22 yielded a sequence 100% identical to Genbank accession numbers: AB824730.1, AB824731.1, AB824855.1, AB824856.1, confirming amplification of the gene of interest.

LB-suspected skin lesions

In our tertiary Lyme center, we offer more extensive diagnostic service than primary care centers will perform based on the current Dutch national guideline for LB (CBO richtlijn Lyme,www.nvvg.nl/images/stories/Richtlijn_lymeziekte_definitief_18_juli_2013.pdf). Occasionally, biopsies of skin lesions are taken to confirm or rule out LB, which enabled us to retrospectively investigate the presence of *B. miyamotoi* in these lesions. Biopsies from skin lesions in patients suspected to have LB were in retrospect analyzed for the presence of *B. miyamotoi*. Our dermatology department gathered 4 mm skin biopsies under local lidocaine anesthesia from 34 patients between 2009 and 2013, which were taken from the edge of suspected (atypical) EM lesions or central in suspected acrodermatitis chronica atrophicans (ACA) lesions. Biopsies were used for PCR and sent for pathologic examination when clinically indicated according to the treating dermatologist. A *B. burgdorferi* s. l.-specific PCR was initially performed, and 31/34 extracted DNA samples were included, while three DNA samples and corresponding patient cases were excluded from analysis due to RT-PCR inhibition. Patient records and PCR results were retrospectively reviewed to obtain a final diagnosis and to categorize the skin lesions into: 1. EM; 2. Multiple EM; 3. ACA; 4. LB-suspected skin lesion after previous treatment for LB, but active LB excluded 5. LB-suspected skin lesion without previous treatment for LB, but active LB excluded. C6 enzyme-linked immunofluorescence assays and RT-PCR for *B. burgdorferi* s. l. and *B. miyamotoi* were performed as previously described [416, 424].

Statistical analysis

Confidence intervals (95%) were calculated using a Fisher's exact test. The Chi-square test was used to assess the correlation between *B. miyamotoi* infections and *B. burgdorferi* s. l. infection in ticks based on a 2x2 contingency table with *B. miyamotoi* infection and *B. burgdorferi* s. l. infection as determining binary conditions.

RESULTS

Ticks

Questing *I. ricinus* nymphs (n=3360) were collected from 11 different areas in the Netherlands between 2014 and 2015. Of these nymphs 313 (9.3%) were positive for *B. burgdorferi* s. l. only and 70 (2.1%) were positive for *B. miyamotoi* only, while 14 nymphs (0.4%) were co-infected with both pathogens (Table 1). Thus, 14/327 (4.3%) *B. burgdorferi* s. l. positive nymphs were also positive for *B. miyamotoi,* and co-infection of *B. burgdorferi* s. l. with *B. miyamotoi* occurred significantly (p=0.03) more often than expected, suggesting the existence of mutual reservoir hosts.

Vertebrate hosts

Seven out of 74 (9%) examined rodent spleens were RT-PCR positive for *B. miyamotoi*. Three out of 21 (14%) wood mice (*Apodemus sylvaticus*), one out of 8 (13%) common voles (*Myodes arvalis*) and 3 out of 34 (9%) bank voles (*M. glareolus*) (Table 2) were found to be infected with *B. miyamotoi*. All 10 roe deer spleen samples were negative for *B. miyamotoi* DNA (Table 2). Two out of 26 studied birds (8%) were found RT-PCR positive for *B. miyamotoi*, namely a great tit (*Parus major*) and a European greenfinch (*Carduelis chloris*) (Table 2).

LB-suspected skin biopsies

Nine out of 31 (29%) patients with LB-suspected skin lesions who were referred to our tertiary LB center were PCR positive for *B. burgdorferi* s. l. in a skin biopsy: 3 patients were diagnosed as having ACA, 1 as multiple EM and 5 were diagnosed with definite EM (table 3). One patient was PCR-negative (possibly because the biopsy was taken after initiation of doxycycline treatment), but clinically diagnosed with EM by the dermatologist. None of these biopsies were taken from febrile patients, and none were concurrently positive for *B. miyamotoi.* In seven patients that had previously been diagnosed with LB, who were now presenting with persisting skin conditions, active LB was excluded by PCR and pathology. In three of these patients, another dermatological diagnosis could be made (progressive purpura pigmentosa, nummular eczema and morphea cutis). In none of these patients, *B. miyamotoi* could be identified as the cause of the symptoms. Finally, the remaining 14 patients with LB-suspected skin lesions tested negative for *B. burgdorferi* s. l., but combined with pathology results, other dermatological diagnoses were made in 11 patients (angioma serpiginosum, contact dermatitis, toxicodermia, granuloma annulare, pityriasis lichenoides chronica, dermatomycosis corporis, eczematous dermatitis, chilblain lupus, erythema annulare centrifugum, and hypersensitivity to a tick bite and a spider bite). These, including, the five patients without a definite diagnosis all retrospectively tested negative for *B. miyamotoi* (Table 3).

Table 1: *Borrelia burgdorferi* s. l. and *B. miyamotoi* infection in questing *Ixodes ricinus* nymphs

***Ixodes ricinus* (n=3360)**	**Positive (n)**	**Percentage (CI)**
B. burgdorferi s. l.	327	10% (9-11% CI*)
B. miyamotoi	84	2.5% (2-3% CI*)
B. burgdorferi s. l. and *B. miyamotoi* co-infection	14	0.4% (0.2-0.7% CI*)

*CI: 95% confidence interval

Table 2: *Borrelia miyamotoi* in spleen tissue from different rodents, roe deer and bird species

Species	**Total (n)**	**Positive (n)**	**Positive (%)**
Apodemus flavicollis	2	0	0
Apodemus sylvaticus	21	3	14
Cocidura russula	4	0	0
Myodes arvalis	8	1	
Myodes glareolus	34	3	
Sorex araneus	5	0	0
Capreolus capreolus	10	0	0
Carduelis chloris	4	1	25
Coccothraustes coccothraustes	2	0	0
Fringilla coelebs	3	0	0
Parus major	2	1	50
Phylloscopus trochilus	1	0	0
Pyrrhula pyrrhula	1	0	0
Turdus iliacus	5	0	0
Turdus merula	2	0	0
Turdus philomelos	6	0	0

Table 3: *Borrelia miyamotoi* detected retrospectically in skin lesion biopsies from suspected Lyme borreliosis patients

Patient category	**Total patient number (N)**	**Presenting May to September**	**Fever**	**PCR *B. burgdorferi* s.l.**	**PCR *B. miyamotoi***	**C6 ELISA n/N***
EM	6	5	0	5	0	0/5**
Multiple EM	1	0	0	1	0	1/1
ACA	3	2	0	3	0	3/3
LB- suspected skin lesion after previous treatment for LB; Active LB excluded	7	1	0	0	0	4/7
LB- suspected skin lesion without previous treatment for LB; Active LB excluded	14	8	1	0	0	1/12
Total	31	16	1	9	0	9/28

* n/N: positive/total tested. Not all samples were analyzed with ELISA
** 1/5 dubious. 2/5 were re-tested at a later stage, both of which seroconverted

DISCUSSION

In this study we have evaluated the prevalence of *B. miyamotoi* in *I. ricinus* ticks, wild animals and LB-suspected skin lesions in the Netherlands. Previous studies in the Netherlands revealed that 2-4% [84, 95] of questing *I. ricinus* ticks were infected with *B. miyamotoi*, and around 4% [85] of ticks that were collected from humans, which corresponds well with the incidence in other endemic areas [406]. We found that *B. miyamotoi* infection in *I. ricinus* nymphs was present more often when ticks were infected with *B. burgdorferi* s. l. than in *B. burgdorferi* s. l. uninfected ticks (4.3% versus 2.3%, p=0.03), suggesting similar reservoir hosts. Indeed, we identified *B. miyamotoi* in rodents (9%) and birds (8%). The role of these animals in the transmission cycle is not clear; they could be amplifying hosts, a transitory or dead-end host for this spirochete, warranting further investigation.

Previously, several studies have shown wild rodents and small mammals to be infected with *B. miyamotoi* in up to 3.7% of animals [409-411]. Because of vertical transmission, larvae can be infected with *B. miyamotoi* and are thought to play an important role in transmission [407, 425]. Indeed, we have recently described field-collected *I. ricinus* larvae to be able to transmit *B. miyamotoi* to laboratory-bred Naval Medical Research Institute (NMRI) mice [420]. In addition to vertical transmission, the relative contribution of mammalian reservoir hosts to the transmission cycle is not fully understood. Mice seem to clear *B. miyamotoi* from their blood by VMP-specific antibodies, and only SCID mice have so far been demonstrated to experience persistent spirochetemia [426, 427].

Interestingly, we identified two *B. miyamotoi* infected birds, namely a great tit and a European greenfinch. Previous studies revealed a high prevalence (58%) of *B. miyamotoi* in wild Tennessee turkeys (*Meleagris gallopavo*), while experimental infection with field-collected *I. ricinus* nymphs did not succeed in common European songbirds (*Parus major*) [408, 428]. Further studies should reveal which bird species are susceptible to *B. miyamotoi* infection, the relationship between species-specific complement sensitivity and the ecological implications of bird infections.

After establishing the presence of *B. miyamotoi* in ticks and wild animals from the Netherlands, we investigated if *B. miyamotoi* infections were missed in patients previously examined in our tertiary LB clinic. *Borrelia miyamotoi* was not found as a co-infection or primary explanation for 31 LB-suspected skin lesions examined in our clinic. These patients were mostly afebrile, while *Borrelia miyamotoi* infection is currently characterized by fever and generalized symptoms several weeks after a tick bite [412, 413, 417, 429, 430] or by chronic meningoencephalitis in immunocompromised patients [415, 416]. Unfortunately, due to the retrospective nature of our study, no blood samples were available to establish serologic evidence or absence of infection with *B. miyamotoi*, and *Borrelia miyamotoi* infection in these patients can therefore not be definitely excluded. Nonetheless, this is the

first study investigating the presence of *B. miyamotoi* DNA in LB- suspected skin biopsies, and our findings support the hypothesis that *B. miyamotoi* is not associated with LB-related skin manifestations. However, more studies, with larger patient populations and with multiple body fluids and tissues, should be performed to corroborate our findings.

Considering the 2.5% *B. miyamotoi* prevalence in ticks in the present study, combined with over a million tick bites per year in the Netherlands [431], an estimated 8% transmission efficiency to humans, elevated seroprevalences in Dutch high-risk populations and the incidence of *Borrelia miyamotoi* infection described in prospective studies in Russia and the U.S.A., we postulate that the diagnosis *Borrelia miyamotoi* infection is currently being missed in Dutch patients [412, 413, 418, 419]. Therefore, we suggest that a prospective clinical study in Dutch patients presenting with fever after a tick bite is needed in order to assess the incidence of *Borrelia miyamotoi* infection and the occurrence of *B. miyamotoi* in blood and other tissues or body fluids of these patients.

CHAPTER 10

High seroprevalence of *Borrelia miyamotoi* antibodies in forestry workers and individuals suspected of Human Granulocytic Anaplasmosis in the Netherlands

S. Jahfari, T. Herremans, A. E. Platonov,
H. Kuiper, L. S. Karan, O. Vasilieva,
M. P. Koopmans, J. W. Hovius and H. Sprong

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ABSTRACT

Substantial exposure to *Borrelia miyamotoi* occurs through bites from *Ixodes ricinus* ticks in the Netherlands, which also transmit *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum.* Direct evidence for *Borrelia miyamotoi* infection in European populations is scarce. A flu-like illness with high fever, resembling Human Granulocytic Anaplasmosis, has been attributed to *Borrelia miyamotoi*-infections in relatively small groups. *Borrelia miyamotoi*-infections associated with chronic meningoencephalitis have also been described in case reports. Assuming that an IgG antibody response against *Borrelia miyamotoi* antigens reflects (endured) infection, the seroprevalence in different risk groups was examined. Sera from nine out of ten confirmed *Borrelia miyamotoi* infections from Russia were found positive with the recombinant antigen used, and no significant cross-reactivity was observed in secondary syphilis patients. The seroprevalence in blood donors was set at 2.0% (95% CI 0.4–5.7%). Elevated seroprevalences in *erythema migrans* patients 5.6% (3.0-9.2%) and in individuals with serologically confirmed 7.4% (2.0-17.9%) or unconfirmed 8.6% (1.8-23%) Lyme neuroborreliosis were not significantly different from blood donors. The prevalence of anti-*Borrelia miyamotoi* antibodies among forestry workers 10% (5.3-16.8%) and in patients with serologically unconfirmed but suspected Human Granulocytic Anaplasmosis 14.6% (9.0-21.8%) were significantly higher compared the seroprevalence in blood donors. Our findings indicate that infections with *Borrelia miyamotoi* occur in tick-exposed individuals in the Netherlands. In addition, *Borrelia miyamotoi* infections should be considered in patients reporting tick bites and febrile illness with unresolved etiology in the Netherlands, and other countries where *Ixodes ricinus* ticks are endemic.

INTRODUCTION

Borrelia miyamotoi belongs to the relapsing fever group of the *Borrelia* genus [432]. Agents of relapsing fever spirochetes are transmitted between vertebrates by different vectors for instance; *Borrelia duttonii* and *Borrelia hermsii* are transmitted by soft ticks and *Borrelia recurrentis* is transmitted by the human body louse [407, 433, 434]. Interestingly, *B. miyamotoi* is transmitted by the same vectors as *Borrelia burgdorferi* senso lato. and *Anaplasma phagocytophilum*, the causative agents of Lyme borreliosis (LB) and Human Granulocytic Anaplasmosis (HGA), respectively [407, 425]. In Europe, Asia and North America, *B. miyamotoi* infection rates in *Ixodes persulcatus, I. scapularis, I. pacificus* and *I. ricinus* range between 0.5% and 5% [425, 432, 435-439]. In the Netherlands, the infection rate of *I. ricinus* is 2.4-4.7% in all three life-stages [440]. The presence of *B. miyamotoi* in wild rodents indicates enzootic circulation in the Netherlands (unpublished observations). Furthermore, a recent study estimated that in the Netherlands annually, approximately 200.000 people are bitten by ticks infected with *B. burgdorferi* s. l. and 36.000 by *B. miyamotoi* infected ticks [440]. Additionally, exposure to both Lyme and relapsing fever *Borrelia* by co-infected ticks occurs in at least 9,000 people annually. This substantial exposure raises the question to what extent *B. miyamotoi* leads to human disease in the general population.

Currently, the clinical symptoms of *B. miyamotoi*-infections are not well-known, and well-defined and validated supportive laboratory diagnostic tests are lacking. Infections with other members of the relapsing fever *Borreliae* are characterized by influenza-like illness and one or more relapse episode(s) of bacteraemia and fever. *Borrelia miyamotoi* infections in humans were first reported in Russian patients suspected of LB (Table 1). Fifty-one LB-suspects had amplifiable *B. miyamotoi* DNA in venous blood samples, and most tested positive by commercial IgM and IgG serology assays used for LB diagnosis. This test consisted of a mixture of whole cell antigens from *B. afzelii, B. burgdorferi,* and *B. garinii* [412]. A potentially severe complication of *B. miyamotoi* infection is meningoencephalitis. The first American and European meningoencephalitis case reports of well-documented *B. miyamotoi* infection were described in severely immune-compromised patients [415, 416] and *B. miyamotoi* was detected in cerebrospinal fluid with the use of microscopy and polymerase-chain-reaction (PCR) assays. In another study, two cases of *B. miyamotoi* infection were initially mistaken for HGA [430] on the basis on their clinical manifestations after a tick-bite (Table 1). Taken together, disease caused by an infection with *B. miyamotoi* may be confused with other tick-borne pathogens, either because of its comparable symptoms or because of misinterpretation of a serological reaction against a (endured) co-infection.

Patient categories that have an (endured) infection with this relapsing fever spirochete will have a higher seroprevalence than the general population. For other relapsing fever *Borreliae* the majority of antibodies are directed towards the Variable Major Proteins (VMP) [441]. However, antibodies to VMP have been described to be cross-reactive to *B. burgdorferi* s. l.

antigens. Furthermore, VMP is a highly variable protein that could give false-negative results in serological tests [441]. On the other hand, Glycerophosphodiester phosphodiesterase (GlpQ) appears to be highly conserved among all members of the relapsing fever *Borreliae*, including *B. miyamotoi,* but distinct for the spirochetes causing LB and their near relatives. In addition, GlpQ is immunogenic in humans and shows negative results when testing sera from LB and syphilis patients [441-445]. More recently, an American study showed that serum samples from 1 to 3% of residents of New England were reactive in an experimental serologic assay targeting the *B. miyamotoi* GlpQ antigen [414].

The long-term objective of our studies is to gain more insight in the public health risk of *B. miyamotoi.* As a first attempt to describe the exposure of *B. miyamotoi* in the Netherlands, using a newly developed serological assay based on the GlpQ antigen, we determined here the seroprevalence of anti-*B. miyamotoi* antibodies in different risk groups within the general population. Apart from important epidemiologic insights, our findings will facilitate the future identification of the clinical symptoms of *B. miyamotoi* infections and might serve as a starting point for further development of serological assays.

MATERIAL AND METHODS

Antigen preparations and biochemicals

A DNA sequence encoding for the *Borrelia miyamotoi* GlpQ protein was amplified from an *Ixodes ricinus* lysate, cloned, sequence-verified, expressed and purified from an *E. coli* construct (Scottish Biomedical, Glasgow, United Kingdom). Purified GlpQ was coupled to activated carboxylated microspheres by using a two-step carbodiimid reaction with an antigen to bead ratio of 50 µg/6.25×10^6 [446]. The beads were incubated in the dark under constant rotation at 25 rpm for 2h at room temperature. The beads were washed three times with PBS and stored in 500 µl PBS containing 0.05% (w/v) sodium azide and 1% (w/v) BSA at 4 °C in the dark until used [447-449]. Biochemicals and reagents used were from Sigma (St. Louis, MO), Pierce (Rockford, IL), Bio-Rad Laboratories (Hercules, CA) and Merck (Darmstadt, Germany) and were used in the highest purity available.

Serum samples

Sera from 150 blood donors were used as negative controls to determine the background in the general Dutch population. Ten sera of PCR-confirmed *B. miyamotoi* patients were used as positive controls and were described previously [412]. These sera were also positive in a anti-glpQ serologic assay developed and performed in Russia (not shown). Sera from patients infected with *Treponema pallidum* were included to test for possible cross-reactivity [450]. In total 120 serum samples from forestry workers were used as a group with high exposure to tick bites [451, 452]. Other serum specimens from patients were obtained from the residuals of sera submitted to the RIVM for routine microbiological investigations,

Table 1: Case reports of *Borrelia miyamotoi* infections associated with disease.

Reference	Methods	Patient description	*Erythema migrans*	Clinical manifestations
Russia [412]	PCR, IgM-positive	(n=51)	4/51	Fever, headache, chills, fatigue, vomiting and myalgia.
USA [414]	Seroconversion	Previously healthy individuals (n=3)	2/3	Fever, chills, sweats, headache, neck stiffness, fatigue, myalgias, arthralgias, abdominal pain, a cough, a sore throat, and right inguinal lymphadenopathy
USA [415]	PCR, microscopy	80-yr women, (treated) non-Hodgkin's lymphoma	0/1	Meningoencephalitis, progressive decline in mental status, wobbling gait, and difficulty hearing, weight loss.
USA [430]	PCR	61-yr male, anorexia	Not mentioned	Severe frontal headaches, photophobia, myalgia, and arthralgia, pain across the chest, muscles were tightening, sweats and episodes of fever with shaking chills
USA [430]	PCR	87-yr male, previously healthy	0/1	Severe fatigue, malaise, short of breath with activities, chills, fever and loss of appetite.
Netherlands [416]	PCR, microscopy	70-yr male, (treated) B cell non-Hodgkin lymphoma stage IV	Not mentioned	Meningoencephalitis, complaints of confusion, altered personality and a disturbed gait, unexplained chronic diarrhea and bradyphrenic.

provided patients did not object to this use by indicating this on the diagnostic request form. Sera with no or doubtful epidemiological data and repetitive sera were excluded. Selected sera were divided in different patient groups: 251 individuals with *erythema migrans* (EM), 54 individuals with serologically confirmed Lyme neuroborreliosis (LNB) based on the detection of local antibody production in the paired liquor sample and 35 individuals with serologically unconfirmed LNB. In addition, 130 samples from patients sent in to our laboratory for HGA serology, but who tested negative for IgG and IgM-specific HGA, were also examined. Serological confirmation for Lyme borreliosis was based on positive test results of *Borrelia burgdorferi* s. l. Immunoblot (in house), and C6-ELISA (Immunetics, Boston, MA). Immunofluorescence assays against *Anaplasma phagocytophylum* and *Ehrlichia chaffeensis* (Focus Technologies, Cypress, CA) were used. Serological unconfirmed was defined as negative test results in these serological assays.

Serological analysis

Antibodies to the GlpQ protein of *B. miyamotoi* were determined by an in house Luminex-assay. Serum-dilutions and conjugate concentrations in the Luminex-assay were optimized using checkerboard titrations. Sera, positive and negative control samples were tested in duplicate or triplicate and were diluted 1:200 in 25ul PBS containing 0.1% (v/v) Tween-20 and 3% (w/v) BSA. Serum samples were mixed with an equal volume of GlpQ-conjugated microspheres (4000 beads/region/well) in a 96-well Multiscreen HTS filter plate (Millipore Corporation, Billerica, MA) and incubated on a plate shaker at 600 rpm in the dark for 45 min at room temperature. Blank and control sera were included on every plate. The beads were collected by filtration using a vacuum manifold and washed three times with 100 µl PBS. To each well 50 µl of a 1:200 dilution of R- phycoerthyryn-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA) in PBS was added and the plate was incubated for 30 min with continued shaking. After a second wash, the beads were mixed in 100 µl PBS and shaken before analysis with a Bio-Plex 200 in combination with Bio-Plex Manager software version 4.1.1 (Bio-Rad Laboratories).

Statistical analysis

The Kolmogorov-Smirnov test was used to test null hypotheses that the LogOD values of different groups were normally distributed. Fisher's exact test was used to calculate the 95% confidence intervals. A one-tailed Fisher's exact test was used to compare pairwise the frequency of *B. miyamotoi* seropositive and seronegative subjects in different groups. The level of significance was set at $p < 0.05$. Moreover, Taylor series was used to calculate Odds Ratio with a 95% confidence limits testing.

RESULTS

All serum samples (n=764) were tested for their reactivity against the GlpQ protein of the European *B. miyamotoi*. The LogOD values of the samples from 150 blood donors were

normally distributed (p=0.56), whereas the LogOD values of 590 samples from the risk groups were not (p<0.004), indicating that the latter distribution is the mixture of two distributions (Figure 1). In order to strive for a relatively high specificity, a cut-off value of two standard deviations above the average of the blood donor group (n=150) was chosen. This is in line with recommendations by the Centres for Disease Control (CDC) to confirm the diagnosis of Tick Borne Relapsing Fever (TBRF) in the USA. With this chosen cut-off (LogOD 3.50), the seroprevalence of *B. miyamotoi* IgG antibodies in blood donors was 2.0% (Table 2) and nine out of the ten sera from confirmed *B. miyamotoi*-infected patients from Russia were also serologically positive. Only one out of 24 serum samples from patients infected with an unrelated spirochete, *Treponema pallidum*, showed reactivity against the GlpQ-antigen, and the seroprevalence in this category was comparable to that of the blood donors. Although an increase in seroprevalence was observed comparing blood donors to EM patients, confirmed and unconfirmed LNB patients, these seroprevalences did not differ significantly from the blood donors (Table 2). In the serologically unconfirmed but suspected HGA group a significantly higher seroprevalence was observed compared to that in the blood donor panel. In addition, the seroprevalence in forestry workers was significantly higher than the seroprevalence in blood donors (Table 2).

Table 2: Seroprevalences against *Borrelia miyamotoi* GlpQ.

Panels of serum samples logOD =3 .50	Test-ed (n)	Pos-itive (n)	Pos-itive (%)	Fisher Exact 95% Confidence		Difference from blood donors, p-value (1-tail), Fisher Exact test	Odds Ratio	Confidence limits	
Blood donors (Background)	150	3	2,0%	0.4%	5.7%	NA	NA		
Forestry workers (High risk)	120	12	10%	5,3%	16,8%	**0.005**	**5.44**	**1.5**	**19.76**
EM patients	251	14	5,6%	3,0%	9,2%	0.067	2.90	0.82	10.24
LNB; serologically confirmed	54	4	7,4%	2,0%	17,9%	0.08	3.92	0.85	18.12
LNB; serologically unconfirmed	35	3	8,6%	1,8%	23,0%	0.08	4.60	0.89	23.81
HGA; serologically unconfirmed	130	19	14,6%	9,0%	21,8%	**< 0.001**	**8.40**	**2.42**	**29.05**
T.pallidum infection, confirmed	24	1	4.2%	0.2%	18.9%	0.45			

Significant p-values (Fisher's exact test, level of significance was set at $p < 0.05$) and Odds Ratio's (Taylor series, with 95% confidence limit) in bold. NA; not applicable.

DISCUSSION

This study provides serological evidence of *B. miyamotoi* exposure in humans in the Netherlands using the GlpQ antigen of the *B. miyamotoi* in a newly developed Luminex-assay. Based on the significantly higher anti-*B. miyamotoi* antibodies in forestry workers and the serologically unconfirmed but suspected HGA group, *B. miyamotoi* infection appears not only to occur in immunocompromised patients - as has recently been described in the Netherlands [416]- but also seems to affect populations without underlying or chronic disease, which is in line with recent studies [412, 414, 430].

The seroepidemiology of the prevalence of IgG antibodies against *B. miyamotoi* as evidence for (endured) infection was examined in this study. Therefore, the study was setup to determine the seroprevalence in the different populations at a given moment, a so-called cross-sectional design, determining *B. miyamotoi* exposure rather than infection. No discrimination between endured or active infection was made, we did not determine a rise, decline or persistence of specific antibody production in these individuals nor did we determine the presence of anti-*B. miyamotoi* IgM antibodies. According to the Centers for Disease Control, to confirm the clinical diagnosis of other Tick Borne Relapsing Fever (TBRF) caused by *B. hermsii, B. parkerii* or *B. turicatae*, specific antibody titers should increase 4-fold between acute and convalescent serum samples. Because of the cross-sectional design of this study, no convalescent serum samples were tested and therefore we were not able to assess the course of the serological response over time. Patients with other TBRF may have false-positive tests in indirect immunofluorescence assay (IFA) or whole lysate enzyme-linked immunosorbent assay (ELISA) for LB because of the similarity of proteins between the two organisms. Because of this cross-reactivity and the fact that cultivating *B. miyamotoi* - as a definite proof of a *B. miyamotoi* infection - has been a genuine challenge until now, a serological test such as the one we describe here is an important step towards a new diagnostic tool.

Our aim was to identify risk groups with significant more exposure that the general population. As recommended by the CDC we used a cut-off two standard deviations above the average of the blood donor group in order to strive for a relatively high specificity. This is higher than the mathematical optimal cut-off value (LogOD 3.45), which gives the best discrimination between positive and negative serum samples, yet without taking false-positivity into account (data not shown). Nonetheless, assays for exposure to *B. miyamotoi*, such as described here and for other recombinant GlpQ antigen ELISA and immunoblot described before [414], need to be more extensively validated and tested to determine the extent of cross-reactivity and sensitivity. In the Netherlands, we have not identified another relapsing fever spirochete other than *B. miyamotoi* in ticks obtained from the environment, thus far. Therefore, we assume cross-reactivity between different relapsing fever GlpQ [441] has no or an insignificant influence in the Dutch setting. In addition, no significant cross-reactivity was observed with *Treponema pallidum* positive patients.

An increased seroprevalence was observed in EM patients and unconfirmed and confirmed LNB patients, which might be attributed to exposure to ticks infected with both *B. burgdorferi* s. l. and *B. miyamotoi* [416, 440]. Whether co-infection with *B. miyamotoi* exists and may alter the clinical manifestations of LB or LNB remains to be answered, our serological evidence suggests that it can occur in the same patients, assuming patients were diagnosed correctly. From our data we cannot conclude whether these were concomitant or serial infections. It should be mentioned that, in analogy to *B. burgdorferi* s.l. infection in Europe, it might also be that anti-*B. miyamotoi* antibodies are a result of a previous asymptomatic infection. To answer these questions a longitudinal approach would be required.

Previous studies conducted in the Netherlands showed that forestry workers have an increased risk of acquiring infections transmitted by ticks [451-454]. In this study, 11.6% (6.5-18.8%) of the forestry workers tested positive for anti-*B. miyamotoi* IgG antibodies. These findings indicate that in addition to LB and other tick-borne pathogens, forestry workers also run an occupational risk of acquiring infection with *B. miyamotoi* or at least exposed to this tick-borne pathogen. Furthermore, based on the findings presented here, demonstrating that the HGA suspected group of patients have a significantly higher seropositivity compared to blood donors, suggests that *B. miyamotoi* might also cause a febrile illness comparable to HGA. This is in line with previous case reports [430]. Indeed, the clinical manifestations of HGA might be similar to those caused by *B. miyamotoi* infection, including (high-grade) fever, chills, myalgia, nausea and headache a few weeks after a tick bite. Therefore, clinicians should include *B. miyamotoi* in the differential diagnosis of patients with a febrile illness caused by an unknown etiological agent when there is evidence of tick exposure.

In conclusion, a Luminex-assay was developed for seroepidemiologic screening of IgG antibodies against the GlpQ protein of *B. miyamotoi*. We observed an increase in seroprevalence from blood donors, to EM patients and unconfirmed and confirmed LNB patients. However, only the forestry workers and the serologically unconfirmed but suspected HGA group consistently showed significantly higher seroprevalence compared to the blood donor panel. It could be that some patients identified as having LB and definitely some that were suspected of having HGA, substantiated in part by nonspecific serological tests such as a whole cell ELISA or not substantiated at all, may actually have been infected with this relapsing fever spirochetes. Furthermore, our study suggests that individuals that are (occupationally) exposed to ticks in the Netherlands, such as forestry workers, are potentially at risk for *B. miyamotoi* infection. Interestingly, in light of the popularity of outdoor recreational activities among Dutch people, it is to be expected that this will certainly predispose a large number of people to the risk of infection with *B.*

miyamotoi, amongst other tick-borne pathogens. Therefore, *B. miyamotoi* infection should be included in the differential diagnosis when forestry workers or patients who engage in outdoor recreational activity present with fever after a tick-bite. In addition, in patients with unexplained fever or HGA-like symptoms after exposure to ticks physicians should consider *B. miyamotoi* infection. Until better validated diagnostic tests become available it should be recommended to consult academic referral centers when there is a suspicion on *B. miyamotoi* infection.

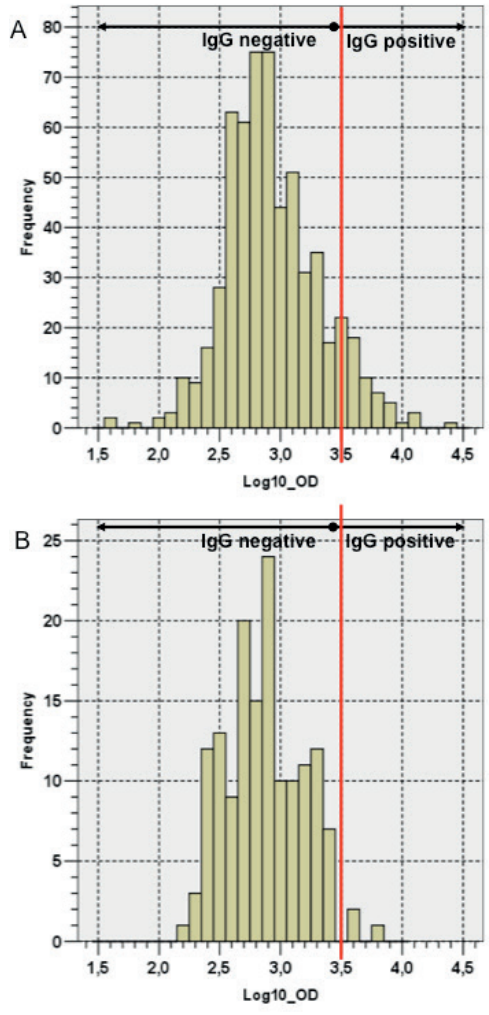
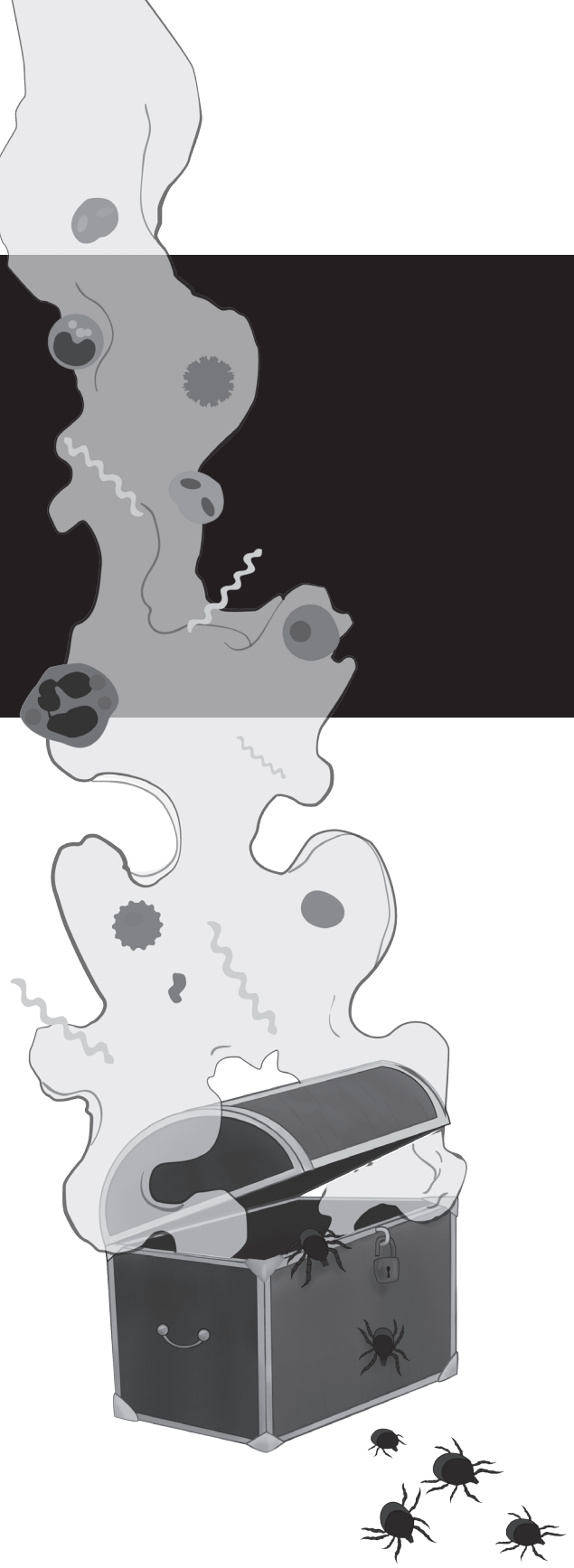


Figure 1: Distribution of LogOD-values
A: Serum samples (n=590) from the five risk groups. B: Serum samples from only blood donors. Red line depicts the selected cut-off value.



CHAPTER 11

Synthesis

Ticking all the boxes: from ticks, pathogens, vertebrate host to human disease

Partially based on 'Emerging tick-borne pathogens: Ticking on Pandora's Box',
S. Jahfari and H. Sprong
'Ecology and the prevention of Lyme borreliosis', 4th volume of Ecology and Control of
Vector Borne diseases, published by Wageningen Academic Publishers (2016)

SUMMARY

There have been marked increases in the incidence of Lyme borreliosis and tick-borne encephalitis over the past ten to twenty years, in most European countries [12-14]. In the Netherlands, this upsurge has been evident from both a continuing increase in consultations for tick bites and *erythema migrans* [9, 24]. In addition, substantial disease burden is caused by either Lyme borreliosis or Lyme borreliosis related manifestations. Patients with Lyme-related persisting symptoms are the majority of this burden [455]. The increasing number of tick bites reflects a growing risk of humans contracting Lyme borreliosis, but also other of tick-borne diseases. Still, these other tick-borne diseases are rarely diagnosed in the Netherlands. The results presented in this thesis, provide insights into presence and public health risk of other tick-borne diseases and could be used for public health decision-making, disease risk assessment and risk management in the field of tick-borne diseases.

In part I, the different tick-borne pathogens in the Netherlands are studied; several knowledge gaps about their enzootic cycles are filled in. In **Chapter 2**, we calculated the prevalence of the different tick-borne pathogens in ticks, while we tested whether these pathogens might share similar enzootic cycles. We studied patterns of co-infection and spatial and seasonal dynamics of infection in questing *Ixodes ricinus* nymphs. *Rickettsia helvetica* (31.1%) and *Borrelia burgdorferi* sensu lato (11.8%) had the highest overall prevalence and were detected in all areas. *Candidatus* Neoehrlichia mikurensis (5.6%), *Anaplasma phagocytophilum* (0.8%), and *Babesia* species (1.7%) were detected in most, but not all areas. The prevalence of pathogens in ticks varied among the study areas from 0 to 64%, while the density of questing ticks varied from 1 to 179/100 m^2. Overall, 37% of the ticks were infected with at least 1 pathogen, and 6.3% with more than 1 pathogen. One-third (1/3) of the *Borrelia*-positive ticks were infected with at least one other pathogen. Coinfection of *Borrelia afzelii* with *Candidatus* Neoehrlichia mikurensis and with *Babesia* species occurred significantly more often than expected, implying shared reservoir hosts for these pathogens. Conversely, coinfection of *Rickettsia helvetica* with either *Borrelia afzelii* or *Candidatus* Neoehrlichia mikurensis occurred significantly less frequent than expected.

In **Chapter 3,** we focused on the prevalence of *Candidatus* Neoehrlichia mikurensis in different tick species in Europe. By using an in-house developed real-time PCR assay, we showed that *Candidatus* Neoehrlichia mikurensis is present in questing *Ixodes ricinus* ticks throughout the Netherlands, Belgium, and other European countries but described under different names. In addition, we further characterized this emerging pathogen molecularly by using *groEL*, *16S rRNA* and *gltA* as molecular markers. We propose that *Ixodes ricinus* can transstadially but not transovarially transmit this microorganism and that different small mammal species act as reservoir hosts. Our data imply that humans are frequently exposed to *Candidatus* Neoehrlichia mikurensis-infected ticks through tick bites.

In **Chapter 4**, we showed that *Anaplasma phagocytophilum* has at least four differential enzootic cycles (ecotypes), and that the *groEL* gene is a suitable molecular marker to identify these different clusters. All human cases described clustered in only one of these ecotypes (ecotype I), suggesting that only one ecotype is zoonotic. Furthermore, the zoonotic ecotype has the broadest range of wildlife hosts. The significant population expansion – as shown by sequencing – of the zoonotic *A. phagocytophilum* ecotype indicates a recent increase of the acarological risk of exposure of humans and animals.

To date, tick-borne encephalitis virus (TBEV) was never shown to occur in the Netherlands. By using roe deer as sentinels and screening their sera for neutralizing TBEV antibodies, and using molecular methods in questing ticks in **Chapter 5**, we showed that there is at least one region in the Netherlands where tick-borne encephalitis is endemic.

In **Chapter 6**, we assessed the relative contribution of one of the most common animal urban dwellers - the European hedgehog - in the maintenance of several tick-borne pathogens and their enzootic cycles in (sub)urban areas. We conclude that hedgehogs and their ticks contribute to transmission and spreading of *Borrelia bavariensis*, *Borrelia spielmanii*, *Borrelia afzelii*, *Anaplasma phagocytophilum* and *Rickettsia helvetica* in (sub)urban areas.

In part II, we aimed to translate these environmental findings of tick-borne pathogens, to human exposure, infection and disease. There are two critical issues in disease risk assessment and risk management for Lyme borreliosis; 1) differentiating between the spirochetes that only cause localized skin infection from those that cause disseminated infection, 2) and tracing the group of medically important spirochetes to a specific vertebrate host species. In **Chapter 7,** we directly link a transmission and/or amplification host for *Borrelia burgdorferi* s. l. to specific clinical manifestations of Lyme borreliosis. To show this, isolates from Lyme borreliosis cases with distinct clinical manifestations (*erythema migrans*, Lyme neuroborreliosis, acrodermatitis chronica atrophicans, and Lyme arthritis) and isolates from *Ixodes ricinus* ticks feeding on rodents, birds and hedgehogs were typed to the genospecies level by sequencing part of the intergenic spacer region. In addition, more in-depth molecular typing was performed by sequencing eight additional loci with different characteristics (plasmid-bound, regulatory, and housekeeping genes). We showed that *Borrelia afzelii* was the most common genospecies in acrodermatitis patients and engorged ticks from rodents. *Borrelia burgdorferi* sensu stricto was widespread in *erythema migrans* patients. *Borrelia bavariensis* was widespread in neuroborreliosis patients and in ticks from hedgehogs, but rare in *erythema migrans* patients. *Borrelia garinii* was the dominant genospecies in ticks feeding on birds. Spirochetes in ticks feeding on hedgehogs were overrepresented in genotypes from spirochetes in *erythema migrans* and acrodermatitis patients. Spirochetes from ticks feeding on birds were overrepresented in genotypes from spirochetes in neuroborreliosis patients. Overall, the analyses of our datasets support the

existence of at least three transmission pathways (1. ticks feeding on hedgehogs and Lyme neuroborreliosis patients, 2. ticks feeding on birds and Lyme neuroborreliosis patients, 3. and ticks from rodents and hedgehogs and acrodermatitis chronica atrophicans patients) from an enzootic cycle to a clinical manifestation of Lyme borreliosis.

In **Chapter 8**, in search of indirect proof of human exposure to, and infection with other tick-borne pathogens, we tested ticks that fed on humans. And blood of a cohort study of humans with tick-bites and/or *erythema migrans*, for the presence of *Borrelia burgdorferi* s. l., *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, spotted fever *Rickettsia*'s, tick-borne encephalitis virus and *Babesia* genospecies using molecular detection techniques. Half of the ticks removed from humans tested positive for *Borrelia burgdorferi* s. l., *Anaplasma phagocytophilum, Candidatus* Neoehrlichia mikurensis*, Rickettsia helvetica, Rickettsia monacensis, Borrelia miyamotoi* and several *Babesia* species*.* Among the *Borrelia burgdorferi* s. l. positive ticks, 33% carried another pathogen from a different genus. Based on molecular detection techniques, the probability of infection with a tick-borne pathogen other than Lyme spirochetes after a tick bite is roughly 2.4% in the Netherlands. Similarly, among patients with *erythema migrans*, the probability of a co-infection with another tick-borne pathogen was approximately 2.7%. Collectively, these data show that exposure to pathogens other than *Borrelia burgdorferi* s. l. and co-infections are more common than assumed and warrant further investigation.

In **Chapter 9**, we investigated a new kid on the block, the relapsing fever spirochetes *Borrelia miyamotoi* in more detail. We showed significant association of *Borrelia burgdorferi* s. l. with *Borrelia miyamotoi* in nymphs implying the existence of mutual reservoir hosts. Indeed, we demonstrated the presence of *Borrelia miyamotoi* DNA in different organs of rodents, suggesting systemic infections. On top of this, we showed that birds also can be infected with this pathogen. It has been postulated that *Borrelia miyamotoi* is able to cause an *erythema migrans*-like skin lesion. Another, perhaps more plausible, hypothesis is that patients with *Borrelia miyamotoi* disease - characterized by fever, malaise and myalgia - with a skin lesion are co-infected with *Borrelia burgdorferi* s. l.. We did however, not find evidence for *Borrelia miyamotoi* infection when testing biopsies of Lyme borreliosis-suspected skin lesions, suggesting that *Borrelia miyamotoi* is not associated with Lyme borreliosis-associated skin manifestations.

Based on the infection rate of *Ixodes ricinus* ticks with *Borrelia miyamotoi* in the Netherlands, we hypothesized that various populations are exposed to *Borrelia miyamotoi*. Therefore, in **Chapter 10**, we serologically tested the exposure of different risk groups to *Borrelia miyamotoi* in the Netherlands, by an in-house developed Luminex assay using a fragment of glycerophosphodiester phosphodiesterase (GlpQ) as antigen. Assuming that an IgG antibody response against *Borrelia miyamotoi* antigens reflects (endured) infection,

the seroprevalence in different risk groups was examined. Sera from nine out of ten PCR-confirmed *Borrelia miyamotoi* infections from Russia were found positive with the recombinant antigen used, and no significant cross-reactivity was observed in secondary syphilis patients. The seroprevalence in blood donors was set at 2.0% (95% CI 0.4–5.7%). The seroprevalence of antibodies to *Borrelia miyamotoi* GlpQ was elevated in *erythema migrans* patients 5.6% (3.0-9.2%) and in individuals with serologically confirmed 7.4% (2.0-17.9%) or unconfirmed 8.6% (1.8-23%) Lyme neuroborreliosis, but these rates were not significantly higher than the percentage blood donors testing positive. The prevalence of anti-*Borrelia miyamotoi* antibodies among forestry workers 10% (5.3-16.8%) and in patients with serologically unconfirmed but suspected Human Granulocytic Anaplasmosis 14.6% (9.0-21.8%) were significantly higher compared to the seroprevalence in blood donors. Our findings indicate that infections with *Borrelia miyamotoi* occur in tick-exposed individuals in the Netherlands. In addition, *Borrelia miyamotoi* infections should be considered in patients reporting tick bites and febrile illness with unresolved etiology in the Netherlands, and other countries where *Ixodes ricinus* ticks are endemic.

GENERAL DISCUSSION

The findings presented in this thesis contribute to a better understanding of the complicated relationship between ticks, pathogens, vertebrate hosts, and human infection, filling in several important knowledge gaps. However, I realize that the research presented in my thesis is only a small part of a larger puzzle. There is much research to be done, many stones are left unturned. In this synthesis, I will put my findings into a perspective, discuss their relevance and relate to previously published work. I start with discussing my findings on different tick-borne pathogens in the Netherlands, and then continue to discuss the most prevalent tick-borne disease: Lyme borreliosis and linking the causative agent *Borrelia burgdorferi* s. l. in relationship to vertebrate hosts and human disease. Second, I connect vertebrate host to ticks and pathogens in section 3 and 4. In the following section, I continue arguing how disease risk and surveillance in the field of tick-borne pathogens can be used in public health strategies. In section 6, I discuss my findings on human exposure, infection and disease of different tick-borne pathogens. In my concluding remarks, I recommend and speculate about policy and future research.

1. Ticks and Pathogens: Ticks contain more pathogens than Lyme spirochetes alone

As described in this thesis, also in the Netherlands, *Ixodes ricinus* is not only the transmitter of *Borrelia burgdorferi* s. l., but also of other tick-borne pathogens, namely *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *Rickettsia monacensis*, several *Babesia* species and tick-borne encephalitis virus. To study and understand the biology, disease dynamics and human health risk of tick-borne diseases, a multidisciplinary approach is required, studying 1) the composition and prevalence of pathogens carried by the vector in the different life stages, 2) the different animal reservoirs

and their contribution in the maintenance of the pathogen, and 3) exposure, infection and importantly disease in humans.

The risk of human infection with tick-borne pathogens after an *I. ricinus* bite depends to a large extent on the prevalence and phenotypic but also genetic make-up of the different pathogens in the different stages of the tick, and abundance and distribution of the reservoir host at a geographical location. By determining the prevalence and distribution of tick-borne microbes in questing ticks, linking that to reservoir hosts and enzootic cycles, we can get an insight into the biological and ecological drivers of these pathogens. Connecting this to epidemiological data on human exposure, infection and disease can give an insight into risk on a population scale, and possibly find novel ways to control the disease.
In the Netherlands, more than one-third of questing ticks harbored one or more of the tested tick-borne pathogens consisting of *B. burgdorferi* s. l., *R. helvetica, Candidatus* Neoehrlichia mikurensis*, A. phagocytophilum* and *Babesia* species. Furthermore, these pathogens have a ubiquitous distribution in the Netherlands, in a variety of habitats, from open areas such as dune and heather to deciduous or coniferous forests (**chapter 2**). This same pattern of distribution was seen in the relatively newly discovered pathogen, *B. miyamotoi* (**chapter 9**). Evidently, the presence of *I. ricinus* is inherently linked to the presence of these pathogens. In addition, we show that some pathogens share the same reservoir host by using data on mixed infections in questing ticks. Some combinations of mixed infection in a tick are found at significantly higher rates than others, indicating a common reservoir host. The idea that some pathogens have a common reservoir host, was confirmed by experimental studies, where infected wild rodents have proven to be competent hosts that can transmit *Candidatus* Neoehrlichia mikurensis, as well as *B. miyamotoi* to laboratory ticks [61].

2. Connecting *Borrelia burgdorferi* sensu lato to vertebrate host and human disease

Lyme borreliosis can be considered a collective name for different clinical manifestations and diseases in humans caused by spirochetes from the *B. burgdorferi* s. l. complex. At least five genospecies of *B. burgdorferi* s. l. complex have been shown to be pathogenic to humans, namely *B. burgdorferi* s. s., *B. afzelii, B. garinii*, *B. spielmanii* and *B. bavariensis* [10, 19]. The wide variety of clinical outcomes is related to genetic variation of the spirochetes of the *B. burgdorferi* s. l. ([456] **chapter 7**). It has been postulated that this genetic diversity is at the base of the multiple clinical manifestations that infection with these bacteria can cause [347-349]. This was shown in **chapter 7** and in other research we have conducted [19]. Not all the *Borrelia* genotypes within the known pathogenic *B. burgdorferi* s. l. genospecies can cause Lyme borreliosis [117]. What genetic information determines the differential invasiveness of *Borrelia* genospecies, but also of genotypes within a genospecies is not known. Successful colonization of the human host by bacterial pathogens, like *Bordetella pertussis*, *Streptococcus mutans* and *Neisseria meningitidis* requires that bacteria overcome limitations in iron and other essential elements imparted by the host [457, 458]. Based on

the knowledge that genes involved in efficient uptake of metals and ions from the host are responsible for virulence and pathogenesis, a set of genes involved in the regulation of genes located on plasmids [383, 459, 460] and chromosomal virulence genes were selected and characterized to look for possible explanations for the observed clinical differences [369, 370, 380]. We could not prove this by the chosen approach, as the data showed that the differences in pathogenicity and clinical manifestations of the Lyme spirochete are not associated with genetic differences in the virulence genes and plasmid-based genes. Second, we hypothesized that a zoonotic transmission cycle for a genotype is one factor that determines the clinical manifestation of Lyme borreliosis. Indeed, we showed that clinical outcomes could be linked to different zoonotic transmission cycles characterized by a defined genotype-host association: each *B. burgdorferi* s. l. genospecies able to cause Lyme borreliosis is maintained in nature through a distinct enzootic cycle with different vertebrates acting as reservoirs. This knowledge about enzootic cycles and the different clinical consequences that each *B. burgdorferi* s. l. genospecies is associated with, can prove to be helpful in disease risk assessment and risk management.

Yet we could not link all *B. burgdorferi* s. l. genospecies (*B. spielmanii* and *B. burgdorferi* s. s.) to an enzootic cycles. We did detect *B. spielmanii* genospecies in hedgehogs and their ticks (**chapter 6**), while small rodent of the Gliridae family (which are extremely rare in the Netherlands) have been described in the literature as the main reservoir hosts [319]. Infection with *B. spielmanii* in humans, however, is rare and therefore we could not study the host reservoir relationship for this pathogen. Thus far, *B. spielmanii* was only found in patients with *erythema migrans* [19].

Interestingly, in hedgehogs we found all mammal reservoir-associated *B. burgdorferi* s. l. genospecies except for *B. burgdorferi* s. s.. In North America the *B. burgdorferi* s. s. causes Lyme arthritis and rodents are its reservoir host. For the European strains of *B. burgdorferi* s. s. we did not find the same clinical manifestations (e.g. causing Lyme arthritis) and rodents as reservoir hosts (**chapter 7**, [19]). To be more specific, no other genospecies than *B. afzelii* are detected in ticks from rodents in the Netherlands, in the more than thousand rodent samples tested to date (data not shown). This suggests that there is a real difference in maintenance of *B. burgdorferi* s. s. in nature between the two continents. In addition, the low prevalence of *B. burgdorferi* s. s. (0.2%) in **chapter 2** suggest that this genospecies is either maintained in a cryptic cycle involving a vector other than *I. ricinus*, or has a specific reservoir host that is not very abundant in the Netherlands and/or was not include in our study. This discrepancy of prevalence in questing ticks and presence in human disease is most obvious in the case of *B. bavariensis,* with less than 1% of the questing *I. ricinus* ticks being positive for this genospecies but almost 60% of all the Lyme neuroborreliosis cases we tested. In addition, since our findings indicate that neuroborreliosis caused by *B. bavariensis* will not always be preceded by *erythema migrans* as a first symptom of infection, the

current medical guidelines may need to be re-evaluated. Current recommendation advises antibiotic treatment of *erythema migrans*, as it commonly prevents the development of late and more severe disease stages [10], but this approach will not prevent all cases of Lyme neuroborreliosis. Clinicians should therefore be aware of this finding. That said, treating all patients that present with a tick bite with antibiotics, will lead to overtreatment and is not desirable in this era where antibiotics-resistance is one of our main public health challenges.

There is another side of the Lyme borreliosis coin, genetic variation of the pathogen, and reservoir host association alone cannot fully explain the observed variation in the clinical manifestations observed in humans. The ability to cause disease depends not only on the genetic make-up of the Lyme borreliosis spirochetes, but also on extrinsic factors, for example genetic factors, the immune status of its host and the human immune responses. Indeed, most if not all tissue damage results from host inflammatory reactions. The intensity of the inflammatory response varies according to the *B. burgdorferi* s. l. genospecies that causes an infection [461, 462]. Host genetic factors have an important role in the expression and severity of infection in animals, host factors being certain SNP's Toll-like receptors, NOD-like receptors [463], as well as *HLA-DR* allele associated differences in the development of antibiotic refractory Lyme arthritis [464]. Thus, the extensive array of disease manifestations in Lyme borreliosis patients reflects most probably the interplay between *B. burgdorferi* s .l. genospecies virulence and host immune responses.

3. Vertebrate host their ticks and pathogens

The role of hedgehogs and Ixodes hexagonus in pathogen maintenance and transmission to Ixodes ricinus

Other tick species maintain their specific set of pathogens in enzootic cycles. However, they may still play a key role in transmitting those pathogens to the generalist feeder *I. ricinus* via a shared vertebrate host. As *I. ricinus* is a generalist feeder, it shares vertebrate hosts with other tick species. *Ixodes ricinus* ticks are therefore exposed to other tick-borne pathogens, and could be able to transmit these pathogens to humans or animals (Figure 1). *Ixodes hexagonus* is a host specialist, feeding primarily on hedgehogs. Despite this, it is known to occasionally bite humans, companion animals and other hosts [323, 324]. In **chapter 6** we show that hedgehogs and *I. hexagonus* contribute to the spread and persistence of ticks and their pathogens in a (sub)urban habitat via secondary enzootic cycles. This is especially of significance since hedgehogs are one of the most successful urban adapters and can reach up to nine times higher densities in urban areas with parks and garden, than in rural areas [329-331].

The presence of different *Borrelia* genospecies in ticks from hedgehogs (**chapter 6**) indicates that infection rates of questing ticks are possibly higher in areas where hedgehog densities are high - like in (sub)urban areas - in comparison to other areas like in forests and rural

areas [329-331]. As hedgehogs carried many adult *I. ricinus* ticks, it might very well be that a host community composed only of small or medium sized vertebrates such as rodents, birds and hedgehogs (such as in (sub)urban area's and parks) is already sufficient to complete the life cycle of *I. ricinus*. Therefore, the general idea that ticks cannot be sustained in (sub)urban areas is not accurate and this should be considered in design of disease risk and control models. These models should incorporate the effect of individual variation on disease emergence and super spreader effects of hosts [465, 466].

In **chapter 7**, we show by using quantitative molecular epidemiologic approach that hedgehogs have significant associations with the clinical Lyme borreliosis manifestations *erythema migrans*, acrodermatitis chronica atrophicans and Lyme neuroborreliosis. Dr. Coipan suggests in her thesis that mammal-associated *B. burgdorferi* s. l. genospecies, such as *B. afzelii*, *B. bavariensis*, and *B. spielmanii*, are more often isolated from patients than avian-associated *Borrelia* genospecies (*B. garinii* and *B. valaisiana*) [19]. She hypothesizes that the reason why mammal associated *Borrelia* are significantly more often retrieved from humans than bird-associated *Borrelia* is that transmission of the bacteria is easier between vertebrates of the same class (i.e. mammals) than between vertebrates of different classes (i.e. birds and mammals). The factors that trigger the specificity of *Borrelia* for small rodents could be the same ones that are responsible for facilitating the establishment of localized infection with these bacteria in humans, for instance outer surface protein B and other factors [467]. Since we show that *B. afzelii*, *B. bavariensis*, and *B. spielmanii* are all associated with hedgehogs, this hypothesis that mammal-associated pathogens are more likely to infect humans than pathogens associated with ectothermic animals, could also very well hold true for hedgehog-associated pathogens including *A. phagocytophilum* and *R. helvetica*. More research is needed, however, to elucidate the role of hedgehogs in the life cycle of *I. ricinus*, and its role in the epidemiology of human tick-borne diseases especially in (sub)urban area's and parks.

4. Connecting pathogens to vertebrate hosts

To survive in the different environments of a tick vector and of a vertebrate host, tick-borne pathogens undergo substantial selection pressures. In the tick, pathogens must survive great fluctuations in pH, temperature, and other factors related to biology of the tick [468], and in the host, pathogens must survive immunological and inflammatory defenses [469]. Strain diversity is likely to be an outcome of this selective pressure. Diversity allows the pathogen to evade immune responses of the host and also increases the number of different host species that can be infected [155]. Therefore, we were interested in strain diversity of *Anaplasma phagocytophilum*, an established tick-borne pathogen.

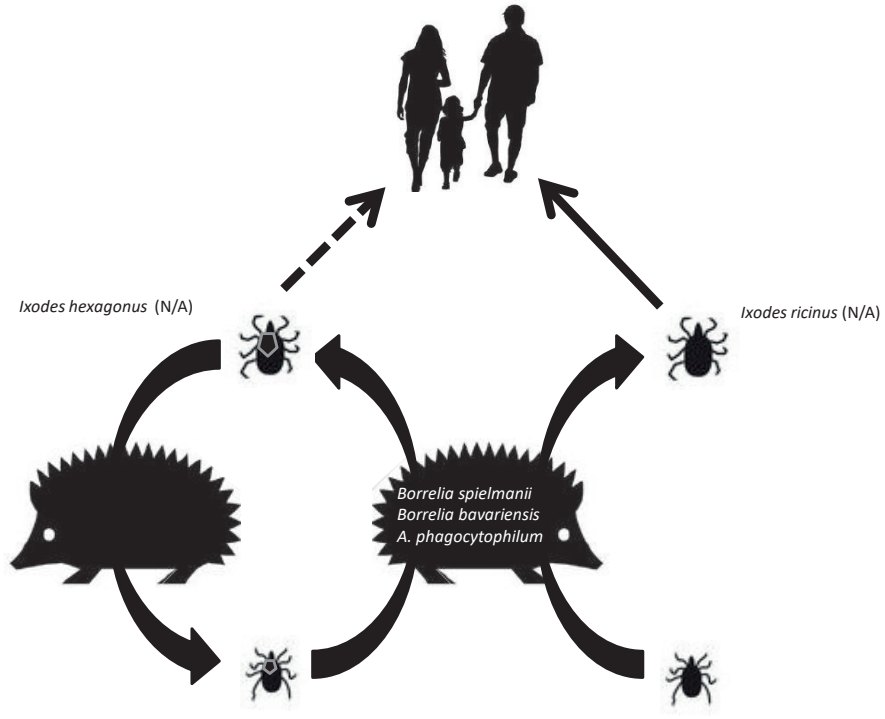


Figure 1. The cryptic cycle of human pathogens via *Ixodes hexagonus* and its host, the hedgehog. When feeding on an infected hedgehog, the generalist tick *Ixodes ricinus* gets infected with pathogens and transmits them to another host species, for example humans. L/N is larva/ nymph and N/A is nymph/ adult.

Strain diversity of Anaplasma phagocytophilum

Various reservoir hosts are implicated to play a role in the maintenance of the endemic life cycle of *A. phagocytophilum* in nature. The animals identified as reservoir hosts range from domestic and wild ungulates, to small mammals like rodents and insectivores, to birds and lizards [1, 36]. To study the enzootic cycles of *A. phagocytophilum* in nature, different genetic markers are used in literature. Sequences of the *groEL* gene (part of the heat-shock protein operon) have shown more clearly delineated genetic variants of *A. phagocytophilum* than have sequences of other genes [470]. Using this genetic marker, in **chapter 4** we show four different ecotypes of *A. phagocytophilum* in vertebrates and vectors in Europe, each with their particular enzootic cycle. We hypothesize that each of these ecotypes has its own main vector and therefore its own ecological niche and that these ecotypes are maintained in their own cycle. Vectors and pathogens have coevolved with specific host species, and therefore these host species are likely to be the preferred hosts for both vector and pathogen. We hypothesize that: 1) The main vector of ecotype II, could be the deer-ked or another deer specific ecto-parasite. Alternatively, *I. ricinus* could still be the main vector for this ecotype

since, the chance of feeding on roe deer in a subsequent life stage is fairly high [18]. 2) The main vector of the rodent ecotype (ecotype III) is *Ixodes trianguliceps* species that feeds only on rodents and shrews and is thought to be a driver of *A. phagocytophilum* and *B. microti* [247, 471] and 3) Ecotype IV, the bird associated type has the *Ixodes frontalis* as its main vector. The notion of different ecotypes is supported by experimental findings that point to variations in the susceptibility of different mammalian species to *A. phagocytophilum* strains [47]. The generalist *I. ricinus* tick species and (perhaps the hedgehog tick *I. hexagonus*) is the main vector for ecotype I, the only ecotype that is considered zoonotic (**chapters 4** and **8**). It is possible that ecotype I is zoonotic due to the great strain diversity, because this diversity could allow the pathogen to evade immune responses of the host and also increases the number of different host species that can be infected, a strategy used by numerous so called multihost pathogens, like influenza A and *Salmonella typhimurium* [472, 473]. Alternately and equally possible is that the strain diversity of this ecotype is a reflection of to population expansion. This particular ecotype showed significant population expansion, implying that either expansion occurred in the vector, or the vertebrate host, with a third option of expansion occurring in both the vector as the vertebrate host, which in turn could also explain strain diversity. Whether the other ecotypes can cause infection in humans remains to be determined.

Other tick-borne pathogen in which strain diversity could play a role in virulence and the ability to cause human disease is *R. helvetica.* As shown in **chapter 2**, *R. helvetica* is widespread in the questing tick population, with infection rates ranging from 3% to 64%, with an overall average of 31%. These numbers suggest that there is considerable exposure to *R. helvetica* after a tick bite. Still, only a few human cases have been described so far, in the Netherlands. *Rickettsia helvetica* was identified using serological and molecular methods in skin biopsies of *erythema migrans* patients. In addition, co-infection with *R. helvetica*, was also found in Lyme neuroborreliosis patients [117, 118]. Combining this, with the knowledge gathered in this thesis and elsewhere, I suggest that there are at least two distinct ecotypes of *R. helvetica* one associated with birds [106] and the other associated with hedgehogs (**chapter 6**). If this hypothesis holds true, the question would be which of the potential ecotype can cause infection in humans? It is imaginable that mammalian associated ecotypes are more suitable to infect humans. In addition, since we show that both birds as well as hedgehogs are amplification host of their own *Borrelia* genospecies that can cause Lyme neuroborreliosis (*B. garinii* and *B. bavariensis*, respectively), this could explain the co-infection with *R. helvetica* in the Dutch patient groups.

5. Unraveling disease risk; many scenarios, many actors at play

Acarological risk

When assessing disease risk of a tick-borne pathogen the term “acarological risk” is often used (likewise in **chapter 2**). This means that the level of the risk depends on the level of exposure of an individual to the density of infected questing ticks, infected with tick-borne pathogens. Often a so-called “DIN” or density of infected nymphs is calculated (DIN= Density of questing nymphs x (times) Nymphal infection prevalence) when assessing disease risk in different areas. Especially the latter needs to be used with caution in the case of tick-borne pathogens. First, adult (female) ticks can also transmit all the mentioned pathogens, and all tick-borne pathogens except *B. burgdorferi* s. l. and *Candidatus* Neoehrlichia mikurensis are thought to be transmitted transovarially, even though with different efficiencies. In other words, larvae do not play a major role in transmitting *B. burgdorferi* s. l. and *Candidatus* Neoehrlichia mikurensis to humans, but could transmit other tick-borne pathogens. Consequently, the contribution of larvae to the disease burden should be re-evaluated for each individual tick-borne pathogen. Second, not all microorganisms have the same ability to infect and cause disease in humans. Therefore, it is important to differentiate between the infectious and non-infectious pathogen strains, genospecies and genotypes, and between the invasive and non-invasive ones (**chapter 4**, **chapter 6** and **chapter 7**). For instance, the discrepancy between prevalence of *B. bavariensis* in questing *I. ricinus* nymphs and its ability to cause severe disseminated neurological disease cannot be captured in this simple DIN calculation. Two other factors are also important to incorporate in the analysis, when calculating acarological risk: the vertebrate source, meaning the main vertebrate or reservoir host(s) of the pathogen, and the abundance of that vertebrate source in a habitat or environment. When people are exposed to an infected tick in an urban environment, it is likely that the composition of the pathogens carried by that tick is different from ticks from dunes, heather and forests (**chapters 6** and **7**). In this regard, it is interesting to know that 31% of the people in the Netherlands reported to have obtained their tick bites from their gardens [345]. Furthermore, it is possible that we have underestimated the number of people bitten by the hedgehog tick, *I. hexagonus* while working in their gardens [324]. For laypersons, it is very difficult to distinguish between *I. ricinus* and *I. hexagonus* ticks, because the macroscopic differences are small. This underscores that understanding enzootic transmission cycles is important to understand and estimate risks better. All tick-borne pathogens described in this thesis have their own specific enzootic cycle and environmental drivers. Therefore, a customized approach is required for each pathogen when the disease risk is analyzed. In different environments and at each life stage, an *I. ricinus* bite has an impact on the risk of tick-borne infection and co-infection for humans.

Surveillance in ticks, the canary in the coalmine

Surveillance systems monitoring the prevalence of infectious disease in humans, domestic animals and wildlife, provide early warning systems of emerging infectious (zoonotic) diseases. For most vector-borne disease surveillance, we rely on surveillance of health indicators, e.g. trend in mortality or disease syndromes, or laboratory submissions, coupled with registration of specific diagnoses [474]. Unfortunately, these surveillance methods are reactive at best, and detect emerging pathogens only when the disease burden in the disease host is increasing, like in the initial outbreak cases of West-Nile virus, Bluetongue and Schmallenberg virus [474]. Thus we are in need of proactive systems allowing for early warning for emerging vector-borne infectious diseases, which may trigger implementation of containment measures at the source; being the tick in this case.

For the discovery of the first patients for both *B. miyamotoi* and tick-borne encephalitis virus in the Netherlands, the finding of pathogens in questing ticks preceded the discovery of infection in humans. *Borrelia miyamotoi* was first found in questing ticks and vertebrate hosts (**chapter 9**) after this finding was communicated to Amsterdam Multidisciplinary Lyme borreliosis Center the first European patient with relapsing fever *B. miyamotoi* disease was retrospectively identified [95], an immunocompromised patient with a chronic meningoencephalitis. In the seroprevalence study of **chapter 10**, we subsequently showed that sero-responses in the high-risk groups like forestry workers and of people that showed presumably anaplasmosis-like illness after a tick bite was significantly higher than the control group (blood donors). With this study, we facilitated the identification of the clinical symptoms of *B. miyamotoi* infections at the time. Showing that *B. miyamotoi* infections should be considered in patients reporting with a febrile illness or anaplasmosis-like symptoms after a tick bite, or even with fever of unresolved etiology.

Another striking example of pathogen surveillance in the environment was the discovery of tick-borne encephalitis virus (TBEV) in the Netherlands (**chapter 5**). By using roe deer sera as sentinels for flavivirus (TBEV) antibodies, we could target our surveillance to one specific national park and showed the presence of TBEV RNA in two questing ticks (a nymph and an adult tick). The National Institute for Public Health and the Environment (RIVM) provided this finding to both medical professionals and the public at large. Within a week, this communication resulted in the report of an autochthonous TBE infection in the Netherlands [475]. After two months, the second Dutch patient was found [476]. This patient lives near the area where we described TBEV positive questing ticks. This example further underscores the value of proactive monitoring and communication. We do not think that the introduction of TBEV is a recent event in the Netherlands, since all the roe deer sera were collected in 2010, whereas the ticks were collected 5 years later in 2015. This 5-year gap implies that TBEV has been circulating in the Netherlands for some time and that perhaps human have not correctly been diagnosed, since the medical professionals

were unaware of the circulation of TBEV in the Netherlands and did not include it in their differential diagnosis. As most cases of TBEV are asymptomatic and when symptomatic are most likely to have a self-limiting course, with or without long-term sequelae, this is not unlikely that patients have been missed.

Even though less obvious than the previous examples, the same sequel of events was followed in the case of *Candidatus* Neoehrlichia mikurensis (**chapters 2** and **3**), *R. helvetica* (**chapters 2** and **6**) and *Babesia divergens* (**chapter 2**). Presence of the pathogens in questing ticks was shown and enzootic cycles were unraveled before the first evidence of infection in humans was found in the Netherlands (**chapter 8**, [118]).

6. Human exposure, infection and disease

Over the past two decades, several European countries have reported marked increases in the incidence of Lyme borreliosis [9, 12, 24, 162]. In the Netherlands in 1994, the incidence for GP diagnosed *erythema migrans* increased from 39 per 100,000 inhabitants to 134 per 100,000 inhabitants in 2009, adding up to a total of 22,000 patients with *erythema migrans* in the Netherlands [9]. These number have stabilized from 2009 to 2014 [25]. It most likely is not just an increase in reported cases as the prevalence of *B. burgdorferi* s. l. in ticks also increased in the same period [5]. In 2009, the total estimated number of tick bites was a dashing 1.1 million tick bites in the Netherlands alone [9]. It is likely that other tick-borne pathogens followed the same upward trends as the Lyme borreliosis spirochetes. However, in Europe, information on the incidence of human infection with tick-borne pathogens other than Lyme borreliosis and TBE is almost completely lacking and disease in humans has largely been derived from occasional case reports.

Based on the results from **chapter 8**, we estimated the number of annual human exposure to tick-borne pathogens in the Netherlands to be roughly 3500 for *B. divergens,* 10,500 for *A. phagocytophilum*, 25,500 for *B. miyamotoi*, 60,000 for *Candidatus* Neoehrlichia mikurensis, up to 207,000 persons for *R. helvetica.* Among the 322,000 persons exposed to *B. burgdorferi* s. l. through a tick bite, roughly 105,000 are simultaneously exposed to another pathogen. In addition to this number, exposure to more than one tick-borne pathogen can happen when people have more than one tick bite at once or several consecutive tick bites. It is clear that not all exposure leads to infection and disease. Based on the development of an *erythema migrans* or seroconversion, the risk of infection with *B. burgdorferi* s. l. after tick bites was estimated to be 5.1% [254]. In **chapter 10**, we show that *B. miyamotoi* infection (by measuring IgG levels) is indeed more widespread than the single case report [95] and occurs is different risk groups, not only in immunocompromised patients.

Co-infection in humans

Ticks retrieved from humans frequently contain multiple pathogens (**chapter 8**). This means that the risk of exposure to one or multiple tick-borne pathogens is substantial. Especially, due to their omnipresence, we underline the need to consider all of these pathogens (*B. miyamotoi*, *A. phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *R. helvetica*, *R. monacensis*, and *Babesia* species) when evaluating the risks of infection and subsequently of disease following a tick bite. Therefore, it is quite possible that infections with tick-borne pathogens other than Lyme spirochetes have been common, but have not been distinguished from other infections. Alternatively, it is possible that infections and symptoms with these tick-borne pathogens have all been categorized - and in some cases treated as - under the collective name of Lyme borreliosis. In **chapter 8**, we show that the probability of infection with a tick-borne pathogen other than Lyme spirochetes after tick bites in the Netherlands is roughly 2.4% (95%CI 1.1% – 4.5%). This number is similar to the probability of a co-infection with another tick-borne pathogen in patients with *erythema migrans* (2.7%, 95%CI 1.3% – 5.2%). Interestingly, one patient in our study had a co-infection with *A. phagocytophilum* and *B. divergens*, confirming that co-infection with other tick-borne pathogens also is possible even though it has rarely been reported before. Co-infections could affect the severity of disease and influence clinical outcomes, especially since some tick-borne pathogens such as *A. phagocytophilum* may modulate host immunity [155, 166, 477]. Moreover, mixed infections of *B. burgdorferi* s. l. with different tick-borne pathogens could be partly responsible for the wide variety of reported clinical manifestations of Lyme borreliosis [392]. Physicians and others sometimes attribute clinical manifestation such as fever and viral-like symptoms to Lyme borreliosis.

How pathogen biology affects detection methods in the clinical samples

The studies in **chapter 8** and **chapter 9** also show that for every pathogen a different detection method and consequently different diagnostic method may be required. Although culturing is considered the most reliable method in proving the presence of living microorganisms and viruses, it is time consuming, costly and often not possible for all pathogens. Therefore, the preferred laboratory diagnostic methods for most pathogens are either serology or PCR. Compared to DNA amplification with PCR, available serological tests generally have a low specificity and/or sensitivity, particularly during the early phase of infection. Still there are technical detection limitations to PCR-based methods, such as the timing of sample collection after a tick bite, as well as the pathogen tissue tropism that strongly affect the ability of pathogen detection [395, 396]. For instance, in patients' blood, *B. burgdorferi* s. l. DNA was detected in only one out of the 291 patients with an *erythema migrans* (**chapter 8**), confirming that the chance of detecting *B. burgdorferi* s. l. DNA in blood samples of confirmed Lyme borreliosis patients is low [395]. This shows that serology is still the preferred approach when it comes to Lyme borreliosis diagnostics. *Rickettsia helvetica* is another example of the influence of microbial tropism on diagnostics.

Despite the high prevalence of *R. helvetica* in ticks, this bacteria was not detected in the 626 examined blood samples (**chapter 8**) whereas molecular evidence for their presence was found in cerebrospinal fluid of Lyme neuroborreliosis patients and in a skin sample of an *erythema migrans* patient [117, 118]. Perhaps this finding is not surprising knowing that *R. helvetica* has a tropism for endothelial cells [396]. Because PCR on blood is shown to be a sub-optimal method, I would recommend the development of serological test in the case of *R. helvetica*.

Similarly, in **chapter 9** *B. miyamotoi* DNA was not detected in 31 *erythema migrans* skin samples, even though 10% of all *B. miyamotoi* patients present with an *erythema migrans* lesion. This finding shows that the chosen detection method - in this case PCR on skin tissue - was sub-optimal for *B. miyamotoi* detection. *Borrelia miyamotoi*, like *Candidatus* Neoehrlichia mikurensis, *A. phagocytophilum,* and *Babesia* species are all pathogens that can be expected in blood because of their biology and tropism [36, 58, 83, 119]. Therefore, in the case of these specific pathogens, the use of PCR-based methods on blood samples is a recommended approach when it comes to diagnostics (**chapter 8**). The data presented in this thesis shows that there is no one-size-fits-all approach when it comes to diagnosis of infection with tick-borne pathogens.

How genetic diversity affects diagnostic laboratory tests

Strain variation can have important implications for development of diagnostic tests, for both serological and molecular tests. In the case of serological assays, if variable antigens are used as a target in the test, these antigens need to be tested if they are multivalent for all the relevant strains. For instance, in the case of *A. phagocytophilum,* the main diagnostic laboratory method now is still serology. When testing for *A. phagocytophilum* infection, commercially available IFA tests with a North American strain (HGA-1 strain) is used in the Netherlands and other European countries, even though cross-reactivity between the North American and European strain has not been evaluated. Moreover, when novel diagnostic laboratory tests are developed (serological and molecular tests) for the *A. phagocytophilum* the zoonotic ecotype (ecotype I) should be used since, the other ecotypes have not been proven to cause disease or to be infectious for humans (**chapter 4**).

In the case of *B. burgdorferi* s. l. as discussed above and in **chapter 7**, five particular genospecies seem to be responsible for the clinical manifestations in humans. This presents greater challenges for interpretation and development of diagnostic laboratory methods in Europe in comparison to North America where only one genospecies seems to cause disease. Especially with regard to serologic testing, determining the best combinations of epitopes to include in an enzyme-linked immunosorbent assay (ELISA) or similar assay for optimal sensitivity and specificity is challenging. It should be noted that there is a high level of cross-reactive antibodies to the various *B. burgdorferi* s. l. genospecies. Currently,

a combination of various selected recombinant and native antigens are used of three *B. burgdorferi* s. l. genospecies (*B. burgdorferi* s. s., *B. afzelii*, *B. garinii*) [478]. Current guidelines for serological diagnosis should follow the principle of a two-step procedure: a sensitive ELISA as the first step, if reactive followed by immunoblot (IgM and IgG) [479]. Not surprisingly, the overall sensitivity for this two-tier testing is high for the late clinical manifestations that are caused by *B. burgdorferi* s. s. and *B. afzelii,* namely acrodermatitis chronica atrophicans and Lyme arthritis (~95%) [480]. The sensitivity for diagnosis of Lyme neuroborreliosis is significantly less (~77%) [480]. Perhaps choosing to test for antigens of the genospecies that cause neuroborreliosis (as shown **chapter 7**) - *B. garinii* and *B. bavariensis* - could increase the specificity and sensitivity to certain extend. The sensitivity for diagnosis early in the course of the disease, (the *erythema migrans* stage) is low with only ~50% [480]. As shown in this thesis, the different *B. burgdorferi* s. l. genospecies can be considered as separate pathogens, with each their own enzootic cycle and disease manifestation in humans, therefore perhaps choosing to specifically test for the genospecies involved with the different clinical manifestations might show to be more fitting. Antigens could be an array of proteins that are reactive in different stages of the infection. Preferably, one multiplex serological test would be developed so all the different tick borne pathogens can be tested for at once, as has proven to be valuable with other pathogens like flaviviruses, coronaviruses and influenza [481-484].

7. Concluding remarks

Many clinicians have limited awareness of and experience in recognizing or managing tick-borne pathogens other than Lyme borreliosis, let alone co-infections of different tick-borne pathogens. When patients report tick bites, clinicians should consider additional laboratory testing or differential diagnoses for patients that display an intense or persistent array of aspecific, viral-like symptoms, especially fever, chills, and headache with or without signs of Lyme borreliosis [155]. Medical professionals should therefore be aware of and consider the likelihood of infection with tick-borne pathogens other than Lyme spirochete and co-infections with different tick-borne pathogens when pursuing laboratory testing or selecting therapy for patients with tick-borne diseases [155], especially since protozoan and viral infections are nonresponsive to antibiotics. Furthermore, infections with pathogens like *A. phagocytophilum* and other intracellular bacteria - like *Candidatus* Neoehrlichia mikurensis and *Rickettsia* - should be considered when (suspected) Lyme borreliosis patients fail to respond to β-lactam antimicrobial therapy (like amoxicillin) [155]. Ampicillin, ceftriaxone, and amikacin have shown to be not active when tested *in vitro* on different *A. phagocytophilum* strains [485]. These compounds are also ineffective in treatment of infections caused by a range of obligatory intracellular pathogens, including rickettsioses, Q fever (caused by *Coxiella burnetii*), and ehrlichioses [486]. Most diagnostic tests currently available are not specific to European (strains of) tick-borne pathogens. Furthermore, some tick-borne pathogens have not yet been cultivated in the laboratory - like *Candidatus*

Neoehrlichia mikurensis - therefore serological assays like whole-cell IFA and ELISA tests are not yet available. Taking all together, more specific laboratory tests need to be developed so that estimates of the infection/disease incidence and disease burden of tick-borne diseases can be made in different population categories, and clinicians can be supported in selecting appropriate therapy. Ideally, we would go towards one multiplex molecular test and one multiplex serological protein test where all relevant tick borne pathogens can be tested for at once.

The control of tick-borne diseases requires a multidisciplinary and interdisciplinary approach. This means that different approaches are required to gain greater insight into the drivers at play in vector-, and disease dynamics. Clinical studies are needed to gain a better picture of the clinical spectrum of these often newly emerging or re-emerging pathogens, and to understand to what extent they affect the diagnoses and the etiology of Lyme borreliosis. Especially in Europe, large-scale or systematic surveys of infection with tick-borne pathogens are lacking. The prospective, clinical study the LymeProspect study and other future studies might help provide some insights into the subject of infection, symptoms and disease. To assess the occurrence and burden of other tick-borne diseases, future studies should also assess patients suffering from fever following a tick bite. To allow this, improved laboratory diagnostic tools are needed, and strict case definitions for each individual tick-borne disease would be helpful to measure the (medical) impact. Furthermore, surveillance and epidemiological studies for newly emerging or re-emerging pathogens should be integrated with ecological and biological driving factors since, especially since we have shown that this umbrella approach is successful in this thesis. Such an integrative approach will allow the relative public health and animal health burden of each pathogen to be assessed appropriately. If the upward trend of Lyme borreliosis is a good predictor for all tick-borne diseases, it is quite possible that we have in fact witnessed the beginning of what might be an upsurge of human disease caused by tick-borne pathogens.

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CO-AUTHORS AND AFFILIATION

Catherine M. Butler
School of Veterinary Medicine, St. George's University, True Blue, Grenada, West indies

Elena Claudia Coipan
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Arieke Docters van Leeuwen
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Gilian van Duijvendijk
Laboratory of Entomology, Wageningen University, the Netherlands

Gábor Földvári
Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent Istvan University, Budapest, Hungary

Manoj Fonville
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Ewa Frazer-Mendelewska
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Joke van der Giessen
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Steven Van Gucht
Belgian Scientific Institute of Public Health (WIV-ISP), Communicable and Infectious Diseases, Brussels, Belgium.

Paul Hengeveld
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Tineke Herremans
The Medicines Evaluation Board, Utrecht, the Netherlands

Dieter Heylen
Evolutionary Ecology Group, University of Antwerp, Belgium

Paul Heyman
Research Laboratory for Vector Borne Diseases, Queen Astrid Military Hospital, Brussels, Belgium

Agnetha Hofhuis
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Joppe W. Hovius
Center for Experimental and Molecular Medicine/ Amsterdam Multidisciplinary Lyme Borreliosis Center. Academic Medical Center, the Netherlands.

Ryanne Jaarsma
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Liudmila Karan
Central Research Institute of Epidemiology, Moscow, Russia

Jenny Kleve
National Hedgehog and Squirrel Asylum, Naarden, the Netherlands.

Marion Koopmans
Erasmus Medical Centre, Viroscience, Rotterdam, the Netherlands

Aleksandra Krawczyk
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Herman Kuiper
Flevoziekenhuis, Almere, the Netherlands

Cees van Maanen
Animal Health Service Deventer, the Netherlands

Catharina B. Maassen
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Jolyon M. Medlock
Medical Entomology & Zoonoses Ecology Group, Microbial Risk Assessment, Emergency Response Department Health Protection Agency, Porton Down, Wiltshire, UK.

Wilfrid van Pelt
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Alexander E. Platonov
Central Research Institute of Epidemiology, Moscow, Russia

Chantal Reusken
Erasmus Medical Centre, Viroscience, Rotterdam, the Netherlands

Jolianne M. Rijks
Dutch Wildlife Health Centre, Utrecht University, the Netherlands

Barry Rockx
Erasmus Medical Centre, Viroscience, Rotterdam, the Netherlands

Sanne C. Ruyts
Forest & Nature Lab, Department of Forest and Water Management, Ghent University, Belgium

Ernst-Jan Scholte
National Centre for Monitoring of Vectors (CMV), Netherlands Food and Consumer Product Safety Authority (NVWA), the Netherlands.

Lodewijk Spanjaard
Department of Medical Microbiology. Academic Medical Center, the Netherlands.

Hein Sprong
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands and Laboratory of Entomology, Wageningen University, the Netherlands

Markus Starink
Amsterdam Multidisciplinary Lyme Borreliosis Center/ Department of Dermatology, the Netherlands.

Sándor Szekeres
Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent Istvan University, Budapest, Hungary

Wesley Tack
Laboratory of Forestry, Department of Forest and Water Management, Ghent University, Belgium

Willem Takken
Laboratory of Entomology, Wageningen University, the Netherlands

Katsuhisa Takumi
Centre for Infectious Disease Control, National Institute for Public Health and Environment (RIVM), the Netherlands

Olga Vasilieva
Central Research Institute of Epidemiology, Moscow, Russia

Harry Vennema
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Kris Verheyen
Forest & Nature Lab, Department of Forest and Water Management, Ghent University, Belgium

Ankje de Vries
Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment (RIVM), the Netherlands.

Henry J. C. de Vries
Amsterdam Multidisciplinary Lyme Borreliosis Center/ Department of Dermatology, the Netherlands.

Alex Wagemakers
Center for Experimental and Molecular Medicine. Academic Medical Center, the Netherlands.

Bob de Wever
Department of Medical Microbiology. Academic Medical Center, the Netherlands.

Sipke E. van Wieren
Resource Ecology Group, Wageningen University, the Netherlands

NEDERLANDSE SAMENVATTING

In de meeste Europese landen is er de afgelopen decennia een duidelijke toename in de incidentie van de ziekte van Lyme en tekenencefalitis waargenomen. Ook in Nederland zien we zowel een aanhoudende toename van consulten voor tekenbeten bij de huisarts als een toename in erythema migrans, het eerste stadium van de ziekte van Lyme. De ziektelast veroorzaakt door de ziekte van Lyme of Lyme gerelateerde verschijnselen is aanzienlijk, in het bijzonder door patiënten met langdurig aanhoudende Lyme-gerelateerde symptomen. Lyme-borreliosis heeft een breed scala aan klinische manifestaties. De meest voorkomende en eerste manifestatie is een groeiende rode ring op de plaats van de tekenbeet (erythema migrans). Wanneer deze rode ring niet adequaat wordt behandeld kan dit in sommige gevallen resulteren in gedissemineerde ziekte. De infectie verspreidt zich dan verder en beïnvloedt het zenuwstelsel, gewrichten en huid van een patiënt, en, in zeldzame gevallen, het hart of de ogen. De pathogenen, of ook wel ziekteverwekkers genoemd, van de ziekte van Lyme zijn spiraalvormige bacteriën die behoren tot de *Borrelia burgdorferi* sensu lato (s. l.) complex. Deze bacteriën worden overgedragen door de schapenteek, *Ixodes ricinus,* en worden in stand gehouden in enzoötische cycli met verschillende vertebraten gastheren. Doordat deze tekensoort geen specifieke voorkeur voor een gastheer heeft, bijt het allerlei soorten gastheren om zich te kunnen voeden inclusief mensen.

Behalve de *Borrelia burgdorferi* s. l. genospecies, kan deze tekensoort een heel scala aan pathogenen overdragen inclusief virussen, bacteriën en parasieten. In Nederland gaat het dan vooral om *Borrelia miyamotoi*, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *Rickettsia helvetica*, *Rickettsia monacensis*, verschillende *Babesia* soorten, en tekenencefalitis virus. In 2009 was het geschatte aantal tekenbeten in Nederland 1,1 miljoen. Toch worden ondanks de hoge blootstelling via tekenbeten, infecties, laat staan ziektes, met deze pathogenen zelden gediagnosticeerd. De gepresenteerde resultaten in dit proefschrift geven inzicht in aanwezigheid van tekenoverdraagbare pathogenen en hun risico voor de volksgezondheid. Verder kunnen deze bevindingen worden gebruikt voor risico analyses op het gebied van tekenoverdraagbare ziekten ten behoeve van de besluitvorming binnen het volksgezondheidsbeleid.

In deel I van dit proefschrift worden de verschillende tekenoverdraagbare pathogenen in Nederland bestudeerd en uiteenlopende kennishiaten met betrekking tot hun enzoötische cycli ingevuld. In **hoofdstuk 2** berekenden we de prevalentie van de verschillende tekenoverdraagbare pathogenen in gastheerzoekende teken, en onderzoeken of deze pathogenen enzoötische cycli delen. We bestudeerden patronen van co-infectie, ruimtelijke en seizoensgebonden dynamiek van infectiegraden in zoekende *Ixodes ricinus* nimfen.
De infectiegraden van teken waren het hoogst voor *Rickettsia helvetica* (31.1%) en *Borrelia burgdorferi* s. l. (11.8%) en veel lager voor *Candidatus* Neoehrlichia mikurensis (5.6%), *Anaplasma phagocytophilum* (0.8%), en *Babesia* species (1.7%). De infectiegraad van teken

varieerde niet alleen tussen pathogenen, maar ook tussen de bestudeerde gebieden van 0 tot 64%. *Rickettsia helvetica* en *Borrelia burgdorferi* s. l. werden ook in alle onderzochte gebieden gedetecteerd, terwijl de anderen in de meeste (maar niet alle) gebieden werden gedetecteerd. De dichtheid van gastheerzoekende teken varieerde tussen de 1 tot 179/100 m^2. Algeheel genomen was 37% van de teken geïnfecteerd met tenminste 1 pathogeen en 6,3% met meer dan 1 pathogeen. Eén-derde (1/3) van de *Borrelia burgdorferi* s. l. positieve teken was geïnfecteerd met ten minste één ander pathogeen. Co-infectie van *Borrelia afzelii* met *Candidatus* Neoehrlichia mikurensis en met *Babesia* soorten kwamen significant vaker voor dan verwacht op basis van kans, wat impliceert dat deze pathogenen reservoirgastheren delen. Het omgekeerd was het geval voor co-infecties van *Rickettsia helvetica* met ofwel *Borrelia afzelii* of *Candidatus* Neoehrlichia mikurensis, die significant minder frequent voorkwamen dan verwacht.

In **hoofdstuk 3** hebben we ons gericht op de infectiegraad van *Candidatus* Neoehrlichia mikurensis in verschillende tekensoorten in Europa. Door het testen van teken met behulp van een zelf ontwikkelde real-time PCR-test, toonden we aan dat *Candidatus* Neoehrlichia mikurensis aanwezig is in *Ixodes ricinus* teken door heel Nederland, België en andere Europese landen, maar dat dit pathogeen onder verschillende namen beschreven is in de internationaal gepubliceerde artikelen. Daarnaast hebben we dit opkomende pathogeen moleculair gekarakteriseerd door gebruik te maken van *groEL*, 16S rRNA en *gltA* als moleculaire markers. Wij stellen vast dat verschillende kleine zoogdieren reservoirgastheren zijn van dit pathogeen. Onze gegevens laten ook zien dat mensen regelmatig worden blootgesteld aan *Candidatus* Neoehrlichia mikurensis via tekenbeten.

In **hoofdstuk 4** laten we zien dat *Anaplasma phagocytophilum* ten minste vier verschillende enzoötische cycli (ecotypes) heeft, en dat het *groEL* gen een geschikte moleculaire marker is om deze verschillende ecotypes te identificeren. Alle humane infecties clusteren in maar één van deze ecotypen (ecotype I), wat suggereert dat slechts één van deze ecotypen zoönotische is. Verder laten we ook zien dat dit zoönotische ecotype de grootste scala aan gastheren heeft. Door de significante populatie-expansie - zoals blijkt uit moleculaire markers - van het zoönotische *Anaplasma phagocytophilum* ecotype neemt het risico voor mensen en dieren toe.

In **hoofdstuk 5**, hebben we in reeën-serum neutraliserende antilichamen tegen tekenencefalitis virus (TBEV) gemeten. Vervolgens hebben we, met behulp van moleculaire methoden, TBEV in gastheerzoekende teken uit één gebied aangetoond. Hierdoor hebben we voor het eerst aangetoond dat TBEV endemisch is in Nederland.

In **hoofdstuk 6** onderzochten we de rol van de Europese egel - één van de meest voorkomende dieren in het stadse omgeving- in het onderhouden van een aantal tekenoverdraagbare

pathogenen in de enzoötische cycli in (sub-)stedelijke gebieden. We concluderen dat egels, en hun teken bijdragen aan de overdracht en verspreiding van *Borrelia bavariensis*, *Borrelia spielmanii*, *Borrelia afzelii*, *Anaplasma phagocytophilum* en *Rickettsia helvetica* in deze gebieden.

In deel II wilden we deze bevindingen met betrekking tot de ecologie van tekenoverdraagbare pathogenen vertalen naar blootstelling, infectie en ziekte bij mensen. Hiervoor was het belangrijk dat er onderscheid gemaakt wordt tussen de Lyme-bacteriën welke alleen lokale huidinfecties veroorzaken en de bacteriën welke gedissemineerde infecties kunnen veroorzaken. Daarnaast is het essentieel dat de vertebraten gastheren van medisch belangrijke spirocheten geïdentificeerd werden. In **hoofdstuk 7** hebben we aan de hand van i) isolaten van Lyme-borreliosis gevallen met verschillende klinische verschijnselen (erythema migrans, Lyme neuroborreliosis, acrodermatitis chronica atrophicans, en Lyme artritis) en ii) isolaten uit *Ixodes ricinus* teken die aangehecht waren aan knaagdieren, vogels en egels, verschillende spirocheten genospecies kunnen koppelen aan specifieke klinische verschijnselen van Lyme-borreliosis en aan specifieke gastheren.
Alle isolaten zijn eerst getypeerd op niveau van genospecies door het sequensen van een gedeelte van de 'intergenic spacer' regio. Een meer diepgaande moleculaire typering is uitgevoerd door het sequensen en analyseren van acht extra loci met verschillende eigenschappen.
Hiermee hebben we aangetoond dat *Borrelia afzelii* de meest voorkomende soort is in acrodermatitis patiënten en in teken aangehecht aan knaagdieren. *Borrelia burgdorferi* sensu stricto is wijdverspreid gevonden in erythema migrans patiënten. *Borrelia bavariensis* is wijdverspreid gevonden in Lyme neuroborreliosis patiënten en in teken van egels, maar zeldzaam in erythema migrans patiënten. *Borrelia garinii* was de dominante genospecies in teken van vogels. Specifieke genotypen afkomstig van teken die op egels voedden waren oververtegenwoordigd in erythema migrans en acrodermatitis patiënten, terwijl genotypen afkomstig van teken die op vogels voedden waren oververtegenwoordigd in Lyme neuroborreliosis patiënten. Kortom, de analyse van onze datasets ondersteunt het bestaan van '*spilover*' van ten minste drie verschillende enzoötische cycli van genospecies die ten grondslag liggen van de verschillende klinische manifestaties van Lyme-borreliosis: 1) teken van egels en Lyme neuroborreliosis patiënten, 2) teken van vogels en Lyme neuroborreliosis patiënten, en 3) teken van knaagdieren en egels en acrodermatitis chronica atrophicans patiënten.

In **hoofdstuk 8** zijn we op zoek gegaan naar bewijs van de blootstelling van mensen aan, en infectie met tekenoverdraagbare pathogenen. Daarom testten we zowel teken, die gevoed hebben op mensen, als het bloed van mensen uit een cohortstudie van mensen met tekenbeten en erythema migrans, op de aanwezigheid van een heel scala van pathogenen (*Borrelia burgdorferi* s. l., *Borrelia miyamotoi*, *Anaplasma phagocytophilum*,

Candidatus Neoehrlichia mikurensis, 'spotted fever' *Rickettsia*'s, tekenencefalitis virus en *Babesia* genospecies). De helft (53%) van de op mensen gevoede teken testte positief voor *Borrelia burgdorferi* s. l., *Anaplasma phagocytophilum, Candidatus* Neoehrlichia mikurensis*, Rickettsia helvetica, Rickettsia monacensis, Borrelia miyamotoi* en verschillende *Babesia* soorten. Drieëndertig procent (33%) van de *Borrelia burgdorferi* s. l. positieve teken droeg ook nog één ander pathogeen van een ander genus met zich mee. Op basis van moleculaire detectietechnieken is de kans op infectie met een teken overdraagbare pathogenen (Lyme spirocheten uitgesloten) na een tekenbeet ongeveer 2,4% in Nederland. Evenzo bij Lyme patiënten met erythema migrans is de waarschijnlijkheid van co-infectie met een ander teken pathogeen ongeveer 2,7%. Uit deze gegevens blijkt dat de blootstelling aan tekenoverdraagbare pathogenen en infectie met andere dan *Borrelia burgdorferi* s. l. ziekteverwekkers en co-infecties vaker voorkomen dan voorheen aangenomen. Dit gegeven verdient daarom verder onderzocht te worden: Vervolg onderzoek moet laten zien hoe vaak infecties met deze ziekteverwekkers, al dan niet als co-infectie met Lyme-bacteriën, ziekte veroorzaken.

In **hoofdstuk 9** onderzochten we een voor Nederland nieuw tekenoverdraagbare pathogeen, namelijk de 'relapsing fever' spirocheet *Borrelia miyamotoi*. We hebben dit pathogeen in verschillende organen van knaagdieren aangetoond wat duid op systemische infecties. Daarnaast hebben we laten zien dat vogels ook besmet kunnen worden met dit pathogeen. Bovendien hebben we aangetoond dat er een significante associatie is tussen *Borrelia burgdorferi* s. l. en *Borrelia miyamotoi* in co-infecties van nimfen. Dit impliceert dat deze twee pathogenen gezamenlijke reservoirgastheren hebben.
Het frequent voorkomen van co-infecties van deze twee pathogenen heeft onze interesse gewekt of erythema migrans-achtige huidklachten bij van Lyme verdachte patiënten misschien zijn te wijten aan een co-infectie met *Borrelia miyamotoi*. Wij hebben echter geen bewijs kunnen vinden voor een *Borrelia miyamotoi* infectie bij het testen van huidbiopten van Lyme-borreliosis verdachte patiënten, deze bevinding suggereert dat *Borrelia miyamotoi* niet geassocieerd is met Lyme borreliosis-geassocieerde huid manifestaties. En dat patiënten met *Borrelia miyamotoi* infectie - gekenmerkt door koorts, malaise en myalgie - met een huidletsel gelijktijdig geïnfecteerd zijn met *Borrelia burgdorferi* s. l ..

Op basis van de infectiegraad van *Borrelia miyamotoi* (2 tot 5%)in *Ixodes ricinus* teken in Nederland ontstond de vraag hoe vaak dat verschillende bevolkingsgroepen worden blootgesteld aan dit pathogeen? Daarom hebben we in **hoofdstuk 10** de blootstelling van verschillende risicogroepen aan *Borrelia miyamotoi* serologisch getest. Hiervoor ontwikkelde we Luminex test gebaseerd op een fragment van *Borrelia miyamotoi* glycerophosphodiester fosfodiesterase (GlpQ) als antigeen. Onder de aanname dat een IgG antilichaamreactie tegen antigenen een (doorstaande) infectie weerspiegelt hebben we aan de hand van een sero-prevalentie studie de blootstelling aan verschillende risicogroepen onderzocht. Sera

van negen van de tien PCR bevestigde *Borrelia miyamotoi* patiënten uit Rusland werden positief met het gebruikte recombinante antigeen. Er werd geen significante kruisreactiviteit waargenomen bij de controle groep (secundaire syfilis patiënten). De seroprevalentie in bloeddonoren was 2,0% (95% CI 0,4-5,7%). De seroprevalentie van antilichamen tegen het gebruikte antigeen was verhoogd in 5,6% (3,0-9,2%) van de erythema migrans patiënten en in 7,4% (2,0-17,9%) van de individuen met serologisch bevestigde en in 8,6% (1,8-23%) van de onbevestigde Lyme neuroborreliosis. Echter waren deze waarden niet significant in vergelijking tot het percentage van seropositieve bloeddonoren. De prevalentie van anti-*Borrelia miyamotoi* antilichamen onder Nederlandse boswachters was 10% (5,3-16,8%) en onder patiënten met serologisch onbevestigde maar vermoed van humane Anaplasmosis 14,6% (9,0-21,8%). Deze waarden zijn significant hoger bevonden dan die van bloeddonoren. Onze bevindingen wijzen erop dat infecties met *Borrelia miyamotoi* optreden bij personen die in Nederlands aan teken zijn blootgesteld. *Borrelia miyamotoi* infecties kunnen waarschijnlijk ook een rol spelen bij patiënten met een tekenbeet en koorts met onopgeloste etiologie in andere landen waar *Ixodes ricinus* teken endemisch zijn.

In het bovenstaande onderzoek hebben we laten zien dat in Nederland het risico van blootstelling en infectie met een tekenoverdraagbare pathogeen aanzienlijk is. Ten eerste hebben we laten zien dat meer dan één-derde van gastheerzoekende teken en meer dan de helft van teken die op mensen voeden één of meerdere van de geteste tekenoverdraagbare pathogenen bij zich dragen. Bovendien hebben we laten zien dat mensen inderdaad worden geïnfecteerd met een groot scala van deze pathogenen. Het is heel goed mogelijk dat in de kliniek verschillende infecties met tekenoverdraagbare pathogenen niet goed onderscheiden worden en allen onder de grotere noemer van Lyme-borreliosis geschaard worden, simpelweg omdat de meeste van deze ziekteverwekkers vaak niet-specifieke symptomen vertonen.

Veel artsen hebben beperkte kennis van en ervaring in het herkennen en behandelen van tekenoverdraagbare infecties anders dan Lyme-borreliosis, laat staan van co-infecties met verschillende pathogenen. Clinici moeten, wanneer patiënten zich melden met tekenbeten, rekening houden met deze andere infecties in hun differentiaal diagnose. Ze zouden extra laboratoriumonderzoek kunnen aanvragen vooral voor patiënten met een intense of aanhoudende reeks van aspecifieke symptomen, in het bijzonder koorts, koude rillingen, malaise, myalgie en hoofdpijn met of zonder tekenen van Lyme borreliosis. Hierbij is het belangrijk te onderkennen dat antibiotica bij protozoïsche en virale infecties niet effectief zijn. Bovendien, infecties met intracellulaire bacteriën - zoals *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis en *Rickettsia*'s - moet worden overwogen bij (vermeende) Lyme patiënten die niet reageren op β-lactam antimicrobiële therapie (zoals amoxicilline).

De meeste diagnostische tests, die momenteel beschikbaar zijn, zijn niet specifiek voor de Europese stammen van tekenoverdraagbare pathogenen. Verder zijn voor sommige tekenoverdraagbare pathogenen serologische tests als 'whole cell' IFA en ELISA ook niet beschikbaar omdat deze pathogenen nog niet gekweekt kunnen worden in het laboratorium. Investeringen in het ontwikkelen van specifieke laboratoriumtests zijn essentieel voor schattingen van de infectie/ziekte-incidentie en ziektelast van tekenoverdraagbare pathogenen kunnen worden gemaakt in verschillende bevolkingsgroepen, en zodat clinici kunnen worden ondersteund in het selecteren van de juiste therapie. De komst van één moleculaire multiplex test en één serologische proteïne multiplex test waarbij alle relevante teken overdraagbare ziekteverwekkers in één keer kunnen worden getest zou ideaal zijn.

Een multi-causaal probleem als door tekenoverdraagbare infecties vereist een multidisciplinaire en interdisciplinaire benadering. Dit betekent dat verschillende benaderingen nodig zijn om meer inzicht te krijgen in de drijvende krachten die een rol spelen in vector- en ziektedynamiek. Er zijn klinische studies nodig om een beter beeld te krijgen van het klinische spectrum van deze infecties, en om te begrijpen in welke mate ze invloed hebben op de diagnoses en de etiologie van Lyme-borreliosis. Vooral in Europa ontbreken er grootschalige of systematische onderzoeken van infecties met tekenoverdraagbare pathogenen. De prospectieve, klinische studie de LymeProspect en andere studies kunnen helpen om inzicht te krijgen in de infectie, symptomen en ziekte. Deze studies zouden dan vooral de patiënten die lijden aan koorts na een tekenbeet verder moeten vervolgen. Hiervoor zijn verbeterd diagnostische laboratorium testen en strikte case definities noodzakelijk. Bovendien moeten surveillance en epidemiologische studies betreffende deze pathogenen worden geïntegreerd met ecologische en biologische studies. We hebben in dit proefschrift laten zien dat deze aanpak succesvol is. Bij een dergelijke integrale aanpak zal de relatieve ziektelast binnen volksgezondheid en/of diergezondheid van elk pathogeen op passende wijze kunnen worden beoordeeld. De opwaartse trend van de Lyme-borreliosis is een goede indicator voor wat ons in de toekomst staat te wachten voor de andere tekenoverdraagbare ziekten.

ABOUT THE AUTHOR

Setareh Jahfari was born on August 27, 1984 in Tehran (Iran). In 1988, her family fled the country due to opposing political ideologies and subsequently migrated to the Netherlands after receiving UN political refugee status. Seta grew up in Drachten and obtained her high-school diploma in 2003 from the Drachtster Lyceum. Following her graduation, she studied different topics, traveled and worked. In 2011, she received her Bachelors in Life Sciences with a major in Biomolecular Research and minor in Business Engineering from the Utrecht University of Applied Sciences. Seta completed her Master's degree in Biomedical Sciences specializing in infection and immunity at the University of Amsterdam. After an internship at the vector-group of the National Institute for Public Health and the Environment (RIVM) in 2012, she was offered a PhD position. The research project in tick-borne infections and public health was a joint effort between two RIVM Centers: Zoonoses and Environmental Microbiology (Z&O) and Infectious Diseases Research, Diagnostics and Screening (IDS), which led to this doctoral dissertation. In early 2015, Seta volunteered to work in a mobile laboratory in Sierra Leone to provide diagnostic support for the Ebola Virus outbreak. In 2017, she was selected for the Young Talent Program of Xendo, a life sciences and pharmaceutical consultancy.

PHD PORTFOLIO

Name:	Seta Jahfari
Research school:	Molecular Medicine Postgraduate School, Erasmus MC Rotterdam
Research group:	Department of Viroscience
PhD period:	2012-2016
Promotor:	Prof. dr. M.P.G. Koopmans (Erasmus MC Rotterdam)
	Prof. dr. J.W. Hovius (University of Amsterdam)
Co-promotor:	Dr. H. Sprong

COURSES

- Virology (MolMed, Erasmus MC) 2012
- Introductory course on statistics & survival Analysis (MolMed Erasmus MC) 2012
- Analysis of Serological Data (RIVM) 2012
- Masterclass Scientific Integrity (RIVM) 2012
- Advanced Course Molecular Immunology (MolMed Erasmus MC) 2013
- Research management for PhD-students (MolMed, Erasmus MC) 2013
- Computational Molecular Evolution (online course, Technical University of Denmark) 2013
- Advanced Immunology course (Utrecht University, Graduate School of Life Sciences) 2014
- Infection Biology course (Utrecht University, Graduate School of Life Sciences) 2014
- Masterclass Scientific Integrity (Utrecht University) 2014
- Molecular Biology of the Cell (University of Amsterdam) 2014
- Advanced Medical Microbiology (University of Amsterdam) 2014
- Advanced Immunology (University of Amsterdam) 2014
- Biosafety level course (RIVM) 2015
- Biomedical English writing course (MolMed Erasmus MC) 2015
- Save the Children Ebola deployment course (London) 2015
- Dutch mobile lab Ebola deployment course (Erasmus MC) 2015

SCIENTIFIC CONFERENCES AND MEETINGS

- ECDC Expert consultation on laboratory testing of Lyme borreliosis, Amsterdam 2013
- Dutch Society of Medical Microbiology, Utrecht 2013
- TickTactics II, Amsterdam 2014
- Conference Louis Pasteur, Emerging Infectious Diseases, Paris 2014
- The Society for Applied Microbiology- MedVetNet Association conference, Brighton 2014
- Symposium on Zoonoses Research and International Conference on Emerging Diseases, Berlin 2014
- International Meeting on Emerging Diseases and Surveillance, Vienna 2014
- International meeting on Lyme borreliosis and other tick-borne diseases, Vienna 2015
- TickTactics III, Wageningen 2016

MISCELLANEOUS

- Supervision/ guidance of (foreign) Bsc-, Msc-, PhD-students, and technicians 2012-2016
- Volunteer to support diagnostic activities during the Ebola Virus outbreak and facilitate capacity building at the local hospital laboratory, Freetown, Sierra Leone 2015
- Organizing committee member of the Scientific symposium 'Ticktactics' 2014-2016
- Reviewer for 'Parasites and Vectors' 2015-2017
- Reviewer for 'Parasitology Open' 2016-2017

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